AURORA KINASES: CLASSICAL MITOTIC ROLES, NON-CANONICAL FUNCTIONS AND TRANSLATIONAL VIEWS

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AURORA KINASES: CLASSICAL MITOTIC ROLES, NON-CANONICAL FUNCTIONS AND TRANSLATIONAL VIEWS

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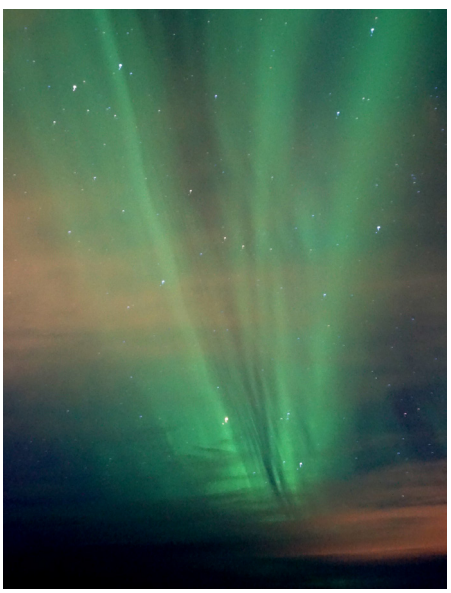

Aurora northern lights.

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Aurora kinases are key mitotic regulators that have also been associated with tumor development and progression. The interest on this highly conserved family of protein kinases has grown exponentially since they were discovered in the 1990s.

Despite the steady increase in the number of laboratories involved and the consequent boost of the volume of research output during the last years, the study of Aurora kinases remains a very dynamic area in which new discoveries frequently keep coming to light.

In this Frontiers Research Topic, we have aimed to not only review and revisit different aspects of the functions and regulation of Aurora kinases but also provide a forum for the publication of new developments in the field. Thanks to the excellent work of the authors and reviewers of this eBook, which includes some of the most experienced voices in the field, we hope that this collection will inspire new research projects that will lead to a better understanding of the role of these kinases in cancers.

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EDITORIAL
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Editorial: Aurora Kinases: Classical Mitotic Roles, Non-Canonical Functions and Translational Views

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Editorial on the Research Topic

Aurora Kinases: Classical Mitotic Roles, Non-Canonical Functions and Translational Views

Aurora kinases are key mitotic regulators that have also been associated with tumor development and progression. The interest on this highly conserved family of protein kinases has grown exponentially since they were discovered in the 1990s. Despite the steady increase in the number of laboratories involved and the consequent boost of the volume of research output during the last years, the study of Aurora kinases remains a very dynamic area in which new discoveries frequently keep coming to light. From a clinical perspective, the interest on Aurora kinase biology stems from their identification as targets for drug development; an increasing number of Aurora kinase inhibitors are being tested in preclinical projects and clinical trials. In this Frontiers Research Topic, we have aimed to not only review and revisit different aspects of the functions and regulation of Aurora kinases but also provide a forum for the publication of new developments in the field. We have been privileged to count on contributions from authors and reviewers that include some of the most experienced voices in our research area.

In their introductory article to the Research Topic, two old-timers in the field, David Glover and Bill Earnshaw, have provided a historical perspective of Aurora Kinase research. By looking at the field's origin using genetic screens in *Drosophila* and yeast and cell biological studies in vertebrates that led to the identification of Aurora kinases and their partner proteins, the authors give us a unique first witness account of the development of the field (Carmena et al.).

Several articles in this Research Topic focus exclusively upon a single member of the Aurora kinase family. Among the contributions focused on Aurora A, Garrido and Vernos review the regulation of this kinase by TPX2, discussing its relevance as well as its (weak) conservation throughout evolution and potential key role in tumorigenesis. Two manuscripts from the Guarguaglini and Medema labs explore the interplay between Aurora A kinase and PLK1 and how this contributes to the regulation of different processes in mitosis (Asteriti et al.; Bruinsma et al.). Moving forward to the final stage of mitosis, Reboutier et al. report on the emerging role of Aurora A on the regulation of mitotic exit. The Research Topic also explores the mitotic and non-mitotic roles of Aurora A in the context of oncogenic transformation and tumor progression (D'Assoro et al.); this includes a review on the interactions between the kinase and the tumor suppressor p53 and the possible consequences for their signaling pathways in tumor cells (Sasai et al.). Finally, we have a report on recently described non-canonical roles of Aurora A kinase in DNA replication (Tsunematsu et al.).

On the other side of the coin, among the articles focusing upon Aurora B kinase, two explore its roles in specific stages of mitosis and cytokinesis: Krenn and Musacchio offer a detailed review on the role of Aurora B in chromosome bi-orientation and spindle checkpoint signaling; whereas D'Avino and Capalbo take us through a thorough analysis of the later roles of Aurora

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kinase within the CPC, focusing on its roles in controlling the assembly of the cleavage furrow, central spindle, and midbody and analyzing the function of the complex in the control of abscission timing. Finally, the chapter by Lindon et al. reviews the ubiquitin-dependent proteolytic regulation of Aurora B in mitosis.

The lesser known member of the Aurora family, Aurora C kinase, is the subject of two reports that center in its role in meiosis (Yang et al.; Quartuccio and Schindler), the latter one also exploring in this context the significance of its expression in cancer cells (Quartuccio and Schindler).

As an important part of this Research Topic, we wanted to include in an overview of different aspects of the use of Aurora kinase as targets for drug development. D'Assoro et al. have reviewed the potential of Aurora A as a therapeutic target in cancer, while Bavetsias and Linardopoulos have summarized the properties of the known Aurora kinase inhibitors currently in the clinic and have discussed current and future directions of such research. The contribution of de Groot and colleagues has given us an invaluable study of 10 commercially available Aurora inhibitors, including a set of "guidelines" for their efficient use in cell biology experiments (de Groot et al.). A group from Lilly Research Laboratories (Marugán et al.) contributes a useful technical manuscript describing phenotypic screening assays to develop Aurora kinase inhibitors. Niu et al. from Takeda Pharmaceuticals review the use of Alisertib (the highly specific Aurora A kinase inhibitor in advanced trials) in cancer therapy. Finally, in this section, the contribution from Nikonova et al. explores the potential of combined therapy directed against both Aurora A and EGFR for the treatment of autosomal-dominant polycystic kidney disease.

We also wanted to have a flavor of some of the non-canonical roles of Aurora kinases outside of mitosis. We have managed to capture some of this activity in the article by Hascoet et al., who have reviewed some of these unconventional functions of Aurora kinases in kidney tumorigenesis.

We hope that the efforts of our authors and referees will find value in the field as we feel that this series of articles "Aurora kinases: classical mitotic roles, non-canonical functions, and translational views" represents an impressive snapshot of our current knowledge of the different functions of Aurora kinases. Written by experts in the field, we hope that this collection will inspire new research projects that will lead to a better understanding of the role of these kinases in cancers.

AUTHOR CONTRIBUTIONS

The authors are the editors of the Research Topic "Aurora kinases: classical mitotic roles, non-canonical functions, and translational views." The four have equally contributed to this editorial.

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REVIEW
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The Dawn of Aurora Kinase Research: From Fly Genetics to the Clinic

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Aurora kinases comprise a family of highly conserved serine-threonine protein kinases that play a pivotal role in the regulation of cell cycle. Aurora kinases are not only involved in the control of multiple processes during cell division but also coordinate chromosomal and cytoskeletal events, contributing to the regulation of checkpoints and ensuring the smooth progression of the cell cycle. Because of their fundamental contribution to cell cycle regulation, Aurora kinases were originally identified in independent genetic screens designed to find genes involved in the regulation of cell division. The first *aurora* mutant was part of a collection of mutants isolated in C. Nusslein-Volhard's laboratory. This collection was screened in D. M. Glover's laboratory in search for mutations disrupting the centrosome cycle in embryos derived from homozygous mutant mothers. The mutants identified were given names related to the "polar regions," and included not only *aurora* but also the equally famous *polo*. Ipl1, the only Aurora in yeast, was identified in a genetic screen looking for mutations that caused chromosome segregation defects. The discovery of a second Aurora-like kinase in mammals opened a new chapter in the research of Aurora kinases. The rat kinase AIM was found to be highly homologous to the fly and yeast proteins, but localized at the midzone and midbody and was proposed to have a role in cytokinesis. Homologs of the equatorial Aurora (Aurora B) were identified in metazoans ranging from flies to humans. Xenopus Aurora B was found to be in a complex with the chromosomal passenger INCENP, and both proteins were shown to be essential in flies for chromosome structure, segregation, central spindle formation and cytokinesis. Fifteen years on, Aurora kinase research is an active field of research. After the successful introduction of the first anti-mitotic agents in cancer therapy, both Auroras have become the focus of attention as targets for the development of new anti-cancer drugs. In this review we will aim to give a historical overview of the research on Aurora kinases, highlighting the most relevant milestones in the advance of the field.

Keywords: Aurora kinase, mitosis, chromosomal passenger complex, anticancer drugs, cytokinesis, centrosome

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The *aurora* gene was first discovered in the late 1980s as part of a search for *Drosophila* genes regulating cell cycle progression (Glover, 1989; Glover et al., 1989, 1995). Since then, Aurora kinases have emerged as essential players in the regulation of cell division (for review see Carmena et al., 2009). The initial steady flow of publications soon accelerated as paralogs in different species were discovered and new functions assigned to them. The finding of elevated levels of Auroras in cancer cells soon stimulated the development of small molecule inhibitors of these kinases (Hauf et al., 2003; Harrington et al., 2004). This too was to become a field in which research output has

increased exponentially in a race to develop new drugs for cancer therapeutics (Lens et al., 2010; Goldenson and Crispino, 2015; Malumbres and Pérez de Castro, 2015). Today, the study of the Aurora family of protein kinases continues to be a highly dynamic and interactive field of research, many of whose aspects will be covered in the articles comprising this Research Topic.

The discovery and functional characterization of Aurora kinases is only a part of the explosion in our knowledge of the molecular mechanics of mitosis over the past quarter of a century. As with all studies of mitosis, the principal findings have been rooted in observations made through microscopy; this is hardly surprising as mitosis is possibly the most spectacular event in a cell's natural life cycle. The events of mitosis were first described in any detail by Flemming (1882) who named the mitotic phases as we still know them today. This was also the time when Boveri and van Benenden independently discovered the centrosome (Boveri, 1887; Van Beneden and Neyt, 1887). However, it was more than a century later that a true genetic dissection of the events of the cell cycle was first undertaken in the pioneering genetic screens of Hartwell and colleagues in their search for cell division cycle (cdc) genes in the budding yeast, Saccharomyces cerevisiae. These famously led to the discovery of *cdc28*, which was later revealed to be the gene encoding the first identified cyclin-dependent kinase (Cdk) whose activity is needed at START, the point at which nutritional, hormonal, and cell size controls regulate cell cycle progression (Hartwell et al., 1970). Taking a similar approach in the fission yeast Schizosaccharomyces pombe, Nurse and coworkers set out to find rate-controlling factors in cell division in this organism (Nurse, 1975). Their work uncovered *cdc2*, the fission yeast counterpart of *cdc28,* a gene with a key role in mitotic entry. In addition, these studies were soon to demonstrate the dramatic extent of conservation of the Cdc28/Cdc2 kinase by showing that its human ortholog could rescue the fission yeast mutant (Lee and Nurse, 1987). Around the same time Hunt and his co-workers were performing studies on protein synthesis during early embryonic development in marine invertebrates that led to the identification of key partner proteins of Cdc28/Cdc2. The newly discovered proteins accumulated each cell cycle and were destroyed at the end of mitosis and so were named cyclins (Evans et al., 1983). It was not until Masui's mysterious factor able to promote progression through the meiotic cycle in frogs (*maturation promoting factor-MPF*) was eventually purified in the lab of Jim Maller (Gautier et al., 1988; Lohka et al., 1988) that the partnership of the "Cdc2" kinase and cyclins was appreciated. The 1980s also saw other genetic screens in fission yeast, notably those of Mitshuhiro Yanagida's group that focused upon identifying genes essential for mitosis in fission yeast by visually classifying the mitotic defects of mutants (e.g., Toda et al., 1983; Hirano et al., 1986). Thus, the stage was being set for the concerted application of genetics and biochemistry to analyse the molecular mechanisms regulating cell division. This marked a fundamental change in the way that the fields of genetics, molecular biology, and biochemistry interacted with each other.

Around the same time, similar plots were also being hatched to use Drosophila melanogaster as a model in which to study metazoan cell division. Fruit flies had an almost century-long genetic tradition and characteristics of their life cycle made them particularly useful for cell cycle studies. A series of screens reported by Gatti and Baker at the Crete Drosophila meeting in 1982, but not published until some 7 years later (Gatti and Baker, 1989), exploited the fact that cell division cycle mutants tended to die in the late larval or early pupal stages. This is because the maternal contribution of cell cycle proteins supports the rapid syncytial nuclear division cycles and the subsequent embryonic cell cycles. Development through the larval stages then has little demand upon mitosis. Instead it requires that many tissues undergo endoreduplication cycles to produce large cells with "giant" chromosomes. The great majority of mitotic divisions in larvae occur in tissues required after metamorphosis to make the adult fly, including neuroblasts and imaginal discs. Thus, as long as heterozygous mothers provide enough wild type products for early development, animals homozygous for mutations in essential mitotic genes can survive into the late larval/pupal stage. Gatti and Baker had the clever idea of screening through collections of late lethal mutants for mitotic defects in the larval central nervous system and showed that indeed these were a rich source of essential cell cycle genes (Gatti and Baker, 1989).

One of us (DMG) took a complementary approach to search for Drosophila's cell cycle regulatory genes. Because Drosophila embryos are loaded with maternal products that are required for the 13 rounds of rapid nuclear division cycles of the syncytial embryo, a search began for mutations that when homozygous in mothers would result in embryos that failed to develop because of mitotic abnormalities. A short-term EMBO Fellowship took DMG off to Christiane Nusslein-Volhard's laboratory in Tubingen to screen her collection of maternal-effect mutants. Mitotic structures including the centrosome could be tracked in embryos using antibodies from a library of monoclonals raised against Drosophila embryonic proteins by Harald Saumweber's lab also in Tubingen (Frasch et al., 1986). The analysis of mitotic phenotypes in mutant embryos led to the identification of genes required for the embryonic syncytial divisions. First came *gnu*, a gene that specifically regulates the onset of the mitotic division cycles in the embryo and whose mutant phenotype is endoreduplication at the expense of mitosis (Freeman et al., 1986). This was soon followed by hypomorphic mutant alleles of genes required in all cell division cycles (Glover, 1989; Glover et al., 1989). A particular interest in the centrosome cycle in the embryonic divisions led to the identification of mutant embryos showing abnormalities in the spindle poles. Among these were the genes *polo* and *aurora,* named after the geomagnetic poles of the earth and their associated phenomena (Sunkel and Glover, 1988; Glover et al., 1995).

Embryos derived from females homozygous for the original *aurora* mutant, a weak hypomorphic allele, displayed defects consistent with defective centrosome separation in embryonic mitoses. As further *aurora* alleles were uncovered, it could be seen that they affected development in different ways. The *aurora* gene mapped within a small genetic interval that had been studied by Gausz and colleagues in Szeged, Hungary (Gausz et al., 1981). Complementation tests with the original maternal effect *aurora* mutant led to the identification of amorphic alleles of the gene. Larvae homozygous for amorphic alleles showed late

larval lethality, and their brains displayed monopolar spindles and enlarged centrosomes reflecting a failure of centrosome disjunction in mitosis. The cloning of the *aurora* gene, in those days a drawn-out, labor-intensive process, revealed it to encode a Ser-Thr protein kinase with a conserved C-terminal kinase domain related to other known kinases but with a divergent N-terminal domain (Glover et al., 1995). It was soon found that the Aurora kinase was in fact localized at centrosomes, not only in fly but also in mammalian cells (Kimura et al., 1997) and Xenopus (Roghi et al., 1998).

Saccharomyces cerevisiae Aurora/Ipl1 was also originally found in a genetic screen, in this case designed to identify factors required for correct chromosome segregation (Chan and Botstein, 1993). A careful phenotypical analysis of *ipl1* mutants revealed that while sister chromatid separation was normal, chromosome segregation was defective. Although Ipl1 was found to be a cell cycle regulated protein associated with spindle microtubules, *ipl* mutants neither showed any defects in spindle formation, breakdown, or morphology nor showed problems with spindle pole duplication or separation. On the other hand, *ipl* mutants were found to interact genetically with CBF3 components and show defective kinetochore function, likely through the kinetochore protein Ndc10p (Biggins et al., 1999). As the phosphatase Glc7p had been previously shown to oppose Ipl1 activity (Francisco et al., 1994) and also to regulate Ndc10p, Biggins and coworkers proposed that Ipl1 had a function in regulating kinetochore/microtubule attachments through Ndc10p. This work highlighted the importance of reversible phosphorylation by Aurora kinases as a crucial mechanism in the regulation of mitotic events, a subject that has been the focus of numerous studies throughout the history of Aurora research.

Several protein kinases related to Aurora and Ipl1 were soon identified in other model organisms including Caenorhabditis elegans, Xenopus, mouse, rat, and human (Giet and Prigent, 1999). The discovery of the rat protein AIM-1 (Aurora and Ipl1-like midbody-associated protein) was of particular importance. In contrast to the centrosomal localization of metazoan Aurora kinases discovered up until that time, AIM-1 was found at the midzone in anaphase and then in the midbody in cytokinesis. Overexpression of a dominant negative form of AIM-1 disrupted formation of the cleavage furrow in late anaphase and resulted in cytokinesis failure. These cells did not show any defects in the formation of the bipolar spindle or chromosome segregation (Terada et al., 1998). Terada and coworkers proposed that AIM-1 was probably not a true functional homolog, but rather a protein related to Aurora kinase and therefore that there were at least two different Auroras in mammalian cells: one involved in the regulation of the spindle pole and the other required for cytokinesis. Importantly, they also pointed out that the differences in location and function between the two Auroras were more likely due to their divergent N-terminal region.

Two AIR (Aurora/Ipl1 related) kinases were also identified in *C.elegans*, and their functions were analyzed by RNA-mediated interference (RNAi). AIR-1 was shown to be associated with mitotic centrosomes and to be required for embryogenesis (Schumacher et al., 1998a). The second ortholog, AIR-2 was described to have a very particular pattern of localization during mitosis: it associated with the metaphase chromosomes but translocated to the microtubule spindle in anaphase and remained in the midbody at cytokinesis (Schumacher et al., 1998b). As AIR-2 RNAi embryos displayed defects in cytokinesis, it was proposed that the protein could be involved in coordinating chromosomal events with cytokinesis. Noticeably, this localization and function of *C. elegans* AIR-2 were reminiscent of those of another protein, at the time not suspected to have any link to Aurora, the Inner Centromere Protein, INCENP.

INCENP had been identified a decade before in the Earnshaw lab (Cooke et al., 1987) in a monoclonal antibody screen aimed at identifying components of the mitotic chromosome scaffold. INCENP exhibited a unique dynamic localization in mitosis, repositioning from centromeres to the central spindle and then to the cleavage furrow. Because of this behavior, one of us (WCE) proposed that INCENP defined a new class of proteins called "chromosomal passengers" that associated with chromosomes to "... position themselves properly in order to fulfill their roles after anaphase onset" (Earnshaw and Cooke, 1991). Subsequent studies using expression of dominant mutants gave the first indications that INCENP played an important role in mitotic regulation (Mackay et al., 1998). The link between INCENP and a second Aurora kinase was firmly established when Richard Adams and colleagues in the Earnshaw lab found that both proteins formed part of an 11S complex stockpiled in Xenopus egg extracts (Adams et al., 2000). The two proteins were also shown to interact *in vitro* in *C. elegans*, where they were proposed to function in resolution of sister chromatid cohesion and in the assembly of the spindle midzone (Kaitna et al., 2000). Eventually, the confusing nomenclature of the field would be rationalized by renaming the centrosomal associated enzyme as Aurora A, the chromosomal passenger kinase as Aurora B, and a third enzyme—a passenger kinase found in the male and female germline of mammals -as Aurora C (Adams et al., 2001a; Nigg, 2001).

Analysis of the function of the Drosophila homologs of INCENP (Adams et al., 2001b) and Aurora B (Adams et al., 2001b; Giet and Glover, 2001) provided definitive evidence of the participation of the complex in the regulation of multiple processes in cell division. Cells in which INCENP or Aurora B levels had been knocked down by RNAi were defective in chromosome structure, condensation, congression to the metaphase plate, segregation, and cytokinesis. Post-translational modifications (i.e., phosphorylation of Histone 3 in Serine 10) and specific changes in the localization of proteins associated with these processes (i.e., Barren/DCapH, Pavarotti/MKLP1) were also shown to be dependent on the correct function of INCENP/Aurora B (Adams et al., 2001b; Giet and Glover, 2001; Murnion et al., 2001). This work also demonstrated that the proteins depend on each other for their correct localization and function (Adams et al., 2001b). Later studies revealed that INCENP and Aurora B are associated with two more proteins, Survivin and Borealin/Dasra to form the Chromosomal Passenger Complex (Wheatley et al., 2001; Gassmann et al., 2004; Sampath et al., 2004). In this complex the proteins INCENP,

Survivin, and Borealin are targeting and activating subunits of the kinase Aurora B. The multiple functions of the CPC have been the subject of numerous studies in the last 15 years (for examples Carmena et al., 2012; van der Horst and Lens, 2014).

Aurora A also has a range of interaction partners; notably its binding to TPX2 results in a conformational change that promotes activation by auto-phosphorylation and hinders the inhibitory activity of PP1 (Protein phosphatase 1). Both Aurora A and Aurora B kinases are highly conserved in their C-terminal domains and it is their divergent N-terminal domains that determine their interactions with different partners in the cell. Curiously, a single amino acid change (G198N) in human Aurora A makes it localize like Aurora B, interact with its partners INCENP and Survivin and rescues the function of an Aurora B knock-down (Fu et al., 2009; Hans et al., 2009).

Study of the human Aurora kinases has been linked to cancer research from its beginnings (for review see Malumbres and Pérez de Castro, 2015). Human Aurora 1 (Aurora B) and 2 (Aurora A) were identified in a PCR-based screen designed to identify novel colon cancer-associated kinases (Bischoff et al., 1998). A previous study had found a partial sequence of a breast tumor-associated kinase BTAK that was later identified as a fragment of Aurora B (Sen et al., 1997). In addition, Aurora A was found very early on to be overexpressed in colorectal carcinomas, and the Aurora A gene was mapped in a region that is amplified in a great variety of cancers (Bischoff and Plowman, 1999). Although its function as an oncogene is disputed, it has been proposed that Aurora A has a dual role in tumorigenesis (for review see Malumbres and Pérez de Castro, 2015): firstly inducing aneuploidy through its function in centrosome maturation/separation, and secondly through interactions with p53. Aurora B is also overexpressed in a wide range of cancers and may participate in tumorigenesis through the induction of tetraploidy (and consequent genetic instability). Because of these roles, both Auroras have been used as targets for the development of new anti-cancer therapies. At present numerous (>70) clinical trials have been carried out with Aurora kinase inhibitors. Although the first trials were marred by the high toxicity of the compounds on trial, there is now renewed optimism arising from the results of the use of Aurora inhibitors in combination with cytotoxic therapies (taxanes, HDAC inhibitors, etc).

In this Research Topic, we will showcase the latest advances in the research on the roles of Aurora kinases in the tumor cell. Contributions will include analysis of their roles in mitosis and meiosis but also new approaches to study the non-canonical roles of Aurora kinases.

AUTHOR CONTRIBUTIONS

MC drafted the manuscript. MC, DG, and WE revised the manuscript and approved for submission.

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Non-centrosomal TPX2-Dependent Regulation of the Aurora A Kinase: Functional Implications for Healthy and Pathological Cell Division

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Aurora A has been extensively characterized as a centrosomal kinase with essential functions during cell division including centrosome maturation and separation and spindle assembly. However, Aurora A localization is not restricted to the centrosomes and compelling evidence support the existence of specific mechanisms of activation and functions for non-centrosomal Aurora A in the dividing cell. It has been now well established that spindle assembly involves an acentrosomal RanGTP-dependent pathway that triggers microtubule assembly and organization in the proximity of the chromosomes whether centrosomes are present or not. The mechanism involves the regulation of a number of NLS-containing proteins, generically called SAFS (Spindle Assembly Factors) that exert their functions upon release from karyopherins by RanGTP. One of them, the nuclear protein TPX2 interacts with and activates Aurora A upon release from importins by RanGTP. This basic mechanism triggers the activation of Aurora A in the proximity of the chromosomes potentially translating the RanGTP signaling gradient centered on the chromosome into an Aurora A phosphorylation network. Here, we will review our current knowledge on the RanGTP-dependent TPX2 activation of Aurora A away from centrosomes: from the mechanism of activation and its functional consequences on the kinase stability and regulation to its roles in spindle assembly and cell division. We will then focus on the substrates of the TPX2-activated Aurora A having a role in microtubule nucleation, stabilization, and organization. Finally, we will briefly discuss the implications of the use of Aurora A inhibitors in anti-tumor therapies in the light of its functional interaction with TPX2.

Keywords: Aurora A kinase, TPX2, spindle, RanGTP, microtubule, cell division, importin, phosphorylation

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Cell cycle progression is crucial for cell viability. During mitosis, most cellular components undergo a dramatic reorganization. In particular, the relatively stable interphase microtubule (MT) network disappears and highly dynamic MTs organize the bipolar spindle, the molecular machine that provides the support and forces for chromosome segregation. The progression and coordination of the events that drive spindle assembly and culminate with the birth of two daughter cells rely on complex regulatory networks involving several kinases. One of them is Aurora A (1), a kinase originally

Abbreviations: γTuRC, γ-tubulin ring complex; APC/C, anaphase-promoting complex; K-Fiber, kinetochore-fiber; MAP, microtubule-associated protein; MT, microtubule; NLS, nuclear localization signal; SAF, spindle assembly factor.

identified in *Drosophila* (2). In higher organisms, Aurora A is a member of the Aurora kinase family consisting of three serine–threonine kinases whose expression and kinase activity peak in M phase (**Figure 1**). Aurora kinases have essential roles during cell division and in particular in centrosome duplication and separation, spindle assembly, chromosome alignment, spindle assembly checkpoint, central spindle assembly, and cytokinesis (3–5).

The potential link between the Aurora kinases and tumor initiation and/or development has fueled the interest in understanding their function and regulation over the last years. Indeed, Aurora A gene is located in a region of chromosome 20 that is frequently overexpressed in human cancers (6, 7), and it is found in higher levels in many tumor types (8–10). Moreover, it shows oncogenic properties (3, 11, 12). Aurora A gene is also a candidate low penetrance cancer-susceptibility gene (13, 14). Aurora A is therefore considered as a potentially useful molecular therapeutic target, and several specific small molecule inhibitors are currently being tested in clinical trials (15–18).

Although the three Aurora kinases share a conserved catalytic domain, a few critical amino acid substitutions in their catalytic domains confer activator specificity. Moreover, divergent N- and C-terminal domains provide specificity at least in part through protein–protein interactions and distinct subcellular localizations during mitosis. While Aurora B and C localize to the kinetochores and the anaphase central spindle as part of the chromosomal passenger complex (19), Aurora A localizes to the centrosome throughout cell division and is often described as a centrosomal kinase (**Figure 1**) (20). However, Aurora A also localizes along the spindle MTs and performs essential functions unrelated to its centrosomal localization. Here, we will focus on the TPX2-dependent regulation and function of acentrosomal Aurora A during cell division.

AURORA A KINASE ACTIVATION

The activity of Aurora A is regulated by phosphorylation–dephosphorylation (21, 22). In particular, the autophosphorylation of Thr288 (in humans), a residue residing within the activation loop of the catalytic domain, has been described as critical for kinase activity (11). In addition, other kinases may phosphorylate Thr288, and *in vitro* assays showed that PKA phosphorylates Aurora A on at least three residues, including Thr288 (11, 22). Specific anti-Phosho-Thr288 antibodies have been useful to monitor when and where Aurora A is active in tissue culture cells, revealing that the kinase is activated at the centrosomes and the spindle microtubules proximal to the poles during prometaphase and metaphase (23). However, some controversy regarding Aurora A activation has recently emerged because phosphorylation on Thr288 alone was shown to be insufficient for the kinase to adopt a fully active conformation (24). On the other hand, there is evidence that activation may occur in the absence of Thr288 phosphorylation (see below).

Aurora A activation can also be triggered through allosteric interactions with a number of proteins such as Ajuba, Bora, protein phosphatase inhibitor-2, nucleophosmin, and PAK (25–29). A specific mechanism drives Aurora A activation in a RanGTP-dependent manner in dividing cells (21, 30, 31) (**Figure 2A**). This mechanism involves TPX2, a cell cycle regulated nuclear protein essential for chromosome and RanGTP-dependent MT nucleation (32, 33) and bipolar spindle assembly whether centrosomes are present or not (34–36) (**Figure 1**). TPX2 release from importins is triggered by RanGTP in the proximity of the chromosomes and enables its interaction with Aurora A thereby promoting its local activation in a centrosome-independent manner (34).

CONSEQUENCES OF TPX2 INTERACTION WITH AURORA A

The interaction between TPX2 and Aurora A has several important functional consequences including the targeting of Aurora A to the spindle microtubules (37) and the assembly of spindles of the correct length that faithfully segregate chromosomes (38). Mechanistically it drives the activation of Aurora A (21, 30) through a direct interaction between the catalytic domain of Aurora A and the first 43 residues of TPX2 in humans (39 residues in *Xenopus*) (30, 37). Despite the high degree of conservation

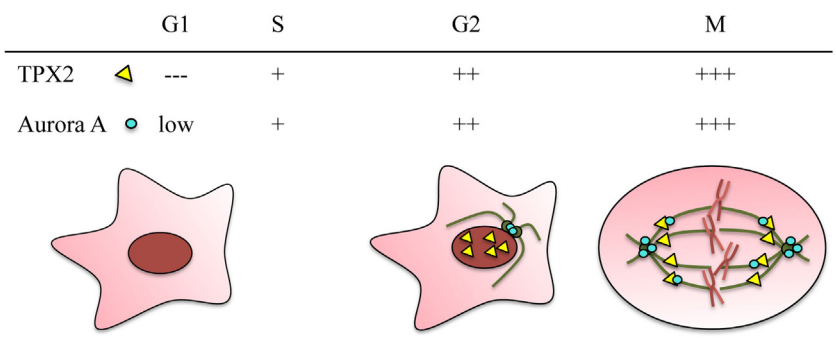

FIGURE 1 | TPX2 and Aurora A are cell cycle regulated proteins. Both proteins accumulate during G2/M and are degraded through the APC/C proteasome pathway at the end of mitosis. The relative protein levels of TPX2 (yellow triangle) and Aurora A (blue circle) in the different cell cycle phases are represented at the top. The localization of the two proteins during these cell cycle phases is represented in the drawings. In G2, TPX2 accumulates inside the nucleus whereas Aurora A accumulates at the centrosomes. During mitosis, both proteins co-localize along the spindle microtubules, and Aurora A also accumulates at the centrosomes.

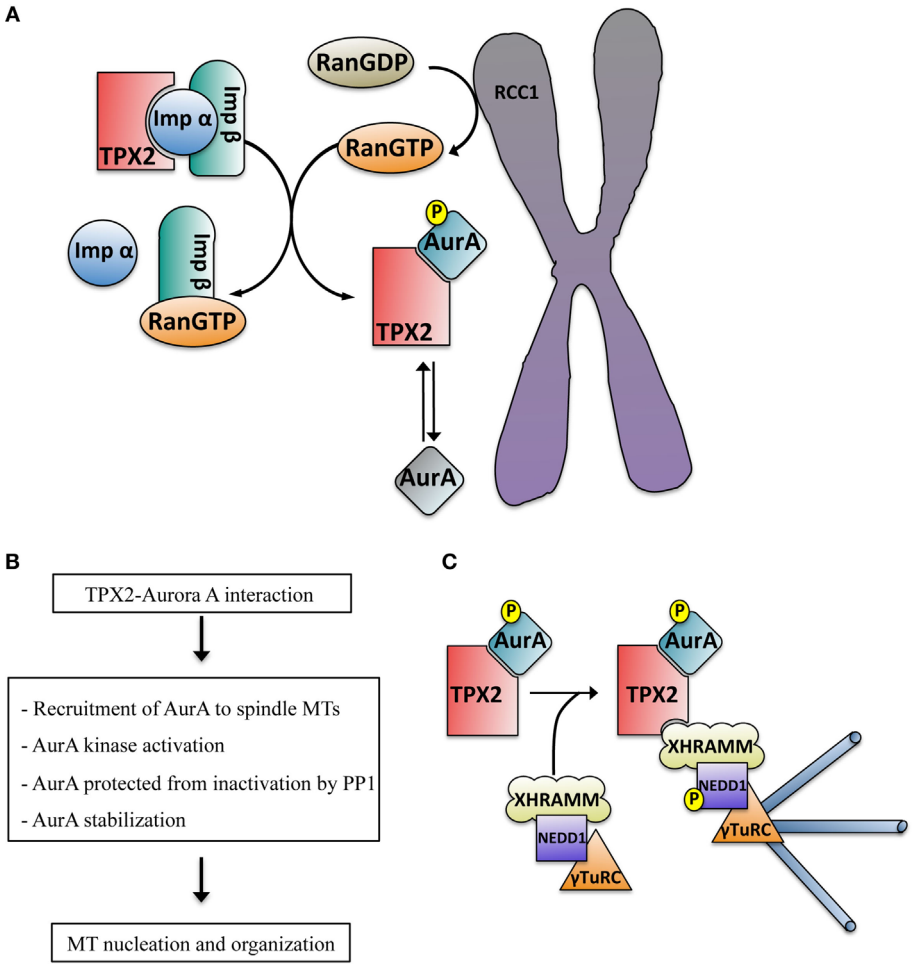

FIGURE 2 | **(A)** Schematic representation of the RanGTP-dependent interaction between TPX2 and Aurora A in the proximity of the chromosomes. The exchange factor RCC1 associated with the chromosomes generates a peak of RanGTP that releases TPX2 from the importin alpha and beta complex. TPX2 can then bind to Aurora A, promoting its autophosphorylation on Thr288 and kinase activation (blue color). The phosphatase PP1 can inactivate TPX2 free active Aurora A (mainly at the centrosome, gray color) through dephosphorylation but not the TPX2-activated Aurora A (blue color). **(B)** Direct consequences of the TPX2–Aurora A interaction. **(C)** Schematic representation of the mechanism driving acentrosomal RanGTP MT nucleation triggered by the complex TPX2–Aurora A. The TPX2–Aurora A complex associates with another specific complex containing XRHAMM-NEDD1–γ-TurC. In this macro complex the activated Aurora A phosphorylates NEDD1 at Ser405, an essential prerequisite for MT nucleation.

between the catalytic domains of Aurora A and B, the interaction between Aurora A and TPX2 is highly specific. Indeed, a single amino acid difference in the catalytic domain of Aurora B is sufficient to impair its interaction with TPX2 (39).

Structural studies showed that the binding of TPX2 to Aurora A promotes a conformational change in its catalytic domain involving the reorganization of the activation segment, providing a good binding platform for substrates (30). This also triggers Aurora A autophosphorylation at Thr288 in human cells (Thr295 in *Xenopus laevis*) (21) contributing to its activation (**Figure 2A**). Although it has been shown that TPX2 can fully activate Aurora A in the absence of Thr288 phosphorylation (40), other authors have proposed that Aurora A Thr288 phosphorylation and TPX2 binding act synergistically for the full kinase activation (41).

The conformational change induced by the binding of TPX2 to Aurora A results in the change in position of Thr288 that moves it into a buried position inaccessible to inactivating phosphatases (30). Therefore, TPX2 not only activates Aurora A but it "locks" the kinase into an active conformation that cannot be readily inactivated by PP1 like TPX2 free Aurora A (**Figure 2A**). Interestingly, the phosphatase PP6 was recently shown to specifically target the Aurora A–TPX2 complex triggering the dephosphorylation of the protected Thr288 thereby regulating Aurora A activity and consequently, spindle formation (42).

Finally, TPX2 protects Aurora A from degradation that occurs under normal conditions at the end of mitosis through the cdh1 activated APC/C proteasome pathway (43). Certainly, TPX2 depletion promotes a premature decrease of Aurora A levels in prometaphase (44) (**Figure 2B**).

Other functional implications of the TPX2–Aurora A interaction may derive from the phosphorylation of TPX2 itself. Indeed, TPX2 is a substrate of Aurora A. *Xenopus* Aurora A

phosphorylates TPX2 on three serine residues (Ser48, Ser90, and Ser94) (21, 45). In HeLa cells Aurora A phosphorylated TPX2 was shown to control mitotic spindle length (46). However, the specific function of the Aurora A-dependent phosphorylation of TPX2 is still not entirely clear. In addition, TPX2 phosphorylation by the essential mitotic kinase polo-like kinase 1 (Plk1) was reported to increase its ability to activate Aurora A (47) while ckd1/2-dependent TPX2 phosphorylation was shown to regulate TPX2 localization impacting spindle assembly via Aurora A and Eg5 (48).

FUNCTIONAL RELEVANCE OF TPX2-DEPENDENT AURORA A PHOSPHORYLATION DURING MITOSIS

TPX2 and Aurora A both perform essential functions during cell division although not all of them are dependent on their interaction. Aurora A null mouse embryos, similar to TPX2 ablation, are embryonic lethal failing to undergo the morula-blastocyst transition due to defects in mitosis (49–51).

The functional consequences of Aurora A activation by TPX2 in the dividing cell have to be examined in the context of the function and regulation of TPX2 during cell division. In *Xenopus* egg extract and in mammalian cells TPX2 is essential for acentrosomal MT assembly driven by the chromosome-dependent RanGTP pathway in M phase (33, 52). In turn, this pathway is essential for the assembly of a functional spindle that can drive faithful chromosome segregation to the daughter cells (35, 53–55).

Ran cycles between an inactive GDP-bound state and an active GTP-bound state, which is controlled by regulatory proteins. The Ran exchange factor RCC1 localizes to the mitotic chromosomes whereas other factors that promote RanGTPase activity (RanGAP1 and RanBP1) are cytosolic. This promotes the formation of a RanGTP gradient centered on the chromosomes that has been directly visualized in *Xenopus* egg extracts (56–58) and in mammalian cells (59, 60). In the dividing cell, RanGTP provides a spatial signal that triggers MT assembly in the proximity of the chromosomes and their organization into a bipolar spindle [reviewed in Ref. (61)]. In mammalian cells the system may however be more complex since it has been shown that components of the Ran system, including RanGTP, localize to the centrosome and play an important role in MT nucleation (62–64). One essential target of the RanGTP pathway away from the centrosome is the nuclear protein TPX2. Work performed in *Xenopus* egg extracts showed that RanGTP promotes the dissociation of TPX2 from inhibitory interactions with importin-α/β in the vicinity of chromosomes (52, 65). This release enables the interaction of TPX2 with Aurora A leading to its activation. Therefore, it is tempting to speculate that the RanGTP gradient translates into an Aurora A-dependent phosphorylation signaling network.

Some functional implications of the TPX2-dependent interaction with and activation of Aurora A have been recently uncovered through the characterization of the mechanism underlying RanGTP-dependent acentrosomal MT nucleation in *Xenopus* egg extract (32). In higher eukaryotes MT nucleation is driven by the γ-tubulin ring complex (γ-TuRC), a multi-subunit complex consisting of multiple copies of γ-tubulin and a number of associated proteins named as gamma-tubulin complex proteins (GCPs) (66, 67). Together with the adaptor protein NEDD1, γ-TuRC is required for all the MT nucleation pathways described in mitosis (68, 69). Another specific requirement for the RanGTP pathway is TPX2 (65). Recently, we showed that RanGTP promotes the association of TPX2 with a XRHAMM-NEDD1– γ-TuRC complex that includes Aurora A. We also showed that within this complex the TPX2-activated Aurora A phosphorylates NEDD1 on Ser405 an essential step for RanGTP-dependent MT nucleation (32, 70) (**Figure 2C**).

Another RanGTP-dependent protein complex containing TPX2 and Aurora A was previously identified in *Xenopus* egg extract (71) and shown to be required for RanGTP-dependent MT organization. This complex includes the tetramic plus-end directed motor Eg5, XMAP215 and the RanGTP target HURP. In *Xenopus* egg extract and in mammalian cells, TPX2 regulates Eg5 activity through a direct interaction (72, 73). Although Aurora A phosphorylates Eg5 (74) no function for this phosphorylation in spindle formation was identified in *Xenopus* egg extracts (75). On the other hand, HURP is necessary for K-fiber stabilization in mammalian cells (76–78) and its phosphorylation by Aurora A is required for MT binding (79). Altogether these data suggest that some proteins may be specific substrates of the TPX2-Aurora A complex. However, further work is needed to test this idea.

The dual role of TPX2 in activating and localizing Aurora A to the spindle microtubules through an allosteric interaction is not unique. For example, besides the classical activation of the MAPK p38α by MAPKK, p38α can be activated by TAB1 [transforming growth factor-β-activated protein kinase 1 (TAK1)-binding protein 1] as well (80). The binding of TAB1 to p38α promotes its autophosphorylation and consequently, its activation. Concerning the targeting role, a similar mechanism is at play for the A-kinase anchoring proteins (AKAPs). AKAPs bind directly PKA and recruit it to specific subcellular localizations where the kinase activity is required (81). AKAPs also function as scaffold proteins to facilitate the formation of multiprotein complexes. TPX2 may also provide a scaffolding activity. It may have a critical role for the recruitment of the MT nucleation complex and NEDD1 phosphorylation by Aurora A. Similarly, it may also act as a scaffold for the HURP containing complex whose formation and function depends on Aurora A activity, and consequently on TPX2 (71).

CONSERVATION OF THE TPX2–AURORA A MODULE?

Aurora kinases are found in a wide range of organisms from yeast to humans and they have conserved functions during cell division. TPX2 orthologs have also been identified in a variety of genera and different kingdoms. Interestingly, the *tpx2* knockout mice display severe developmental defects and embryonic lethality (82) and similar phenotypes were described for a *tpx2* knockout in *Arabidopsis thaliana* (83).

Aurora A was identified in *Drosophila*. However, it is only recently that Ssp1/Mei-38 was proposed to be a putative TPX2 ortholog (84). Although the effects of loss-of-function of this

protein are less severe than in the case of human TPX2, Ssp1/Mei-38 shows similar localization to spindle microtubules. Moreover, it also contains a sequence conserved in human TPX2 that confers the microtubule-binding and bundling activities. However, Ssp1/Mei-38 lacks an Aurora A binding domain suggesting that it does not fulfill the same role as the vertebrate TPX2 during cell division and therefore it may not be a true TPX2 ortholog.

Caenorhabditis elegans has two Aurora-like kinases. A putative ortholog of TPX2 was recently identified and named TPX2-like protein (TPXL-1) (85). Although TPXL-1 activates and localizes Aurora A to the mitotic spindle and not to the centrosome, it actually does not share other essential features and functions of TPX2 like its RanGTP regulation and its role in microtubule nucleation (86).

These data suggest that different evolutionary modules may exist to control the localization and activation of the Aurora kinases during cell division. Vertebrates seem to have developed a unique module to integrate the control of localization and activation of Aurora A by the chromosomal RanGTP-dependent pathway through a single interacting protein, TPX2.

CANCER AND THERAPEUTICS: TPX2 AND AURORA ARE OVEREXPRESSED IN DIFFERENT TUMORS

Aurora A and TPX2 are overexpressed in several types of tumors and have been implicated at different levels in cancer. Although the mechanism underlying the role of TPX2 and Aurora A in tumorigenesis may be at least in part independent, there are data to suggest a role for the complex. TPX2 was initially identified as a proliferation marker with a potential role in human cancer (87). It is indeed overexpressed in many tumor types (88). High levels of Aurora A were detected in many cancer types including prostate cancer, gastric carcinoma, breast carcinoma (89), ovarian cancer, laryngeal carcinoma, bladder cancer, and pancreatic carcinoma, among others (90). Moreover, both genes are part of the chromosomal instability signature that was found to predict clinical outcome for different cancers with TPX2 having the highest CIN score (91).

Interestingly, both TPX2 and Aurora A genes are located on chromosome 20q, whose amplification is found in tumors and moreover, co-expression of TPX2 and Aurora A has been observed in some tumors (92). For instance, Aurora A and TPX2 were found overexpressed in lung cancer cells (93), different colon cancers (94, 95) and neuroblastoma (96). Based on the correlation of co-expression it was in fact proposed that TPX2 and Aurora A might act as a functional unit (90). Interestingly, a mutant of Aurora A (S155R), that is unable to interact with TPX2, has been identified in colon cancer (97), suggesting that the misregulation of Aurora A localization and/or activity may also be deleterious for the cell. It is also interesting to note in this context that the tumor suppressor p53 is regulated by both TPX2 and Aurora A in *Xenopus* (98).

Some data suggest that the increased levels of Aurora A in various tumors may be the consequence of protein stabilization rather than gene amplification. Indeed, phosphorylation of Aurora A Ser51 inhibits its degradation via the cdh1 activated ubiquitin ligase APC/C at the end of mitosis and Aurora A constitutively phosphorylated at Ser51 was shown to be present in neck and head cancer tissues with Aurora A overexpression (99). Although no direct connection has been reported yet, it is interesting to note here that TPX2 protects Aurora A from degradation potentially contributing to the maintenance of high levels.

The clear implications of Aurora A in cancer have promoted the intensive search for small molecule inhibitors for their potential therapeutic use (100). Some of them already show interesting potential in clinical trials but a further optimization may be required. Targeting specifically the TPX2-activated Aurora A may open new strategies in cancer therapy.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Cross-Talk between AURKA and Plk1 in Mitotic Entry and Spindle Assembly

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The Aurora kinase A (AURKA) is involved in different aspects of mitotic control, from mitotic entry to cytokinesis. Consistent with its pleiotropic roles, several AURKA interactors are able to modulate its activity, the best characterized being the microtubule-binding protein TPX2, the centrosomal protein Cep192, and Bora. Bora has been described as an essential cofactor of AURKA for phosphorylation-mediated activation of the mitotic kinase polo-like kinase 1 (Plk1) at the G2/M transition. A complex AURKA/Plk1 signaling axis is emerging, with multiple involved actors; recent data suggest that this control network is not restricted to mitotic entry only, but operates throughout mitosis. Here, we integrate available data from the literature to depict the complex interplay between AURKA and Plk1 in G2 and mitosis and how it contributes to their mitotic functions. We will particularly focus on how the activity of specifically localized AURKA/Plk1 pools is modulated in time and space by their reciprocal regulation to ensure the timely and coordinated unfolding of downstream mitotic events.

Keywords: kinases, mitosis, G2/M transition, centrosomes, spindle

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INTRODUCTION

About 20 years ago, two loci encoding for serine–threonine kinases required for correct spindle pole assembly were described in *Drosophila* and named "polo" and "aurora" (1–3); these were the forefathers of the corresponding kinase families, now well characterized as key regulators of the cell cycle and mitotic division. Aurora and polo kinases are evolutionary highly conserved, from yeast to mammals (4, 5), and homologs of the originally identified *Drosophila* genes were described in humans as Aurora2 (now AURKA) and polo-like kinase 1 (Plk1), respectively (6–9). Besides the spindle pole phenotypes, several common features led to association of the two kinases, since their discovery. Both display cell cycle-regulated expression (6, 9), with upregulation of mRNAs in the late S and G2 phases ensured by shared transcriptional mechanisms, such as activation by E2F factors (10, 11) and G1-specific repression through CDE/CHR elements (12, 13). Protein levels peak at G2 and mitosis, paralleled by the activation of kinase enzymatic function (9, 14), and drop in a highly coordinated manner at mitotic exit by proteasome-dependent degradation (15). Both kinases localize at centrosomes and spindle poles, although they also display nonoverlapping localization sites, with AURKA associated to spindle pole microtubules, and Plk1 residing at kinetochores; both are also found at the spindle midzone and midbody at ana–telophase (16, 17). Functionally, both AURKA and Plk1 are involved in control of mitotic entry, with an essential role during recovery from DNA damage checkpoint-mediated G2 arrest, and in several aspects of mitotic progression

(18–21). Finally, ever since their discovery it has been evident that cancer cells frequently display altered levels of AURKA and Plk1 (7–9, 22) and that downregulating their expression yields antiproliferative effects (23–25); indeed, both kinases are actively studied as potential anticancer targets (26, 27). All these similarities suggested direct links between AURKA and Plk1, which started to come out only in the last 10 years. Here, we review data about the interplay of AURKA and Plk1, focusing on the emerging view of how this can contribute to AURKA activation at distinct subcellular sites and in different cell cycle windows, thus finely coordinating downstream mitotic events.

ACTIVATION MECHANISMS FOR AURKA AND Plk1

Phosphorylation of a threonine residue within the activation loop of AURKA and Plk1 kinases, Thr-288 and Thr-210, respectively, is crucial for their enzymatic activity (28, 29). Phosphorylation of Plk1$^{Thr\text{-}210}$ occurs upon release of an inhibitory intramolecular interaction between the N-terminal catalytic domain and the C-terminal "polo-box" domain (PBD). The latter is a phosphoserine/threonine recognition domain; its binding to target phosphopeptides, mainly generated by the cdk1 kinase, impairs the interaction with the catalytic domain, thus triggering Plk1 activation (30, 31). Plk1 activation mechanism, thus, relies on making the region where Thr-210 lies accessible; Thr-210 can then be phosphorylated by an upstream kinase (see the following sections).

Data collected so far indicate a more complex mechanism for AURKA activation. AURKA$^{Thr\text{-}288}$ lies within an AURKA consensus motif and is therefore regarded as an autophosphorylation site. It is still debated whether autophosphorylation is achieved by an intra- or intermolecular reaction, and conformational shifts as well as dimerization appear to underlie different activation states (32–34). Indeed, data in the literature indicate multiple binding partners (see the following sections) that are able to stimulate AURKA activity without a direct enzymatic action but rather by inducing specific conformational transitions. These observations suggest that cells need to manage distinct pools of AURKA, acting at distinct subcellular sites and displaying different extents of activity.

Interestingly, although activation mechanisms for AURKA and Plk1 are distinct, coupling intracellular localization with function appears to be a conserved feature: for Plk1, the PBD is also required for correct targeting of the kinase to centrosomes, kinetochores, and spindle midzone (35, 36), and the major AURKA activators, namely Cep192 and TPX2, mediate AURKA binding to centrosomes and microtubules, respectively (37–39).

THE AURKA/Plk1/BORA AXIS AND MITOTIC ENTRY

The direct link between AURKA and Plk1 came with the identification of AURKA as the upstream kinase responsible of phosphorylation of Thr-210 in the Plk1 activation loop, an event requiring the presence of the coactivating protein Bora (19, 40) (**Figure 1**, upper box). Distinctly from other AURKA activators, Bora does not modify AURKA activity *per se* but rather interferes with the intramolecular interaction between the catalytic domain of Plk1 and the PBD, so to render Thr-210 accessible (40). Consistently, Bora does not significantly increase AURKA activity toward substrates other than Plk1 (19, 40), and the extent of activation of AURKA coimmunoprecipitated with Bora, as assessed by p-Thr-288, is by far lower than that associated with the fractions immunoprecipitated with TPX2 or Cep192 (41, 42). Although low, this activity may suffice to trigger what was defined as the "outer feedback loop" through which AURKA, Plk1, and cdk1 activate each other (43). Phosphorylation of Bora at Ser-252 (human) by cdk1 creates a PBD-docking site and promotes Bora/Plk1 interaction (**Figure 1**); consistently, phosphorylation of Bora by cdk1 enhances its ability to stimulate AURKA-mediated Plk1 activation (41, 44). A second residue on human Bora, i.e., Thr-52, is responsive to cdk1: GST-tagged human Bora carrying Thr-52 substitution to alanine is destabilized in CSF-arrested *Xenopus* oocytes extracts (45), thus suggesting that cdk1 phosphorylation plays also a role in protecting Bora from degradation. An opposite effect is mediated by Plk1 in that Plk1 phosphorylation of Bora in the 496-DSGYNT-501 degron triggers Bora degradation through the SCF-β-TrCP pathway (41, 46) about 2 h before mitotic entry (45, 47). Consistently, a decreased interaction between Plk1 and Bora, by mutating the previously mentioned Bora$^{Ser\text{-}252}$ to Alanine, influences Bora stability: (i) it prevents GST-Bora degradation in CSF extracts (45) and (ii) in human cells, it impairs the interaction between Bora and β-TrCP (41). In addition, it prevents Bora accumulation induced – as a result of a dominant-negative effect – by kinase-dead Plk1 (41). The opposite effects of cdk1-mediated phosphorylation of Bora on Thr-52 and Ser-252 suggest that timely degradation of Bora constitutes a strictly controlled event; the balance between phosphorylation of Thr-52 by cdk1 and on the degron sequence by Plk1 may determine when the switch toward SCF-β-TrCP-mediated degradation of Bora occurs (**Figure 1**).

The Spatiotemporal Level of Bora/AURKA/Plk1 Regulation

The bulk of cycB1/cdk1 complexes is cytoplasmic until prophase, when it promotes its own translocation to the nucleus (48, 49). On the other hand, although Thr-210-phosphorylated Plk1 is first detected at centrosomes, results obtained using a FRET biosensor suggest that Plk1 kinase activity first increases in the nucleus and raises in the cytoplasm only 2 h before mitotic entry (19, 45, 47), at a time that coincides with the onset of Bora degradation (45, 47). Together with the recent observation that Bora is prevalently cytoplasmic in mammalian cells (47), these data suggest that cdk1 and Plk1 activities antagonistically modulate Bora levels, with cdk1-mediated Thr-52 phosphorylation protecting Bora from degradation until cytoplasmic Plk1 activity raises. A potential player in this regulatory mechanism is the peptydylprolyl isomerase Pin1, a modulator of the G2/M transition, which promotes Bora degradation (50) and whose activity and stability are controlled by AURKA and Plk1, respectively (50, 51); further studies are needed to understand how these molecular events interplay in regulating mitotic entry. Phosphatases acting both

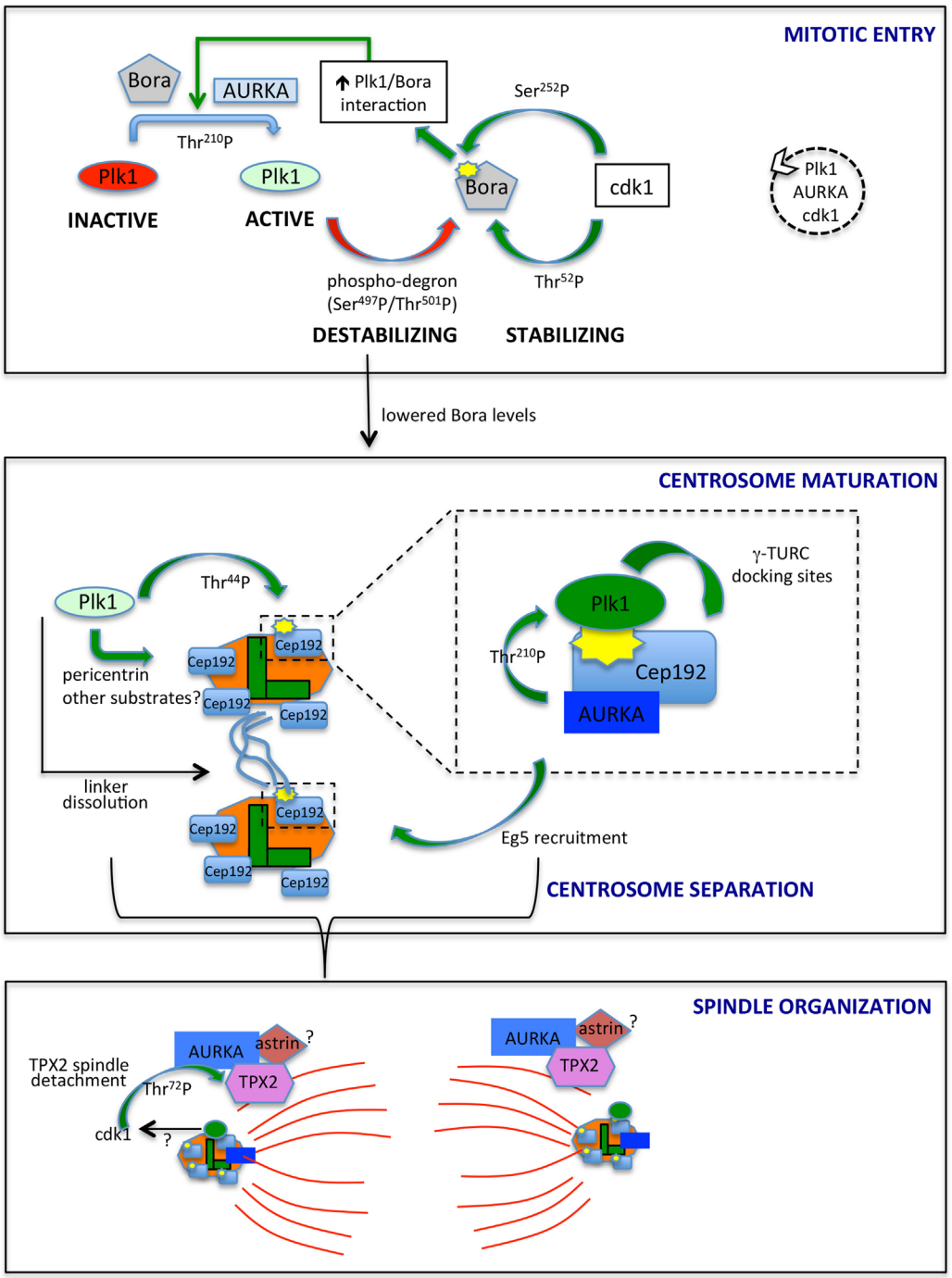

FIGURE 1 | AURKA and Plk1 in mitotic entry and spindle formation. The best characterized links between AURKA and Plk1 are schematized. In mitotic entry (upper box), the combined action of AURKA and Bora activates Plk1, while antagonistic phosphorylation events by Plk1 and cdk1 control Bora stability. The dashed circle on the right indicates the ongoing feedback loop leading to the activation of Plk1, AURKA, and cdk1. Lowered Bora levels enable the interaction of AURKA with Cep192 (central box) and TPX2 (lower box), at centrosomes (centrioles, green; PCM, orange) and microtubules (red), respectively. The enlargement in the central box depicts the scaffolding function of Cep192, leading to recruitment of AURKA and Plk1, activation of the latter and generation by activated Plk1 of γ-TURC-docking sites, with consequent centrosome maturation. Note that Cep192-bound AURKA is activated in a dimeric form, although not represented here to simplify the scheme. Cep192/Plk1/AURKA also contributes to centrosome separation via Eg5 recruitment, and Plk1 independently participates to this process by triggering centrosome linker (light blue lines) dissolution. Separated centrosomes nucleate spindle microtubules that are organized, among others, by AURKA/TPX2 complexes, possibly bound to astrin (lower box). cdk1 phosphorylation of TPX2, possibly influenced by Plk1 activity, yields decreased binding to microtubules. Centrosomal proteins in the lower panel are schematized as in the upper ones, although for space reasons their names are not indicated. The yellow symbols identify PBD-docking sites. Green arrows indicate positive regulatory events, while red arrows represent negative ones. Phosphorylated residues or domains are indicated on the arrows. The different intensities of colors for Plk1 and AURKA denote a different extent of activity.

on kinases themselves and on their substrates, with time- and space-dependent selectivities (52), are also expected to play a role in this fine-tuned regulation. The key serine–threonine phosphatases that counteract mitotic kinase activity are PP1 subunits and PP2A complexes (53, 54). Potentially relevant to Bora degradation, PP2A activity, which is able to counteract Plk1 and cdk1 substrate phosphorylation, is inhibited in the cytoplasm by the Mastl/Greatwall kinase before mitotic entry (52). Translocation of nuclear Greatwall to the cytoplasm is promoted by both cdk1 and Plk1 (55, 56): this mechanism may ensure that phosphorylation of Plk1 cytoplasmic substrates, such as Bora, only accumulates subsequent to Plk1 activation in the nucleus and to cdk1 nuclear import. Whether a differential specificity of action of phosphatases on the different Bora residues phosphorylated by cdk1 and Plk1 exists is an open question that may provide further hints on the time-dependent regulation of Bora stability. As also recently proposed by Bruinsma and colleagues (47), differentially localized phosphatase activity may generally contribute to time-dependent compartmentalization of Plk1 activity, thus explaining why the latter is first observed in the nucleus, although Bora is reported to be strictly cytoplasmic and the extent and timing of AURKA nuclear entry is poorly characterized. We also noticed that the NLS sequences described for Plk1 fall within the catalytic and polo-box domains (57, 58) (**Figure 2**), raising the possibility that formation of import complexes in the cytoplasm impairs Plk1 kinase function, which would be only released in the nucleus. Modulated interaction between Plk1 and importins may therefore contribute to the switch to cytoplasmic Plk1 activity 2 h before mitotic entry: indeed Ser-137 within one of the NLS sequences (**Figure 2**) is phosphorylated *in vivo* and this is described as an activating event for Plk1, although so far described only in late mitosis (29, 59). Alternatively, over time, increased cdk1-generated PBD-docking sites on Plk1 cytoplasmic substrates could retain Plk1 in the cytoplasm by competing with importins for Plk1 binding.

Changing Interactors for Progressing Through Mitosis

What is the functional significance of Bora degradation before mitotic entry by the same protein (Plk1) that it activates? A possible explanation is that the cdk1/AURKA/Plk1 signaling cascade generating the mitotic entry signal (43) must timely switch toward other pathways to sustain spindle assembly and mitotic progression. Evidence summarized below supports the notion that lowering Bora levels is necessary to make AURKA available to other partners. Immunoprecipitation experiments indicate that AURKA complexes containing Bora or TPX2 are distinct and that artificially increasing Bora levels – through Plk1 inactivation – changes the stoichiometry and decreases the amount of TPX2 bound to AURKA (41). In addition, AURKA localization to spindle poles, mediated by Cep192 and TPX2 (see below), is altered when Bora levels are increased by overexpression or by Plk1 inactivation (41). This is likely accounting for the proposed role of Plk1 in AURKA centrosomal localization (38, 60) and further indicates that the Bora/Plk1 complex is able to compete with other AURKA activating/localizing partners.

Together, these observations suggest that AURKA activity initially needs to be focused toward the Plk1 kinase; this activates the AURKA–Plk1–cdk1 loop, until a threshold is reached and the cell is committed toward mitosis (43, 61). Now AURKA and Plk1 kinases must be properly redirected toward their mitotic activators and substrates to coordinate mitotic entry with centrosomal and spindle processes (**Figure 1**). How does Plk1 remain active in mitosis when Bora is degraded? On the one hand, the accessibility of Thr-210 may not represent a limiting factor in mitosis, when high cdk1 activity creates abundant PBD-docking sites. On the other hand, recent data indicate that although Bora levels are strongly reduced in mitosis, a residual fraction exists (45), and it is responsible of Plk1 activation throughout the division process (62). An independent protein, Furry, has been described to activate Plk1 through AURKA, with a mechanism comparable to Bora (63). It will be interesting to investigate whether this redundancy underlies subcellular, temporal, or cell-type specificity. Most importantly, Cep192 emerging scaffolding functions may bypass the requirement for Bora in the AURKA/Plk1 axis at centrosomes.

THE AURKA/Plk1/Cep192 AXIS CONTROLS CENTROSOME MATURATION AND SEPARATION

The drop in Bora levels following Plk1 activation may ensure that centrosomal processes leading to spindle assembly, depending on other AURKA containing complexes, start only when the mitotic entry signaling cascade is fully active. The centrosomal protein Cep192, involved in both centrosome maturation and separation (39, 64), appears as a key coordinator of AURKA and Plk1 activity at this stage. Cep192 was first shown to trigger dimerization-driven AURKA activation at centrosomes in *Xenopus* egg extracts (65) and was later confirmed as a key AURKA centrosomal activator in mammalian cells (42, 66). Cep192-bound AURKA is highly active compared to the Bora- or TPX2-bound pools (42, 65). In human cells, the interaction between AURKA and Cep192 is reported from S phase (42); the strong increase in centrosomal Cep192 at mitotic entry, just before centrosome separation (39, 64), suggests that more Cep192–AURKA centrosomal complexes exist at this stage, in agreement with the proposed requirement of freeing AURKA from Bora-containing complexes. Importantly, Plk1 has recently been shown to be a part of the AURKA/Cep192 axis driving centrosome maturation (**Figure 1**, central box): Cep192 acts as a scaffold for both Plk1 and AURKA and is the key recruiting factor for the kinases at centrosomes, with Plk1 binding following that of AURKA (39, 42, 66). Cep192 brings AURKA and Plk1 in close proximity thereby enabling $Plk1^{Thr\text{-}210}$ phosphorylation (42, 66). AURKA-activated Plk1 creates its own PBD-docking site on Cep192 by phosphorylating $Cep192^{Thr\text{-}44}$ (42, 66); a subsequent AURKA-independent PBD-docking site centered on $Cep192^{Ser\text{-}995}$ has been reported (42), although the separation of the functional roles of Thr-44 and Ser-995 needs further investigation.

It could be speculated that preceding activation by Bora/AURKA generates the low Plk1 activity required for initial phosphorylation of Thr-44, while ensuing stabilization of Cep192/Plk1/AURKA complexes (42), where AURKA activity is higher,

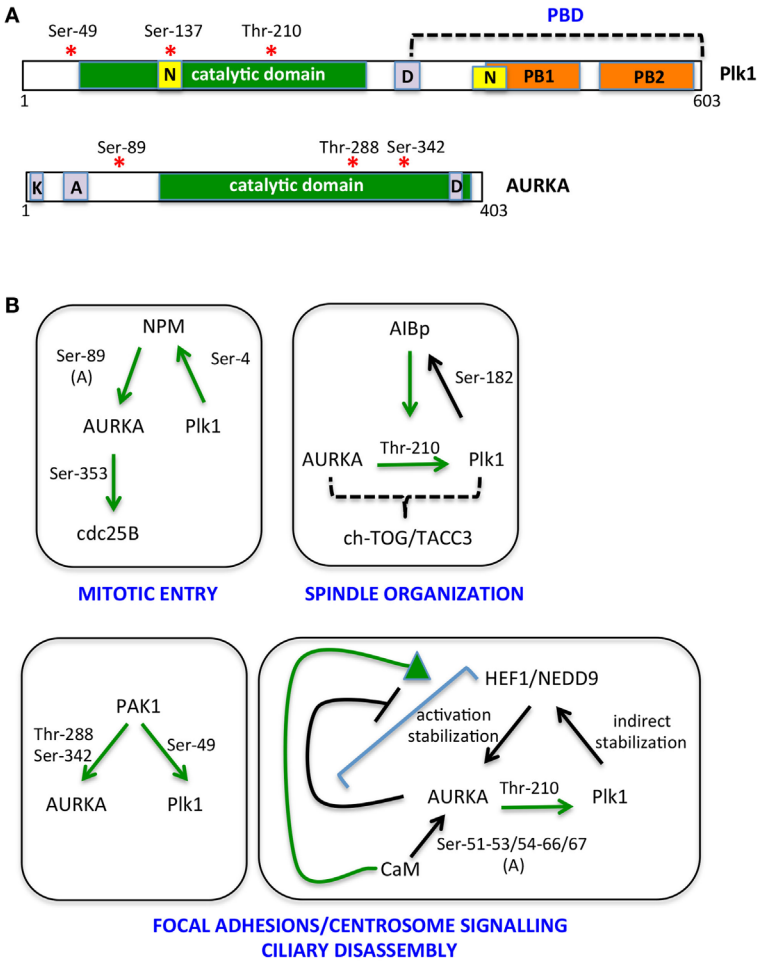

FIGURE 2 | The multiple AURKA and Plk1 activating networks. **(A)** A schematic representation of Plk1 and AURKA kinases is shown with activating phosphorylation sites indicated by red asterisks. Yellow boxes represent nuclear localization sequences (N); orange ones are polo boxes (PB1 and 2); and violet ones are degradation motifs (D, destruction box; K, KEN box; A, A box). Catalytic domains are in green. **(B)** Networks involving NPM, AIBp, PAK1, HEF1/NEDD9, and calmodulin (CaM) as activators and/or substrates of AURKA and Plk1 are represented. Green arrows indicate direct and clarified activating events; phosphorylated residues are indicated; and **(A)** denotes induction of an autophosphorylation event. Downstream substrates and proposed regulated processes are included.

boosts the signaling cascade leading to centrosome maturation. Plk1 is required for Cep192 centrosomal localization, partly through phosphorylation of pericentrin (67, 68), supporting the hypothesis that an initial Cep192-independent Plk1 activation triggers a subsequent and more sustained Cep192-mediated one. Cep192/AURKA-activated Plk1 in turn phosphorylates Cep192 to generate γ-TURC-docking sites and induce the sudden increase in pericentriolar material (PCM) characterizing centrosome maturation (42, 66) (**Figure 1**, central box).

Centrosome separation requires linker dissolution and Eg5-mediated centrosome movement, both involving Plk1 (69–71). While linker dissolution does not require Cep192, the observation that loss of Cep192 impairs Eg5 centrosomal localization and centrosome separation (39, 66) suggests that the role of Plk1 in centrosome movement passes through the Cep192/AURKA axis, with a key upstream involvement of centrosomal cyclin B2/cdk1 (72) (**Figure 1**, central box).

Cep192 complexes identify an AURKA pool clearly distinct from the microtubule- and TPX2-bound one: (i) the AURKA/Cep192 interaction occurs also in the absence of microtubules (42); (ii) Cep192 and TPX2 bind to the same region of AURKA and are detected in independent AURKA complexes (65); (iii) Cep192-loaded beads recapitulate in CSF-arrested *Xenopus* oocytes extracts the functions as microtubule-organizing center (MTOC) of AURKA-loaded beads but not their ability of RanGTP-induced spindle organization (66, 73). TPX2 is a RanGTP-regulated factor (74); these observations together suggest that the pools of AURKA bound to Cep192 and TPX2 are functionally separated and involved in centrosome maturation and spindle assembly, respectively. The observation that both Cep192/AURKA and TPX2 regulate Eg5 activity (66, 75) may reflect independent functions in centrosome separation or an interplay of the two pools of AURKA in this process yet to be unveiled.

MICROTUBULE-ASSOCIATED AURKA POOLS AND SPINDLE ORGANIZATION

Microtubule-organizing functions of AURKA are less obviously linked to Plk1 activity. AURKA localization to microtubules is mediated by the microtubule-binding protein TPX2 (37, 38), which also activates AURKA by stabilizing the active conformation and making AURKA$^{Thr\text{-}288}$ inaccessible to the PP1 phosphatase (76). In addition, TPX2 protects AURKA from APC/C^{Cdh1} proteasome-dependent degradation in G2 and early mitosis, with TPX2 depletion impairing accumulation of high levels of AURKA in prometaphase (77). *Xenopus* Plx1 has been shown to phosphorylate TPX2 on Ser-204, with a positive effect on TPX2-mediated AURKA activation (78). A corresponding mechanism has not been explored in mammalian cells given the poor conservation of the phosphorylated site. Yet, phosphoproteomic screenings identified TPX2 *in vivo* phosphosites that are likely to be phosphorylated by Plk1 (79, 80). Furthermore, TPX2 abnormally accumulates at spindle poles in Plk1-interfered mitoses (38), and recent data show that cdk1-mediated phosphorylation of TPX2$^{Thr\text{-}72}$ negatively modulates TPX2 association to the mitotic spindle (81). It is, therefore, conceivable that Plk1 activity at mitotic centrosomes, through its effects on cdk1, influences TPX2 mobility at spindle poles (**Figure 1**, lower box).

Astrin is an independent regulator of AURKA localization at microtubules, with no effect on the kinase activity (82); RNA interference-mediated depletion of astrin induces spindle defects reminiscent of those observed following AURKA inactivation (82, 83). Astrin localization to the spindle is in turn mediated by TPX2 (82). Interestingly, Plk1 has also been detected in astrin–kinastrin complexes in mitotic cell extracts (84).

Together these observations suggest that exploring the interplay between AURKA, TPX2, Plk1, and astrin deserves further investigation and may improve our understanding of AURKA spindle-organizing functions.

THE GROWING NETWORK OF AURKA ACTIVATORS

Additional activators of AURKA at centrosomes and spindle poles have been described, many being also functionally linked to Plk1 (**Figure 2**).

Nucleophosmin (NPM) activates AURKA by stimulating a newly identified autophosphorylation event, on Ser-89 (85). Phosphorylation of NPM by Plk1 is required for its mitotic functions (86), while NPM depletion does not affect Plk1$^{Thr\text{-}210}$ phosphorylation (85). These observations suggest that NPM-activated AURKA is generated when Plk1 activation has become prevalently AURKA independent; alternatively, since the only AURKA substrate affected by NPM depletion is so far CDC25B, NPM may provide AURKA specificity of action toward a limited set of substrates.

The AIBp protein, colocalizing with AURKA at centrosomes and spindle poles, has recently been reported as an AURKA regulator, relevant for Plk1 activation and in turn a substrate of it; the observation that localization of the downstream AURKA targets TACC3 and ch-TOG is affected by AIBp depletion, while PCM recruitment is not, together with the associated spindle pole phenotypes, suggest an involvement of AIBp in the spindle-organizing functions of AURKA (87).

AURKA activators also include proteins that localize both at focal adhesions and centrosomes, in particular the PAK1 kinase and the HEF1/NEDD9 scaffolding protein (88, 89). PAK1 promotes AURKA activation by directly phosphorylating Thr-288 and Ser-342 (89) and also phosphorylates Plk1$^{Ser\text{-}49}$, an event that contributes to its activation (90). HEF1/NEDD9 promotes the catalytic activity of AURKA (88) and also stabilizes it (91); the interaction between AURKA and HEF1/NEDD9 is favored by CaM (92), while it is inhibited by AURKA phosphorylation of HEF1/NEDD9 (88), indicating the presence of a negative feedback loop. Plk1 in turn indirectly regulates HEF1/NEDD9 stability, with deriving increased AURKA activity signaling back on Plk1 activation (93). The focal adhesion localization of PAK1 and HEF1/NEDD9 suggests that they define a pool of AURKA responsible of a signaling path that links loss of cell adhesion – typical of the cell division process – with mitotic centrosomal events and mitotic entry (94, 95). This pool appears also to be involved in the non-mitotic role of AURKA in cilia disassembly at cell cycle reentry from G0 (92, 96), a process that also requires Plk1 activity (93). An additional interactor of AURKA involved in both cell–cell adhesion and cell proliferation and survival (97) is Ajuba. The interaction between AURKA and Ajuba was first described in human cells (98), where it was shown as a key AURKA-activating step at G2 centrosomes (98). Recent data suggest that the activation mechanism relies on the ability of Ajuba, upon binding to AURKA N-terminus, to prevent an inhibitory intramolecular interaction between the N- and C-termini of the kinase (99); in addition, the subsequent binding of a distinct Ajuba domain to the C-terminus of AURKA directly stimulates kinase activity (99). A role of Ajuba in AURKA regulation has been confirmed in *Drosophila* neuroblasts, although data indicate an effect on localization, rather than activation, of the kinase (100). Organism and/or cell-type specificity may account for the observed differences, although cell cycle- (G2 vs. mitosis) or reporter- (phospho-AURKA/phospho-H3 vs. phospho-TACC3) dependent effects may also be envisaged.

CONCLUSION

Several AURKA activators have been described at centrosomes and microtubules, and evidence exist that they create independent complexes with the kinase. The scaffolding functions of some of them and the finding of specific phospho-AURKA fractions depending on the bound activator suggest that distinct interactors define specific AURKA pools with differential kinase activity and/or substrate specificity. More interconnected analyses of the different AURKA pools and a better spatiotemporal resolution of their formation during the cell cycle are expected to uncover in the next years how they ensure tight coordination of downstream events. Plk1 is a key substrate of AURKA and at the same time a major regulator of the multiple AURKA activators: besides contributing to generate an activation feedback loop that reinforces AURKA and Plk1 activities at mitotic entry, this is also emerging as a mechanism to impart time-dependent regulation

to the unfolding of AURKA-regulated events. Exploring the contribution of the AURKA/Plk1 axis in mitotic control, including in newly identified mitotic functions of AURKA (101–104), is a promising field of investigation for the future.

AUTHOR CONTRIBUTIONS

IAA critically analyzed the literature, contributed to article writing, and prepared figures. FDM critically analyzed the literature and discussed extensively review structure, contents, and models. GG conceived the structure of the review, critically analyzed the literature, and wrote the article.

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Spatial separation of Plk1 phosphorylation and activity

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Polo-like kinase 1 (Plk1) is one of the major kinases controlling mitosis and cell division. Plk1 is first recruited to the centrosome in S phase, then appears on the kinetochores in late G2, and at the end of mitosis, it translocates to the central spindle. Activation of Plk1 requires phosphorylation of T210 by Aurora A, an event that critically depends on the co-factor Bora. However, conflicting reports exist as to where Plk1 is first activated. Phosphorylation of T210 is first observed at the centrosomes, but kinase activity seems to be restricted to the nucleus in the earlier phases of G2. Here, we demonstrate that Plk1 activity manifests itself first in the nucleus using a nuclear FRET-based biosensor for Plk1 activity. However, we find that Bora is restricted to the cytoplasm and that Plk1 is phosphorylated on T210 at the centrosomes. Our data demonstrate that while Plk1 activation occurs on centrosomes, downstream target phosphorylation by Plk1 first occurs in the nucleus. We discuss several explanations for this surprising separation of activation and function.

Keywords: plk1, aurora kinase, cell cycle, checkpoint recovery, bora

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Introduction

Polo-like kinase 1 (Plk1) is an important kinase during the cell cycle. It controls several key processes that drive cells into and through mitosis such as centrosome maturation, spindle assembly, sister chromatid cohesion, cytokinesis, and recovery from a DNA damage-induced arrest (1). To carry out these specific functions, Plk1 is recruited to very specific subcellular sites throughout the cell cycle. Plk1 is predominantly localized at the centrosomes during S-phase, G2, and mitosis. In addition, Plk1 localizes to the kinetochores during G2 and mitosis and at the end of mitosis when the chromosomes segregate Plk1 translocates to the spindle midzone (2). This localization of Plk1 depends on its Polo-box domain that can efficiently bind to pre-phosphorylated substrates (3, 4) and can target Plk1 to its various subcellular localizations in the cell (5–9). Plk1 is first activated in G2, by phosphorylation on its T210-residue (10). The Aurora A kinase is responsible for this phosphorylation event and requires binding of the co-factor Bora in order to be able to phosphorylate Plk1 (11, 12). The notion that Plk1 is activated in G2 is well established; however, the exact location in the cell where Aurora A phosphorylates Plk1 is not clear. Both proteins localize to centrosomes and T210-phosphorylated Plk1 has also been observed at centrosomes (1, 12, 13). However, antibodies targeting T210-phosphorylated Plk1 have been shown to recognize off-target epitopes, making analysis based on these signals ambiguous (14). In addition, measuring Plk1 activity in living cells using a FRET-based biosensor specifically regulated by Plk1 showed that

substrate phosphorylation by Plk1 activity is first observed in the nucleus at around 5 h before mitosis, when S-phase is completed, with activity spreading to the cytoplasm approximately 2 h before mitosis (12, 15). A straightforward interpretation of this observation is complicated by the fact that the FRET-based biosensor is diffusible and active transportation of the FRET-based biosensor could contribute to the observed effects. Moreover, the localization of the co-factor Bora is not extensively studied. In *Drosophila*, where Bora was first identified as a co-factor for Aurora A, it was shown that Bora is located in the nucleus and translocates to the cytoplasm in early prophase (16). However, localization of Bora during the cell cycle in human cells seems to be regulated differently, although this is mainly based on exogenous Bora (17).

Here we show, using a nuclear localized FRET-based biosensor, that initial substrate phosphorylation by Plk1 in the nucleus is not the result of a diffusing FRET-probe, but that substrate phosphorylation by Plk1 initially occurs in the nucleus. However, we find that sequestration of Plk1 in the nucleus prevents phosphorylation of T210. This is in contrast to sequestration of Plk1 to the centrosomes, where Plk1 can get phosphorylated on T210. Thus, our data show that Plk1 is phosphorylated and activated at the centrosome, but Plk1 activity is first seen to rise in the nucleus. We were unable to reconstitute Plk1 function by mutants of Plk1 that strictly localized at the centrosomes or in the nucleus, indicating that Plk1 needs to be able to diffuse from the centrosomes to the nucleus in order to be fully functional. Finally, we show that Bora localizes strictly in the cytoplasm in human cells and Bora degradation is induced approximately 2 h before cells enter mitosis. This degradation does not completely remove all Bora, as we have shown previously, and Bora/Aurora A continue to activate Plk1 also in mitosis (14, 17). Taken together, our data show that Plk1 is activated in the cytoplasm where both Bora and Aurora A are localized; however, translocation of Plk1 to the nucleus seems to be required for the establishment of target phosphorylation, as it is where Plk1 activity first appears.

Materials and Methods

Cell Culture, Antibodies, and Reagents

Human osteosarcoma U2OS cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 6% FCS (Lonza), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cell lines expressing LAP-Plk1, AKAP-LAP-Plk1, H2B-LAP-Plk1, and GFP-Bora under the control of tetracycline-inducible were cultured in DMEM containing Tet system approved fetal bovine serum (Lonza). Antibodies that were used were directed against Plk1 (18, 19), Plk1, Cyclin B1, Actin (all from Santa Cruz), GFP (Roche), Plk1-pT210 (BD), Tubulin (Sigma), Bora (17), Aurora A (Cell Signaling) Histone H3-pS10, and H2AX (both from Upstate). The following drugs were used: BI 2536 (100 nM, Boehringer Ingelheim Pharma), MLN8054 (1 μM, Millennium Pharmaceuticals), thymidine (2.5 mM, Sigma), caffeine (5 mM, Sigma), adriamycin (0.5 μM, Sigma), nocodazole (250 ng/ml, Sigma), PI (Sigma) puromycin (Sigma, 2 μg/ml), and tetracyclin (Sigma, 1 μg/ml).

Cloning and Generation of Stable Cell Lines

H2B-tagged versions of LAP-Plk1 and the FRET-based biosensor were generated in the following manner: H2B was amplified by PCR using the forward primer 5′-AAGCTTATGCCAGAGCCAGCGAAGTC-3′ and the reverse primer 5′-AAGCTTAGATCCTTAGCGCTGGTGTACTTGG-3′ and ligated into either the FRET-based biosensor or the LAP-Plk1 construct using the restriction enzyme *Hin*dIII (NEB). AKAP-LAP-Plk1 was generated in the following manner: the AKAP centrosomal binding doamina was amplified by PCR using the forward primer 5′-AAGCTTGCCACCATGGCCAACATTGAAGCC-3′ and the reverse primer 5′-CTTAAGCTTCTCATGCCAGCATGAAATTG-3′ and ligated into the LAP-Plk1 consruct using the restriction enzymes *Hin*dIII and *Eco*RI (NEB). The pTON-GFP-Bora construct has been described previously (12). U2OS-derived U2TR cells stably expressing LAP-Plk1 have been described previously (12). U2TR cells stably expressing AKAP-LAP-Plk1, H2B-LAP-Plk1, and GFP-Bora were generated by calcium phosphate transfection of the constructs, selection of stable clones by zeocin (400 mg/ml, Invitrogen) treatment for 2 weeks followed by clonal selection. Stable clones were grown in media containing tetracycline system approved fetal bovine serum (Lonza). To induce expression, cells were treated for indicated times with tetracycline (1 mg/ml).

Transfections, Cell Synchronization, and FACS

Cells were transfected using calcium phosphate transfection of plasmids. For selection of transfected cells with pSuper constructs, GFP-spectrin was co-transfected for FACS or with pBABE-puro followed by puromycin treatment for western blot analysis. For analysis of checkpoint recovery, cells were synchronized at the G1/S-border by thymidine (2.5 mM) for 24 h followed by a 6 h release and 1 h incubation with Adriamycin (0.5 μM). Afterwards, cells were kept for 16 h in nocodazole (250 ng/ml). Recovery was induced by adding caffeine (5 mM). Unperturbed mitotic entry was assayed by a 24 h thymidine block followed by a release into nocodazole. For reconstitution assays, expression was induced by addition of tetracycline (1 mg/ml) at the indicated times. For FACS analysis, cells were transfected were harvested by trypsinization and fixed with ice-cold 70% ethanol. Cells were stained using the anti-Histone H3-pS10 antibodies (Millipore) and Alexa488-conjugated secondary antibodies (Molecular Probes). DNA was stained using propidium iodide and samples were analyzed on a FACSCalibur flow cytometer (BD biosciences). Cell cycle distribution was determined by flow cytometry counting 10^4 events of cells positive for GFP-spectrin.

FRET- and Live Cell Imaging

For time-lapse microscopy, cells were grown on LabTek II chambered coverglasses in Leibovitz's L-15 medium (Gibco) supplemented with 6% FCS (Lonza), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin, and were imaged with DIC on a Zeiss Axiovert 200M using 20 × 0.75NA objectives or on a Deltavision imaging system using 20 × 0.75NA objectives. Images were taken every 20 min. GFP-Bora levels were quantified by measuring the integrated density of the GFP signal in cells. Background was subtracted using an area that contained no cells.

The FRET-based biosensor for monitoring PLK1 activity has been described previously (12, 20). The CFP/YFP emission ratio after CFP excitation of U2OS cells stably expressing the FRET-based biosensor was monitored on a Deltavision Elite imaging system, using a 20 × 0.75NA objective. Images were acquired every 20 min. The images were processed with ImageJ using the Ratio Plus plug-in (http://rsb.info.nih.gov/ij/).

Cellular Fractionation Immunoprecipitations and Western Blotting

Chromatin fractionation was performed as described (21). Soluble cytosolic proteins were extracted from U2OS cells by incubating cells in buffer A (10 mm HEPES, pH 7.9, 10 mm KCl, 1.5 mm MgCl2, 0.34M sucrose, 10% glycerol, 1 mm DTT, 0.1% Triton X-100, and protease inhibitor cocktail) at 4°C for 10 min and spinning down at 1500 × *g* for 2 min. Soluble nuclear fraction was obtained by extraction of pelleted nuclei with an equal amount of buffer B (10 mm HEPES, pH 7.9, 3 mm EDTA, 0.2 mm EGTA, 1 mm DTT) and spinning down at 2000 × *g* for 2 min. Insoluble chromatin was washed with buffer B and finally resuspended in SDS sample buffer. For immunoprecipitations, cells were lysed in with 1 ml lysis buffer (1% NP-40, 50 mM Hepes pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM MgCl2, 1 mM NaF, 1 mM Na3VO4, 25 mM β-glycerophophatese, 1 tablet of complete EDTA-free per 50 ml) on ice for 10 min. The lysate was cleared by centrifugation and 10% of supernatant was used for whole cell lysate. Immunoprecipitations were performed using S-protein beads (Novagen) or with antibodies bound to Dynabeads Protein A (Life Technologies). Beads were washed with TBST and incubated with the rest of the lysate at 4°C for 24 h. Beads were washed extensively with lysis buffer, after which bound protein was eluted with Laemmli sample buffer.

Results

The Plk1 FRET-Probe Is Phosphorylated in the Nucleus

Plk1 localizes both at the centrosomes and kinetochores during G2. While most of the literature suggests that Plk1 is activated at the centrosomes, this is mainly based on immunofluoresence using anti-phospho-T210 antibodies (12), and the fact that Aurora A is localized at the centrosomes in G2 (13). However, using a FRET-based biosensor to measure the Plk1 activity in real-time, we found that Plk1 activation is first visible in the nucleus approximately 5 h before cells entered mitosis [**Figure 1A**; (12)]. Since this probe is not tethered, we wondered whether shuttling of an activated probe between the cytoplasm and the nucleus might account for this observation. To test this, we generated an H2B-tagged FRET-based biosensor for Plk1 that is localized exclusively in the nucleus (**Figure 1B**). We observed that cells expressing this construct entered mitosis normally and displayed similar kinetics of Plk1 activation in the nucleus (**Figures 1C,D**). In addition, pharmacological inhibition of Plk1 with the small molecule inhibitor BI 2536 (22) led in both cases to inhibition of Plk1 activity, except for a small signal in mitosis (**Figures 1A–D**), which we have previously shown to be dependent on the mitotic kinase Mps1 (14). In addition, inhibition of Aurora A with the small molecule inhibitor MLN 8054 (23) also led to similar kinetics of Plk1 activation where the initial activation is repressed (**Figures 1A–D**). Activation of Plk1 during the later stages of G2 is also dependent on Aurora A, but inhibition of Aurora A through MLN 8054 is not penetrant enough to achieve complete inhibition of its activity, as we have shown previously (14). Taken together, these results show that immobilization of the FRET-based biosensor for Plk1 does not affect the timing of its phosphorylation, demonstrating that substrate phosphorylation by Plk1 first becomes apparent in the nucleus, approximately 5 h before cells enter mitosis.

Dynamic Localization of Plk1 is Important for Checkpoint Recovery

Since substrate phosphorylation by Plk1 is first observed in the nucleus during G2, we next asked if Plk1 needs to be on the centrosomes or in the nucleus in order to promote entry into mitosis during recovery from a DNA damage-induced arrest. To this end, we generated stable cell lines expressing tetracycline-inducible and RNAi-resistant variants of Plk1 that were either freely diffusible, or exclusively localized at centrosome or in the nucleus. For this purpose, we used EGFP-TEV-S (LAP)-tagged wild-type Plk1 (12, 24), an AKAP-LAP-Plk1 fusion that is physically tethered to the centrosome through fusion to the centrosomal targeting domain of AKAP450 (25) and an H2B-LAP-Plk1 fusion in which Plk1 is fused to H2B and is therefore located exclusively in the nucleus (**Figure 2A**). Using a short hairpin that targets endogenous Plk1 (26), we depleted the endogenous protein and used tetracycline-induced expression of the exogenous proteins for protein replacement (**Figure 2B**). We first analyzed if these fusion proteins could be phosphorylated at T210 during checkpoint recovery. To this end, we synchronized the cells in G2 and induced DNA damage by Adriamycin. As a consequence of checkpoint activation, these cells remain arrested in G2, and we subsequently induced checkpoint recovery by adding caffeine for 8 h. During this time, Plk1 gets activated through phosphorylation of T210 and cells resume the cell cycle and enter mitosis (12, 26). Indeed, LAP-Plk1 was efficiently phosphorylated at T210 after the addition of caffeine (**Figure 2C**). In accordance with the hypothesis that Plk1 is activated at centrosomes, we found that AKAP-LAP-Plk1 was also phosphorylated at T210. Although the phosphorylation of AKAP-LAP-Plk1 was less prominent than LAP-Plk1, we could not detect any phosphorylation of T210 on H2B-LAP-Plk1, suggesting that activation of Plk1 occurs outside of the nucleus (**Figure 2C**). Furthermore, inhibition of Aurora A affected phosphorylation of Plk1 at T210 in both LAP-Plk1 as well as LAP-AKAP-Plk1 showing that this phosphorylation is dependent on Aurora A (**Figure 2D**). Next, we wondered if the centrosomal- and nuclear-tethered Plk1 versions could induce checkpoint recovery, a well-established function of Plk1 (12, 26). To this end, we depleted endogenous Plk1 by RNAi and reconstituted Plk1 expression with the exogenous proteins prior to induction of recovery. We subsequently determined the amount of mitotic cells after 8 h of caffeine treatment as a measure of checkpoint recovery. Expression of the exogenous versions of Plk1 did not affect recovery in the presence of the endogenous Plk1 and we clearly observed a reduction in cells entering mitosis

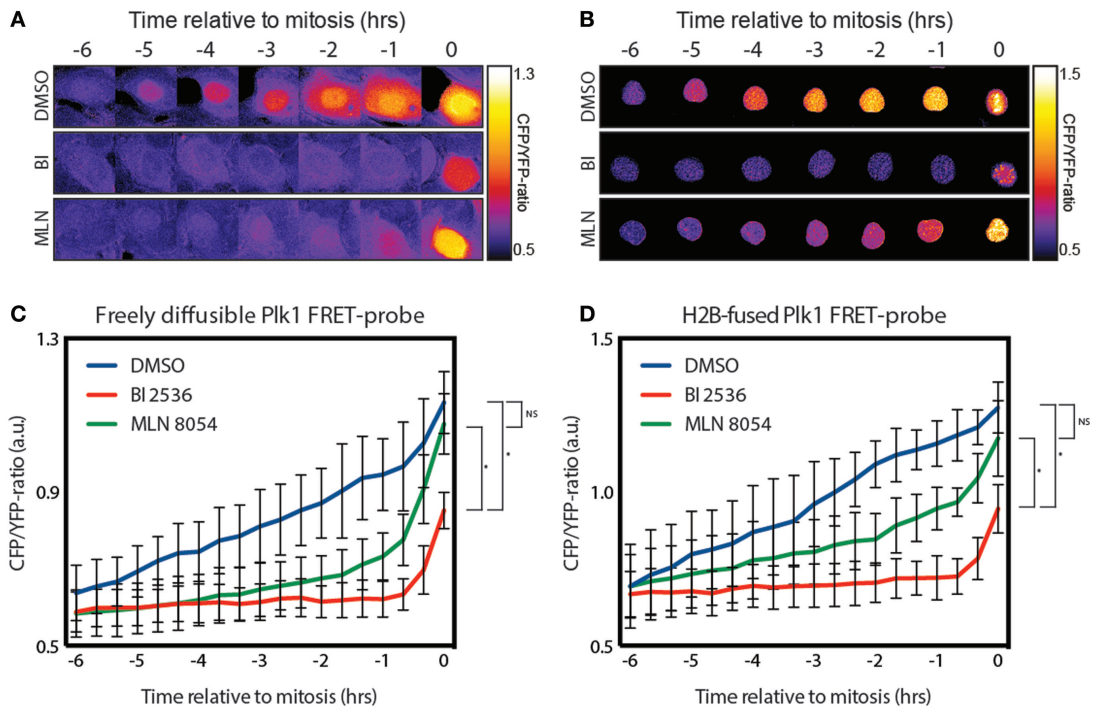

FIGURE 1 | Plk1 activity is first seen in the nucleus. **(A)** Stills from a movie showing false color-coded CFP/YFP emission ratios. The stills show control-, BI 2536-, and MLN 8054-treated U2OS cells expressing the diffusible FRET-based biosensor for Plk1 activity while entering mitosis. BI 2536 was used at a concentration of 100 nM; MLN 8054 at a concentration of 1 μM. **(B)** Stills from a movie showing false color-coded CFP/YFP emission ratios of U2OS cells expressing the H2B-tagged FRET based biosensor were treated as in **(A)**. **(C)** Quantification of CFP/YFP-ratio of cells shown in **(A)**. Error bars represent the SD of 10 individual cells. **(D)** Quantification of CFP/YFP-ratio of cells shown in **(B)**. Error bars represent the SD of 10 individual cells; $*p < 0.0001$.

when Plk1 was depleted (**Figure 2E**). Reconstitution of LAP-Plk1 rescued recovery, albeit not completely (**Figure 2E**). However, reconstitution of AKAP-LAP-Plk1 or H2B-LAP-Plk1 was unable to significantly increase the fraction of cells that could recover (**Figure 2D**). These results indicate that Plk1 function requires free diffusion of Plk1 between nucleus and cytoplasm, not only to be efficiently phosphorylated at T210 but also to be able to promote recovery from a DNA-damage-induced arrest.

Bora Localizes in the Cytoplasm

Activation of Plk1 is carried out by Aurora A, which phosphorylates T210 in G2. This phosphorylation event requires the co-factor Bora (11, 12). To further study Plk1/Bora complex formation, we synchronized cells at the G1/S border and performed a time course. We immunoprecipitated Plk1 and analyzed the amount of Bora that co-immunoprecipitated to see when these proteins started to interact. We observed that interaction between Plk1 and Bora occurs already early after thymidine release, possibly reflecting Plk1 functions during replication (27), while phosphorylation of Plk1 at T210 accumulates later in G2 (**Figure 3A**). Despite our best efforts, we were unable to detect any Aurora A in these co-immunoprecipitation experiments, which may indicate that the interaction of Aurora A with the Plk1-Bora complex might be extremely transient (data not shown). Since available antibodies that recognize Bora are not suitable for immunofluorescence, we were unable to determine the exact localization of endogenous Bora in cells (data not shown). Therefore, we generated a tetracycline-inducible GFP-Bora cell line to study Bora localization (17). Induction with tetracycline resulted in efficient induction of GFP-Bora expression (**Figure 3B**). In addition, GFP-Bora could efficiently co-immunoprecipitate Plk1 and this interaction increases when recovery is induced by the addition of caffeine (**Figure 3B**). We next monitored the localization of GFP-Bora. In *Drosophila*, Bora has been shown to initially localize in the nucleus, then transfer to the cytoplasm in early prophase until nuclear envelope breakdown (16). However, we were unable to detect any substantial nuclear signal of GFP-Bora in line with an earlier report (17); instead, we clearly observed that Bora was persistently cytoplasmic throughout interphase. When cells enter mitosis, Bora is targeted for degradation in a Plk1- and βTrCP-dependent manner (17–19). To see if GFP-Bora behaves in a similar manner, we filmed cells expressing GFP-Bora entering mitosis. Indeed, we observed a reduction in GFP-Bora expression approximately 2 h before cells entered mitosis, similar to a recent report (**Figures 3C,D**) (17). In accordance with the literature, degradation of Bora was abrogated when Plk1 was inhibited. In addition, inhibition of Aurora A had a similar effect on the stability of Bora [**Figures 3C,D**; (17)], which is consistent with the continuous activation of Plk1 by Aurora-A during mitosis (14).

Because we were unable to monitor endogenous Bora by immunofluorescence, we performed a fractionation assay to

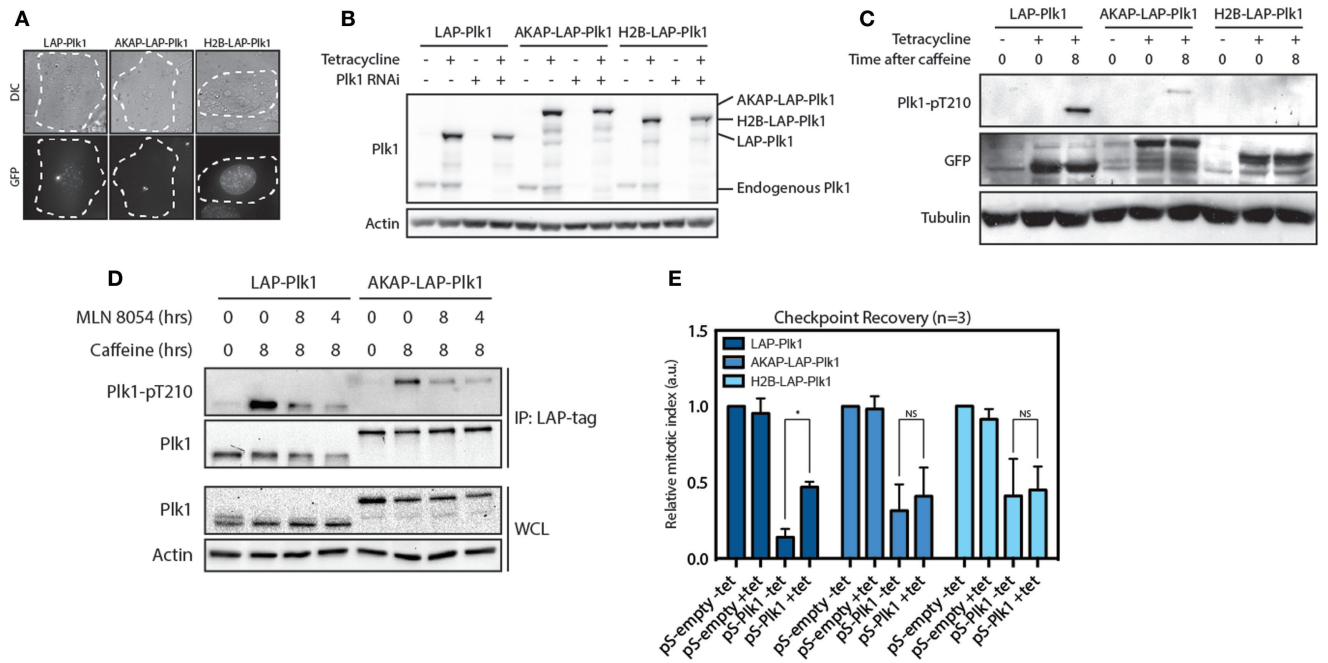

FIGURE 2 | Plk1 is activated at the centrosomes but needs to be dynamically localized. **(A)** Expression of RNAi resistant LAP-Plk1, AKAP-LAP-Plk1 or H2B-LAP-Plk1 was induced in U2TR stably expressing these constructs by addition of tetracycline. DIC- and GFP-images were taken of representative cells. **(B)** Tetracycline inducible U2TR cells stably expressing RNAi resistant LAP-Plk1, AKAP-LAP-Plk1 or H2B-LAP-Plk1 were transfected with an empty pSuper, or a pSuper targeting endogenous Plk1. Cells were synchronized in G2 and damaged with 0.5 μM adriamycin for 1 h and expression was induced where indicated using tetracycline. 16 h after induction of DNA damage cell were harvested and analyzed by western blotting. **(C)** Tetracycline inducible U2TR cells stably expressing RNAi resistant LAP-Plk1, AKAP-LAP-Plk1 or H2B-LAP-Plk1 were synchronized in G2 and damaged with 0.5 μM adriamycin for 1 h and expression was induced where indicated using tetracycline for 16 h. Cells were harvested at the indicated time points after addition of caffeine and analyzed by western blotting. **(D)** Cells were treated as in C. MLN 8054 was added at a concentration of 1 μM either for 8 h together with caffeine or during the last 4 h of caffeine. LAP-tagged proteins were immunoprecipitated with S-protein agarose beads and analyzed by western blot. **(E)** Tetracycline inducible U2TR cells stably expressing RNAi resistant LAP-Plk1, AKAP-LAP-Plk1 or H2B-LAP-Plk1 were transfected with an empty pSuper, or a pSuper targeting endogenous Plk1. Cells were synchronized in G2 and damaged with 0.5 μM adriamycin for 1 h and expression was induced where indicated using tetracycline. Cells were arrested for 16 h, recovery was induced by caffeine addition for 8 h and the mitotic index was determined, based on the percentage of Histone H3-pS10 positive cells, using FACS. Error bars represent the SD of three independent experiments; $^*P < 0.001$.

separate cytoplasmic proteins from the nuclear proteins (21). We synchronized cells in G2 and induced DNA damage. About 16 h after the DNA damaging insult, we induced recovery by addition of caffeine and harvested cells after 1, 2, and 4 h to monitor T210 phosphorylation. Similar to our observations with the GFP-Bora-expressing cell line, endogenous Bora appeared to be strictly cytoplasmic (**Figure 3E**). Interestingly, Aurora A was clearly present in both the nucleus and the cytoplasm. Nuclear enrichment of GFP-Aurora-A next to its well-known centrosomal localization during G2 was also observed by monitoring a GFP-Aurora A expressing U2OS cell during mitotic entry (**Figure 3F**). Although the general idea is that Aurora A localizes predominantly at the centrosomes, nuclear localization has also been reported by overexpression studies as well as on endogenous levels (15, 28). Phosphorylation of Plk1 at T210 did not seem to be preferentially present in the cytoplasm or in the nucleus as the signal appeared in both places at 4 h after caffeine addition. These results, combined with the data presented in **Figure 2**, suggest that Plk1 is phosphorylated at T210 at the centrosomes from where active Plk1 subsequently can translocate to the nucleus.

Discussion

Plk1 localization is highly dynamic during the cell cycle. Activation starts in G2, presumably at the centrosomes, but activity monitored by a FRET-based biosensor is first observed in the nucleus approximately 5 h before mitosis (12, 14). Similarly, Plk1 localization to kinetochores, which depends on Plk1 activity, occurs at the S/G2 transition (15). These observations raise questions about the exact location where Plk1 is initially activated.

Here, we provide proof that stable phosphorylation of Plk1 targets first occurs in the nucleus. Since the H2B-tagged and diffusible probes showed similar profiles, we ruled out the possibility that detection of Plk1 activity was affected by diffusion or active import of the phosphorylated FRET-probe from the cytoplasm (**Figure 1**). This, in combination with our observation that centrosome-tethered Plk1 is phosphorylated at T210 while H2B-tethered Plk1 is not, implies that Plk1 needs to be in the cytoplasm for its initial activation and subsequently move into the nucleus to phosphorylate its targets. Neither the centrosome-tethered variant nor the nuclear-restricted variant of Plk1 is able to rescue

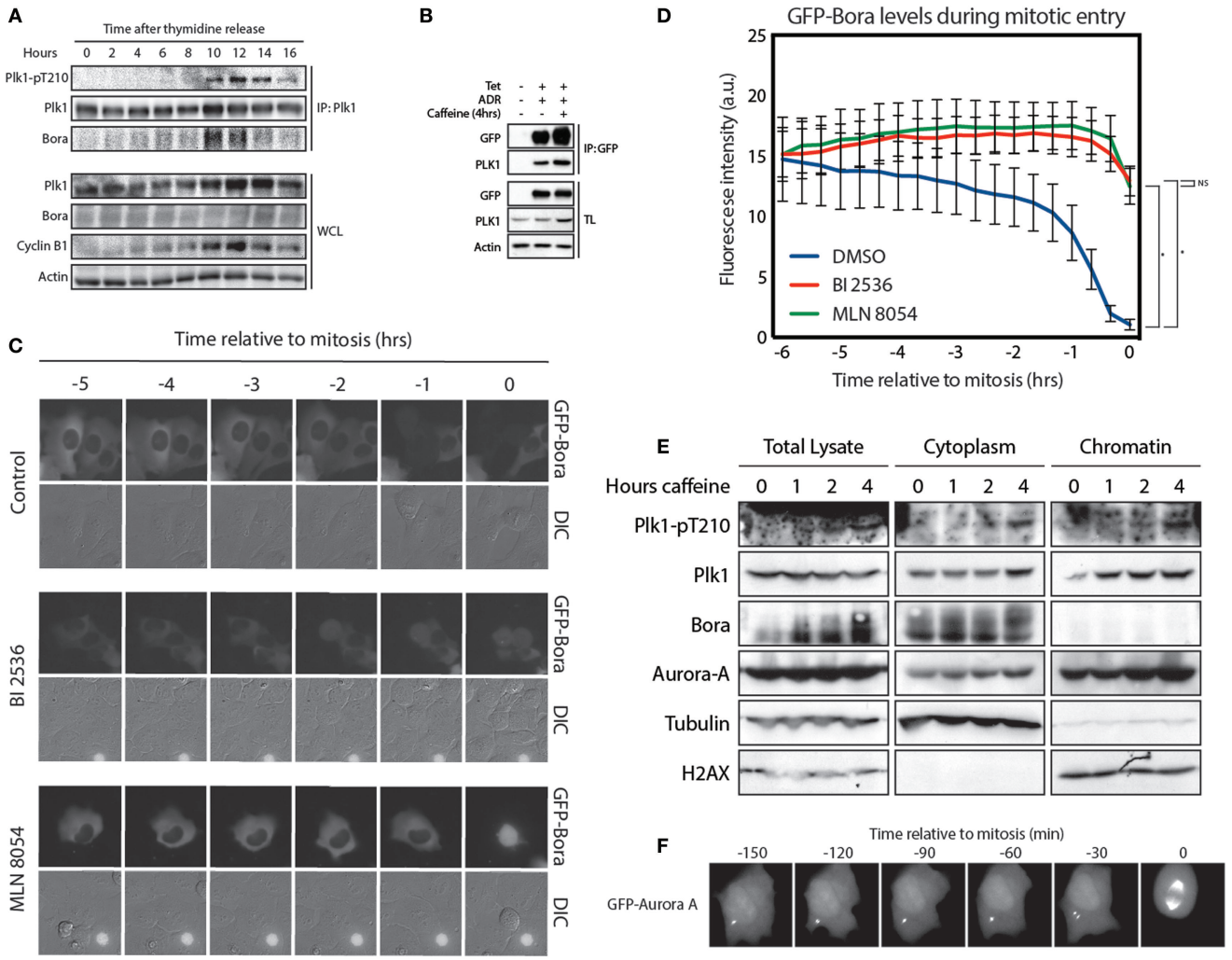

FIGURE 3 | Bora localizes in the cytoplasm and is degraded 2 h before mitosis. **(A)** U2OS cells were synchronized at the G1/S border using a 24-h thymidine block and harvested at the indicated times after thymidine release. Plk1 was immunoprecipitated and protein levels were analyzed by western blot. **(B)** Tetracycline inducible U2TR cells stably expressing GFP-Bora were synchronized in G2, DNA damage was induced for 1 h using 0.5 μM adriamycin. Tetracycline was added where indicated and cells were arrested for 16 h. Caffeine was added for 4 h where indicated. Cells were harvested and GFP-Bora was immunoprecipitated. Protein levels were analyzed by western blot. **(C)** Tetracycline inducible U2TR cells stably expressing GFP-Bora were filmed asynchronously and expression was induced by tetracycline. Cells were treated as indicated with 100 nM BI 2536 or 1 μM MLN 8054. Stills from a movie showing DIC- and GFP-images are shown of cells entering mitosis. **(D)** Quantification of 3C, total GFP expression levels were measured over time. Error bars represent SEM of 10 individually quantified cells. $^*p < 0.0001$. **(E)** U2OS cells were arrested for 16 h after induction of DNA damage in G2 with 0.5 μM adriamycin. Recovery was induced with caffeine for the indicated times. Total lysates were obtained as well as cytoplasmic and chromatin fractions using the cell fractionation protocol from Méndez and Stillman (21). **(F)** Stills from real time imaging of a GFP-Aurora A-expressing U2OS cell entering mitosis.

recovery from a DNA damage-induced arrest in cells depleted of endogenous Plk1 (**Figure 2**), further supporting a model in which centrosomal activation is followed by translocation to the nucleus in order for Plk1 to execute its function in regulating mitotic entry. Finally, we show that Bora localizes exclusively in the cytoplasm and its degradation is induced approximately 2 h before cells enter mitosis. Our data suggest that Plk1 is phosphorylated at T210 at the centrosomes but phosphorylation of Plk1 targets is somehow inhibited in the cytoplasm, whereas activated Plk1 that translocates to the nucleus can phosphorylate its targets (**Figure 4**).

There are several possible explanations for the preferential target phosphorylation by Plk1 in the nucleus. Phosphorylation of the FRET-based biosensor can easily be reversed as Plk1 inhibition decreases the CFP/YFP-ratio to basal levels in approximately half an hour (14). This observation shows that phosphatases are also at play and dephosphorylate the FRET-probe. Thus, preferential substrate phosphorylation by Plk1 in the nucleus could either be due to accumulation of active Plk1 to the nucleus or it could be due to higher phosphatase activity directed toward Plk1 targets in the cytoplasm. Our observation that the relative level of T210-phosphorylation is similar in the cytoplasm compared to the

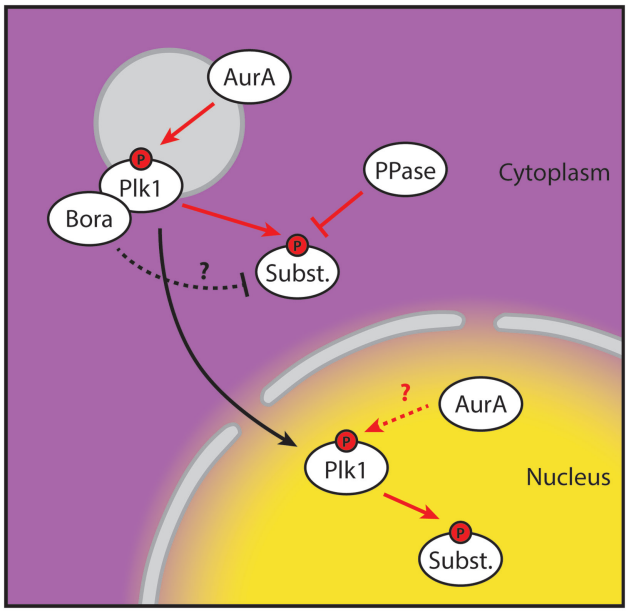

FIGURE 4 | Spatial separation of Plk1 phosphorylation and activity. Proposed model for Plk1 activation during G2. Plk1 binds to its co-factor Bora and is subsequently activated at the centrosome on T210 by Aurora A. Activity of the T210-phosphorylated Plk1 is inhibited in the cytoplasm, possibly by Bora or by phosphatase activity directed at its substrates. T210-phosphorylated Plk1 then translocates to the nucleus where the inhibitory factors of Plk1 activity are absent and Plk1 starts phosphorylating its substrates. The contribution of nuclear Aurora A is currently unclear. Yellow and purple indicate high and low levels of Plk1 substrate phosphorylation, respectively. Red arrows indicate phosphorylation events. The "P" indicates phosphorylation of T210.

nucleus suggests that preferential substrate phosphorylation in the nucleus is due to high phosphatase activity in the cytoplasm. One of the candidate phosphatases that could suppress the activity of Plk1 toward its cytoplasmic substrates is PP2A/B55 that is highly active in cytoplasm until its inhibition by Greatwall kinase and endosulfine shortly before mitotic entry (29–31). In addition, PP2A/B55 has recently been shown to counteract Plk1 activity through dephosphorylation of T210 after DNA damage (32).

We find that a version of Plk1 that is tethered to the centrosome can be phosphorylated at T210, albeit less than the wild type version of Plk1. However, it is not possible to functionally rescue Plk1 function when Plk1 is tethered to the centrosome, something others have observed as well (33). In addition, forced nuclear localization did not result in phosphorylation at T210 during recovery nor did it result in a functional rescue. These results suggest that dynamic localization of Plk1 during the cell cycle is of utmost importance to carry out its functions. Activity of Plk1 can direct it to different subcellular sites. For instance, Plk1 is recruited in a Cdk-dependent manner to the centrosomes by hCenexin1 (9) and to kinetochores by BubR1, Bub1, or INCENP (8, 34, 35). In addition, Plk1 can also create its own docking site to target itself to the kinetochores through PBIP1 (5) or mediate its translocation to the central spindle in anaphase through PRC1 (7). These reports and our current results are clear indications that dynamic localization of Plk1 is indispensible for proper execution of its functions during G2 and mitosis.

Phosphorylation of Plk1 at T210 by Aurora A requires Bora (11, 12). Our data strengthen the idea that initial phosphorylation occurs at the centrosomes as we observe that Bora is strictly cytoplasmic as opposed to phosphorylation in the nucleus. Bora binds to the Plk1 Polo-box domain in a Cdk-dependent manner (18). This not only allows Aurora A to phosphorylate Plk1 at T210 but additionally targets Bora itself as a substrate of Plk1 (18, 19), since phosphorylation by Plk1 targets Bora for βTrCP-dependent proteasomal degradation. While the literature so far has suggested that this takes place in mitosis, we observed that Bora levels already diminish at 2 h before cells enter mitosis (17). This observation coincides roughly with the time that substrate phosphorylation by Plk1 is first observed in the cytoplasm. Since Bora most likely interacts with Plk1 in the cytoplasm, it is tempting to speculate that despite the T210-phosphorylation that occurs in the cytoplasm, Plk1 target phosphorylation is somehow inhibited until Plk1 translocates to the nucleus where it cannot bind Bora anymore (**Figure 4**). This could either take place by direct inhibition of phosphorylation by Plk1, or as mentioned earlier, by rapid reversion of phosphorylation of Plk1 targets by a phosphatase that is enriched in the cytoplasm. As the degradation of Bora before mitotic entry coincides with Plk1 target phosphorylation in the cytoplasm, it is a distinct possibility that Bora can function as an inhibitor of Plk1 activity, at least toward its other substrates, in the cytoplasm. It would therefore be interesting to test if binding of Bora to Plk1 can prevent efficient binding to its other substrates, for example by occupying the Polo box domain binding site. Further analysis of the Bora-Plk1 complex and the effect of Bora-binding to Plk1-dependent phosphorylation of other Plk1 targets will be required to answer these questions.

In addition to the downstream regulation, upstream regulation of Aurora A activity during G2 and how this relates to specific timing of T210-phosphorylation on Plk1 is currently unclear. Aurora A relies on several co-factors to exert its functions and we have previously shown that the Aurora A co-factor TPX2 does not contribute to the activation of Plk1 during G2 and mitosis (14). However, it will be interesting to investigate other Aurora A co-activators and recruiters such as Ajuba (36) or CEP192 (37) and study their impact on timely activation of Plk1. More detailed analysis of timing and activation events of Plk1, Bora, and Aurora A will be required to elucidate the complex spatiotemporal regulation of Plk1 activation during G2.

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REVIEW
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Aurora A's Functions During Mitotic Exit: The Guess Who Game

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Until recently, the knowledge of Aurora A kinase functions during mitosis was limited to pre-metaphase events, particularly centrosome maturation, G2/M transition, and mitotic spindle assembly. However, an involvement of Aurora A in post-metaphase events was also suspected, but not clearly demonstrated due to the technical difficulty to perform the appropriate experiments. Recent developments of both an analog-specific version of Aurora A and small molecule inhibitors have led to the first demonstration that Aurora A is required for the early steps of cytokinesis. As in pre-metaphase, Aurora A plays diverse functions during anaphase, essentially participating in astral microtubules dynamics and central spindle assembly and functioning. The present review describes the experimental systems used to decipher new functions of Aurora A during late mitosis and situate these functions into the context of cytokinesis mechanisms.

Keywords: mitosis, cytokinesis, Aurora kinase, central spindle, cancer, microtubule

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INTRODUCTION

Aurora A and Aurora B are two major serine/threonine kinases participating in mitosis regulation. From an evolutionary point of view, equatorial Aurora B kinase likely appeared before polar Aurora A kinase (1). Although the latest is a derivative of Aurora B, it possesses its own expression pattern and its own crucial mitotic functions. Aurora A was discovered in the 90s by Glover and colleagues in a screen designed to identify genes that affect centrosomes cycle in *Drosophila* (2). Since this first study, Aurora A has been the focus of many attentions in fundamental and medical research, because the loss of control of its expression or activity has been directly linked to cancer. Several functions of Aurora A kinase during mitosis have been well established. Aurora A regulates mitotic entry through phosphorylation of CDC25B phosphatase (3) or PLK1 kinase (4, 5). Aurora A also contributes to DNA damage (6) and to spindle assembly checkpoints (SAC) (7). Once the cell is engaged into mitosis, Aurora A participates in mitotic spindle assembly and functioning. Aurora A triggers centrosome maturation by recruiting NDLE1 (8) and TACC3 (9). In prometaphase, Aurora A participates in the regulation of microtubule dynamics and contributes to the recruitment of factors involved in the dynamic instability of microtubules, including DDA3 (10), MCAK (11), ch-TOG (12, 13), and KIF2A (14). Aurora A is also involved in the recruitment of proteins that move along microtubules, for example, Kinesin 5 (Kif11) (15) and p150Glued (16). Lastly, Aurora A has been shown to be involved in chromatin driven microtubules nucleation through NEDD1 phosphorylation (17). These functions of the kinase are closely related to its localization. Indeed, Aurora A is located to centrosomes in G2 and both to centrosomes and to mitotic spindle poles during mitotic spindle assembly. Interestingly, the kinase is also found associated with the central spindle and later on the midbody during mitotic exit. In spite of the description of these late mitotic localizations, there was no formal data demonstrating the involvement of Aurora A into

mitotic exit until recently, mainly because of technical limitations. Indeed, studies investigating the functions of Aurora A have involved modifying Aurora A activity by RNA interference depletion of the protein (siRNA), by over expression (18, 19) and/or by the use of mutants (active, inactive, hyperactive, or non-degradable) (7, 20–24). The major outcome of such experiments is the failure of centrosome maturation (23). During G2, the cell prepares to enter mitosis and numerous proteins required for microtubule nucleation are recruited to centrosomes to participate in the mitotic spindle assembly. Defects in centrosome maturation frequently result in a longer G2/M transition and perturb the mitotic spindle assembly, thus maintaining the SAC active. The active SAC prevents the metaphase/anaphase transition, thereby most of the time impedes the investigation of Aurora A functions beyond this step. In view of the crucial role of Aurora A in spindle organization before anaphase and its post-metaphase localization, an implication of Aurora A in the regulation of the spindle during mitotic exit would not be surprising. In order to better understand late mitotic events, potential late mitotic functions of Aurora A should be investigated. Indeed, in the early 2000s, studies that had resulted in only partial perturbation of the activity of the kinase have pointed out some late mitotic functions for Aurora A. The specific involvement of Aurora A during mitotic exit was confirmed only recently by the use of pharmacological inhibition of the kinase. The present review focuses on the experimental systems that have been used to decipher late mitotic functions of Aurora A and discusses these functions in the context of mitotic exit.

THE FIRST CLUES OF THE LATE MITOTIC INVOLVEMENT OF AURORA A

Targeting of the Kinase by Cellular Microinjection of Anti-Aurora A Antibodies

The first study that brought some insight in the involvement of Aurora A in mitotic exit was led by Marumoto and colleagues (24). The aim of this study was to understand the physiological functions of human Aurora A. In this context, the authors first depleted the kinase by siRNA in HeLa cells. In cells reaching the best depletion efficiency, they observed a classical absence of mitotic entry. Yet, when only partial depletion was achieved, they observed chromosomes misalignment and some cells presented multiple nuclei that are often synonymous of cytokinesis failure. To pin point the specific role of Aurora A throughout the different phases of mitosis, the authors inhibited the kinase by microinjection of affinity purified anti-Aurora A polyclonal antibodies at different time of mitosis progression. Injection of HeLa cells with the antibodies in late G2 triggered a delay in mitotic entry, a prolonged duration of early (prometaphase and metaphase) and late mitosis (anaphase and telophase), a defect in chromosomes congression, the appearance of mitotic spindle with multiple spindle poles, and an unequal chromosomes segregation. All these phenotypes have now commonly been described as typical of the inhibition of Aurora A. Instead, microinjection of anti-Aurora A antibodies after centrosomes separation and chromosomes alignment onto metaphase plate triggered a cytokinesis defect. Albeit sister chromatids separated and the cleavage furrow formed, meaning that the acto-myosin ring assembled and could contract, cytokinesis aborted, and daughter cells fused. These data, which strongly suggested for the first time that the Aurora A kinase could be involved in mitotic exit, were reinforced by the first demonstration that Aurora A was not only localized on the centrosomes and the mitotic spindle poles but also on the central spindle and the midbody. This pioneer study was particularly interesting since it was the first time that Aurora A was inhibited in a precise window of time, which specifically targeted mitotic exit. Yet, effects of antibodies microinjections are difficult to interpret because there is no real negative control that assesses putative off target effects. Moreover, since the antibodies used to inhibit Aurora A were obtained after injection of the regulatory domain of the kinase into a rabbit (amino acids 1–129), the catalytic domain of the kinase is most likely not targeted. As the authors did not test the effect of this polyclonal antibody onto Aurora A's catalytic activity, the real effect of the injection into culture cells is thus difficult to assess and cellular data have to be examined cautiously.

Indirect Stabilization of the Kinase

More recently, a second study brought interesting data concerning putative late mitotic functions of Aurora A through indirect action on the stability of the kinase. In HeLa cells, Floyd and colleagues explored the time course of APC/C^{Cdh1} activity and functioning (25). As previously described in *Xenopus* cell free extracts (26, 27), authors observed that it was involved in Aurora A kinases degradation. Indeed, they found that siRNA-mediated Cdh1 depletion led to a stabilization of Aurora A and B that were not degraded anymore during mitotic exit. In parallel, Floyd and colleagues examined whether APC/C^{Cdh1} could be involved into mitotic exit through time-lapse recordings of Cdh1-depleted cells. They measured the time taken from anaphase onset to cleavage furrow ingression completion and found that, after knockdown of Cdh1, this time was reduced. Authors also observed that sister chromatids segregation occurred more rapidly, likely indicating that microtubules dynamics was modified. This was indeed the case, since in Cdh1-depleted cells, the robustness of the central spindle was weaker, and there was an exaggerated growth of astral microtubules at the spindle poles, which persisted abnormally during telophase and abscission. To test whether the Aurora kinases were responsible for such phenotypes, the authors then expressed non-degradable forms of Aurora A or B. They found that expression of each one could mimic the depletion of Cdh1. Interestingly enough, the authors also remarked that overexpression of a non-degradable version of Aurora B reduced the degradation of Aurora A. As Aurora A appears to be degraded earlier than B, the authors suggested that destruction of Aurora A in anaphase may be sufficient to prevent proper anaphase spindle organization, and Aurora A is the likely critical target of APC/C^{Cdh1} at anaphase onset. To confirm this hypothesis, Floyd and colleagues then tested whether depletion of Aurora A by siRNA could rescue Cdh1 depletion. In agreement with previously published data, cells depleted for Aurora A were delayed in prometaphase and when they entered into metaphase, they frequently presented fragmented poles. When Cdh1 and Aurora A were depleted simultaneously, the over elongation of anaphase spindle observed when Cdh1 was depleted alone was partially

reduced, thus confirming a role played by Aurora A in central spindle dynamics.

To further investigate the mechanisms that could be responsible for spindle over elongation, Floyd and colleagues analyzed the distribution of Aurora kinases in Cdh1-depleted cells. The authors first observed a persistence of the Aurora A staining at spindle poles from anaphase to G1 stage. This persistence of Aurora A correlated with the increase of astral microtubules density indicating that the degradation of Aurora A at this stage of the mitosis might be used to regulate astral microtubules dynamics. Concomitantly with the polar stabilization of the kinase, Cdh1 depletion also triggered the polar retention of TPX2, which is a well-characterized Aurora A activator involved into mitotic spindle microtubules nucleation in a Ran-GTP-dependent manner. The staining of EB1 protein, which localized at the plus tip of growing microtubules, was not modified, indicating that Cdh1 likely regulates global microtubules stability rather than microtubules growth. In parallel to these results, the authors also observed that Cdh1 depletion altered not only the stability but also the distribution of Aurora B. Instead of being localized to the central spindle midzone, authors found that the kinase was weakly localized to the midzone and rather accumulated to a diffuse band in the region of the equatorial cortex. Mklp2, which mediates the localization of Aurora B to the central spindle midzone, was also relocalized to the equatorial cortex. In contrast to MKLP1, a component of the Centralspindlin complex, PRC1 and PLK1, was correctly localized. The authors proposed that alteration of the central spindle density and structure could be due to the weak localization of MKLP2 and Aurora B to the midzone, but MKLP1, PRC1, and PLK1 are sufficient to drive the assembly of a weak spindle midzone that allows the initiation of the cleavage furrow.

Altogether, these results suggest a predominant role of Aurora A in the regulation of early anaphase spindle dynamics, notably in the stabilization of astral microtubules, whereas Aurora B would be involved later, likely in central spindle stability. However, in this experimental system, a function for Aurora A in central spindle assembly cannot be definitively ruled out since it may be hidden by the phenotype triggered by the mislocalization of Aurora B. Another possibility could be that Aurora A and B share common substrates and could participate in the same pathways during mitotic exit.

Conditional Knock-Out of the Kinase

Work by Hégarat and colleagues (28) has pursued on the notion of cooperation between Aurora A and B, a few years later. In their paper, the authors explored Aurora A's functions through conditional knock-out of the protein. They took advantage of the DT40 chicken cells to set up a system in which the two WT alleles of Aurora A were disrupted. This system was chosen to ensure complete Aurora A depletion and avoid the potential side effects triggered by kinase inactivation and protein removal. Using this strategy, the authors first confirmed previous results: they observed mitotic cells with unaligned chromosomes, mitotic spindle with reduced volumes, and defective PLK1 activation in G2 phase. Interestingly, the simultaneous impairment of Aurora A expression and chemical inhibition of Aurora B (with 60 nM AZD1152, a potent Aurora B inhibitor) triggered a complete absence of chromosomes segregation followed by their decondensation. This defect was accompanied by the persistence of long and stable MT fibers in Aurora A^{KO}/Aurora B inhibited cells, whereas Aurora B inhibited cells presented the classical spindle contraction typical of anaphase onset. In Aurora A^{KO} cells, astral microtubules appeared partially stable, but chromosomes finally separated. Curiously, the authors did not mention any further effect in later phases of mitosis. Altogether, these results suggested a collaborative role for Aurora A and Aurora B in chromosomes segregation during early anaphase, through control of mitotic spindle microtubules stability. This observation could be the result of substrate or pathway redundancy and point to the complex interplay between centrosomal and centromeric functions in regulating mitotic spindle dynamics [for further information, see the review by Hochegger and colleagues (1)]. Even though the experimental system used by Hégarat and colleagues allowed a real-specific targeting of Aurora A, it did not allow the inhibition of the kinase within an accurate window of time. Consequently, many events that require the presence of Aurora A or its activity remained inaccessible. This drawback was solved 2 years later through pharmacological inhibition of the kinase.

THE VALIDATION OF AURORA A's LATE MITOTIC INVOLVEMENT THROUGH PHARMACOLOGICAL INHIBITION

The Chemical Genetics Strategy

The best way to address the late mitotic functions of Aurora A is to pharmacologically target the kinase just after the metaphase to anaphase transition, once the SAC is satisfied. Our group was the first who succeeded in developing such an approach (29). To perform this task, we used chemical genetics techniques that consist in modifying the catalytic domain of the kinase to make it sensitive to an ATP analog that has no effect on the WT Aurora A kinase. This system thus, in addition to allow the timely control of Aurora inhibition, enables us to detect any off-target effects of the ATP analog by using the WT kinase as a negative control. To generate an Aurora A variant with an enhanced sensitivity to ATP analogs, we have modified the specificity of the ATP-binding pocket of the kinase by converting leucine 210 into an alanine [L210A Aurora A mutant referred to hereafter as analog-sensitive Aurora A (as-AurA)]. *In vitro*, recombinant as-AurA was as active as the WT version of the kinase (wt-AurA) but was specifically inhibited by the ATP analog 1-Na-PP1 that had no effect on wt-AurA. We generated stable U2OS human cell lines, expressing RNA interference resistant GFP-tagged versions of wt-AurA or as-AurA alleles under the Aurora A minimal promoter (30). In these cells, as-AurA localized similarly to what has been previously described for WT Aurora A and was able to rescue endogenous Aurora A depletion, indicating it is fully functional. Treatment of cells only expressing wt- or as-AurA with 1-Na-PP1 for 24 h, substantially increased the percentage of multipolar or fragmented spindle poles in as-AurA expressing cells [as previously described by Asteriti and colleagues (12)], whereas it had no effect in wt-AurA expressing cells. Altogether, these data show that our chemical genetics system is valid.

To study the effect of Aurora A inhibition just after the metaphase to anaphase transition, we applied 1-Na-PP1 in a timely fashion on wt- or as-AurA cells. When Aurora A was inhibited in metaphase, most cells were blocked and the mitotic spindle collapsed with the two spindle poles closely juxtaposed to the chromatin. When Aurora A was inhibited within the first few seconds of anaphase, the chromosomes separated, but rapidly stopped and cells did not undergo telophase or cytokinesis, leading to the generation of binucleated cells. In these cells, the central spindle was largely disorganized or even absent, leading to the absence of anaphase B. Clearly, these results indicate that Aurora A is both involved in mitotic spindle stabilization during metaphase and later in central spindle assembly during anaphase.

We then searched to identify the defective molecular mechanism leading to anaphase spindle abortion. Central spindle assembly is a complex process involving diverse molecules with highly specific functions. The evolutionarily conserved Centralspindlin complex is a major player in this process. Appropriate localization of Centralspindlin in *Drosophila* depends on the dynactin complex and depletion of the dynactin subunit p150Glued in *Drosophila* S2 cells perturbs Pav-KLP (the ortholog of MKLP1) localization and central spindle organization (31). Our data showed that inhibition of Aurora A during early anaphase-triggered mislocalization of MKLP1 and the accumulation of p150Glued at mitotic spindle poles. Furthermore, we investigated the molecular mechanism involving p150Glued and found that it was phosphorylated by Aurora A on serine 19. This residue belongs to the microtubule-binding domain of p150Glued that is known, in *Drosophila*, to be phosphorylated by Aurora A in pre-anaphase stages (16). Moreover, in interphasic human cells, p150Glued phosphorylation by the PKA kinase has previously been shown to regulate its affinity for microtubules (32). Interestingly, the mutation of serine 19 into an alanine (S19A, which is non-phosphorylable) mimics the inhibition of Aurora A, whereas the mutation into aspartic acid (S19D, that mimics a constitutive phosphorylation) partially rescues Aurora A inhibition.

Currently, the exact mechanism involving Aurora A and p150Glued in central spindle assembly remains to be deciphered. The p150Glued protein can interact with EB1, a microtubule-associated protein involved in microtubule nucleation (33, 34). This interaction, between p150Glued and EB1, is necessary for microtubule binding to centrosomes (33) and for microtubule nucleation (35–37). The C-terminus of EB1 binds to the N-terminus of p150Glued, and this event decreases microtubule shortening and increases rescue frequency and the growth rate of microtubules, thereby favoring microtubule elongation (33, 36). Aurora A depletion results in the disconnection of centrosomes from mitotic spindle poles in *Drosophila* (16), and inhibition of Aurora A seems to be involved in central spindle microtubule nucleation (29). Both of these effects resemble those of EB1 inactivation (33, 35–37). Consequently, phosphorylation of p150Glued serine 19 by Aurora A could be involved in central spindle assembly through an EB1 function. Another hypothesis involves Kinesin 5. Uteng and colleagues have shown that the dynein/dynactin complex is responsible for the transport of the kinesin 5 motor toward the poles (38). As kinesin 5 is required for accurate central spindle assembly (39–42), a defect in p150glued localization during early anaphase could trigger Kinesin 5 mislocalization and concomitant defects in central spindle assembly.

Targeting of Aurora A by a Small Molecule Inhibitor

During the same period, Lioutas and Vernos also demonstrated the involvement of Aurora A in central spindle assembly by using the small molecule inhibitor MLN8237 (43). MLN8237 is a selective Aurora A inhibitor that has >200-fold higher selectivity for Aurora A than Aurora B in cell free assay. In HeLa cells, the authors determined that 250 nM MLN8237 was the concentration that gave the best inhibitory effect on Aurora A without any effect on Aurora B. Similarly to our results, when cells were treated with MLN8237 during metaphase, mitotic spindle collapsed with both centrosomes traveling toward each other, confirming that Aurora A activity is required for mitotic spindle stability. When cells were treated with MLN8237 at anaphase onset, they progressed through anaphase until cytokinesis but with a slower kinetics than control cells. Moreover, Aurora A inhibited cells presented several chromosome segregation defects, including chromatin bridges and lagging pieces of chromosomes. As Aurora A is an important regulator of microtubule stability during mitotic spindle assembly, the authors examined whether the microtubule function was compromised. The pole-to-pole distance during chromosomes segregation was strongly reduced, due to a decrease in central spindle elongation. Additionally, central spindle appeared weaker and more disorganized than in control, while kinetochore fibers appeared to shorten slightly faster than in control cells. Overall, these data indicated that Aurora A activity is involved in chromosomes segregation and is strongly required for central spindle microtubules assembly and organization during mitotic exit.

TACC3 is a substrate of Aurora A that is involved in microtubules stabilization in partnership with chTOG/XMAP215. It is phosphorylated by the kinase on serine 558 during mitotic spindle assembly (9, 44). Immunofluorescence experiments showed that phosphorylated TACC3 is localized on mitotic spindle poles in pre-anaphase and on the poles and the central spindle during mitotic exit. Moreover, the phospho-TACC3 signal was strongly reduced when cells were treated with MLN8237. Depletion of TACC3 by siRNA-triggered effect similar to those induced by Aurora A inhibition: the progression of cells through mitotic exit was slower, the elongation of central spindle was decreased, and the microtubule fluorescence intensity of central spindle was reduced when compared to control. Interestingly, while the exogenous expression of the WT version of TACC3 partially rescued the depletion of TACC3, it was not the case for the non-phosphorylable S558A mutant version. The authors finally realized depolymerization and regrowth assays to further characterize the role of Aurora A and TACC3 into the regulation of central spindle dynamics. When cells were incubated on ice, depolymerization occurred faster in MLN8237-treated cells or in TACC3-depleted cells than in control. Interestingly, for TACC3-depleted cells treated with MLN8237, depolymerization was not enhanced when compared with TACC3-depleted cells alone, likely meaning that Aurora A and TACC3 act in a similar pathway. Similarly, whereas in control cells microtubule regrowth was very

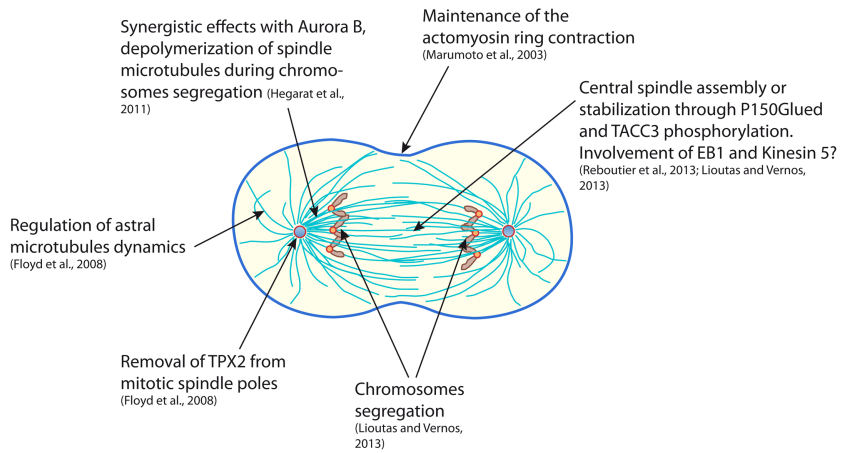

FIGURE 1 | Scheme representing a mammalian cell in early cytokinesis and summarizing the functions of the Aurora A kinase during mitotic exit.

efficient, in MLN8237-treated or TACC3-depleted cells, microtubule regrowth was strongly delayed. Altogether, these data strongly confirm the late mitotic involvement of Aurora A by using a small molecule pharmacological inhibition of the kinase.

CONCLUSION

Historically, mitotic spindle assembly is considered as a critical event for proper chromosome segregation and mitosis progression. Nonetheless, cytokinesis is also emerging as a crucial event of cell division. Even though a dividing cell manages to correctly build its metaphase spindle, a subsequent cytokinesis failure would also lead to polyploidy or aneuploidy and cause genome instability. Whereas the pre-anaphase functions of the Aurora A kinase are extensively documented, studies deciphering the cytokinetic functions of Aurora A remained limited until recently. The various works presented in this review confirm that Aurora A cytokinetic functions are not anecdotal, and understanding these functions is of critical importance for the comprehension of cytokinesis.

Despite the highly different approaches that were used in the works presented here, Aurora A clearly appears to be directly involved in astral microtubules stability and central spindle robustness, both being determinant for an accurate cytokinesis (**Figure 1**). The few discrepancies observed in the different studies may mainly reflect the heterogeneous means that were used to target Aurora A: partial or total depletion, indirect stabilization or pharmacological inhibition of the kinase. Moreover, Aurora A has been shown to also perform functions that are independent of its kinase catalytic activity, thus carrying out a depletion or an inhibition of Aurora A may target different function of the kinase and result in a different outcome (45, 46). According to the studies described in the present review, Aurora A likely exerts many functions from the "dawn to the dusk" of cytokinesis. Some of the events participating in cytokinesis are very dynamic and last only few minutes (for example, central spindle assembly). Under these circumstances, *in vitro* experimental systems could in the future be highly valuable, notably "artificial centrosomes" that are constituted of Aurora A coated beads nucleating aster-like structures in *Xenopus* egg extract (47). This system enables to reassemble an anaphase spindle showing interesting features in terms of size, shape, and biochemistry (48). However, the highly dynamic nature of the remodeling of the mitotic spindle also calls for live cell video-microscopy approaches in order to decipher Aurora A's late mitotic functions. Even though the chemical genetics system that we have developed was up to date and the only way to evaluate the effect of a real-specific inhibition of the kinase, the emergence of small molecule inhibitors that appear more and more specific should soon open the way to extensive study of the hidden functions of Aurora A kinase.

Its function in pre-anaphase stages of mitosis has made Aurora A as a potentially interesting target for cancer therapy and has led to the development of Aurora A-specific pharmacological inhibitors. The Aurora A inhibitor, MLN8237 (also known as Alisertib), is now in clinical phase III study (49). Paradoxically, the understanding of Aurora A's functions, during interphase, asymmetric division or mitotic exit, is at its dawn. Moreover, we now know that both a gain and a loss of activity of Aurora A can lead to carcinogenesis, depending on the mode of cell division (50–53). The fact that Aurora A appears more and more as a pleitropic protein should thus lead to consider cautiously the opportunity to inhibit its activity to treat cancer.

AUTHOR CONTRIBUTIONS

DR conceived the structure of the review, critically analyzed the literature, wrote the manuscript, and prepared figures. CB discussed extensively review structure and contents, contributed to manuscript writing. CP critically analyzed the literature, contributed to manuscript writing.

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REVIEW
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Aurora-A Kinase as a Promising Therapeutic Target in Cancer

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Mammalian Aurora family of serine/threonine kinases are master regulators of mitotic progression and are frequently overexpressed in human cancers. Among the three members of the Aurora kinase family (Aurora-A, -B, and -C), Aurora-A and Aurora-B are expressed at detectable levels in somatic cells undergoing mitotic cell division. Aberrant Aurora-A kinase activity has been implicated in oncogenic transformation through the development of chromosomal instability and tumor cell heterogeneity. Recent studies also reveal a novel non-mitotic role of Aurora-A activity in promoting tumor progression through activation of epithelial–mesenchymal transition reprograming resulting in the genesis of tumor-initiating cells. Therefore, Aurora-A kinase represents an attractive target for cancer therapeutics, and the development of small molecule inhibitors of Aurora-A oncogenic activity may improve the clinical outcomes of cancer patients. In the present review, we will discuss mitotic and non-mitotic functions of Aurora-A activity in oncogenic transformation and tumor progression. We will also review the current clinical studies, evaluating small molecule inhibitors of Aurora-A activity and their efficacy in the management of cancer patients.

Keywords: mitotic kinase, cell cycle, cancer, tumor progression, targeted therapy

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INTRODUCTION

Cell division in normal cells is a tightly regulated process by which replicated DNA is equally distributed into two daughter cells (1). Key players that orchestrate cell division are the centrosomes and mitotic spindles that ensure correct chromosome alignment on the metaphase plate and equal chromosome segregation, resulting in the maintenance of a genomic stable diploid karyotype (2). Due to the complexity of the mitotic machinery, several checkpoint surveillance mechanisms have evolved to safeguard accurate temporal and spatial coordination of cell cycle events (3). Abrogation of cell cycle checkpoints impairs the fidelity of correct chromosome segregation and induces chromosomal instability (CIN), a driving force of oncogenic transformation and tumor progression (4, 5). Aurora serine/threonine kinases are key mitotic regulators required for the maintenance of chromosomal stability (6). In mammalian cells, Aurora kinases consist of three members termed Aurora-A, -B, and -C that are expressed in a cell cycle-dependent fashion. These mitotic kinases are highly conserved through evolution and guarantee the precise coordination of cytoskeletal and chromosomal events through modulation of centrosome duplication, maturation, and separation, as well as proper mitotic spindle assembly resulting in equal chromosome distribution into daughter cells (7). While all three Aurora kinases are expressed in human cancer cells, Aurora-A and Aurora-B are best characterized because they are expressed at high levels in aneuploid tumors (8, 9). Aurora-A

and Aurora-B share about 70% homology in the carboxyl terminus catalytic domain and three conserved Aurora box motifs in their varying amino terminal domain (10). However, they control cell cycle progression and mitosis by interacting with different proteins. Aurora-A is localized primarily on centrosomes, spindle poles, and transiently along the spindle microtubules as cells progress through mitosis (**Figure 1**) (11, 12). By contrast, Aurora-B interacts with the chromosomal passenger complex (CPC) that localizes to the inner centromere during prophase through metaphase and then moves to the spindle midzone and the midbody during late mitosis and cytokinesis (13). While some studies have shown that Aurora-B kinase is overexpressed in cancer cells (14, 15), it is not clear whether Aurora-B overexpression is merely associated with the high proliferative activity of cancer cells or if it plays a causative role in tumorigenesis. Due to the lack of definitive evidence that Aurora-B strictly functions as an oncogene, Aurora-A kinase represents a better candidate target for cancer therapeutics. In the last decade, several small molecule inhibitors of Aurora kinases have been developed, though only a few are selective for Aurora-A; they represent promising drugs to impair the progression of aggressive tumors (16).

AURORA-A EXPRESSION IN CANCER CELLS

The mammalian Aurora-A protein contains 403 amino acids and has a molecular weight of 46 kDa. Aurora-A was first isolated as the product of gene *BTAK* (breast tumor amplified kinase, also named *STK15*) on chromosome 20q13, a region that is frequently amplified in breast, colorectal, and bladder tumors as well as ovarian, prostate, neuroblastoma, and cervical cancer cell lines (17–21). Although gene amplification represents a well-established mechanism to induce Aurora-A overexpression in cancer cells, transcriptional and post-translational mechanisms also play an important role to enhance Aurora-A expression in the absence of *BTAK* gene amplification. In normal cells, the abundance of Aurora-A is down-regulated through APC/C–Cdh1-dependent, proteasome-mediated proteolysis, leading to the organization of the anaphase spindle at the end of mitosis. APC/C–Cdh1-dependent degradation of human Aurora-A requires a destruction box (D-box) in the C-terminal region and a motif in the N-terminus (A-box) (22). Importantly, the phosphorylation state of a serine residue (Ser51) in the A-box inhibits degradation of Aurora-A, as mutants mimicking constitutive phosphorylation of this site cannot be degraded by the APC/C–Cdh1 (23). Furthermore, *we* have showed that HER-2 oncogenic signaling induces Aurora-A phosphorylation, thereby increasing Aurora-A stability and expression in breast cancer cells (24). These findings indicate a functional link between deregulation of Aurora-A stability and tumorigenesis. Conversely, tumor suppressors involved in the control of cell cycle progression promote Aurora-A degradation. The mitotic checkpoint protein Chfr physically interacts with Aurora-A and ubiquitinates Aurora-A both *in vitro* and *in vivo*, ensuring the proper control of mitotic events and maintenance of chromosomal stability (25). Loss of Chfr expression in cancer cells induces aberrant Aurora-A kinase activity, CIN, and promotes tumorigenesis (26). The tumor suppressor p53 modulates Aurora-A expression via both transcriptional and post-translational regulation. Specifically, p53 knockdown in cancer cells promotes the activation of E2F3

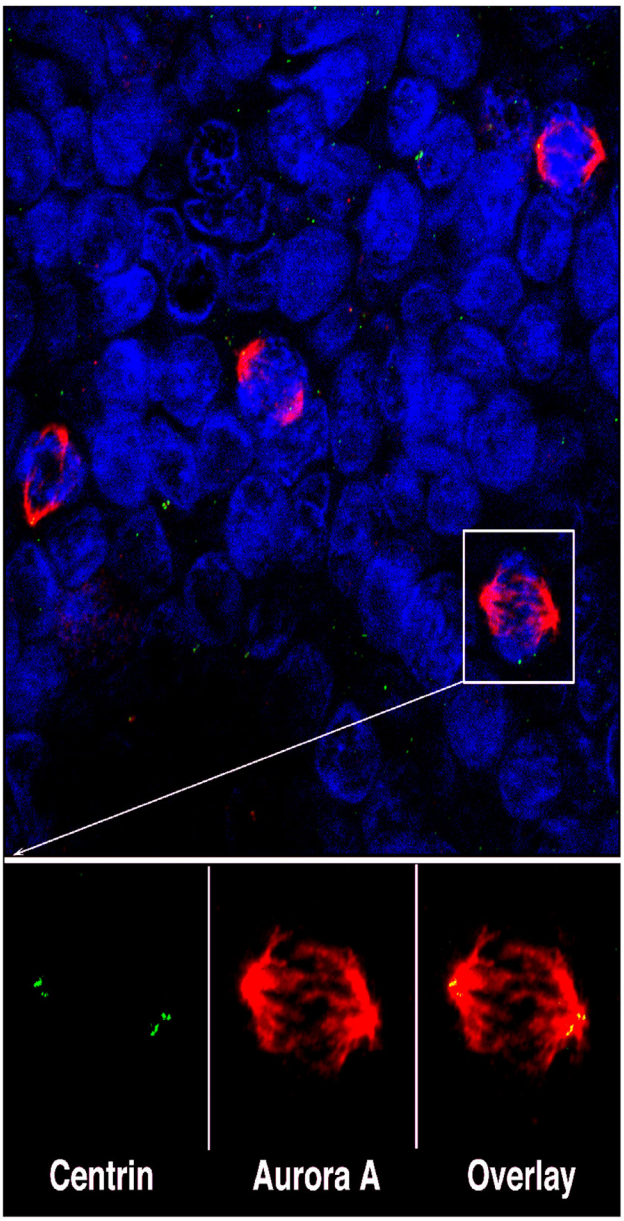

FIGURE 1 | Aurora-A localization in human breast cancer cells: representative image of mitotic figures from MCF-7 breast cancer cell line engineered to express the Raf-1 oncoprotein (vMCF-7^{Raf-1}). Centrosomes are labeled in green with 20H5 centrin mouse monoclonal antibody (Mayo Clinic), mitotic spindles are labeled in red with Aurora-A rabbit polyclonal antibody (Abcam, Cambridge, MA, USA) and nuclei are labeled in blue with DAPI (Thermo Fischer Scientific, Rockford, IL, USA). Centrosomal co-localization of Aurora-A is observed in the overlay image (yellow).

transcriptional factor that in turn induces Aurora-A gene expression. p53 deficiency also increases Aurora-A expression through the downregulation of Fbw7α, a key component of e3 ligase of Aurora-A involved in its degradation (27). A separate study demonstrated that highly invasive primary tumors harboring mutant p53 also exhibited Aurora-A overexpression (28). Taken together, these findings strongly demonstrate that Chfr and p53 are key negative regulators of Aurora-A kinase signaling, and their loss of function promotes a growth advantage for cancer cells through increased expression of Aurora-A.

AURORA-A PROMOTES CENTROSOME AMPLIFICATION, ANEUPLOIDY, AND CIN

Aurora-A is overexpressed in a variety of solid tumors, indicative of the critical role that aberrant Aurora-A kinase activity plays in tumorigenesis. Several studies demonstrate the causative function of Aurora-A overexpression in promoting cell transformation *in vitro* and tumor growth *in vivo* employing NIH 3T3 cells and Rat1 fibroblasts (17, 29). The majority of research aims to identify the mechanisms responsible for Aurora-A-induced tumorigenesis has focused on the role of Aurora-A kinase in the control of centrosome duplication and mitosis. Accurate centrosome duplication plays a central role in the maintenance of a normal diploid karyotype. In order to give rise to a bipolar mitotic spindle responsible for the equal segregation of chromosomes to dividing cells, the centrosome must be duplicated once, and only once during each cell cycle (30). Cell cycle checkpoints are essential surveillance mechanisms that guarantee the coordination between centrosome duplication, DNA replication, and mitosis during cell cycle progression (31). Abrogation of cell cycle checkpoints in cancer cells induces centrosome amplification, a pathological condition characterized by the presence of more than two centrosomes within a cell. Centrosome amplification may result from inactivation of the G1/S checkpoint leading to centrosome overduplication or from abrogation of the G2/M checkpoint leading to cytokinesis failure, endoreduplication, and consequent centrosome accumulation (2). Centrosome amplification due to cytokinesis failure is exacerbated in cancer cells lacking the "G_1 phase post-mitotic checkpoint" that is dependent on the integrity of p53/Rb axis (32–34). One of the major consequences of centrosome amplification is the formation of multipolar or pseudo-bipolar mitotic spindles that will result in unequal chromosome segregation and aneuploidy (35–37). Aneuploidy is characterized by gains and/or losses of whole chromosomes during cell division and occurs in early stages of tumor development, playing a critical role in both tumorigenesis and tumor progression (38). Significantly, while aneuploidy represents the state of an aberrant karyotype, the continuous generation of chromosome variations in cancer cells is defined as CIN that will ultimately drive genetic heterogeneity, tumor recurrence, and poor outcome (39). Several lines of evidence have established that centrosome amplification drives CIN and genetic heterogeneity in aneuploid tumors (40–42). Elegant studies have demonstrated that deregulated expression of Aurora-A is functionally linked to centrosome amplification and CIN (43–45). The major mechanism by which aberrant Aurora-A kinase activity induces centrosome amplification and CIN is through cytokinesis failure and consequent multinucleation leading to centrosome accumulation (46). Aurora-A induces cytokinesis failure and centrosome amplification mainly through its interaction with key tumor suppressor gene products that control cell cycle checkpoints, centrosome duplication, and chromosomal stability. Aurora-A phosphorylates the tumor suppressor p53 on Ser^{215} residue, abrogating the DNA-binding and transactivation activity of p53 that results in the inhibition of the downstream target gene p21 involved in the control of centrosome duplication (47). Moreover, Aurora-A-mediated phosphorylation of p53 on Ser^{315} residue will increase the affinity of p53 with Mdm2 that in turn will promote p53 degradation (48). The tumor suppressors BRCA1 and BRCA2 play a central role in the maintenance of chromosomal stability and germline mutations in BRCA1 and BRCA2 genes have been detected in approximately 90% of hereditary breast/ovarian cancers (49). Specifically, BRCA1 monitors the physical integrity of DNA following genotoxic stress and coordinates DNA replication with centrosome duplication cycle (50). It has been demonstrated that Aurora-A directly binds to BRCA1 and phosphorylates it on Ser^{308} residue. Deregulated Aurora-A-mediated BRCA1 phosphorylation on Ser^{308} residue induces abrogation of the G2/M checkpoint leading to centrosome amplification and CIN (51). Moreover, Aurora-A is required to activate polo-like kinase 1 (PLK1) that plays a key role in promoting centrosome duplication and mitotic entry (52, 53). These findings indicate that Aurora-A overexpression induces aberrant Plk1 activity that will drive centrosome amplification, improper segregation of chromosomes, CIN, and tumorigenesis. Leontovich et al. uncovered a novel mechanism by which Cyclin-A/Cdk2 oncogenic signaling favors Aurora-A centrosomal localization that in turn induces centrosome overduplication in breast cancer cells (54). Taken together, these studies strongly demonstrate that deregulated Aurora-A kinase activity induces centrosome amplification in cancer cells through different mechanisms and results in the development of CIN, a driving force for genetic heterogeneity and tumor progression.

NON-MITOTIC FUNCTION OF AURORA-A IN TUMORIGENESIS

Although Aurora-A-mediated centrosome amplification and CIN represents a well-recognized mechanism that promotes oncogenic transformation, the kinase activity of Aurora-A is essential to acquire a transformed phenotype regardless of the induction of centrosome amplification (55). These findings led to the discovery that Aurora-A kinase also phosphorylates proteins unrelated to centrosome function that play a central role in tumorigenesis. Taga et al. showed in U2OS human osteosarcoma cells that Aurora-A induces phosphorylation of Akt and mTOR oncoproteins that is required to increase U2OS tumorigenicity (56). In agreement with these results, aberrant Aurora-A kinase activity promotes resistance to cisplatin, etoposide, and paclitaxel-induced apoptosis by phosphorylating Akt in wild-type p53 ovarian cancer cells (57). Other studies have revealed the direct role of Aurora-A kinase activity in mediating cancer

cell motility and distant metastases. Aurora-A promotes breast cancer metastases by dephosphorylation of cofilin and activation of cofilin–F-actin pathway, which accelerates actin reorganization and polymerization (58). Furthermore, inhibition of phosphatidylinositol 3-kinase (PI3K) oncogenic signaling blocked Aurora-A-mediated cofilin dephosphorylation, actin reorganization, and cell migration. These results uncover a novel crosstalk between PI3K signaling and Aurora-A in tumor progression. In esophageal squamous cell carcinoma cells, Aurora-A overexpression induces cell migration and invasion as well as secretion and expression of matrix metalloproteinase-2 (MMP-2). This mechanism is mediated by Aurora-A-induced phosphorylation of p38 MAPK and Akt protein kinases (59). Aberrant Aurora-A kinase activity also induces activation of Rap1, a member of the Ras family of small GTPases, leading to the development of distant metastases originating from oral cavity squamous cell carcinomas (60). Du and Hannon demonstrated that Aurora-A kinase activity inhibits the function of Nm23-H1 protein that is involved in the suppression of distant metastases, facilitating tumor progression (61).

Moreover, recent studies revealed a novel function of Aurora-A in the progression of solid tumors through activation of epithelial–mesenchymal transition (EMT) and stemness reprograming. Cammareri et al. demonstrated that Aurora-A overexpression is restricted in colorectal cancer stem cells (CR-CSC), and Aurora-A inhibition restored chemosensitivity and compromised the tumor initiating ability of CR-CSC to form tumor xenografts in immunocompromised mice (62). The causative role of Aurora-A overexpression in promoting EMT and tumor progression through stabilization of Snail transcription factor has been shown in head and neck cancer cells (63). Significantly, we have defined for the first time the essential role of Aurora-A in promoting breast cancer progression through activation of EMT and the genesis of breast cancer stem cells responsible for the onset of distant metastases (24). Moreover, Aurora-A-induced EMT and onset of distant metastases was functionally linked to SMAD5 and SOX2 expression, two master transcription factors involved in the development of EMT, tumor self-renewal, and an invasive, basal-like phenotype. In the same study, we have uncovered the causative role of Aurora-A overexpression in inducing expansion of cancer stem cells through impairment of asymmetric divisions. These results are in agreement with a previous study showing that a phosphorylation cascade triggered by the activation of Aurora-A kinase is responsible for the asymmetric localization of Numb during mitosis (64). Taken together, these studies highlight an essential role of Aurora-A kinase in driving tumor progression by modulating the activity of key oncogenic pathways involved in cell migration, chemoresistance, tumor initiating ability, and onset of distant metastases.

AURORA-A AS A NOVEL BIOMARKER PROGNOSTIC OF POOR CLINICAL OUTCOME

Several studies have shown that Aurora-A kinase is overexpressed in a variety of tumors, suggesting that Aurora-A may represent a promising prognostic biomarker. Reiter et al. reported that increased expression of Aurora-A in head and neck squamous cell carcinomas was significantly associated with shorter disease-free and overall survival of patients (65). Likewise, Aurora-A overexpression is associated with centrosome amplification and shorter survival in an extensive proportion of ovarian tumors (66, 67). Gastrointestinal tumors also display deregulation of Aurora-A expression that is linked to high risk of recurrence and tumor progression. Employing tissue microarrays from a retrospective cohort of 343 patients with colorectal cancer liver metastases, Goos et al. showed that Aurora-A levels were increased in liver metastatic lesions compared to corresponding primary tumors and was associated with poor clinical outcome (68). Wang et al. showed that Aurora-A overexpression was an independent prognostic marker of poor survival in gastric cancer patients without lymph node metastases (69). Samaras et al. performed a comparative immunohistochemical analysis of Aurora-A and Aurora-B expression in 40 patients with primary glioblastomas to identify possible correlations with Ki-67 proliferation index and clinical outcomes (70). While Aurora-A was overexpressed in glioblastomas with high Ki-67 expression and was associated with poor survival, Aurora-B expression was not correlated with Ki-67 expression and patient survival. Aurora-A overexpression has also been established as a valuable biomarker prognostic of poor clinical outcome in breast carcinomas. Nadler et al. demonstrated in a tissue microarray containing primary breast tumor tissue from 638 patients with 15-year follow-up that aberrant expression of Aurora-A, but not Aurora-B, was an independent prognostic marker strongly correlated with decreased survival (71). High Aurora-A expression was also associated with high nuclear grade and elevated HER-2/neu and progesterone receptor expression. In 48 cases of operable triple-negative breast tumors, Yamamoto et al. established that basal-like subtype was significantly associated with high levels of Aurora-A and shorter disease-free and overall survival compare to non-basal-like breast tumors (72). Using microarray-based gene expression data from three independent cohorts of 766 node-negative breast cancer patients, Siggelkow et al. demonstrated that patients harboring high Aurora-A expression had a shorter metastasis-free survival in the molecular subtype estrogen receptor-positive (ER+)/HER2− carcinomas, but not in ER−/HER2− or HER2+ carcinomas (73). A recent study reported, in a cohort of 426 patients with primary breast cancer, that elevated expression of Aurora-A and SURVIVIN, together with *BTAK* gene amplification, is correlated with increased CIN and shorter survival (74). Taken together, these studies highlight Aurora-A as a novel, independent prognostic biomarker of poor clinical outcome that could identify patients at high risk of tumor recurrence or progression.

PHARMACOLOGIC TARGETING OF AURORA-A KINASE ACTIVITY IN CANCER THERAPY

In the last decade, at least 13 different inhibitors of the Aurora kinases have been evaluated in phase I clinical trials in patients with various hematologic and solid tumor malignancies. Nearly all of the initial agents studied were pan-inhibitors of Aurora-A, -B,

and -C, and several of them furthermore inhibited other kinases, such as bcr–abl (T135I), Flt3, VEGFR2, and JAK 2/3. Some of these trials were suspended and not completed or published. Some inhibitors have not continued beyond phase I evaluation due to significant toxicities at clinically effective doses or limited clinical antitumor activity. Only a limited number of these pan-Aurora and multi-kinase inhibitors have been pursued in phase II clinical trials (AT-9283, MK-0457, ENMD-2076, PHA-739358). Three of the Aurora kinase inhibitors developed were selective for Aurora-A (MLN 8054, MLN 8237, TAS-119). Of all the inhibitors, only MLN 8237 (alisertib) has proceeded to phase III evaluation.

The first of the selective Aurora-A kinase inhibitors to enter into human studies was MLN 8054. In Phase I dose escalation studies in patients with advanced solid cancers, the observed dose limiting toxicity (DLT) was reversible somnolence, attributed to $GABA_A$ α-1 benzodiazepine off-target binding (75, 76). With the aim of improving the therapeutic window, the chemical structure of the molecule was modified, and of potential new agents, MLN 8237 (alisertib) was selected for further development based on preclinical evidence demonstrating its increased potency in Aurora-A enzymatic inhibition, reduced degree of brain partitioning, and while $GABA_A$ binding potency was comparable to MLN 8054, alisertib displayed a greater selectivity ratio of Aurora-A inhibition to $GABA_A$ α-1 benzodiazepine site binding affinity (77).

In 2007, the first clinical trial opened to evaluate alisertib, an orally administered, small molecule inhibitor that is selective for Aurora-A kinase. To date, well over 1000 patients with hematological or solid tumor malignancies have participated in clinical trials with the agent as monotherapy or in combination with chemotherapy or other targeted agents (78, 79). In the original phase I trials, different formulations of the drug, doses, and schedules were evaluated (80, 81). Stomatitis and neutropenia were the most common DLTs consistent with its antiproliferative effect. Somnolence was evident in patients receiving once daily dosing of alisertib at the highest dose levels; however, the frequency and severity of these episodes were reduced with twice daily dosing of alisertib at lower individual doses, which reduced peak plasma levels while maintaining overall systemic exposures. Other common low-grade toxicities included alopecia, nausea, diarrhea, anemia, and fatigue. The recommended phase II dose was 50 mg twice daily on days 1–7 of a 21-day cycle, and the preferred formulation was the enteric-coated tablet; both were confirmed in the industry-sponsored study of alisertib as monotherapy in patients with advanced solid tumor malignancies (82). Encouraging clinical activity was demonstrated in this trial. In the cohort of heavily pre-treated women with hormone receptor-positive metastatic breast cancer ($n = 26$), 23% had an objective response (complete or partial response) and 31% achieved stable disease for at least 6 months, resulting in a clinical benefit rate of 54%. Median PFS was 7.9 months. In the chemotherapy-refractory, relapsed small cell lung cancer (SCLC) cohort ($n = 12$), a response rate of 25% was observed with a median duration of response of 4.3 months. A phase II trial of alisertib alone or combined with paclitaxel for second-line therapy of SCLC is currently active (NCT02038647). Based on promising activity observed in relapsed/refractory peripheral T-cell lymphoma (83, 84), a phase III clinical trial of alisertib versus treatment of investigator's choice (NCT01482962) was pursued but subsequently terminated enrollment at a pre-specified interim analysis due to projections that the study was unlikely to meet the primary endpoint of superior PFS.

An alternative 28-day regimen with alisertib given days 1–3, 8–10, and 15–17 was studied in combination with paclitaxel in breast and ovarian cancer models, and it is associated with equivalent drug levels, decreased incidence of dose limiting neutropenia with negligible compromise to efficacy (85). The safety and tolerability of this schedule in combination with fulvestrant

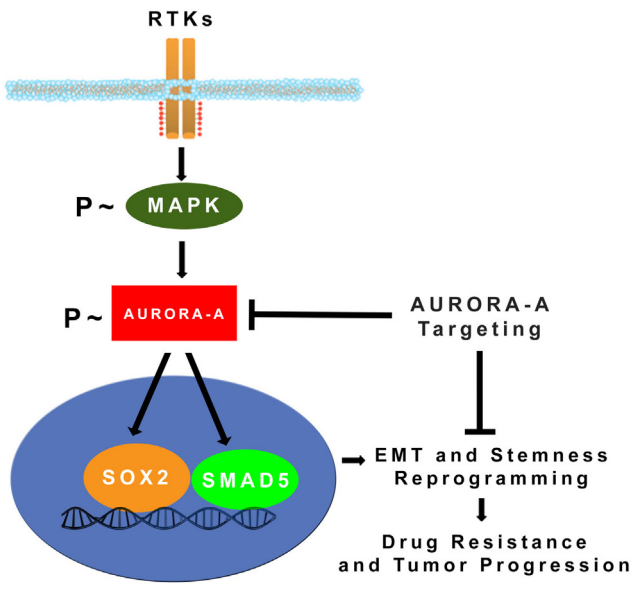

FIGURE 2 | MAPK-induced activation of Aurora-A kinase promotes EMT, stemness, and tumor progression: constitutive activation of MAPK oncogenic signaling during tumor growth leads to stabilization and accumulation of Aurora-A kinase. Aberrant Aurora-A kinase activity induces activation of SMAD5 and SOX2 transcription factors that in turn will orchestrate EMT and stemness reprograming leading to drug resistance and tumor progression (24). Pharmacologic targeting of Aurora-A kinase activity can be effective for eliminating highly invasive cancer stem cells and defeat tumor progression.

TABLE 1 | Aurora kinase inhibitors in clinical trials.

	Inhibitor commercial name	Clinical trials
Pan-Aurora inhibitors	VX-680/MK-0457 (Vertex/Merck)	Phase II (terminated due to toxicity)
	Tozasertib	Phase II
	PHA-739358 (Pfizer/Nerviano)	
	Danusertib	
	PHA-680632 (Pfizer/Nerviano)	Phase I
	CYC-116 (Cyclacel)	Phase I
	SNS-314 (Sunesis)	Phase I
	R763 (Rigel)	Phase I
	AMG-900 (Amgen)	Phase I
	AT-9283 (Astex)	Phase II
	PF-03814375 (Pfizer)	Phase I
	GSK1070916 (GlaxoSmithKline)	Phase I
Aurora-A inhibitors	MLN8237 (Millennium)	Phase II
	ENMD-2076 (EntreMed)	Phase II
	MK-0457 (Vertex)	Phase II

is currently being explored in an ongoing phase I trial in patients with hormone receptor-positive, advanced breast cancer (NCT 02219789).

TAS-119 is the only other selective Aurora-A kinase inhibitor to enter into clinical evaluation. It is being studied as monotherapy and in combination with taxane-based chemotherapy in two separate, active phase I clinical trials (NCT02134067, NCT02448589).

CONCLUSION

One of the major hallmarks of cancer is aneuploidy and the development of CIN characterized by the relentless generation of chromosome variations that will ultimately drive genetic heterogeneity and tumor progression. In normal cells, cell division is monitored by checkpoints that are safeguard mechanisms to guarantee the accurate temporal and spatial coordination of cell cycle events. Abrogation of cell cycle checkpoints induces centrosome amplification that impairs the fidelity of correct chromosome segregation, promoting aneuploidy and CIN. Members of the Aurora serine/threonine kinase family are key mitotic regulators required for the maintenance of chromosomal stability. Overexpression and aberrant activation of Aurora-A kinase has been functionally linked to oncogenic transformation mainly through development of centrosome amplification and CIN. Significantly, recent studies have also demonstrated that Aurora-A kinase mediates MAPK-induced distant metastases through activation of EMT and stemness reprograming (**Figure 2**). Taken together, these findings demonstrate that Aurora-A kinase represents a critical "*druggable target*" in cancer, controlling key oncogenic pathways associated with drug resistance and poor patient outcome. For this reason, several small molecule inhibitors of Aurora-A kinase activity have been developed and their efficacy is being tested in clinical trials (**Table 1**). Significantly, recent studies have highlighted the incremental therapeutic efficacy when combining Aurora-A inhibitors with conventional anti-cancer drugs to restore chemosensitivity and inhibit tumor progression, a strategy expected to further build on the clinical benefit potential of Aurora-A inhibition.

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REVIEW
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Functional Significance of Aurora Kinases–p53 Protein Family Interactions in Cancer

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Aurora kinases play critical roles in regulating spindle assembly, chromosome segregation, and cytokinesis to ensure faithful segregation of chromosomes during mitotic cell division cycle. Molecular and cell biological studies have revealed that Aurora kinases, at physiological levels, orchestrate complex sequential cellular processes at distinct subcellular locations through functional interactions with its various substrates. Aberrant expression of Aurora kinases, on the other hand, cause defects in mitotic spindle assembly, checkpoint response activation, and chromosome segregation leading to chromosomal instability. Elevated expression of Aurora kinases correlating with chromosomal instability is frequently detected in human cancers. Recent genomic profiling of about 3000 human cancer tissue specimens to identify various oncogenic signatures in The Cancer Genome Atlas project has reported that recurrent amplification and overexpression of Aurora kinase-A characterize distinct subsets of human tumors across multiple cancer types. Besides the well-characterized canonical pathway interactions of Aurora kinases in regulating assembly of the mitotic apparatus and chromosome segregation, growing evidence also supports the notion that deregulated expression of Aurora kinases in non-canonical pathways drive transformation and genomic instability by antagonizing tumor suppressor and exacerbating oncogenic signaling through direct interactions with critical proteins. Aberrant expression of the Aurora kinases–p53 protein family signaling axes appears to be critical in the abrogation of p53 protein family mediated tumor suppressor pathways frequently deregulated during oncogenic transformation process. Recent findings reveal the existence of feedback regulatory loops in mRNA expression and protein stability of these protein families and their consequences on downstream effectors involved in diverse physiological functions, such as mitotic progression, checkpoint response pathways, as well as self-renewal and pluripotency in embryonic stem cells. While these investigations have focused on the functional consequences of Aurora kinase protein family interactions with wild-type p53 family proteins, those involving Aurora kinases and mutant p53 remain to be elucidated. This article presents a comprehensive review of studies on Aurora kinases–p53 protein family interactions along with a prospective view on the possible functional consequences of Aurora kinase–mutant p53 signaling pathways in tumor cells. Additionally, we also discuss therapeutic implications of these findings in Aurora kinases overexpressing subsets of human tumors.

Keywords: Aurora kinases, p53 tumor suppressor protein family, chromosome instability, centrosome amplification, pluripotency, tumorigenesis

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INTRODUCTION

Gain-of-function alterations in the Aurora kinase protein family member, Aurora kinase-A (AURKA), due to amplification and/or overexpression of the gene- and loss-of-function changes in the TP53 tumor suppressor protein have been associated with multiple cellular phenotypes of similar nature, such as centrosome amplification, override of spindle assembly, and DNA damage checkpoint response, aneuploidy, and cellular transformation. Induction of such shared cellular phenotypes consequent to AURKA overexpression or functional inactivation of TP53 as well as reported localization of the two proteins at the centrosomes indicate that AURKA and TP53 (hereafter referred to as Aurora-A and p53) are involved in overlapping signaling pathways regulating the abovementioned cancer-associated aberrant cellular phenotypes through direct or indirect functional interactions (1–5). Evidence in support of this concept first became available following demonstration that p53 could suppress Aurora-A's oncogenic potential through physiological interaction in transactivation-independent manner in mammalian cells (6). Similarly, *Xenopus* p53 was shown to inhibit Aurora-A kinase activity, indicating that the inhibitory role of p53 on Aurora-A kinase enzyme activity is conserved among vertebrates (7). Later studies have revealed that p53, besides inhibiting the kinase activity of Aurora-A through direct interaction, also regulates Aurora-A function in transactivation-dependent manner, as discussed below.

In addition to the findings mentioned above, a number of studies have identified Aurora kinases regulating p53 function through phosphorylation-mediated posttranslational modification of either p53 protein directly or a p53 interacting protein at multiple residues with each phosphorylation event having distinct functional consequence. Aurora-A phosphorylates p53 at serine 315, facilitating MDM2-mediated p53 ubiquitination and degradation (8), whereas phosphorylation of serine 215 inhibits p53 DNA-binding and transactivation function (9). These findings demonstrated that Aurora-A phosphorylation of p53 negatively regulates p53 tumor suppressor functions, resulting in abrogation of DNA damage checkpoint and induction of cell death responses in Aurora-A overexpressing cells. As a consequence, Aurora-A overexpressing cancer cells with wild-type p53 acquire cellular phenotypes associated with p53 loss-of-function mutant harboring cancer cells. A more recent finding of a novel Aurora-A phosphorylation residue, serine 106 of p53, was, however, reported to have an opposing effect on p53 stability compared with the destabilization effect of Aurora-A-mediated phosphorylation of p53 at serine 315. Phosphorylation of p53 serine 106 was shown to inhibit the interaction of p53 with MDM2 and prolong the half-life of p53 protein (10). Physiological significance of Aurora-A-mediated p53 phosphorylation at serine 106 *in vivo* and its functional implications in Aurora-A overexpressing tumor cells remain unknown. The possibility of enhanced p53 protein stability in Aurora-A overexpressing tumor cells appears intriguing since steady-state levels of Aurora-A and p53 proteins have been reported to be inversely correlated in most human tumors. Molecular characterization studies have shown that serine 215 phosphorylation is associated with loss of serine 33 phosphorylation of p53, mediated by p38 critical for p53 activation stabilization and induction of apoptosis, indicating that Aurora-A mediates cross-talk between N- and C-terminal posttranslational modifications of p53 (11, 12). In addition, Aurora-A also indirectly compromises p53 function by phosphorylating positive and negative regulators of p53, such as hnRNPK and MDM2 proteins, respectively. The RNA-binding protein, such as hnRNPK, is a p53 transcriptional cofactor that promotes gene expression in response to DNA damage and is also a target of MDM2 (13, 14). While Aurora-A-mediated hnRNPK phosphorylation at serine 379 disrupts its interaction with p53 and impairs DNA damage-induced gene expression, MDM2 phosphorylation at serine 166 enhances its protein stability and in turn destabilizes p53 (15–17). These findings demonstrate that Aurora-A is involved in regulating p53 downstream signaling negatively affecting growth arrest and apoptotic response pathways.

Aurora-B has also been shown to interact with and phosphorylate p53 at multiple residues in DNA-binding domain. Similar to the effect of Aurora-A phosphorylation on p53 activity and stability, Aurora-B phosphorylations of p53 at serine 269 and threonine 284 inhibit p53 transactivation activity, whereas phosphorylations at serine 183, threonine 211, and serine 215 accelerate the degradation of p53 through polyubiquitination-mediated proteasome pathway (18, 19). However, these studies have been performed with phosphor mutants of p53 under conditions of ectopic expression in cells and thus physiological relevance of identical *in vivo* phosphorylations have not been well validated. Further investigations of endogenous protein modifications are required to verify the role of Aurora-B-mediated p53 phosphorylations *in vivo* and to determine how Aurora-A and Aurora-B may be coordinately regulating p53 function through the cell cycle. It is worth noting that exogenously expressed p53 colocalizes with Aurora-B at centromeres during mitosis. This observation may be biologically significant since several spindle assembly checkpoint (SAC) kinases such as MPS1/TTK, BUB1, and BUBR1, localized at kinetochores, have been reported to functionally interact with p53 in activating spindle assembly and postmitotic checkpoint response pathways (20–23). In view of these findings and those demonstrating Aurora kinases regulating functions of p53 family proteins, it is likely that varying levels of Aurora kinases in tumor cells influence the extent of deregulations in checkpoint response pathway activation downstream of p53 family proteins in tumor cells. We discuss the role of Aurora kinases–p53 protein family signaling axis in SAC response pathway later in this review.

Aurora-A involvement in regulating p73 function first became evident from a study in which Aurora-A inhibitor treatment or knockdown of Aurora-A in p53-deficient cells induced p73-mediated expression of apoptosis-related genes and also cell death (16). Further investigation revealed that Aurora-A directly interacts with and phosphorylates p73 at serine 235 in the DNA-binding domain, an equivalent site of serine 215 in p53, resulting in loss of its DNA-binding and transactivation activity. As a result, cells become resistant to DNA damage-induced cell death (24). Importantly, this study uncovered that Aurora-A phosphorylation of p73 leads to the formation

of a large molecular complex that includes the chaperon protein Mortalin promoting translocation of the Mortalin–p73 complex into cytoplasm. Similar cytoplasmic distribution of Aurora-A phosphorylated p53 at serine 215 in a complex with Mortalin was observed as well. As a corollary to this finding, cytoplasmic distribution of p73 was found to correlate with Aurora-A expression levels in human primary pancreatic cancer tissues. Moreover, consistent with the earlier findings that p73 deficiency causes relaxation of the SAC reflected in the mislocalization of BUB1 and BUBR1 at kinetochores and reduced BUBR1 kinase activity (25–27), Aurora-A phosphorylation of p73 in a constitutive manner was found to facilitate accelerated mitotic progression and exit accompanied with relaxation of SAC due to premature dissociation of the MAD2–CDC20 complex in proliferating cells *in vitro*. SAC inactivation correlated with significant increase in multinucleated cells. These findings indicate that the mitotic checkpoint functions of p53 family proteins are regulated in a complex manner involving Aurora kinase-mediated posttranslational modifications during mitotic progression. It is currently unknown whether p73 reciprocally controls Aurora-A kinase function and if Aurora-B and Aurora-C also regulate p73 function.

Along with the discovery of crosstalk between Aurora kinases and p53 family proteins, there is growing evidence that these protein complexes directly or indirectly participate in various cellular processes and inappropriate activation of Aurora kinases can have dominant-negative effects on the phenotypes of normal cells involving pathways regulated by a variety of proteins functionally interacting with p53 protein family (**Figure 1**; **Table 1**). In the following sections, we summarize the current knowledge of Aurora kinases–p53 protein family signaling cascades relevant to the regulation of posttranslational modifications and stability of proteins, activity and integrity of centrosomes, checkpoint pathways in normal and aberrant mitosis, as well as protein–protein interactions and transcription and translation of genes involved in the development of pluripotent embryonic stem cells (ESC) and cancer stem cells (CSC), as outlined in the schematic overview diagram in **Figure 2**.

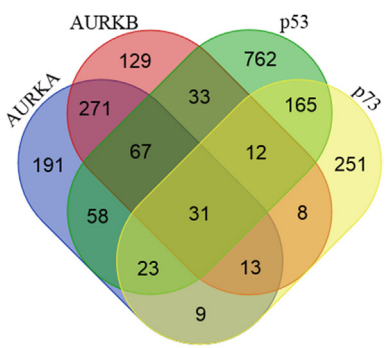

FIGURE 1 | Proteins interacting with Aurora-A, Aurora-B, p53, and p73. Venn diagram showing the number of shared and unique proteins interacting with Aurora-A, Aurora-B, p53, and p73. Protein–protein interaction data were downloaded from the BioGRID (v3.4) and STRING (v9.1) databases.

MECHANISM OF DOWNREGULATION OF AURORA KINASES BY p53

In addition to direct inhibition of Aurora-A by p53 *via* protein–protein interaction, p53 has been shown to downregulate Aurora-A expression, kinase activity and stability through its binding to Aurora-A promoter or transactivation of its target genes including p21, Gadd45a, and Fbxw7α. Genome-wide chromatin occupancy of p53 analyzed by chromatin immunoprecipitation-seq (ChIP-seq) following activation with non-genotoxic molecules and genotoxic chemotherapeutic drugs revealed *AURKA* gene promoter as one of the novel p53 target sequences and that direct p53 binding to the promoter of *AURKA* gene repressed expression in MCF-7 and HCT-116 cells (28). This study also found that STAT3 binds to *AURKA* promoter and antagonizes p53-mediated repression of *AURKA*. Intriguingly, a recent study has shown that Aurora-A promotes STAT3 activity through regulating expression and phosphorylation levels of JAK2 in gastric and esophageal cancers (29), indicating the existence of negative feedback regulation of p53 function by Aurora-A–JAK2–STAT3 axis. These results suggest that the combination of Aurora-A and JAK2 inhibitors with p53 activators might be an effective therapeutic approach for the treatment of cancer. Both p21 and Gadd45a are transcriptionally activated by p53 upon DNA damage and play important roles in DNA repair and cell cycle checkpoint response. The E2F family transcription factor, E2F3 is known to be involved in the transactivation of Aurora-A gene expression during G2–M cell cycle progression (30). Induction of the cyclin-dependent kinase inhibitor, p21 leads to inhibition of Cdk kinase activity resulting in the maintenance of RB1 in hypophosphorylated state in a complex with E2F3, thereby impairing activation of Aurora-A gene expression, an indirect downstream effect of p53–p21 signaling axis. It is noteworthy that Aurora-B phosphorylates RB1 at serine 780, a known inhibitory phosphorylation site for Cdk4. Thus, deregulation of Aurora-B might lead to Aurora-A overexpression through direct downregulation of both p53 and RB1 functions. In fact, co-occurrence of increased gene expression of both Aurora-A and Aurora-B is observed in some human tumors. On the other hand, Gadd45a inhibits Aurora-A kinase activity *via* direct interaction to prevent cells from Aurora-A-induced centrosome amplification and aborted cytokinesis (31). These results indicate that cooperative inhibition of Aurora-A activity by p53 and Gadd45a is important for cells to maintain centrosome number and chromosomal/genomic stability.

Besides regulating Aurora kinase function through transcription-dependent and -independent mechanisms, p53 also downregulates Aurora-A activity by modulating its degradation pathway. Fbxw7α is a p53-dependent haploinsufficient tumor suppressor protein and a component of the SCF-like ubiquitin ligase complex that targets both Aurora-A and Aurora-B for proteasome degradation (32–34). Fbxw7α is frequently mutated or downregulated in tumors. Importantly, Fbxw7α cooperates with PTEN to regulate Aurora-A degradation *via* the PI3K/AKT/GSK3β pathway and Fbxw7α also preferentially degrades active Aurora-A (33, 35). It has been demonstrated that Aurora-A-mediated centrosome amplification and subsequent induction

TABLE 1 | List of proteins interacting with Aurora–p53 family protein complex represented in Venn diagram in Figure 1.

Combination	Qty	Interacting proteins
AURKA/AURKB/p53/p73	31	ATM, BCL2, BIRC5, BRCA2, CCNA2, CCNB1, CCND1, CCNG1, CDC20, CDC25A, CDC25C, CDK1, CDK2, CDK4, CDKN1A, DDB1, GADD45A, HSPA9, LRPPRC, MTOR, MYL9, PCNA, PTEN, PTTG1, RASSF1, RPS27A, SUMO1, TP63, UBC, UBE2I, XPO1
AURKA/AURKB/p53	67	BARD1, BIRC6, BUB1, CDC14A, CDC14B, CDK5, CENPA, CEP55, DDX5, ECT2, FBXW7, FTH1, FZR1, HNRNPA1, HNRNPU, HSP90AA1, HSP90AB1, HSPA1A, HSPA5, IQGAP1, IRS4, MAP9, NCL, NFKBIA, NINL, NPM1, OFD1, PARP1, PBK, PLK1, PLK3, PPP1CA, PPP1CC, PPP3CA, PRRC2C, PSMB3, PSMC3, PSMC5, PSMD10, PSMD11, PSMD4, PSMD6, PSME3, RPS16, RPS27, RPS4X, RRM2, SETD1A, SMARCB1, TCEAL4, TK1, TOP2A, TOP2B, TP73, TTK, TUBA1A, TUBA1C, TUBA4A, TUBB, TUBB2A, TUBG1, UBA52, UBB, UBE2D1, UBE2N, YY1, YY2
AURKA/AURKB/p73	13	BUB1B, CCNA1, CHFR, E2F2, E2F3, FLT3, HIST1H3C, LATS2, MAD2L1, SASS6, TK2, TP53, TSPO
AURKA/p53/p73	23	AKT1, AURKB, CASP1, CDKN2A, CHUK, CSNK2A1, DICER1, EGFR, ESR1, GSK3B, HDAC2, HRAS, IGF2BP1, IKBKB, MDM2, MYC, NEDD8, PIK3CA, PML, RPL11, RPS19, TAF9, WWOX
AURKB/p53/p73	12	AURKA, BRCA1, CHEK1, CHEK2, DNMT1, EP300, EZH2, H2AFX, HDAC1, MAPK8, PPP1R13L, RB1
AURKA/p53	58	ALB, BTRC, CELA2B, CEP120, CEP128, CEP135, CEP152, CSNK1D, CSNK1E, DCAF7, DGCR14, EEF1A1, EEF2, HAUS1, HNRNPA2B1, HNRNPK, HSPA2, HSPA8, IGF2BP3, ITPKC, KLF4, KRAS, LYZ, MFAP4, MRPL24, MRPS22, NFKB1, NIN, NME1, NRAS, PCMT1, PDCD5, PDCD6, REL, RFC4, RPL12, RPL23, RPL27, RPL30, RPLP0, RPLP2, RPS10, RPS14, RPS3, RPS3A, RPS6, SETD2, SIRT7, SKP1, SRPK1, TFAP2A, TNRC6C, TRIM28, TUBB4B, VHL, YBX1, YWHAE, YWHAG
AURKB/p53	33	ABR, CCDC8, CUL7, DOCK7, DTL, GIGYF2, HDAC5, HDAC9, HERC2, MOGS, MRPS27, MYBBP1A, MYLK, NOC2L, PHKB, PRKDC, RANBP2, RAVER1, RPS25, SKP2, SMARCC1, SNW1, SUMO2, SUMO3, TBC1D4, TUBA8, UBR4, UBR5, UFD1L, VIM, VRK1, WEE1, ZWINT
AURKA/p73	9	AZI1, CCNE1, CDH13, CTNNB1, FUS, MYCN, OAZ1, PRKACA, PSRC1
AURKB/p73	8	ANKRD17, AURKC, CDKN1B, DSN1, GNB2L1, LATS1, STAG1, STK3

of aneuploidy is mediated in part through dysfunction of p53–Fbxw7α axis, commonly detected in human tumors and also in mouse models (33, 36). It is relevant in this context to mention that synthetic lethal screening of protein interacting with N-Myc in N-Myc amplified neuroblastoma has identified that Aurora-A stabilizes N-Myc by directing a K48 to K63/K11 switch in its ubiquitylation by Fbxw7α (37). Although this interaction was reported to be independent of Aurora-A kinase activity, recent finding have demonstrated that inhibitor of Aurora-A kinase activity can disrupt interaction between Aurora-A and Fbxw7α, leading to N-Myc destabilization and tumor regression in mouse model of N-Myc-driven neuroblastoma xenograft (38). Similarly, Aurora-B inhibitor treatment also showed profound growth inhibition and tumor regression in N-Myc-driven neuroblastoma, although the underlying mechanism of this finding remains unclear (39, 40).

Recent studies have identified an important role of microRNA functional networks in the control of gene expression and protein stability of Aurora-A and Myc involving the p53–Fbxw7α axis in neuroblastoma and other tumors. A well-characterized tumor suppressor micoRNA, let-7, regulated by p53 directly targets Aurora-A, c-Myc, N-Myc, and RAN-binding protein 2 (RANBP2). In normal cells, let-7-mediated suppression of c-Myc expression helps maintain basal low level expression of Aurora-A mRNA, while miR-25-targeted Fbxw7α regulates basal level protein expression (41–45). In p53-deficient and p53-mutant cells, these regulatory mechanisms are disrupted, and Aurora-A expression and stability are elevated. Functional genomic studies in N-Myc-amplified neuroblastoma have revealed that LIN28B RNA-binding protein promotes RAN level by directly binding to RAN mRNA and *via* RANBP2 by inhibiting let-7 expression, consequently facilitating Aurora-A activation and stabilization which in turn promote N-Myc stabilization (44). It was recently been reported that Aurora-A acts as a transactivating factor for hnRNPK, a known transcriptional cofactor of p53, to promote c-Myc expression and reciprocal c-Myc-mediated transactivation of Aurora-A gene in breast cancer stem-like cells (46). This finding on apparent absence of p53 inhibitory role in Aurora-A–c-Myc positive regulatory circuit is associated with frequent observation of centrosome amplification in N-Myc-amplified neuroblastoma cells compared to non-amplified neuroblastoma cells. Mechanistically, N-Myc directly transactivates MDM2 and Aurora-A stabilizes MDM2 by phosphorylating at Ser-166 both of which impair p53 function, resulting in centrosome amplification (17, 47, 48). Taken together, these data indicate that p53 controls Aurora-A function through multiple inhibitory signaling pathways and lack of p53 function results in deregulation of Aurora-A oncogenic signaling cascades which lead to profoundly aberrant phenotypes associated with tumor cells. Involvement of additional signaling pathways regulating centrosome activity and integrity mediated by Aurora-A–p53 interaction is discussed below.

INVOLVEMENT OF AURORA-A–p53 SIGNALING PATHWAY IN CENTROSOME ACTIVITY AND INTEGRITY

A common phenotypic change in cells with gain of Aurora-A and loss of p53 function is manifested in the form of increased

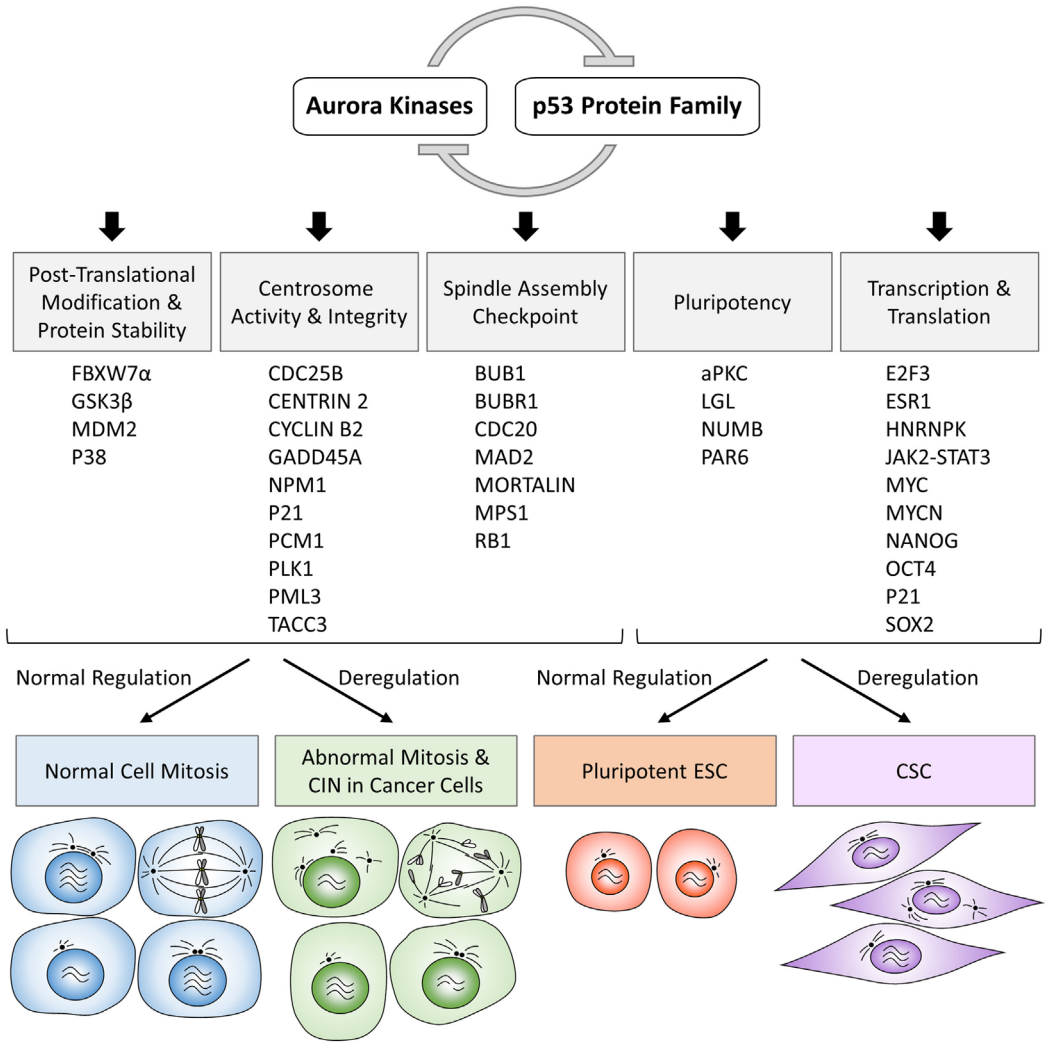

FIGURE 2 | Schematic overview diagram showing phenotypic consequences of physiologically regulated interactions in normal cells and deregulated interactions in cancer cells involving Aurora kinases–p53 protein family. CIN, chromosomal instability; ESC, embryonic stem cells; CSC, cancer stem cells.

number of centrosomes. Multiple investigations have revealed that p53 controls centrosome duplication and separation in both transactivation activity-dependent and -independent manner (**Figure 3**). In transactivation activity-dependent mechanism, p21 expression plays a key role in synchronizing DNA replication and centrosome duplication by inhibiting Cdk2/Cyclin E activity which phosphorylates Nucleophosmin/NPM1 at centrosomes to promote its dissociation from the centrosomes to allow initiation of centrosome duplication (49). On the other hand, p53 downregulates PLK4 gene expression which is essential for centriole biogenesis through regulation of phosphorylations of centrosomal protein GCP6 and STIL (50–52).

In transactivation activity-independent mechanism, centrosomal localization of p53 appears to be critical for negatively regulating centrosome biogenesis and its dissociation from centrosome appears to be sufficient to initiate centrosome duplication. p38–p53 axis was reported to play a central role in inhibition of G1–S cell cycle progression in response to loss of centrosome integrity. Centrosome perturbation caused by depletion of centrosomal proteins such as PCM1, centrobin, and TACC3 promotes the recruitment of both p38 and p53 to centrosomes and facilitate p53 phosphorylation by p38 at serine 33, which in turn transduces the inhibitory signal for cell cycle arrest by inducing p21 expression (53–55). However, the precise function of phosphorylated p53 on centrosome and the molecular mechanism of signal transduction from impaired centrosomes to the nucleus remain unknown. Regarding the mechanism of p53 dissociation from centrosome, a study has revealed that Mortalin through binding to p53 facilitates dissociation of p53 from centrosomes, which in turn results in release of the p53-mediated suppression of centrosome duplication (56). Interestingly, centrosome localization of Mortalin depends on the presence of centrosomal MPS1 kinase which is implicated in the regulation of centrosome duplication and

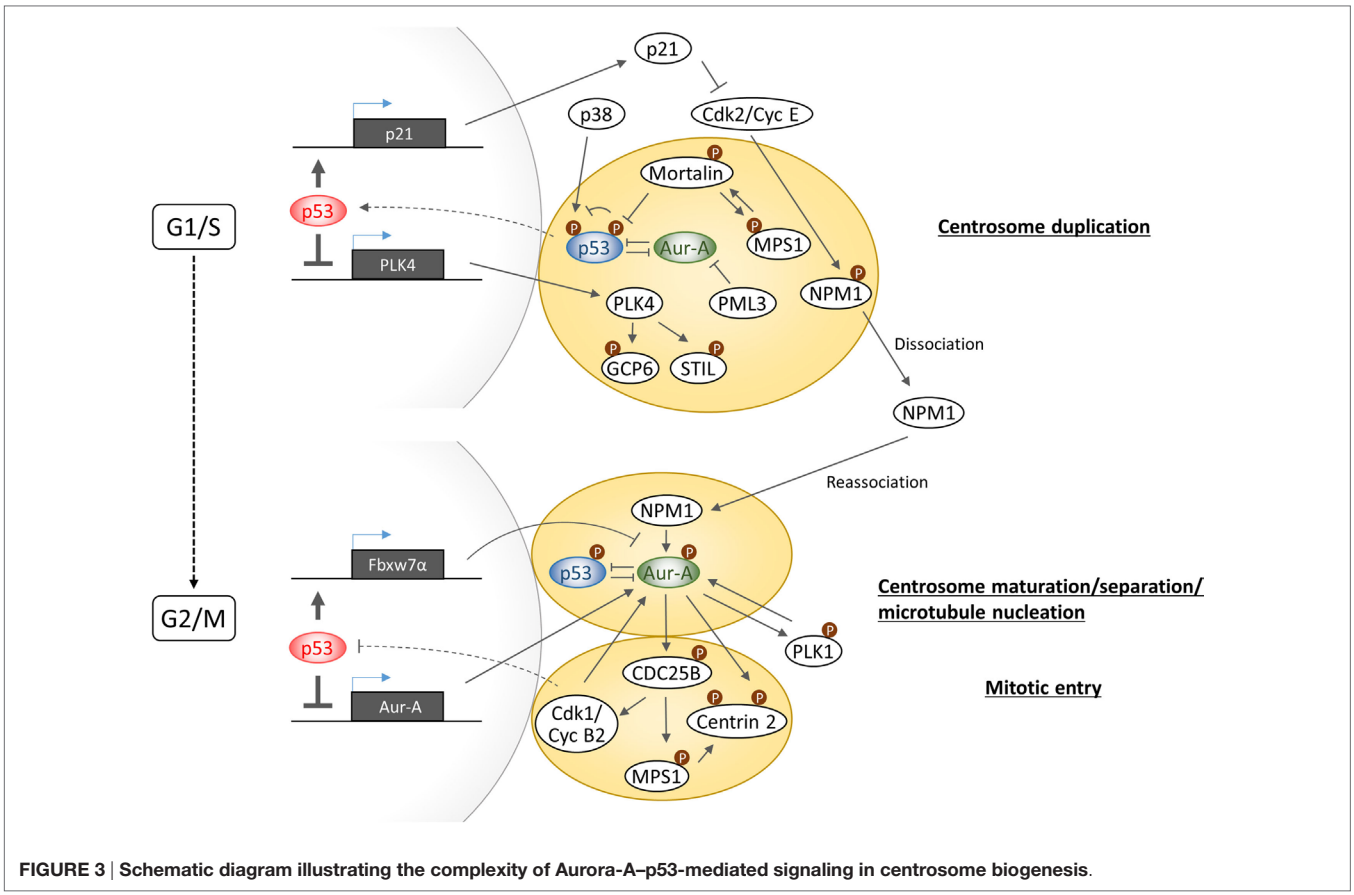

FIGURE 3 | **Schematic diagram illustrating the complexity of Aurora-A–p53-mediated signaling in centrosome biogenesis**.

mitotic spindle checkpoint response (57). MPS1 phosphorylates Mortalin, which in turn hyperactivates MPS1 kinase in a feed-forward regulatory manner. Importantly, Mortalin phosphorylation-activated MPS1 can drive centrosome overduplication. Although MPS1 phosphorylation of p53 positively regulates postmitotic checkpoint response (20), the precise role of MPS1 in the regulation of p53 function at the centrosome remains uncertain. Interestingly, the promyelocytic leukemia gene 3 (PML3) was shown to physically interact with Aurora-A and inhibit its kinase activity, while loss of PML3 shown to increase Aurora-A kinase activity and reduced protein stability of p53 along with decreased p21 expression, leading to activation of Cdk2/Cyclin E activity (58). Therefore, since there is no direct evidence supporting a role of centrosome localized Aurora-A in centrosome duplication, it would be imperative to further investigate whether or not increased p53–Mortalin interaction mediated by Aurora-A promotes p53 dissociation from centrosome and accompanying reduction of serine 33 phosphorylation is a cause of centrosome amplification induced in Aurora-A overexpressing cells.

At G1–S transition phase, Nucleophosmin/NPM1 is dissociated from unduplicated centrosome and at G2 phase is again recruited to duplicated centrosome to activate Aurora-A through phosphorylation of serine 89 (59). Activated Aurora-A cooperates with PLK1 to produce the onset signal for entry into mitosis as well as centrosome maturation. Since PLK1 has been shown to induce p53 degradation through phosphorylation of Topors (60), Aurora-A–PLK1 functional interaction, therefore, could interfere with p53 function on the centrosome at G2/M phase. NPM1-activated Aurora-A has also shown to induce phosphorylation of Centrin 2 at serine170 for stabilization of the protein (61). Phosphorylation of CDC25B at serine 353, which in turn stabilizes MPS1, also leads to stabilization of Centrin 2 through phosphorylation (62, 63). These findings indicate that Aurora-A and MPS1 cooperatively regulates Centrin 2 stability to induce centrosome maturation and separation. Activation of CDC25B is also pivotal for activation of Cdk1/Cyclin B, and a recent study has revealed that Cyclin B2 antagonizes p53 inhibitory activity against Aurora-A to control proper timing of centrosome separation at the onset of mitosis (64). Taken together, Aurora-A signaling branches off from CDC25B toward MPS1 for control of Centrin 2 stabilization regulating centrosome activity and toward Cdk1/CyclinB for positive feedback toward activation of Aurora-A in part by preventing p53 inhibitory action on Aurora-A.

The studies mentioned above clearly present evidence in support of a critical role for p53 signaling in regulating centrosome biogenesis and activity through cell cycle. In view of Aurora-A expression levels correlating with centrosome number and activity as well as known Aurora-A functional interactions with p53, Mortalin, PLK1, CDC25B, and, possibly MPS1, it will be interesting to investigate how the entire signaling axis

involving these proteins maintains centrosomal homeostasis in proliferating cells.

AURORA-A–p73 INTERACTION IN SPINDLE ASSEMBLY CHECKPOINT

A number of studies have shown the association of deregulated Aurora-A expression and activity with SAC override in cells irrespective of the p53 functional status in cells. Therefore, it is currently unclear whether or not p53 is involved in Aurora-A-mediated signal for SAC override. Accumulating evidence consistently suggest that p53 also functions in mitotic cell death and postmitotic checkpoint activated following aberrant mitosis and/or spindle damage through interaction with and phosphorylation by SAC proteins rather than being involved in the activation of SAC (20, 65–68). On the contrary, the role of Aurora-A–p73 interaction in SAC is relatively better defined. *In vitro* studies have shown roles of p73 in G2–M transition, mitotic exit, and mitotic cell death (69–72), while analysis of transgenic mouse lacking transactivation competent p73 (TAp73) revealed frequent occurrence of aberrant spindle structure associated with aneuploidy, chromosome instability, and mitotic slippage with spindle poisons (26). Further biochemical studies have also shown interaction of TAp73 with SAC proteins BUB1, BUB3, and BUBR1, and this interaction is crucial for BUB1 and BUBR1 localization at kinetochores and BUBR1 kinase activity (26, 27). These results suggest that TAp73 is directly involved in regulating SAC pathway to maintain chromosome stability. More recent study has demonstrated that TAp73 interacts with the inhibitory mitotic checkpoint complex of MAD2 and CDC20, preventing activation of the E3 ubiquitin ligase APC/C, and that Aurora-A phosphorylation of TAp73 at serine 235 causes dissociation of the MAD2–CDC20 complex, facilitating mitotic exit (24), suggesting that Aurora-A–TAp73 interaction is essential for a critical step in the SAC inactivation pathway (**Figure 4**). Unlike its effect on MAD2–CDC20 interaction and p73 depletion induced mislocalization of BUBR1 from the kinetochore, phosphorylation of p73 does not affect interaction of BUBR1 with CDC20 and its kinetochore localization, indicating that p73 participates in distinct pathway to control SAC activation. Although serine 235 phosphorylation of p73 enhances its interaction with Mortalin as described above, a more detailed investigation on the role of Aurora-A–Mortalin signaling axis in mitotic progression and SAC is warranted.

Expression level of transactivation-defective ΔNp73 is known to be elevated in many tumors and ectopic expression of transactivation-defective ΔNp73 has been implicated in abnormal mitotic progression accompanied with multipolar spindle and cytokinesis failure resulting in multinucleated cells. However,

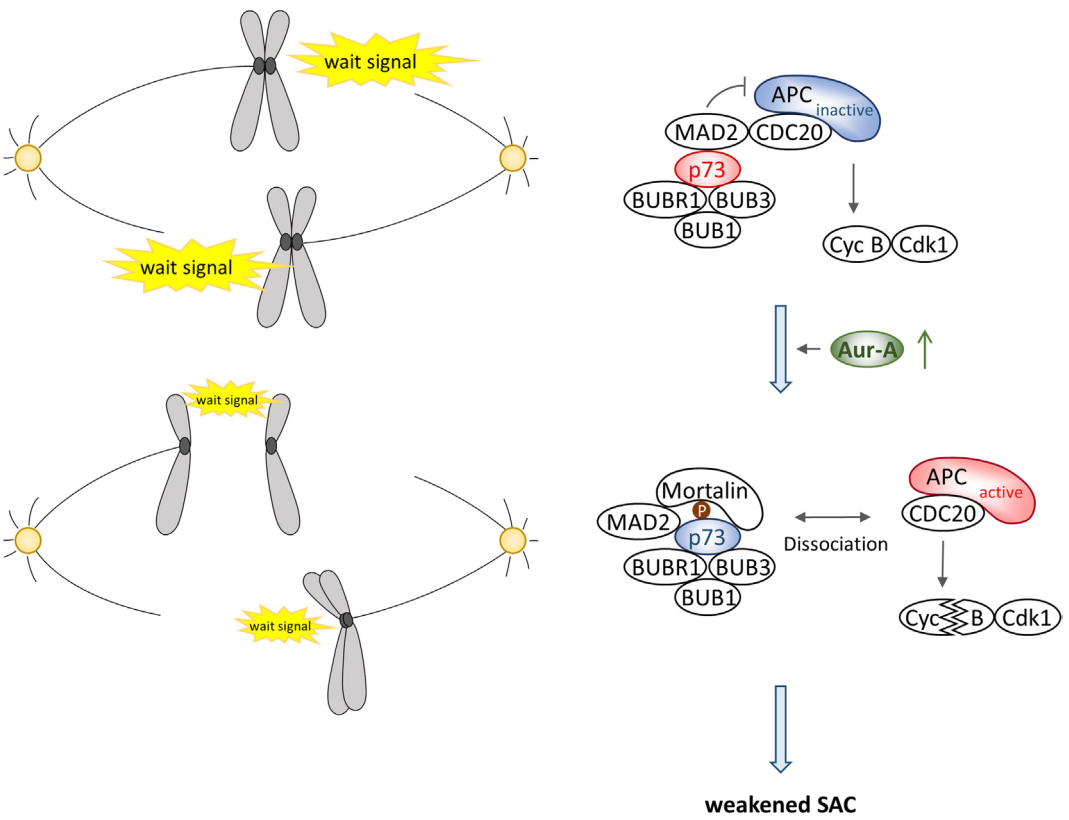

FIGURE 4 | Schematic illustration of Aurora-A–p73 interaction in spindle assembly checkpoint.

ΔNp73 neither affects SAC activation in the presence of spindle poison nor is it known to interact with BUBR1 (26, 73), indicating that expression of ΔNp73 helps bypass SAC. Intriguingly, Aurora-A also interacts with and phosphorylates ΔNp73 with similar efficacy as that of TAp73 but its phosphorylation site is different from TAp73 that remains to be mapped (24). Thus, characterization of physiological role of Aurora-A phosphorylated ΔNp73 could provide evidence of a novel signaling pathway affecting SAC.

AURORA-A–p53 SIGNALING IN PLURIPOTENT CELLS

Aurora-A has been reported to suppresses p53 function *via* phosphorylation of cell-fate determinant protein NUMB. While NUMB interacts with and helps stabilize and activate the tumor suppressor protein p53 (74, 75), Aurora-A initiates a phosphorylation cascade of aPKC–PAR6–Lgl cell polarity complex that ultimately leads to NUMB phosphorylation during mitosis to commit to asymmetric cell division (76–78). A recent study has revealed that phosphorylation of NUMB by Aurora–aPKC cascade disrupts its binding to p53 and promotes MDM2-mediated p53 degradation in cancer initiating cells of liver cancer (79). Thus, Aurora-A also antagonizes p53 activity indirectly through aPKC activation, resulting in maintenance of pluripotent state of cells and possibly promoting tumorigenesis. It would be interesting to examine if Aurora-A phosphorylation of p53 and NUMB synergistically affect disruption of their bindings.

A number of studies on cancer stem-like cells have revealed strong association of Aurora-A expression with gene expression of core stemness markers, such as Myc, Sox2, and Oct4. Additionally, Aurora-A–p53 functional interaction in the regulation of self-renewal and differentiation of mouse embryonic stem cells (mESC) and somatic cell reprograming has also been investigated (80, 81). Loss-of-function screening for protein kinases and phosphatases essential in mESC development and subsequent functional studies revealed strong correlation between elevated expression of Aurora-A and the undifferentiated state of mESC. Furthermore, loss of Aurora-A, but not loss of Aurora-A, mitotic substrates compromised self-renewal and triggered differentiation of mESC, indicating that non-canonical function of Aurora-A, unrelated to its role in mitosis, is possibly involved in regulating self-renewal potential of mESC (82). This observation also showed inverse correlation with p53 activity in mESC and attributed this finding to Aurora-A-mediated inactivation of p53 function. The study also revealed that Aurora-A-mediated serine 215 phosphorylation rather than serine 315 phosphorylation is more critical in antagonizing p53-induced mESC differentiation and p53-mediated suppression of induced Pluripotent Stem Cells (iPSC) reprograming *via* activation of gene expression program associated with pluripotency. Phosphorylation of serine 315, on the other hand, was shown to cause partial impairments of both mESC differentiation and suppression of iPSC reprograming correlating with lower expression of pluripotency markers. The varying degree of downstream effects of the two Aurora-A-mediated p53 phosphorylated residues possibly represents stronger inhibition of p53 function following serine 215 phosphorylation resulting in complete loss of its transactivation activity and cytoplasmic sequestration reflecting the naturally observed localization of endogenous p53 in mESC (83). The study concluded that Aurora-A controls pluripotency through inhibition of p53 target gene expression required for ectodermal and mesodermal differentiation. The observation regarding serine 315 phosphorylation showing less pronounced phenotype in this study appeared conflicting to an earlier report showing elevated serine 315 phosphorylation during mESC differentiation and knockin of serine 315 phosphor-deficient mutant impairing mESC differentiation. Importantly, serine 315 phosphorylation was also reported to enable the recruitment of the corepressor mSin3a to the NANOG promotor, resulting in complete suppression of NANOG transcription and primitive endodermal differentiation (84–86). Serine 315 phosphorylation is known to be mediated not only by Aurora-A but also by Cdk/cyclin complex. In view of the observed loss of serine 33 phosphorylation in serine 215 phosphorylated p53, it is plausible that serine 215 phosphorylation might inhibit serine 315 phosphorylation by Cdk1 or Aurora-A. Alternatively, Aurora-A phosphorylation of the two p53 residues may be playing non-overlapping physiological roles in Aurora-A-mediated cellular processes.

In contrast to the requirement of Aurora-A in maintenance of pluripotency and induction of iPSC state mentioned above, a study reported that loss of Aurora-A function is essential for somatic cell reprograming (87). In this study, authors reported that loss of Aurora-A function following small-molecule inhibitor treatment or siRNA knockdown enhanced efficacy of iPSC generation with cells reaching a fully reprogramed state. The iPSC generated by this approach possessed ability to differentiate into different lineages *in vitro* and *in vivo*. Moreover, p53 depletion could further enhance the effect of loss of Aurora-A function. The underlying reasons for these contradictory findings are not known at this time and need to be investigated.

GENETICALLY ENGINEERED AURORA-A–p53 TARGETED MOUSE MODELS

Comprehensive genomic analyses have identified Aurora-A as a low penetrance tumor-susceptibility gene and elevated expression was reported to play an essential pathological in tumor development correlating with prognosis and resistance to therapy (80, 88–91). Several transgenic mouse models have been developed to gain direct evidence of Aurora-A tumorigenic potential and associated phenotypic alternations *in vivo*, which have yielded somewhat conflicting and distinct results (92–94). While Wap-Cre mouse model system in which Aurora-A was constitutively overexpressed under *CAG-CAT* promoter in mammary gland after one cycle of pregnancy developed hyperplasia in p53 wild-type background and precancerous atypical ductal hyperplasia in

p53–null background (92, 94), *MMTV* promoter-driven mouse model was reported to develop mammary tumors in both p53 wild-type and heterozygous background after four to five cycles of pregnancy (93). Notably, centrosome amplification and chromosome instability were detected in all mouse models, suggesting that Aurora-A overexpression affects p53 function in the maintenance of centrosome homeostasis and chromosomal stability *in vivo*. Consistent with *in vitro* studies, activation of AKT signaling pathway leading to Cyclin D overexpression was seen in the tumors developed in *MMTV*–Aurora-A mice. We have recently reported a mammary gland targeted Aurora-A mouse model in a p53 wild-type background in which Aurora-A expression is driven by ovine *β-lactoglobulin* promoter led to the development of mammary tumors after four to five of pregnancy cycles (95). In addition to genomic instability, reduced expression of p53 protein and activation of AKT signal pathway was detected in tumors similar to *MMTV*–Aurora-A mouse model, again suggesting that elevated levels of Aurora-A can be oncogenic with inhibitory effects on p53-mediated tumor suppressor signaling pathways. It is relevant to mention, in this context, that an inducible gene switch mouse model overexpressing Aurora-A in skin epidermis exposed to tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and the mutagen 7,12-dimethylbenz(*a*)anthracene, developed by us earlier, revealed malignant progression of skin tumors with centrosome amplification, abnormal spindle formation, and genomic instability (96). Expression of p53 protein was lost, and amplification of MDM2 gene was concurrently found in these tumors. Taken together, Aurora-A overexpressing mouse models of organ-specific tumors have revealed loss of p53 expression recapitulating naturally occurring Aurora-A and p53 expression changes seen in human tumors. Further in-depth studies to elucidate the role of Aurora-A–p53 signaling cascades relevant to human tumor development utilizing Aurora-A overexpressing mouse models are warranted.

CONCLUSION

Functional interactions between Aurora kinases and p53 family proteins coordinately regulate diverse cellular pathways by modulating activity and subcellular localization of each other and their downstream effector proteins. Deregulations of these interactions in cells undergoing tumorigenic transformation have significant functional consequences on induction of chromosome instability, development of different tumor-associated phenotypes including resistance to therapy. In addition to Aurora kinase functional interactions with wild-type p53 and p73, there is evidence of Aurora-A interacting with and phosphorylating mutant p53 protein. Physiological function of Aurora-A–mutant p53 interactions have not been elucidated yet. Mutant p53 and transactivation-deficient mutant of p73 also phenocopy some of the Aurora-A overexpression-induced phenotypes. It would be interesting to investigate the functional consequences of Aurora-A phosphorylation of mutant p53 family members in the p53 signaling cascades and their significance in the development of tumorigenic phenotypes.

AUTHOR CONTRIBUTIONS

All authors contributed to writing the review and preparing figures.

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REVIEW
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The Aurora B kinase in chromosome bi-orientation and spindle checkpoint signaling

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Aurora B, a member of the Aurora family of serine/threonine protein kinases, is a key player in chromosome segregation. As part of a macromolecular complex known as the chromosome passenger complex, Aurora B concentrates early during mitosis in the proximity of centromeres and kinetochores, the sites of attachment of chromosomes to spindle microtubules. There, it contributes to a number of processes that impart fidelity to cell division, including kinetochore stabilization, kinetochore–microtubule attachment, and the regulation of a surveillance mechanism named the spindle assembly checkpoint. In the regulation of these processes, Aurora B is the fulcrum of a remarkably complex network of interactions that feed back on its localization and activation state. In this review, we discuss the multiple roles of Aurora B during mitosis, focusing in particular on its role at centromeres and kinetochores. Many details of the network of interactions at these locations remain poorly understood, and we focus here on several crucial outstanding questions.

Keywords: centromere, kinetochore, spindle assembly checkpoint, kinase, phosphatase, Aurora B, chromosome passenger complex, bi-orientation

GENERAL REMARKS

Cells executing mitosis are challenged in ways that can jeopardize their viability and survival (1). The duplicated chromosome pairs (sister chromatids) in the mother cell need to orient on the mitotic spindle so that they can be equally distributed to the two daughter cells after the cohesion that holds them together is dissolved at the metaphase-to-anaphase transition. This process of "bi-orientation" requires that the sister chromatids establish stable "end-on" interactions with microtubules emanating from opposite poles of the mitotic spindle (**Figures 1A,B**) (2–4). Sister chromatids that fail to bi-orient are mis-segregated into the wrong daughter cell, or separated from the bulk of correctly segregated chromosomes forming the primary nucleus of daughter cells and secluded into extra-nuclear structures called micronuclei. Either fate of mis-oriented chromosomes can have dire consequences for cell physiology (5, 6).

Aurora B is a member of the Aurora family of Serine/Threonine (S/T) protein kinases. Originally discovered as a gene required for maintenance of ploidy in *Saccharomyces cerevisiae* and named increase in ploidy-1 (*IPL1*) (8), Aurora B was later found to control several aspects of chromosome segregation in all eukaryotes (9–11). Two additional members of the Aurora family named Aurora A and Aurora C exist in mammals (12, 13). Substrates of these Aurora kinases usually conform to the consensus [RK]-[RK]-X-[TS]-Θ, where X is any residue and Θ is a hydrophobic or aromatic

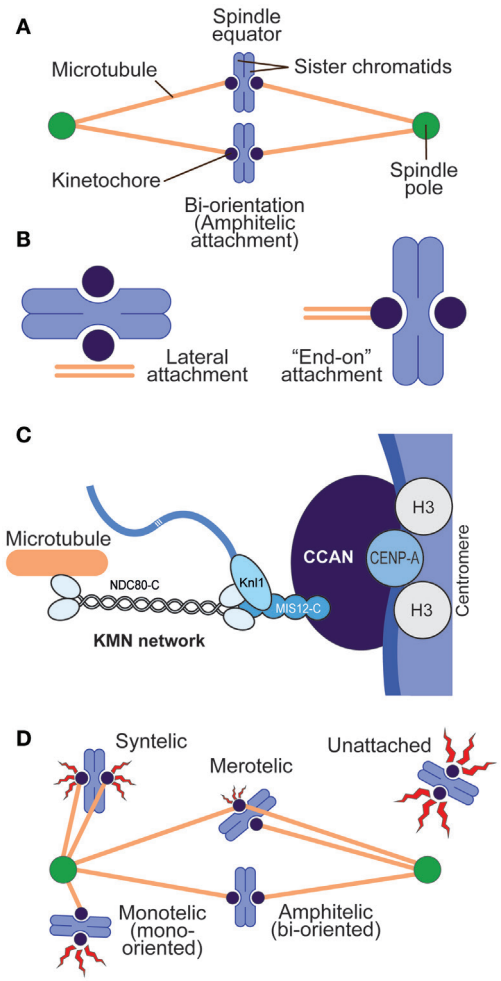

FIGURE 1 | Chromosome–spindle interactions. **(A)** A simple spindle with two chromosomes at metaphase. When chromosomes are bi-oriented, the sister chromatids are attached to microtubules, and the microtubules point to opposite spindle poles. **(B)** Two main modes of kinetochore–microtubule attachment predominate in mitosis. Lateral attachment (left) to the microtubule lattice (as opposed to the microtubule end) is typical of early phases of chromosome congression to the equatorial plane of the mitotic spindle and may not fully engage the core kinetochore machinery devoted to microtubule binding but rather molecular motors (7). "End-on" attachment (right) is typical of the final stages of attachment and involves core kinetochore machinery. **(C)** Schematic depiction of centromeres and kinetochores. Centromeres host CENP-A, the histone H3 variant, at much higher levels than other segments of the chromosome. CENP-A binds to a subset of 16 or 17 CCAN subunits, collectively represented as a blue oval. The KMN network binds directly to microtubules. **(D)** Various types of kinetochore–microtubule attachment modes, including erroneous attachments that require correction and that will engage the spindle assembly checkpoint (red flashes). Different "offenses" may provide a graded checkpoint response (variable size of the red flash), with lack of attachment providing a more robust response and merotelic attachment a weak one.

residue. For instance, Aurora B phosphorylates human KNL1 (CASC5) on the RRVSF motif, which, in the non-phosphorylated version, is a recruitment motif for protein phosphatase 1 (PP1) (14–17). Broad analyses of Aurora B substrates and consensus target sequences have been reported (18, 19). Although Aurora kinases share a similar consensus, distinct subcellular localizations ensure that they deliver activity to distinct substrates and regulate different aspects of mitosis (19, 20).

In this review, we discuss the role of Aurora B in the regulation of chromosome segregation, focusing in particular on the roles of Aurora B during prometaphase, the phase of mitosis during which chromosomes attempt to create stable interactions with spindle microtubules. Readers are also referred to comprehensive reviews that discuss the role of Aurora B also in other phases of mitosis (20, 21).

INTRODUCTORY CONCEPTS I: CENTROMERES AND KINETOCHORES

Sister chromatids interact with spindle microtubules through specialized and structurally complex protein assemblies known as kinetochores (22, 23). On each chromosome, the kinetochore is established on a unique genetic locus named the centromere (**Figure 1C**). Centromeres, which may consist of several million base pairs of DNA in metazoans, are specialized chromatin domains whose hallmark is the enrichment of the histone H3 variant CENP-A (also known as CenH3) (24). At centromeres, CENP-A containing nucleosomes are embedded in histone H3-containing chromatin at a ratio that, even if estimated to be as little as 1 CENP-A nucleosomes over 25 H3 nucleosomes, is greatly superior to that in bulk chromatin (25).

CENP-A acts as a platform for the recruitment of kinetochore proteins collectively defined as the constitutive centromere-associated network (CCAN), most of which localize at centromeres during the entire cell cycle (26). These proteins form the so-called "inner kinetochore." Upon entry into mitosis, an additional protein complex, the Knl1 complex–Mis12 complex–Ndc80 complex (KMN) network, is recruited to the CCAN. The KMN network in the "outer kinetochore" interacts directly with spindle microtubules (27) (**Figure 1C**).

INTRODUCTORY CONCEPTS II: ERROR CORRECTION AND THE SPINDLE ASSEMBLY CHECKPOINT

Two feedback mechanisms control the process of kinetochore–microtubule attachment during mitosis, and Aurora B contributes decisively to both of them. These pathways are named error correction (EC) and spindle assembly checkpoint (SAC, also known as mitotic checkpoint, metaphase checkpoint, or "wait anaphase signal"). Error correction is a "local" mechanism that allows kinetochores selectively to stabilize interactions with microtubules that drive chromosome bi-orientation and to weaken those interactions that do not, such as the erroneous configurations known as syntelic and merotelic attachment (**Figure 1D**) (28). This description of EC summarizes the interpretation of pioneering chromosome micromanipulation experiments carried out over 45 years ago by Bruce Nicklas (29), but is nothing more than a statement of fact, partly because we are still far from a full molecular comprehension of EC. EC is believed to depend

on the ability of the kinetochore–centromere system to detect tension, associated with bi-orientation, or lack of tension, associated with lack of bi-orientation. While tension at the bi-oriented chromatids suppresses error correction, lack of tension may not necessarily require error correction (e.g., when lack of tension is due to lack of attachment), but it will require it when kinetochores that are bound to microtubules fail to build tension (as in the case of syntelic or merotelic attachments, **Figure 1D**).

The KMN complex captures dynamic microtubules to create load-bearing attachments (30, 31). For error correction to occur, kinetochore (KMN)–microtubule interactions need to be sufficiently dynamic to allow the destabilization of erroneous attachments. Aurora B is a key component of the error correction machinery (32, 33). Aurora B inhibition through small-molecule inhibitors or inhibitory antibodies stabilizes incorrect attachments (32–36). Conversely, Aurora B overexpression causes continuous disruption of KT–MT attachments (37), while Aurora B re-activation allows the selective destabilization of incorrect attachments (38–40). Many of the proteins at the interface with microtubules are Aurora B substrates (41).

Similarly to the EC, the SAC also requires kinetochores (42, 43). In contrast to the EC, however, the SAC has the ability to extend into a "global" signal that diffuses away from kinetochores and prevents mitotic exit in the presence of even a single unattached or improperly attached kinetochore (44). The SAC pathway converges on the assembly of a checkpoint effector complex, the mitotic checkpoint complex (MCC), which targets and inhibits the anaphase-promoting complex or cyclosome (APC/C, **Figure 2A**). The activity of this ubiquitin (Ub) ligase targets Cyclin B and Securin, which are, respectively, the activator of the main mitotic "engine", the Cdk1 kinase, and a stoichiometric inhibitor of the protease Separase, which is required for dissolution of sister chromatid cohesion. Proteasome-dependent destruction of Cyclin B and Securin upon their ubiquitination by the APC/C inactivates Cdk1 and activates Separase, respectively, triggering mitotic exit and sister chromatid separation (**Figure 2A**) (4, 45). Cells in which the checkpoint is altered or artificially inactivated undergo precocious mitotic exit in the presence of unattached or incorrectly attached chromosomes and are therefore prone to mis-segregation events (44).

The general role of Aurora B activity in the EC and the SAC has been widely debated (28, 49). Early models based on experiments with attenuated alleles of Aurora B or at non-saturating doses of small-molecule inhibitors identified in Aurora B an exclusive component of the EC machinery (**Figure 2B**). According to these models, Aurora B contributed indirectly to SAC activation by generating unattached kinetochores, which became identified as the only structures capable of activating the SAC (32, 50).

This view has been progressively revised, partly because the molecular evidence in favor of a direct role of Aurora B in SAC control has been growing (34–36, 48, 51–54) and partly because there has been a conceptual evolution regarding what the SAC may be monitoring at kinetochores, with a shift from a pure "microtubule occupancy" model to an "intra-kinetochore tension" model (41, 55–58) (**Figure 2C**). Importantly, results obtained with different experimental approaches have caused the community to oscillate in their preference for a model or the other. However, a full assessment of the virtues and shortcomings of these models remains out of reach, as the molecular understanding of the conditions that lead to EC and SAC activation or silencing remains rudimentary, at least in relation to the considerable complexity of the process. Furthermore, while these two pathways are separable in their downstream components, they may be largely

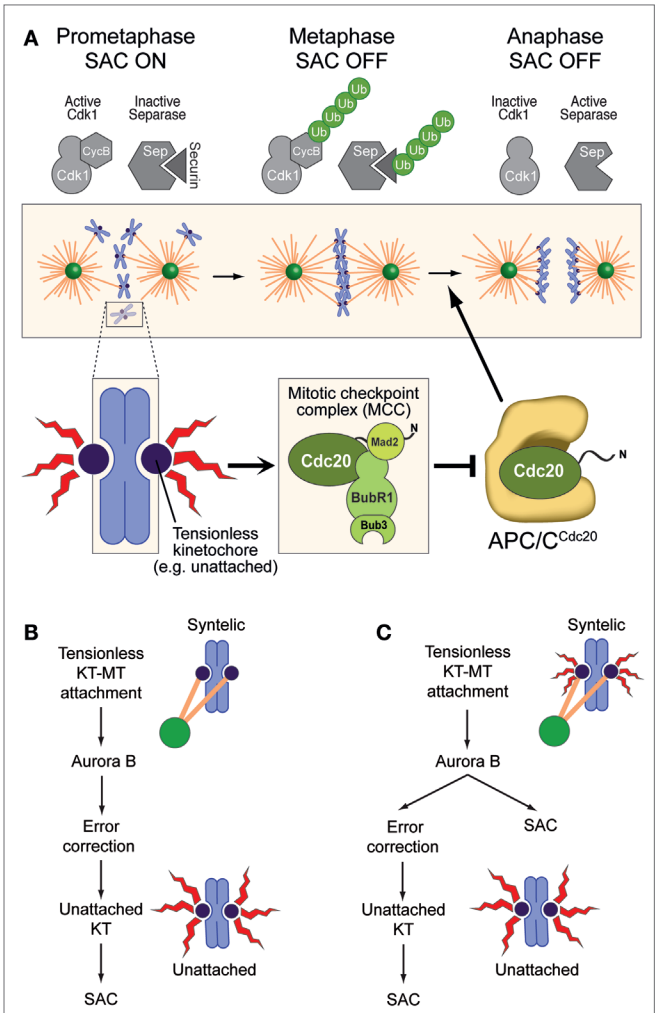

FIGURE 2 | The spindle assembly checkpoint and error correction. **(A)** The SAC pathway originates at kinetochores and converges, through several steps, on the assembly of the mitotic checkpoint complex (MCC), which acts as the SAC effector. MCC has been proposed to target the complex of APC/C pre-bound to a second molecule of Cdc20 (which can act both as an APC/C co-activator and as an MCC subunit) (46, 47). APC/C^{Cdc20} promotes poly-ubiquitylation (Ub) of Cyclin B and Securin, promoting mitotic exit and separation of sister chromatids. MCC inhibits this activity of APC/C^{Cdc20} until all chromosomes have achieved bi-orientation, at which point the SAC becomes "satisfied" (it subsides). **(B)** In this model of Aurora B function, any kinetochore–microtubule interaction, even if erroneous, satisfies the SAC. Aurora B is not a SAC component, but its ability to recognize and correct improper attachment makes it activate the SAC indirectly through generation of unattached kinetochores (as an intermediate in error correction), which are considered the only source of SAC signal. **(C)** In this alternative model, any tensionless kinetochore is a source of SAC signal, albeit of different signal strengths (size of the red flashes). Aurora B is directly required both for error correction and for the SAC. **(B,C)** were adapted from Ref. (48).

or even completely non-separable in the sensory apparatus that activates or switches them off at the "outer kinetochore," where they operate. For instance, *cyclin-dependent kinase 1* (Cdk1), Aurora B, *monopolar spindle 1* (Mps1), and *budding uninhibited by benzimidazoles 1* (Bub1), all mitotic protein kinases, are required to promote correct kinetochore–microtubule-binding configurations, as well as for the SAC response (59). They are likely to regulate both phenomena at the same time and from the same place, the kinetochores. In this review, we focus on some of the molecular details that implicate Aurora B in these two pathways.

AURORA B IS A SUBUNIT OF THE CPC

Aurora B kinase is embedded in a multi-protein complex known as the chromosome passenger complex (CPC), whose subunits are codependent for stability and localization (60–64). The three additional CPC subunits are named inner centromeric protein (INCENP, and known as Sli15p in yeast)], Survivin (Bir1p) and Borealin (also known as CSC-1, Dasra, and Nbl1p) (63, 65–69) (**Figure 3A**). The CPC consists of two functionally distinct modules (20): a module delivering the catalytic activity, composed of Aurora B and a ~50-residue segment at the C-terminal end of INCENP, the so-called IN-box (62, 67, 70–74); and a module mediating localization, consisting of a ~45-residue segment at the N-terminal end of INCENP, Survivin, and Borealin (64, 75–84). The two modules are connected by the central part of INCENP (**Figure 3B** and see below).

Activation of Aurora B kinase arises from multiple regulatory steps, including the binding of the IN-box of INCENP around the Aurora B active site, the Aurora B-mediated phosphorylation of the IN-box on a Thr-Ser-Ser (TSS) motif, and the auto-phosphorylation of Aurora B at Thr232 (abbreviated as AB-T232-P) in the activation segment (70, 72, 85). Thus, Aurora B activation resembles that of many other kinases, in that it requires interaction with a partner protein and phosphorylation. Phosphorylation at the TSS and at the Aurora B activation segment is likely to occur in trans (70) and may therefore be sensitive to the local concentration of the CPC (86, 87).

Besides the intrinsic mechanisms of regulation described above, Aurora B may also be controlled by extrinsic mechanisms. For instance, phosphorylation of Ser311 of Aurora B by checkpoint kinase 1 (Chk1) may promote catalytic activation of Aurora B near kinetochores (88, 89). In addition, phosphorylation of the CPC targeting subunit Borealin by Mps1 has also been proposed to regulate Aurora B activity (90), but this remains controversial as neither Aurora B nor its activity are grossly perturbed by Mps1 inhibition (91–93). Furthermore, the protein TD-60 has been indicated as an additional CPC subunit required for CPC centromere targeting and Aurora B activation (94, 95). A recent study revealed that TD-60 is as a guanine nucleotide exchange factor (GEF) for the small Ras-like GTPase RalA, and that the latter modulates Aurora B activity and localization (96). While the mechanisms through which the RalA GEF activity of TD-60 influences Aurora B localization and activity requires further investigation, it seems now clear that TD-60 is not part of the CPC.

One of the most exciting chapters in the study of Aurora B kinase has been the development of highly specific and selective chemical inhibitors, spurred by the identification of this kinase as a potential target in oncology (97). Leaving clinical implications aside (98), small-molecule ATP-competitive inhibitors such as Hesperadin and ZM447439 proved invaluable tools for acute mitotic inhibition of Aurora B function and for the investigation of its mitotic functions in basic research laboratories (34, 35).

THE LOCALIZATION MODULE OF THE CPC: CENTROMERE LOCALIZATION AND BEYOND

The localization module targets the CPC to the centromere, where the bulk of the CPC localizes during mitosis. Crucial for centromere targeting is the CPC subunit Survivin, a member of a family of inhibitor of apoptosis (IAP) proteins containing a BIR domain (99). While Survivin might have lost its function as inhibitor of apoptosis, typical of other IAPs, its BIR domain has retained the ability of recognizing the N-terminus of target proteins. In Survivin, this ability is leveraged to bind a short N-terminal segment of Histone H3 (78, 80, 81) (**Figure 4A**). In fact, Survivin binds a short N-terminal segment of Histone H3 that must include a phosphorylated version of Thr3 (H3-T3-P) for efficient recognition (76, 78, 80, 81, 83, 84). The kinase responsible for this preeminently mitotic modification of Histone H3 is named Haspin (100). By recognizing H3-T3-P at centromeres, Survivin targets the CPC to the centromere (**Figure 4B**). Whether this H3-T3-P-dependent mechanism operates in yeast has remained unclear, because deletion of the yeast haspin-like kinases does not result in a growth defect phenotype (101). In yeast, a survivin-dependent mechanism may rely on the interaction of the Survivin homolog Bir1 with Ndc10, a subunit of the CBF3 centromeric complex (102, 103).

Besides H3-T3-P, also the phosphorylation of Thr120 of Histone H2A by Bub1 kinase (H2A-T120-P, H2A-S121-P in fission yeast) has been implicated in centromere recruitment of the CPC (81, 104, 105) (**Figure 4B**). The role of this mark, which is detected at kinetochores but not at centromeres, is more elusive. It appears to be crucial to regulate a homeostatic circuit that dynamically controls the activity and abundance of protein kinases, including Plk1 and Aurora B, and protein phosphatases, including members of the protein phosphatase 2A (PP2A) family associated with the B56 regulatory subunit (PP2A-B56), at kinetochores and centromeres. Specifically, H2A-T120-P is believed to promote recruitment of Shugoshin proteins (SGOL1 and SGOL2/TRIPIN in humans) (106, 107). These, in turn, control the recruitment of proteins that play a prominent role in error correction, including kinesin-13 family members such as MCAK, a microtubule depolymerase and Aurora B substrate, and the PP2A-B56 protein phosphatase complex, which balances abundance and activity of Aurora B and Plk1, as well as the phosphorylation of important CPC targets (108–118).

In addition to the recognition of histone marks, other mechanisms have been implicated in CPC centromere recruitment or activation, such as post-translational modifications of Survivin,

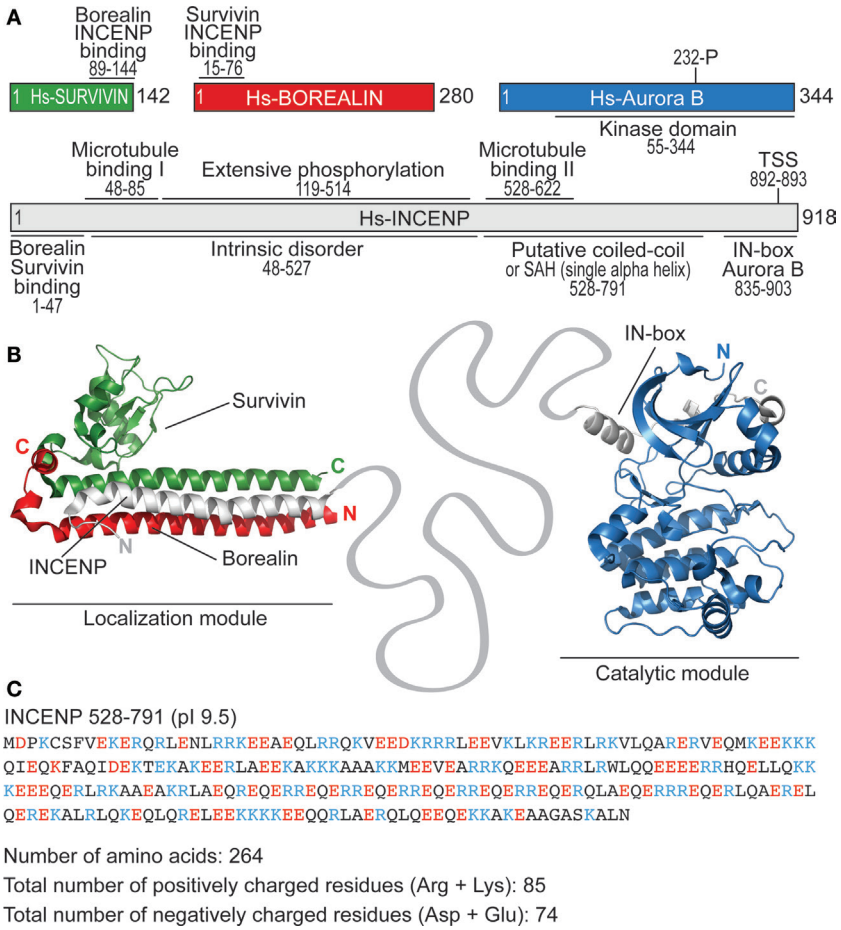

FIGURE 3 | Structural organization of the CPC. **(A)** Schematic representation of human CPC subunits with main structural features. **(B)** Structural organization of the localization module of the CPC (PDB ID 2QFA) (77) and of the catalytic module (PDB ID 2BFX) (70). The linker between the N- and C-terminal regions of INCENP encompasses more than 800 residues. **(C)** Sequence of the putative coiled-coil region of INCENP shows that its features are hardly compatible with coiled-coil folding (due to insufficient number and irregular spacing of hydrophobic residues). pI defines isoelectric point.

Borealin, and INCENP, including phosphorylation by Aurora B itself, Cdk1, Mps1, and Plk1 (90, 119–123). Direct binding of Borealin to double-strand DNA has also been reported (64). Finally, there is evidence that oligomerization of the localization module also contributes to its localization (64, 124). How these features may impinge on CPC centromere targeting remains poorly understood.

INCENP, THE BRIDGE CONNECTING THE TWO CPC MODULES

The functional properties of INCENP that have remained mechanistically obscure are now starting to emerge. INCENP is a rather large protein (918 residues for Isoform 1 in humans; source Uniprot: http://www.uniprot.org) (**Figure 3A**). Large parts of the INCENP primary sequence are low-complexity and unlikely to adopt a defined three-dimensional tertiary (and even secondary) structure. A predicted coiled-coil between residues 528 and 791 of INCENP is often considered an exception. More careful scrutiny, however, leads to exclude that this segment of INCENP is a genuine coiled-coil. It contains too few hydrophobic residues to support coiled-coil oligomerization and its frequent stretches of positively and negatively charged residues (**Figure 3C**) may produce false-positive classifications as coiled-coils. Analysis of INCENP residues 528–791 with REPPER, a program that detects short repeats and predicts periodicities in protein sequences (125), suggests that it lacks the regular sequence pattern typical of coiled-coils. In agreement with this idea, a very recent study on avian INCENP showed that this region (residues 503–715, corresponding to residues 528–791 of human INCENP in **Figure 3A**) folds as a single alpha helix (SAH) domain, rather than as a coiled-coil (126). SAH can unfold reversibly under force, thus extending up to 2.5-fold over their rest length. Because the N-terminal part of the SAH domain contains a second microtubule-binding domain (126), in addition to the one already identified in the N-terminus of INCENP (residues 48–85 of the human protein) (60, 74, 127, 128), it is a potential candidate for regulation by microtubule attachment.

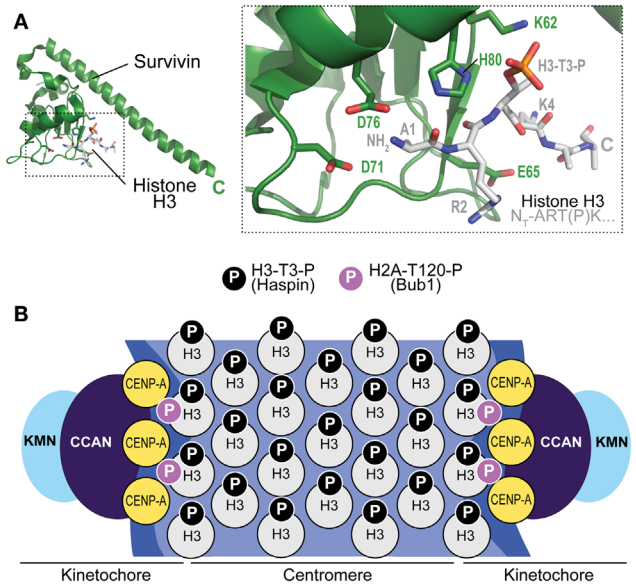

FIGURE 4 | Mechanism of CPC recruitment to centromeres. (A) Crystal structure of the complex of Survivin with a peptide encompassing the N-terminal region of Histone H3 (PDB ID 4A0J). The peptide has sequence Ala-Arg-Thr(P)-Lys, where (P) indicates that Thr3 is phosphorylated. Asp71 (D71) is implicated in the recognition of the free N-terminus of Ala2 (the N-terminal Met1 is removed by an aminopeptidase). **(B)** Haspin kinase phosphorylates Thr3 of histone H3 (H3-T3-P) in the centromere region to allow recruitment of the CPC. Bub1 kinase phosphorylates Histone H2A on Thr120 (H2A-T120-P) near kinetochores (i.e., the modification does not extend to centromeres). In principle, both H3-containing and CENP-A containing nucleosomes may contain this modification.

INCENP also contains a large disordered region (residues 48–527, **Figure 3A**), stuffed with phosphorylation sites. The UNIPROT (http://www.uniprot.org) reports at least 24 phosphorylation sites in residues 119–514 of human INCENP. Phosphorylation of Thr388 in this segment has been implicated in binding and targeting of Plk1 (129). As discussed more thoroughly below, Cdk1-dependent phosphorylation of Thr59 within this segment has important consequences for CPC localization.

THE SPATIAL SEPARATION MODEL OF AURORA B FUNCTION

While it is clear that Aurora B substrates at centromeres and kinetochores undergo dynamic changes in their phosphorylation state during the relatively short time it takes kinetochores to attach to the spindle, it is uncertain to which extent these changes reflect the dynamic regulation of Aurora B activity by the intrinsic and extrinsic mechanisms discussed above (41). Rather, current models of Aurora B function focus primarily on the tension-dependent separation of Aurora B from its substrates as the basis of Aurora B regulation in EC and the SAC (41, 58). To appreciate this argument, it is important to explain the geometry of centromeres and kinetochores, their variation during bi-orientation, and how Aurora B may position itself within this system. The size of kinetochores is roughly equivalent to the wavelength of visible light, and in first approximation kinetochores appear as diffraction limited "spots" in the light microscope. In HeLa cells, the distance between the centroids of "spots" corresponding to inner kinetochore proteins in the two sister kinetochores (inter-kinetochore distance) grows from ~0.9 μm in the absence of microtubule binding in prometaphase (i.e., in the absence of tension) to ~1.4 μm upon bi-orientation at metaphase (when chromosomes are end-on-attached and under full tension) (130) (**Figure 5A**). Similar increases in inter-kinetochore distance have been measured in other cell types: inter-kinetochore distance increases from ~1.1 μm in the absence of microtubule binding to ~2.2 μm upon bi-orientation in newt lung cells (131), and from 0.72 to 0.94 μm in *Drosophila* S2 cells (57).

Thus, tension introduces macroscopic changes in the organization of the inter-kinetochore space between sister kinetochores. Importantly, tension also modifies the internal structure of the kinetochore, a condition referred to as intra-kinetochore stretch (**Figure 5A**). In S2 cells, for instance, the span of the kinetochore [from CENP-A to the centromere-proximal end of the Ndc80 subunit (also known as Hec1), measured along the inter-kinetochore axis] is ~65 nm in the absence of tension, and 102 nm in the presence of tension (57) (**Figures 5B,C**). Similar tension-driven increases in stretch are observed within human and yeast kinetochores (132, 133). The precise structural changes underlying the establishment of intra-kinetochore tension, however, remain unknown.

The spatial separation model builds on the observation that an Aurora B FRET sensor shows constitutive, tension-independent phosphorylation when positioned close to Aurora B at the interface between the centromere and inner kinetochore, but tension-sensitive phosphorylation when positioned more distantly from the kinase (55) (**Figures 5D,E**). More specifically, Aurora B phosphorylates a FRET sensor located at the centromere–kinetochore interface (because fused to the CENP-B protein) regardless of attachment status and despite the very significant increase in inter-kinetochore distance upon bi-orientation. Conversely, Aurora B phosphorylates the same FRET sensor now fused to a subunit of the Mis12 complex, in the outer kinetochore, when chromosomes are not under tension, but does so less effectively when tension is present at metaphase (55). Analogous observations have been made with *bona fide* Aurora B substrates (41, 55–58, 130, 134) and Aurora B substrates located in the outer kinetochore become progressively dephosphorylated during the attachment process (134–136). Furthermore, artificial repositioning of Aurora B to the outer kinetochore prevents dephosphorylation of outer kinetochore substrates (55). The persistence of Aurora B phosphorylation on a sub-class of "proximal" substrates despite full tension suggests that the kinase activity of Aurora B may not *per se* be force dependent.

Thus, it appears that certain substrates, and in particular substrates in the KMN network that mediates the EC and SAC responses, become physically separated from the kinase as tension arises (41). In the absence of tension, such as in syntelic

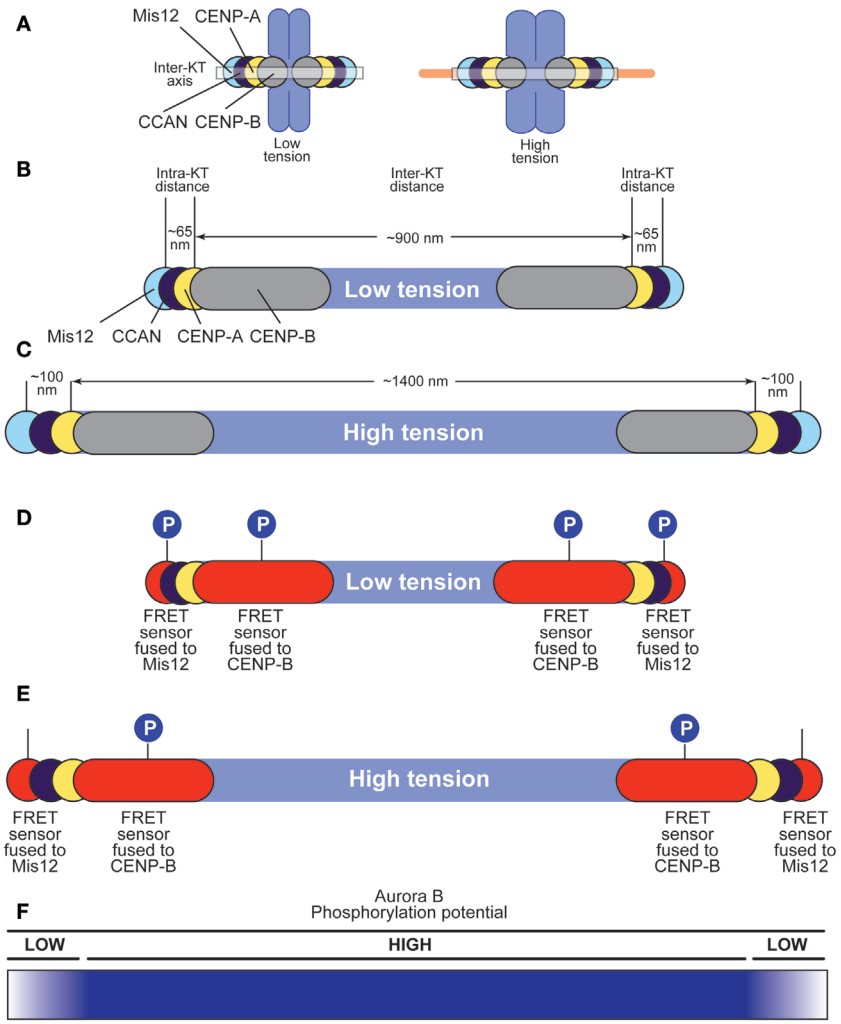

FIGURE 5 | Effects of tension on centromere and kinetochore structure. **(A)** Chromosome lacking tension (left) or under tension (right). The centroid of the distributions of proteins in the kinetochore, including CENP-B, CENP-A, CCAN subunits, and Mis12 (part of the KMN network) is represented as a circle along the inter-kinetochore axis. Each centroid has a defined coordinate along the axis (57). Microtubules cause changes in the position of the centroids. **(B,C)** Under low tension **(B)**, inter-kinetochore distance in HeLa cells is ~900 nm (0.9 μm), whereas the distance between the centroids of the distributions of CENP-A and Mis12 is ~65 nm in *Drosophila* and as little as ~40 nm in human kinetochores (132). CENP-B binds the CENP-B box in alpha-satellite DNA at centromeres, and extends slightly beyond CENP-A toward the centromere (55). Under high tension **(C)**, inter-kinetochore distance grows to 1400 nm (1.4 μm), whereas the distance between the centroids of the CENP-A and Mis12 distributions grows to 100 nm (57). **(D,E)** A FRET sensor responding to Aurora B phosphorylation was fused either to CENP-B or to Mis12 (55). Under low tension **(D)**, the sensor is phosphorylated regardless of its position, suggesting that Aurora B can reach both positions with similar efficiency. Under high tension **(E)**, the outermost sensor cannot be phosphorylated efficiently (possibly because it becomes dephosphorylated), whereas the innermost sensor continues to be phosphorylated. **(F)** The phosphorylation potential of Aurora B decays very rapidly after the position defined by the innermost FRET sensor (fused to CENP-B) when chromosomes are under stretch. This rapid decay takes place in ~200 nm or less along the inter-kinetochore axis.

attachment, substrates remain phosphorylated and attachments intrinsically unstable. In conclusion, the "phosphorylation potential" of Aurora B is dampened with a sharp edge within the very short distance that separates centromeres from kinetochores under tension, whereas it is largely insensitive to the considerable degree of stretching of the inter-kinetochore region (**Figure 5F**). This clearly suggests that Aurora B is able to read intra-kinetochore tension rather than inter-kinetochore tension, but how it achieves this has remained unclear.

READING INTRA-KINETOCHORE TENSION: FROM CENTROMERES?

It was initially hypothesized that the spatial separation that promotes stabilization of kinetochore–microtubule attachment might be linked to the increase in the distance from centromeres, where the bulk of Aurora B is positioned, to kinetochores, where the substrates of Aurora B that mediate microtubule attachment are located (33). With increasing distances, indicative of end-on

attachment, the ability of Aurora B at centromeres to reach its substrates would progressively decrease, allowing a progressive stabilization of the kinetochore–microtubule interface. Implicit in this model is the existence of a sharp and separable centromere–kinetochore boundary, but a precise definition of what this boundary looks like is missing. If we consider that CENP-A is embedded in centromeric chromatin containing abundant histone H3 nucleosomes, H3-T3-P is likely to extend to the immediate periphery of kinetochores, and there is no obvious reason why the CPC should not bind to these H3 nucleosomes. It is unknown whether these nucleosomes become separated from CENP-A containing nucleosomes when tension builds up following microtubule end-on attachment to kinetochores.

Because the subunits of the CPC turn over at centromeres with halftimes ($t_{1/2}$) of <1 min (137–139), an alternative hypothesis is that a gradient of Aurora B substrate phosphorylation may be created by initial recruitment of the CPC to H3-T3-P to the centromere and by subsequent release and diffusion from centromeres (41) (**Figure 6A**). Indeed, this mechanism ("centromere gradient") can create gradients of Aurora B substrate phosphorylation (140–142). We note, however, that such gradients are relatively flat and form over length scales of several micrometers (140–142). It is therefore unlikely that this diffusible gradient of Aurora B would generate the very sharp edge of activity observed within the ~100 nm (0.1 μm) length scale of the kinetochore.

The idea that the phosphorylation gradient of Aurora B is created by centromere recruitment and release of the CPC is also at odds with the results from experiments in which centromere enrichment of the CPC was prevented by targeted mutations in CPC subunits. For example, a Survivin mutant impaired in its ability to bind H3-T3-P supports chromosome segregation and long-term viability of DT40 cells deprived of endogenous Survivin (82). Similarly, the deletion of residues 1–228 of Sli1 (Sli15ΔN) rescues the lethality of *BIR1* in *S. cerevisiae*, even if Sli15ΔN does not localize to centromeres (see below) (101). Furthermore, phosphorylation of an inner kinetochore substrate of Aurora B, Ser7 of CENP-A (CENP-A-S7-P) is unaltered after depletion or inhibition of Haspin with 1 μM 5-ITu (5-iodotubercidin), a concentration of the drug that clears centromeres of H3-T3-P (78, 143). Altogether, these observations suggest that a gradient of Aurora B substrate phosphorylation at the centromere–kinetochore interface can be established also in the absence of Aurora B at centromeres.

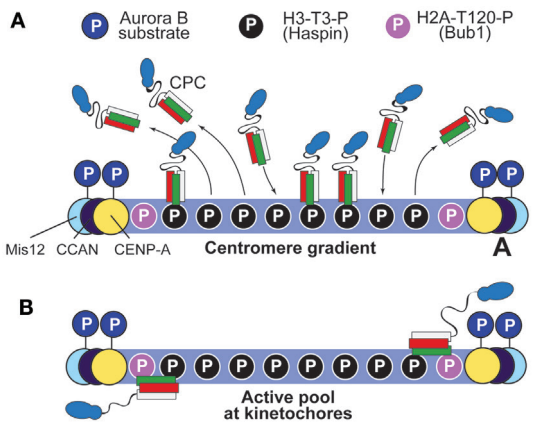

FIGURE 6 | Two possible models to account for the gradient of Aurora B phosphorylation. **(A)** The "centromere gradient" model predicts that the CPC becomes recruited to centromeres as illustrated in **Figure 4B**, and it then dissociates from them, creating a gradient of CPC concentration (and therefore, by inference, of substrate phosphorylation). We note, however, that it is unlikely that this gradient could account for the sharp transition of phosphorylation potential of Aurora B within the very limited scale length of the kinetochore (see **Figure 5**). **(B)** An alternative model posits that an active form of the CPC is anchored near the kinetochore, and that the centromere pool is not strictly required for function (it was therefore omitted from the drawing). Proximity to H2A-T120-P might lead to the activation of this kinetochore pool of the CPC. Interactions with kinetochore subunits are also possible.

… OR FROM KINETOCHORES?

An alternative hypothesis for Aurora B function is that the functionally relevant pool of Aurora B resides near or at kinetochores, rather than centromeres (22, 59). Strikingly, it was shown that centromeric accumulation of Aurora B is subordinate to kinetochore establishment. An ectopic kinetochore built at a chromosome site containing a Lac-O array, by tethering segments of the kinetochore CCAN subunits CENP-C or CENP-T, promotes accumulation of H3-T3-P and of the CPC in an area comprised between the two ectopic tethering sites, suggesting that the ectopic kinetochore dictates the position of the "centromere" (144). Remarkably, the CENP-C and CENP-T segments used in these experiments do not recruit CENP-A, suggesting that the latter is not required for CPC recruitment at the ectopic "centromere." Establishment of this ectopic centromere likely involves kinetochore-associated Bub1, which may promote the recruitment of Sgo1. Sgo1, in turn, plays a crucial role in the establishment and protection of centromeric cohesion (104, 107, 115, 145). In *S. cerevisiae*, the core centromere (a ~125 bp segment on which the kinetochore is built) and two kinetochore proteins, Iml1 and Chl4 (respectively, related to the CCAN subunits CENP-L and CENP-N in humans), are important for the spreading of Sgo1 to pericentromeric regions (146).

Further emphasizing the role of kinetochores in CPC localization is the observation that the abundance of the centromere pool of Aurora B in diploid human cells is controlled dynamically by kinetochore attachment status, with misaligned chromosomes showing an enrichment of Aurora B (135). En passing, this dynamic kinetochore-driven enrichment of Aurora B at centromeres requires Aurora B and Plk1 activity, but not Mps1's (135). The dispensability of Mps1 is further testified by experiments showing that chemical inhibition of Mps1 activity does not affect the total levels of H3-T3-P, the phosphomark that recruits the CPC to centromeres (124). However, Mps1 may modulate the timing of CPC accumulation at the centromere (147).

The role of kinetochores is further supported by the observation that Knl1, one of the outer kinetochore KMN subunits, is required for Aurora B activation, and that the active form of Aurora B (monitored through AB-T232-P) resides at kinetochores rather than at centromeres (148–150). Identifying the precise reason for this is a crucial question for future analyses.

It is plausible that the centromere, contrary to the prometaphase kinetochore, represents a domain of high phosphatase activity that prevents the accumulation of the active form of Aurora B. The kinetochore pool of Aurora B was recently observed under conditions of Haspin inhibition, and was shown to depend on dimerization of Borealin (124).

Sli15ΔN, the Sli15 mutant discussed in the previous section, is also observed at kinetochores in cells depleted of Bir1/Survivin, suggesting that kinetochore localization of this mutant is survivin independent (101). Remarkably, while the Sli15ΔN mutant rescued chromosome segregation and the lethality associated with loss of Bir1/Survivin in *S. cerevisiae*, it was synthetic lethal with two normally non-essential CCAN subunits at the kinetochore, Mcm21 and Ctf19 (respectively, homologous to CENP-O and CENP-P of higher eukaryotes) (101, 151). These two proteins and their binding partners Okp1 and Ame1 (considered to be homologous to CENP-Q and CENP-U, respectively) have been previously shown to promote kinetochore recruitment of the CPC and to be necessary for the error correction activity of the CPC in *S. cerevisiae* (152–154).

How Aurora B is recruited to kinetochores is unclear, but a hint comes from the observation that INCENP is phosphorylated on Thr59 (INC-T59-P) by Cdk1 kinase, the master regulator of cell division (64, 129, 155). A phosphomimetic T59E mutant causes INCENP to persist on chromosomes rather than to become relocated on the central spindle at anaphase (155, 156). With the decline in tension upon sister chromatid dissolution in anaphase, the T59E mutant causes the re-activation of typical Aurora B-dependent events at kinetochores, including the recruitment of Mps1, Bub1, and BubR1. Thus, "stripping" of the CPC from centromeres might be required to prevent EC and SAC re-activation during anaphase. However, retention of CPC localization is *per se* not sufficient for a complete re-activation of these pathways (156–160).

Of note, both H3-T3-P and H2A-T120-P are removed from centromeres at anaphase (81, 161), suggesting that the T59E INCENP mutant may not be retained on chromosomes through these phosphomarks but via a different, currently uncharacterized interaction. Phosphorylation of INC-T59-P may prevent an interaction of INCENP with the MKLP2 kinesin, which is required to relocate the CPC to the central spindle at anaphase (156, 162, 163). Alternatively, it might mediate a direct interaction with one or more kinetochore subunits. This pathway is conserved in *S. cerevisiae*, where dephosphorylation of Cdk1-dependent sites on Sli15 was shown to be important for CPC relocation at anaphase (123).

Thus, the active CPC pool that generates the intra-kinetochore phosphorylation gradient discussed above may reside within kinetochores, rather than being delivered there by a diffusible gradient of the kinase (**Figure 6B**). How does this kinetochore pool of the CPC generate the observed phosphorylation gradient within kinetochores? We have previously proposed that INCENP might act as a flexible arm whose maximal extension limits the reach of Aurora B within kinetochores (22). In this "dog leash" model, intra-kinetochore stretch promoted by microtubule binding might create relative movements of the Aurora B substrates relative to the tethered CPC, until substrates become unreachable by the kinase (**Figure 7**). As discussed above, the coiled-coil domain of INCENP has been recently shown to contain a SAH domain (126). In agreement with the "dog leash" model, it was shown that the length of the SAH domain modulates the ability of Aurora B to reach its substrates in the centromere and in the outer kinetochore (126).

… OR FROM MICROTUBULES?

Yet, another hypothesis is that Aurora B performs its functions from microtubules (101). This theory builds on previous work characterizing INCENP/Sli15 as a microtubule-binding protein

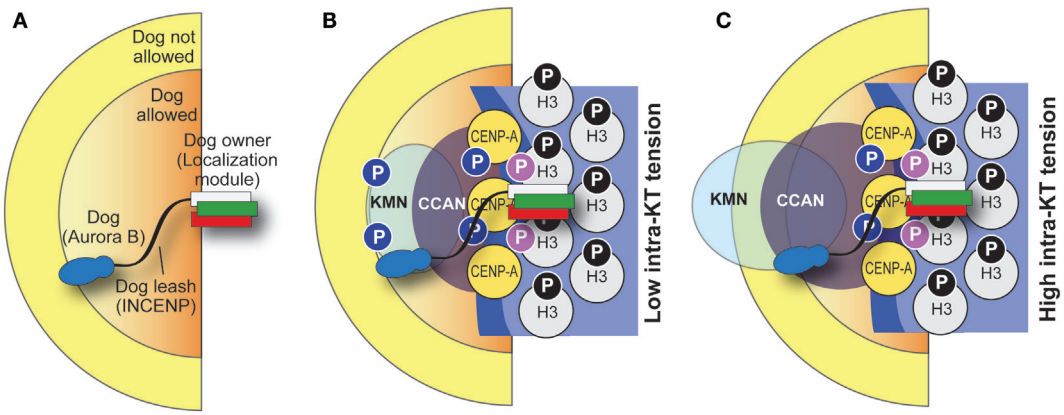

FIGURE 7 | "Dog leash" model of CPC function. **(A)** The idea behind the "dog leash" model is that the localization module of the CPC (the owner) is tethered at the base of kinetochores. INCENP acts as a "dog leash" that allows the "dog," Aurora B, to phosphorylate substrates only within limits defined by the length of the linker (which may vary, e.g., as a consequence of phosphorylation). This defines a boundary between regions where the dog is allowed and regions where it is not. **(B,C)** Application of the dog leash model to kinetochores. Under low tension **(B)**, Aurora B can reach out in the kinetochore and phosphorylate substrates there. Under high tension **(C)**, substrates (e.g., in the KMN network) have crossed the boundary defined by the leash and become unreachable. Note that in this drawing the CPC is tethered at the base of the kinetochore and its position is stationary, but this may not be the case and tension might increase its distance from the CENP-A base of the kinetochore. The function of a phosphatase is implicit in the model.

(discussed above). Indeed, Sli15ΔN, the already discussed deletion mutant rescuing the lethality of the *bir1* deletion in *S. cerevisiae*, localizes strongly to microtubules (101). Another recent study suggests that microtubules regulate Aurora B localization and activity in prometaphase (164). Albeit attractive, the hypothesis that microtubule localization is sufficient for CPC function requires further evaluation, not least because CPC function is delivered also in cells lacking microtubules altogether (e.g., because treated with spindle poisons). We reason that because the Sli15ΔN mutant retains kinetochores localization, the most parsimonious interpretation of its ability to suppress the lethality of the *bir1* deletion is that it does so from kinetochores.

A MODEL FOR CPC LOCALIZATION AND FUNCTION

Based on the discussion above, we propose a tentative model for the mechanism of CPC localization (**Figure 8**). Cdk1-mediated phosphorylation of INCENP may be the initial trigger causing the recruitment of a pool of the CPC to kinetochores through interactions with yet to be identified subunits, possibly within the CCAN at the inner kinetochore. At kinetochores, Aurora B contributes to the recruitment of Bub1 kinase, which creates H2A-T120-P to recruit a kinetochore pool of Sgo1 (107, 115). The latter is responsible for the homeostatic control of phosphorylation at kinetochores through recruitment of PP2A phosphatase and Polo-like kinase 1 (Plk1). How these proteins interact at kinetochores is largely unclear and requires further analysis.

We surmise that execution of this pathway may have two main consequences: (1) limiting the activation of Aurora B to the kinetochore pool and (2) igniting a positive feedback loop that promotes Haspin activation and further CPC accumulation at centromeres via phosphorylation of H3-T3-P in neighboring H3 nucleosomes. Aurora B itself, Plk1, Bub1, and, to a lesser extent, Mps1 may be involved in this positive feedback loop (62, 120, 121, 124, 135, 145, 147, 165). Although Bub1 acts downstream from Mps1 in the SAC pathway (3), there is significant residual Bub1 at kinetochores of cells in which Mps1 activity has been inhibited (91, 92).

Of note, H2A-T120-P is limited to kinetochores (115). Although it has been proposed that the CPC may localize at the intersection of H2A-T120-P and H3-T3-P (81), the overlap between these two marks may be limited to the inner kinetochore, whereas the localization domain of Aurora B is broader and clearly extends to the centromere. However, H2A-T120-P may contribute, by recruiting Shugoshin, to limit the activation of Aurora B to the kinetochore pool (149), although the details of this mechanism remain obscure. Sgo1 may also provide another anchoring point for the CPC at kinetochores, as the BIR domain of Survivin recognizes the N-terminal region of Sgo1 (76).

MECHANISMS OF ERROR CORRECTION

A comprehensive picture of the contribution of Aurora B to the establishment of bi-orientation is still missing, but there has been substantial progress in recent years. Importantly, Aurora B has also been shown to regulate the structural stability of the

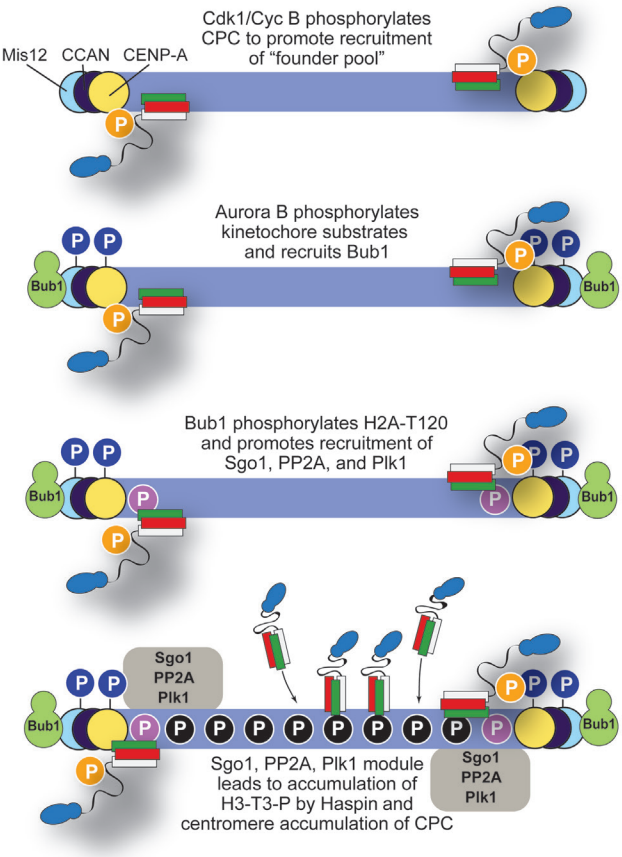

FIGURE 8 | A model for localization of CPC. The initial step in accumulation of CPC to centromeres may involve Cdk1/Cyclin B, which phosphorylates INCENP-T59 and possibly other sites on CPC. Initial CPC activity at kinetochores recruits Bub1, which in turn, after phosphorylating H2A-T120, promotes recruitment of Sgo1, PP2A, and Plk1. These, in turn, promote activation of Haspin and centromere accumulation of the CPC. See main text for additional detail.

kinetochore. For instance, it phosphorylates the CCAN subunit CENP-C/Mif2 to confer robustness to kinetochore function (166). In addition, phosphorylation of human Dsn1/Mis13 at two closely spaced residues (S100 and S109) increases the binding affinity of the Mis12 complex for CENP-C (42, 134, 166–171).

As already discussed above, anaphase retention of the CPC on kinetochores by expression of the T59E INCENP mutant or by suppression of MKLP2 (see above) leads to loss of tension that re-activates Aurora B-dependent pathways, including re-recruitment of Mps1, Bub1, and BubR1 (156). Nevertheless, kinetochores remain attached to their microtubule fibers under these conditions, indicating that re-activation of Aurora B is not sufficient for error correction. The crucial missing factor is the activity of Cdk1, which declines at anaphase due to degradation of Cyclin B. Artificial retention of Cdk1 activity in cells that have undergone sister chromatid separation

leads to extensive destabilization of kinetochore–microtubule attachments (158, 159, 172).

Aurora B contributes at least three, partly related functions, to the process of bi-orientation: (1) the modulation of microtubule-binding affinity of the kinetochore to allow or prevent maturation of attachments; (2) the regulation of microtubule dynamics by controlling the activity and localization of microtubule-associated proteins; (3) the control of the localization of additional proteins involved in the regulation of kinetochore–microtubule attachment, including protein phosphatases that antagonize the phosphorylation of Aurora B substrates (4, 173).

A widely studied example of how Aurora B modulates the affinity of kinetochores for microtubules is the phosphorylation of multiple residues on a disordered and positively charged ~80-residue tail at the N-terminus of Ndc80/Hec1, a subunit of the Ndc80 complex (30, 31, 174–178). This segment of Ndc80 neighbors a calponin-homology (CH) domain that binds directly to microtubules (174, 179). Different models have been proposed for how Ndc80 phosphorylation modulates the binding affinity of the Ndc80 complex for microtubules (174, 175, 178, 180–182). A rigorous recent analysis suggested that each new phosphorylation event on the Ndc80 tail determines a relatively small decrease in microtubule-binding affinity by the Ndc80 complex, regardless of which specific position, among the eight or nine available, becomes phosphorylated (177). In this model, the phosphorylation sites of the Ndc80 tail configure a "rheostat" capable of increasing the microtubule-binding affinity of individual Ndc80 complexes by a factor as small as 20- and as large as 100-fold when transiting from a fully phosphorylated form of the protein to a fully dephosphorylated one (174, 177). Because the degree of phosphorylation of the Ndc80 tail is maximal when tension is low (e.g., in the absence of microtubules) (148), it is plausible that dephosphorylation of the Ndc80 complex is a gradual process that occurs concomitantly with the generation of tension within kinetochores. Consistent with this hypothesis, expression of a non-phosphorylatable mutant of the Ndc80 complex leads to hyper-stretched kinetochore–microtubule attachment and frequent attachment errors (31, 148, 181). Ndc80 has also been shown to have a direct influence on the dynamics of kinetochore microtubules, and Ndc80 phosphorylation may influence this property (183). Importantly, another Aurora family member, Aurora A, has also been very recently implicated in this correction mechanism (184, 185).

In addition to microtubule binding by the KMN network, other Aurora B substrates are important for the stabilization of the kinetochore–microtubule interface. The Dam1 complex in *S. cerevisiae* and the SKA complex in higher eukaryotes are structurally unrelated but may perform analogous functions as stabilizers of kinetochore–microtubule attachment (186–193). Contrary to the Ndc80 complex, both the Dam1 and the SKA complexes are able to form processive, load-bearing attachments to depolymerizing microtubule *in vitro*, and both contribute to retaining the Ndc80 complex at depolymerizing microtubule tips, possibly enhancing the overall processivity of microtubule binding (191–197). Importantly, Aurora B phosphorylation negatively regulates the association of the SKA and Dam1 complexes with Ndc80 (194–196, 198–201). An analogous pattern is also observed for the kinetochore recruitment of another microtubule-binding complex, the Astrin–SKAP complex (202). Thus, recruitment of these additional microtubule-binding complexes likely "seals" the kinetochore–microtubule interface of bi-oriented sister chromatids on which the phosphorylation of Aurora B has already faded.

Aurora B also controls kinetochore localization and activity of the non-conventional kinesin-13 family member mitotic centromere-associated kinesin (MCAK, Kif2C), which plays an important role in error correction as a microtubule depolymerase at microtubule ends (39, 203–210). Kinetochore and centromere recruitment of MCAK requires Aurora B phosphorylation of MCAK (203, 206, 210) and the presence of Sgo2 (113, 114, 118, 211).

Also dependent on Aurora B is the recruitment of CENP-E, a kinesin that plays an important role in the initial, lateral attachment of kinetochores to microtubules that precedes end-on attachment (7, 35, 212, 213). Conversion from an initial lateral attachment to end-on attachment occurs also in budding yeast (214). It has been proposed that lateral attachments may be insensitive to Aurora B activity, and therefore may be able to provide a mechanism for establishment of initial kinetochore–microtubule attachments even when Aurora B activity is high (4, 200).

Finally, Aurora B is in an antagonistic relationship with protein phosphatases, most notably of the protein phosphatase 1 (PP1) and PP2A-B56 families (4). These phosphatases counter phosphorylation by Aurora B kinase and other downstream kinases both in the EC and in the SAC (15, 109, 215–218). Many details of the complex molecular mechanisms subtending to the antagonism of Aurora B and PP1 and PP2A phosphatases remain to be elucidated. The following examples illustrate the complexity of this regulation.

Distinct interactions of the B56 regulators with Sgo1, Sgo2, and with the checkpoint component BubR1 recruit the PP2A holoenzyme to centromeres and kinetochores during mitosis (108, 115, 211, 219). The interaction of PP2A-B56 with BubR1 requires the so-called kinetochore attachment regulatory domain (KARD) motif of BubR1, which undergoes multisite phosphorylation (presumably) at kinetochores, partly mediated by Plk1 (219). Interference with the interaction of PP2A with the KARD domain leads to an elevation of Aurora B substrate phosphorylation in the outer kinetochore and prevents the stabilization of kinetochore–microtubule attachment (219).

Repo-Man, a protein scaffold that interacts with the PP1 phosphatase, is responsible for the clearance of the Haspin-mediated phosphorylation of H3-T3-P (161). Aurora B counteracts the chromatin recruitment of Repo-Man by phosphorylating it on Ser893, thus ultimately preventing the dephosphorylation of H3-T3-P. Dephosphorylation of Ser893, which might follow the release of the CPC from its centromeric localization at anaphase, requires an interaction of Repo-Man with PP2A, which is mediated by a motif closely related to the KARD motif of BubR1 (220).

Kinetochore recruitment of PP1 requires interactions with Knl1 and with CENP-E (15, 16, 221). Aurora B prevents kinetochore targeting of PP1 by phosphorylating a PP1-docking motif on Knl1 (15, 16). In *S. cerevisiae*, a requirement for kinetochore recruitment of PP1 to Knl1 (known as Spc105 in this organism), without which the SAC cannot be silenced, resulting in cell

lethality, can be bypassed if Aurora B activity is compromised (16). Both PP1 and PP2A have been implicated as suppressors of the Mps1-dependent phosphorylation of the multiple Met-Glu-Leu-Thr (MELT) repeats of Knl1 that provide a docking site for the Bub1/Bub3 complex at kinetochores (215, 216).

AURORA B IN THE SAC

The SAC effector MCC consists of three SAC proteins, Mad2 (mitotic arrest deficient 2), Bub3 (budding uninhibited by benzimidazoles 3), BubR1 (Bub1-related 1, the human ortholog of yeast Mad3), and the APC co-activator Cdc20. Additional SAC components are Mad1, the kinases Mps1 (monopolar spindle protein 1), and Bub1 (budding uninhibited by benzimidazoles 1), and, limitedly to metazoans, the components of the Rod-Zwilch-ZW10 complex (RZZ). All SAC components contribute to the formation of the MCC and therefore to APC/C inhibition (3, 45).

The mechanisms through which Aurora B regulates the SAC are likely to be closely interwoven with the mechanisms that trigger error correction. As already pointed out in the previous paragraph, retention of Aurora B activity on chromosomes during anaphase is insufficient to cause error correction, but is sufficient to recruit *bona fide* SAC components, such as Mps1, Bub1, and BubR1, despite the retention of robust kinetochore fibers (156, 158–160, 172). This observation argues that Aurora B plays a direct role in the recruitment of the SAC components also in the absence of error correction and of unattached kinetochores. Incidentally, the observation that Mps1 can be recruited to anaphase chromosomes that have retained kinetochore fibers needs to be reconciled with the recent proposition that microtubules compete directly with Mps1 localization to kinetochores (222, 223).

Aurora B appears to occupy an upstream position in the pathway of recruitment of SAC components, as its inhibition prevents kinetochore recruitment of all other SAC components (35, 48, 52, 224, 225). Co-inhibition of Aurora B and Mps1 has profound synergistic effects in the impairment of SAC signaling (48, 52). Aurora B inhibition prevents Mps1 recruitment, and artificially tethering Mps1 to the kinetochore bypasses the checkpoint requirement for Aurora B in human cells, suggesting that a primary function of Aurora B in the SAC is the recruitment of Mps1 (51, 52). Conversely, when a downstream SAC component, such as Mad1:Mad2 is tethered to kinetochores, the resulting mitotic arrest depends on Aurora B (51, 226, 227).

Mps1 becomes recruited to the Ndc80 complex of the kinetochore (222, 223, 228, 229). The precise role of Aurora B in the recruitment of Mps1 remains unclear, but a role of Ndc80 phosphorylation has been suggested (223, 229). However, the observation that Aurora B activity becomes at least partly dispensable for kinetochore recruitment of Mps1 when the Mps1 TPR region is deleted suggests that Aurora B does not need to generate a docking site for Mps1 on Ndc80 but rather regulates a conformational transition within Mps1 (230).

After its Aurora B-dependent recruitment to kinetochores, Mps1 promotes the recruitment of downstream SAC component by phosphorylating Knl1 on multiple MELT repeats to dock the Bub1:Bub3 complex (231–234). The latter, in turn, elicits the formation of a comprehensive assembly of SAC protein that may facilitate SAC signaling from kinetochores (235–240).

CONCLUSION

Aurora B and the CPC are crucial for successful chromosome segregation during cell division. The two pathways Aurora B controls, error correction and the SAC, are tightly interwoven and interdependent. Both appear to rely on spatial control of Aurora B activity, but the precise molecular basis for this spatial control remains unknown. Future analyses will have to rigorously test the implications of the models that have been proposed to explain the spatial regulation of Aurora B activity, including the "centromere gradient" model and the "dog leash" model. It is hoped that global analyses of Aurora B substrate phosphorylation within the framework of predictable alterations of CPC and kinetochore function will finally shed light on the molecular basis of a mechanism that is indispensable for life.

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New auroras on the roles of the chromosomal passenger complex in cytokinesis: implications for cancer therapies

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The chromosomal passenger complex (CPC), composed of a kinase component, Aurora B, the scaffolding subunit inner centromeric protein, Borealin, and Survivin, is a key regulator of cell division. It controls multiple events, from chromosome condensation in prophase to the final separation or abscission of the two daughter cells. The essential functions of the CPC during metaphase, however, have always hindered an accurate study of its role during cytokinesis. The recent development of small molecule inhibitors against Aurora B and the use of elegant technologies such as chemical genetics have offered new approaches to study the functions of the CPC at the end of cell division. Here, we review the recent findings about the roles of the CPC in controlling the assembly of the cleavage furrow, central spindle, and midbody. We will also discuss the crucial function of this complex in controlling abscission timing in order to prevent abscission when lagging chromatin is present at the cleavage site, thereby avoiding the formation of genetically abnormal daughter cells. Finally, we offer our perspective on how to exploit the potential therapeutic applications of inhibiting CPC activity during cytokinesis in cancer cells.

Keywords: cell division, microtubule, Aurora B, abscission, anticancer therapies

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INTRODUCTION

Faithful chromosome segregation during cell division is crucial for growth, development, and reproduction in many organisms. Defects in this process have been associated with various genetic diseases, including cancer. For example, many cancer cells present chromosomal instability (CIN), which contributes to carcinogenesis by altering the balance of critical growth and death pathways and the overall expression of oncogenes and tumor suppressors. Thus, understanding the mechanisms that control genome segregation during mitosis can reveal some of the processes that promote genomic instability in cancer. Consistent with this, animal models have shown that failure in controlling either chromosome segregation or the final separation of the two dividing cells – cytokinesis – can cause CIN and carcinogenesis (1–4). Moreover, one of the hallmarks of cancer is uncontrolled cell proliferation, and many cell division regulators are validated targets for the isolation of novel chemotherapeutic drugs for the treatment of cancer pathologies. In particular, mitotic serine/threonine kinases have become an intensively studied class of anticancer drug targets, and inhibitors of mitotic kinases, such as Aurora and Polo-like kinases, are currently undergoing clinical trials (5). A comprehensive knowledge of the function of these kinases is therefore crucial to identify new pathways and biomarkers that could aid in the design of better-targeted and less toxic anticancer therapies.

The chromosomal passenger complex (CPC) is one of the major regulators of cell division in all eukaryotes and is composed of four subunits: the scaffolding component inner centromeric protein (INCENP), Borealin, Survivin, and Aurora B kinase [reviewed by Carmena et al. (6)]. The name of the complex reflects its dynamic distribution during mitosis. It localizes to centromeres until chromosome segregation and then relocates to the central spindle, an array of antiparallel and interdigitating microtubules that forms between the separating sister chromatids after anaphase onset (**Figure 1**). The translocation from centromeres to the central spindle depends on the interaction of INCENP's coiled-coiled domain with microtubules and requires the kinesin MKLP2/KIF20A (7, 8). Consistent with this localization, the CPC controls various events throughout cell division, from chromosome condensation in prophase to the final separation or abscission of the two daughter cells. The best-known and most studied role of the CPC is undoubtedly the correction of improper kinetochore–microtubule attachments in prometaphase, but there is a growing evidence that the CPC plays important roles also during cytokinesis. Cytokinesis is mediated by the constriction of an actomyosin contractile ring at the equatorial cell cortex that bisects the dividing cell. As mentioned earlier, after anaphase onset, microtubules reassemble to form the central spindle, an array of antiparallel microtubules that overlap at their plus ends in a region called the spindle midzone (**Figure 2**). Central spindle and astral microtubules cooperate to activate the small GTPase RhoA at the equatorial cortex, which in turn triggers the assembly and constriction of an actomyosin ring responsible for cleavage furrow ingression. Furrow ingression progressively compacts the central spindle to form an organelle known as the midbody, which provides a platform important for the recruitment and organization of many proteins that regulate the final abscission of the two daughter cells (**Figure 2**). Here, we review recent findings about the role of the CPC in controlling cleavage furrow ingression, the formation and dynamics of the central spindle, the architecture of the midbody, and abscission. Finally, we offer our perspective on the possibility of exploiting the roles of the CPC in cytokinesis for anticancer therapy.

THE CPC PROMOTES CENTRAL SPINDLE AND MIDBODY FORMATION

Various microtubule-associated proteins cooperate to regulate the assembly and dynamics of central spindle microtubules [reviewed by Douglas and Mishima (9)]. The CPC has been shown to control the activities of at least two central spindle kinesin motors, MKLP1/KIF23 and KIF4A (**Figure 2**).

MKLP1 is the motor component of the centralspindlin complex, a heterotetramer composed of two MKLP1 subunits and two molecules of RacGAP1/MgcRacGAP/Cyk4 (10). Centralspindlin is required for central spindle formation in many organisms, from nematodes to humans (10–13), but is also known to perform many other crucial roles during cytokinesis. For example, the RacGAP1 subunit interacts with and activates the RhoGEF Ect2, which in turn promotes RhoA activation and contractile ring assembly and constriction (14–17). RacGAP1 also associates with the plasma membrane through its C1 domain, and this interaction provides a crucial link between the midbody and the membrane, important for the final abscission of the two daughter cells (18). Centralspindlin activity is also essential for microtubule bundling (19) and formation of the central spindle and midbody. To exert these functions, centralspindlin complexes need to oligomerize, and

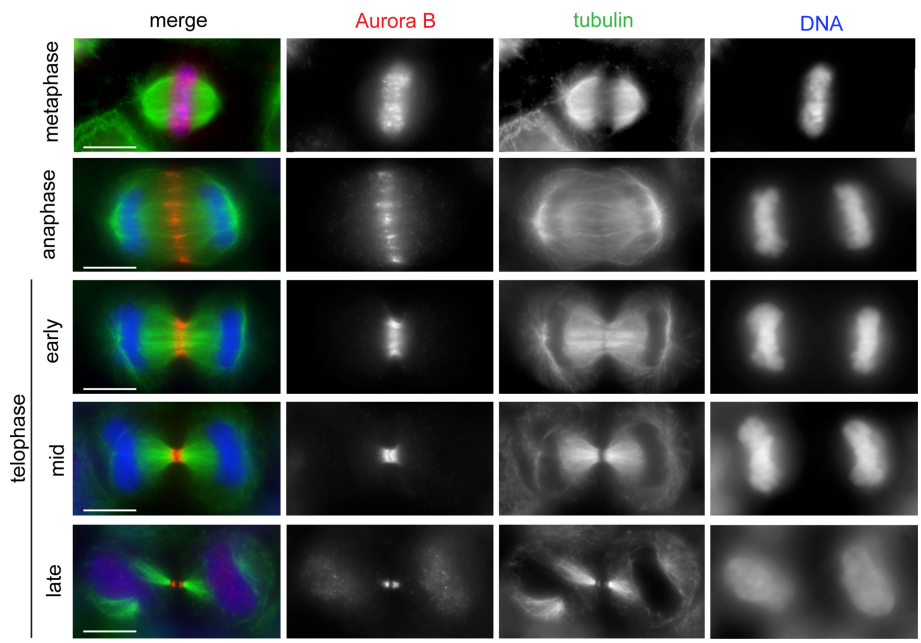

FIGURE 1 | The CPC shows dynamic localization during mitosis and cytokinesis. HeLa cells were fixed and stained to reveal Aurora B (red), tubulin (green), and DNA (blue). The CPC (here represented by Aurora B) translocated from the mitotic chromosomes to the central spindle early in anaphase. In early telophase, the CPC accumulated at the midbody arms. Scale bars: 10 μm.

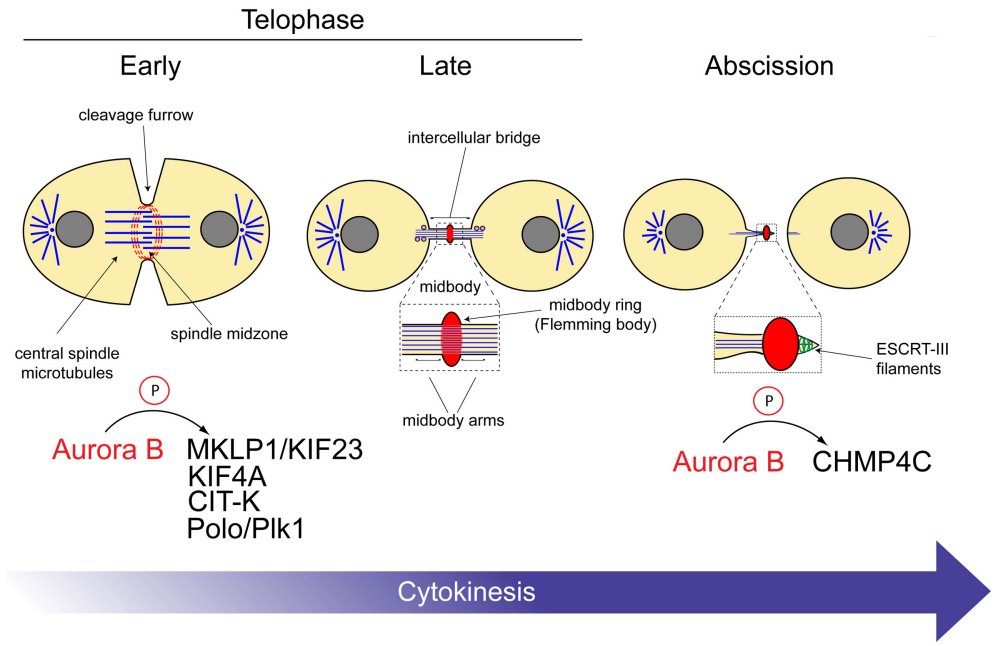

FIGURE 2 | Aurora B substrates during cytokinesis. Schematic diagram illustrating the roles of the CPC at different stages of cytokinesis. The substrates of Aurora B are indicated at the bottom. See text for details.

this clustering is promoted by Aurora B phosphorylation of the serine 708 in the MKLP1 C-terminal tail. This phosphorylation prevents the association of MKLP1 with 14-3-3 protein, which inhibits centralspindlin clustering (20). Thus, Aurora B promotes the assembly of the central spindle via phosphorylation of the kinesin component of the centralspindlin complex.

The kinesin KIF4A and the microtubule-associated protein PRC1 form another complex important for central spindle assembly. PRC1 is able to cross-link and bundle microtubules and is transported to the spindle midzone by KIF4A (21–25). The formation of the PRC1/KIF4A complex is prevented by cyclin-dependent kinase 1 (Cdk1) phosphorylation in metaphase (24), but after anaphase onset, this interaction is instead promoted through Aurora B phosphorylation of KIF4A (26). This phosphorylation also stimulates the microtubule-dependent ATPase activity of KIF4A, which suppresses microtubule dynamics and limits the length of the central spindle (26). Quite interestingly, KIF4A is also responsible for maintaining phosphatase PP2A-B56 at the central spindle, thereby creating a spatially restricted negative feedback loop counteracting Aurora B in cytokinesis (27).

We have recently found that the CPC directly interacts with citron kinase (CIT-K), an important midbody protein that links a network of contractile ring and central spindle proteins, including actin, anillin, myosin, MKLP1, KIF14, and RhoA, in both *Drosophila* and human cells (28–32). The CPC and CIT-K depend on each other for proper localization to the midbody and Aurora B phosphorylates CIT-K to control its localization and interaction with central spindle partners (McKenzie et al., submitted). Thus, a cross-regulatory mechanism between two important kinases seems to regulate proper midbody architecture and successful completion of cytokinesis.

New evidence also involved Aurora B in the regulation of Polo kinase during cytokinesis in *Drosophila*. Polo kinase was the first identified member of the evolutionary conserved family of Polo-like kinases (Plk). All of the members of this family have essential roles during cell division (33). Aurora B phosphorylates Polo on its activation loop to promote its kinase activity in mitosis (34). In cytokinesis, Aurora B-mediated phosphorylation of Polo is responsible for its translocation from central spindle microtubules to the midbody. Failure in this process induces cytokinesis defects (35). Not much is known about Polo function at the end of cytokinesis, but its localization is probably necessary to activate substrates essential for abscission.

In conclusion, together these data indicate that the CPC orchestrates the activity of various proteins to regulate the correct assembly and size of both the central spindle and midbody (**Figure 2**).

IS THERE A ROLE FOR THE CPC IN CLEAVAGE PLANE POSITIONING?

The CPC was found to accumulate at the spindle midzone and equatorial cortex very rapidly after anaphase onset (**Figure 1**) (36), leading to the proposal that this localization could reflect a role for the CPC in positioning the division site and promoting contractile ring assembly. In 2008, a study reported that CPC translocation to the central spindle generates an Aurora B phosphorylation gradient that has its peak at the spindle midzone (37). This Aurora B gradient has been suggested to determine the position of the cleavage plane, but the molecular mechanisms are still lacking. More recently, another group has reported that Aurora B-mediated centralspindlin clustering is important to promote the interaction of the RacGAP1 with the plasma membrane and proposed that this event could promote RhoA activation at the cleavage furrow in both nematodes and human cells (38). In conclusion, although these data point to a potential role for Aurora

B in furrow formation, further studies are needed to define the molecules and mechanisms involved in this process.

THE CPC REGULATES ABSCISSION TIMING

The CPC has been proposed to prevent abscission in the presence of DNA at the cleavage site, thereby avoiding the formation of genetically abnormal daughter cells (39). In this study, it was reported that if lagging chromatin lingered at the cleavage site, Aurora B remained active and stabilized the intercellular bridge. The Aurora B target(s) in abscission, however, have remained elusive until a few years ago when two studies simultaneously showed that one of such targets is the Snf7 component of the endosomal sorting complex required for transport III (ESCRT-III) (40, 41). ESCRT proteins are evolutionarily conserved and best known for catalyzing membrane fission events both in virus budding and in the sorting of receptors into vesicles that bud off into the lumen of the endosome, creating multivesicular bodies (MVBs) (42). The ESCRT-III complex provides the core machinery that mediates membrane deformation and fission events during these events (43) as well as during abscission, which is topologically similar to MVB biogenesis and virus budding (44). Consistent with this, the ESCRT-III Snf7 components (known as CHMP4 proteins in humans) form spiral filaments that appear to remodel and constrict the membrane in order to create the abscission site (45). The CPC has been proposed to regulate abscission timing through direct interaction of the ESCRT-III Snf7 components both in *Drosophila* and humans (40, 41). In human cells, Borealin directly interacts with all three CHMP4 proteins, CHMP4A, CHMP4B, and CHMP4C, and Aurora B phosphorylates the terminal tail of CHMP4C. Two different models have been proposed to explain the regulation of CHMP4 proteins by the CPC. Carlton et al. (41) proposed that Aurora B phosphorylation promotes CHMP4C translocation to the midbody ring, where this ESCRT-III component inhibits abscission. By contrast, we proposed that CPC controls abscission through inhibition of CHMP4 polymerization and membrane association using two concurrent mechanisms: interaction of its Borealin component with all three CHMP4 proteins and phosphorylation of CHMP4C by Aurora B (40). These two concomitant events could preclude the formation of the ESCRT-III filaments essential for the formation of the constriction that physically separate the two daughter cells. In this model, CHMP4 proteins could assemble into spiral filaments only after CPC removal from the midbody. Overall, the CPC-mediated regulation of ESCRT-III has been suggested to act as a surveillance mechanism that prevents abscission in the presence of DNA at the cleavage site (39–41) (**Figure 2**).

TARGETING CPC FUNCTIONS IN CYTOKINESIS: AN ALTERNATIVE TO THE USE OF AURORA B SMALL MOLECULE INHIBITORS IN CANCER THERAPY?

Aurora kinases are overexpressed and amplified in many tumors, and Aurora A, but not Aurora B, displays oncogenic properties (46–48). However, polyploid cells overexpressing Aurora B can induce tumor formation when injected in nude mice, indicating that high levels of this kinase can be tumorigenic when coupled with cytokinesis failure (49). Consistent with this, tetraploid cells are more sensitive to Aurora B inhibition (50). Moreover, overexpression of Aurora B has been correlated with poor prognosis in a large number of cancers, including breast, ovarian, lung, nasopharyngeal, and hepatocellular carcinomas (51–55). This evidence has led to the development of small molecule inhibitors designed to interfere with the ATP-binding pocket of Aurora kinases that are currently in clinical trials for the treatment of various cancer pathologies (5, 56). Aurora B inhibitors have the ability to silence the spindle assembly checkpoint causing premature mitotic exit and consequent chromosome mis-segregation, cytokinesis failure, and nuclear fragmentation. All these defects ultimately lead to cell death, and this antiproliferative effect could potentially affect cancer cells that rely on Aurora B overexpression more than normal cells. However, in the long term, Aurora B inhibitors also interfere with the division of normal cells and indeed clinical toxicity profiles of Aurora inhibitors indicated frequent side effects such as myelosuppression, febrile neutropenia, and gastrointestinal problems (nausea, diarrhea, and mucositis), some of which have been directly attributed to Aurora B inhibition (5, 56). Furthermore, ATP-binding competitors are often not very selective and can inhibit the activity of other kinases. Thus, there is a need to develop alternative, more selective, and less toxic approaches to inhibit Aurora B activity.

There is evidence that targeting mitotic exit without perturbing spindle assembly could potentially be a more effective cancer treatment (57). In addition, low levels of cytokinesis failure do not seem to affect the shape and size of proliferative tissues in invertebrate animal models. For example, actively proliferating tissues such as brains and imaginal disks (i.e., the tissues that give rise to the adult fly) of larvae carrying strong mutant combinations of the *Drosophila* CIT-K homologue are highly polyploid (8N or more), misshapen, and smaller than their wild-type counterparts. By contrast, the same tissues of larvae carrying weaker allelic combinations are mostly tetraploid and normal in shape and size (58). These results indicate that, at least in *Drosophila*, organs can tolerate the presence of a considerable number of tetraploid cells and thus one single event of cytokinesis failure does not appear to significantly interfere with tissue development and function, whereas multiple cytokinesis failures lead to cell death and impair tissue development. Therefore, it is conceivable that inhibition of cytokinesis could selectively affect the proliferation of very actively dividing cells. There is also another motive to hypothesize that cytokinesis failure could selectively eliminate cancer cells. It is well established that many carcinomas present numerical chromosomal abnormalities (aneuploidy and/or polyploidy) and instability (59, 60). Therefore, provoking cytokinesis failure in these already chromosomally abnormal cancer cells could very rapidly increase their genomic content above a threshold compatible with cell viability. Together, these data indicate that targeting the cytokinetic functions of the CPC could be a valid alternative strategy for antiproliferative cancer therapy. Clearly, inhibitors of Aurora B kinase activity cannot be used to impair CPC functions specifically during cytokinesis. However, the use of small peptides

interfering with protein–protein interactions is emerging as a valid alternative pharmacological approach and peptides able to interfere with the interaction between INCENP and Aurora B have already been successfully used to impair CPC activity (61). Therefore, small peptides designed to impair CPC binding to its cytokinesis partners – such as KIF20A, CIT-K, and CHMP4 proteins – could be used to specifically inhibit this complex during cytokinesis and offer an alternative strategy for the development of highly targeted and potentially less toxic anticancer therapies.

CONCLUDING REMARKS

Considerable progresses have been made in the last years to understand the multiple roles of Aurora B and the CPC during the rapid and highly coordinated process of cytokinesis. These studies have indicated that the CPC plays important functions in every step of this process, from the initial determination of the cleavage plane to the final abscission of the two daughter cells. They have also determined that the CPC controls the proper segregation of the genomic material not only in early mitosis by controlling kinetochore–microtubule attachments but also later in cytokinesis by delaying abscission in the presence of lagging chromosomes at the cleavage site. These findings suggest that the CPC probably deserves the appellative of "guardian of genome segregation" for its key role in preventing aneuploidy and CIN. However, important questions still remain open. Do Aurora B and other kinases, such as Plk1 and CIT-K, share common substrates during cytokinesis? How is Aurora B function coordinated with that of other kinases to cooperatively regulate the function of their various substrates during cytokinesis? Elucidating these mechanisms will keep scientist in the field busy for many years to come.

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Ubiquitin-Mediated Degradation of Aurora Kinases

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The Aurora kinases are essential regulators of mitosis in eukaryotes. In somatic cell divisions of higher eukaryotes, the paralogs Aurora kinase A (AurA) and Aurora kinase B (AurB) play non-overlapping roles that depend on their distinct spatiotemporal activities. These mitotic roles of Aurora kinases depend on their interactions with different partners that direct them to different mitotic destinations and different substrates: AurB is a component of the chromosome passenger complex that orchestrates the tasks of chromosome segregation and cytokinesis, while AurA has many known binding partners and mitotic roles, including a well-characterized interaction with TPX2 that mediates its role in mitotic spindle assembly. Beyond the spatial control conferred by different binding partners, Aurora kinases are subject to temporal control of their activation and inactivation. Ubiquitin-mediated proteolysis is a critical route to irreversible inactivation of these kinases, which must occur for ordered transition from mitosis back to interphase. Both AurA and AurB undergo targeted proteolysis after anaphase onset as substrates of the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase, even while they continue to regulate steps during mitotic exit. Temporal control of Aurora kinase destruction ensures that AurB remains active at the midbody during cytokinesis long after AurA activity has been largely eliminated from the cell. Differential destruction of Aurora kinases is achieved despite the fact that they are targeted at the same time and by the same ubiquitin ligase, making these substrates an interesting case study for investigating molecular determinants of ubiquitin-mediated proteolysis in higher eukaryotes. The prevalence of Aurora overexpression in cancers and their potential as therapeutic targets add importance to the task of understanding the molecular determinants of Aurora kinase stability. Here, we review what is known about ubiquitin-mediated targeting of these critical mitotic regulators and discuss the different factors that contribute to proteolytic control of Aurora kinase activity in the cell.

Keywords: Aurora kinase, AURKA, AURKB, ubiquitin-mediated proteolysis, mitosis, APC/C

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INTRODUCTION

Aurora kinases are critical regulators of eukaryotic cell division. Their structure, activities, and functions have been extensively reviewed elsewhere (1–4) and will be mentioned only briefly here. Although Aurora kinases share a high degree of homology in their kinase domains, they play distinct roles in cell division (**Figure 1**). Aurora A (AurA) is an upstream element in the cascade of kinase

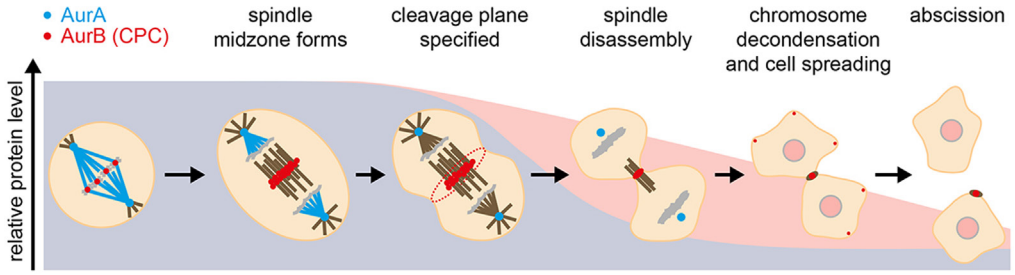

FIGURE 1 | Events during mitotic exit that are influenced by Aurora kinases, illustrated against their degradation profiles. Blue, AurA; red, AurB.

activities that control progression from G2 to M phase (through Bora-mediated activation of Plk1) and further plays direct roles in the maturation of the centrosome, in microtubule (MT) nucleation, and in the activation of other components required to build a bipolar mitotic spindle. AurA has a large number of substrates and interactors and alternative modes of activation, with different partners thought to give rise to distinct pools of active kinase. Aurora B (AurB), on the other hand, resides as an obligatory component of the chromosome passenger complex (CPC; along with INCENP, survivin, and borealin), which is essential for chromosome condensation and organization during mitosis, including a critical role as an effector of the mitotic checkpoint in regulating kinetochore-MT attachments on the mitotic spindle. Both Auroras have a predicted disordered N-terminus. This disordered region is more extensive in AurA and is found to mediate much of the specificity in AurA interactions, including those required for its functions at the centrosome (5).

It is striking that a version of AurA bearing a single-point mutation that switches its major mitotic interaction from TPX2 to INCENP can rescue knockdown of AurB (6, 7), and consistent with this observation, the two kinases appear to have many shared substrates. Other, specific, AurA or AurB substrates are likely to be constrained in their specificity in a cellular context through colocalization with one or other of the Auroras (8–11). Perhaps not surprisingly then, some lower eukaryotes were found to have a single Aurora kinase that carries out roles at both centrosomal and chromosomal locations and which can functionally substitute for either AurA or AurB in mammalian cells (12, 13). Spatial organization of Aurora kinase activity is thus thought to have arisen through the acquisition of different binding partners. The divergence of AurA and AurB functions in higher eukaryotes presents an interesting paradigm of differential regulation of kinase activity at specific subcellular domains. One of the elements contributing to such spatiotemporal regulation is differential targeted proteolysis.

The discovery of the ubiquitin–proteasome system (UPS) for targeted destruction of proteins (proteolysis) provided the framework for understanding how mitotic exit is driven by the activity of a multisubunit ubiquitin ligase complex known as the anaphase-promoting complex/cyclosome (APC/C) (14, 15). Targeting of securin and mitotic cyclins by the APC/C is necessary and sufficient for chromosome segregation and mitotic exit, respectively. Two decades of research on the APC/C have elucidated many features of its action and identified a large number of additional targets, which include the Aurora kinases. How, and why, the APC/C targets many different substrates with high temporal specificity remains an intriguing question in mitosis control. A resetting of the protein landscape of the cell must occur in preparation for interphase, for example, to rid the cell of factors that contributed to the assembly of the mitotic spindle. In some cases, however, it has been shown that the destruction of specific substrates contributes to the orderly progression of mitotic exit (16–19). Aurora kinases are two such substrates whose targeting by the APC/C and its coactivator Cdh1 contributes to the correct dosing, timing, and localization of their activities (17, 19).

There is now a substantial body of literature pointing to additional, non-mitotic roles of AurA, indicating a requirement for regulating Aurora kinase activity in interphase. It seems likely that a substantial fraction of AurA is protected from APC/C–Cdh1 activity in G1 phase, since APC/C–Cdh1 activity is predominantly nuclear (20–22) and AurA largely cytoplasmic (in contrast to AurB, which is strongly localized to the nucleus). Therefore, alternative UPS pathways may regulate cytoplasmic AurA outside of mitosis, and a number of candidate UPS components are reported in the literature.

The importance of regulating Aurora kinase activity is well established. In this review, we will consider the importance of proteolysis for the activity of Aurora kinases in mitosis and in interphase and what is known about mechanisms of Aurora kinase proteolysis. A bias in our review toward AurA reflects the fact that far more is known about proteolysis of this Aurora paralog in higher eukaryotes.

WHY ARE AURORA KINASES TARGETED FOR PROTEOLYSIS?

Spatiotemporal Organization of Aurora Kinase Activity Through the Cell Cycle

Proteolytic pathways have been shown to effect dosage compensation to enforce stoichiometric expression of the components of multiprotein complexes (23), and indeed, both AurA and AurB are destabilized by the loss of respective interaction partners TPX2 and INCENP (24, 25). This observation may be widely applicable to proteins, such as Aurora kinases, containing short linear interaction motifs (SLiMs) (26) within extended

unstructured regions. SLiMs can adopt specific structures upon interaction. Various pools of AurA act through different interactors, generating structures with distinct autophosphorylation profiles (for example, interaction with nucleophosmin generates a phospho-Ser89 epitope in an active pool of AurA distinct from that activated by phosphorylation in the T-loop at Thr288) (27). Destabilization could be a default mode to constrain Aurora kinase activity unless protected by interaction, helping to maintain distinct, spatially defined pools of AurA and AurB activities.

We note that during early mitosis, the APC/C is proposed to play a role in "dosing" spindle-associated factors by eliminating components in excess of those required for the assembly of the correctly sized bipolar mitotic spindle (28). In this model, binding to MTs directly stabilizes proteins, such as HURP, which are otherwise turned over rapidly by the APC/C. We speculate that such default targeting by the APC/C could be a characteristic of mitotic regulators that assists their clearance from the cell as the machinery of cell division disassembles at the end of mitosis.

Execution of Mitotic Exit

Aurora kinases play critical roles in orchestrating events at mitotic exit (**Figure 1**). Elucidating them has been a challenging task, given the multiple functions of the Auroras earlier in mitosis. In recent years, however, the use of chemical genetics and development of specific small molecule inhibitors have helped decrypt roles of Aurora kinases after anaphase onset. Activity of either AurA or AurB is essential for disassembly of the metaphase spindle (29). Furthermore, AurA activity is required for anaphase spindle dynamics and central spindle formation, with AurA inhibition reducing anaphase pole-to-pole separation, resulting in a disorganized midzone with sparse MTs. Although the exact molecular mechanism remains to be elucidated, TACC3 and the dynactin subunit p150Glued have been identified as AurA substrates mediating anaphase spindle elongation (27, 30, 31). AurB plays critical roles during anaphase as the CPC relocates to the midzone of the anaphase spindle and from thence to the equatorial cortex, where AurB activity is essential for furrowing (32, 33). At the completion of cytokinesis, AurB is found on MTs flanking the midbody, where it retains activity to control the timing of abscission through the CHMP4C component of the ESCRTIII complex (34–37).

Downregulating Aurora kinase activity is also important for mitotic exit. The activation of counteracting phosphatases (38) does not appear sufficient to reverse the functional phosphorylation events mediated by the Auroras, since non-degradable versions perturb the organization of mitotic exit. The gain-of-function phenotypes exhibited by non-degraded Auroras could point to kinase-independent roles, but more likely mean that the kinases retain some activity when dephosphorylated in the activation loop (39). We propose therefore that targeted Aurora degradation, as a tool for tuning the activity of the kinases, is a critical element of their functions in mitotic exit.

In Cdh1 knockdown, consequently, anaphase spindle organization is perturbed, and the spindle over-elongated, in a fashion that can be rescued by codepletion of AurA and mimicked by expression of non-degradable AurA (17). AurA, p150, and TACC3 may act to translate the precise downregulation of AurA into remodeling of the anaphase MT network. Cleavage furrow ingression occurs earlier in Cdh1 knockdown than in control cells and is accompanied by the premature appearance of AurB at the equatorial cortex (19). Whether this effect is mediated through stabilization of AurB, through disruption of the central spindle caused by stabilization of AurA, or through a different Cdh1 substrate, is not known. The effect of Cdh1 knockdown on abscission timing has not been reported, but stabilization of AurB is likely to delay this process, as well as contributing to the genomic instability reported in $Cdh1^{-/-}$ MEFs (40).

Establishing Interphase

Several mitotic processes that depend on Aurora activity must be reversed as cells return to interphase. Reorganization of the cell cytoskeleton requires degradation of AurA for disassembly of spindle poles and of AurB for formin-mediated cell spreading (17, 19). For other processes, such as AurA-mediated mitochondrial fissioning (41), the role of Aurora degradation has not yet been established. Ubiquitination of AurB is proposed to be required for its p97-dependent extraction from chromatin to allow chromosome decondensation and nuclear envelope formation (42). More generally, tuning of AurB activity may tie the timing of abscission to the state of the nucleus at the passage to interphase: AurB activity has been proposed to delay abscission in response to delays in nuclear pore assembly (43), and recent studies show that the same ESCRT machinery regulated by AurB in the process of abscission is involved in resealing the nuclear envelope at the start of interphase (44, 45). A gradient of AurB activity emanating from the midzone is proposed to coordinate these events with sister chromatid separation in a checkpoint-like manner (46). What has become increasingly apparent is that AurB acts as both sensor and effector in the transition from mitosis to interphase, with its activity carefully modulated through localization, exposure to phosphatases, and degradation. This role for AurB provides a rationale for the very different degradation kinetics of AurA and AurB observed at the end of mitosis (**Figure 2**).

Regulating Cell Fate

A substantial body of literature points to additional, non-mitotic roles of Aurora kinases. AurA is required for reabsorption of the primary cilium in serum-stimulated quiescent cells and for migration of postmitotic neurons during development (49, 50). Both AurA and AurB are implicated in cell fate decisions, AurA through effects on stability of N-myc and p53, GSK3 signaling, and Notch pathways (51–55) and AurB through modulating the epigenetic states of histone H3, for example, in maintaining the differentiated state of C2C12 myoblasts and in transient transcriptional reprograming of events in interphase nuclei (56–58). As already shown for the role of AurA in postmitotic neurons, the activities of Aurora kinases in each of these processes could be regulated by proteolysis (50).

Proteostasis and Cancer

The systematic overexpression of AurA in cancers was noted early on after the discovery of Aurora kinases (59, 60) and is now recognized as an important driver of many cancer types, often

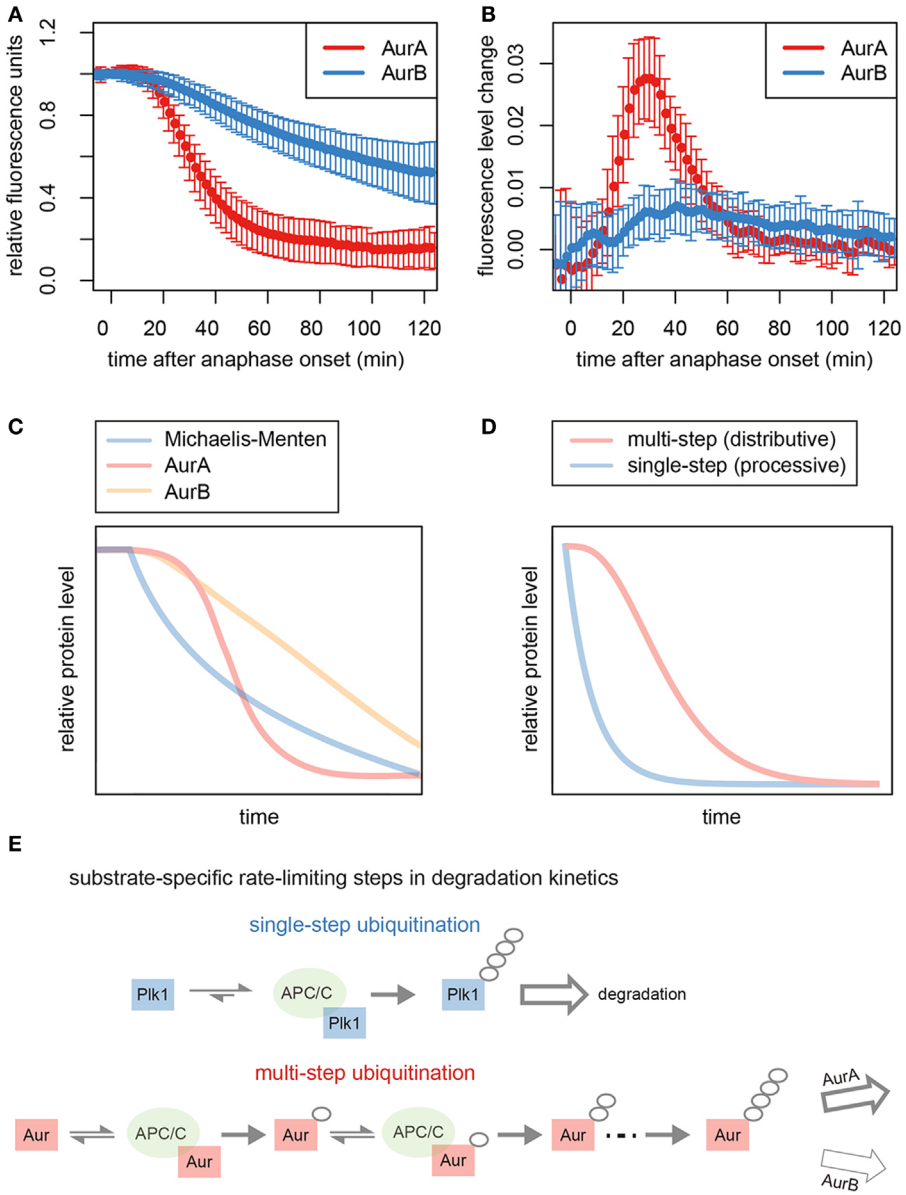

FIGURE 2 | Kinetics of Aurora kinase destruction during mitotic exit. (A) Total levels of Venus-tagged AurA and AurB in cells passing through mitosis. Data taken from Min et al. (47). Fluorescence measurements from single cells were used to generate averaged progress curves for the degradation of each substrate ($n \geq 50$). **(B)** Plots of the changing rate of degradation over time for the averaged progress curves show that the maximum rate of AurA degradation is fivefold higher than that of AurB. **(C)** Simulation of first-order (Michaelis–Menten) kinetics predicts a theoretical degradation curve showing an exponential decrease in substrate levels over time that resembles the degradation curves that we have previously described for other substrates of the APC/C, such as Plk1, RacGAP1, and KIFC1 (16, 48), and is consistent with the idea that for these substrates, ubiquitination is the single rate-limiting step for proteolysis (since the rate of proteolysis depends on the amount of substrate present). **(D)** Modeling of distributive ubiquitination of a substrate, where a threshold number of stepwise ubiquitin modifications is required to generate the product that can be processed for proteasomal degradation, compared to a processive ubiquitination process. The simulated reaction exhibits the sigmoidal/switch-like response that characterizes degradation of AurA. **(E)** Schematic to explain kinetics of degradation of different substrates. Processive ubiquitination of substrates, such as Plk1, is achieved by a single binding event to the APC/C, and substrates rate limited by single-step ubiquitination are degraded with first-order kinetics. By contrast, Aurora kinases bind to the APC/C multiple times to acquire polyubiquitin chains. AurA, rate-limited by this multistep ubiquitination, exhibits switch-like degradation kinetics. Degradation of AurB is likely governed by a post-ubiquitination step.

as a result of amplification of the AurA gene, located on the 20q amplicon (for example, the most common amplicon in colorectal cancer) (61, 62). AurA is thought to contribute to chromosome instability (CIN) during mitosis through its effects on MT dynamics (63), raising the possibility that control of AurA levels is required to protect cells from CIN (64). The functions of Aurora kinases in interphase could also contribute to the tumorigenic nature of AurA overexpression – perhaps more efficiently than

functions in promoting chromosome segregation in mitosis. Notably, kinase-independent roles, such as the protein–protein interaction between AurA and MYCN protein that stabilizes MYCN in neuroblastoma (53), provide a link between regulation of AurA levels and proliferation. Drugs that disrupt the AurA–MYCN interaction may offer a therapeutic route to treating neuroblastoma (65).

To what extent, then, is pathological expression of AurA a problem with regulation of protein stability? One model for conditional AurA overexpression showed that *in vivo* overexpression of mouse AurA from a transgene did not result in increased AurA protein levels, since these were suppressed by proteolysis under physiological conditions (66). A recent proteogenomic survey of colorectal cancer reported that, in general, mRNA overexpression driven by gene amplifications was not reflected in overexpression at the protein level, suggesting that the latter is buffered by posttranscriptional regulation (62). Therefore, overexpression of AurA protein in cancers may indicate changes in the stability of the protein, either changes in AurA or in the pathways that regulate it. For example, stabilization of AurA through constitutive phosphorylation of a critical residue, Ser51, has been reported in head and neck cancers (67). Coexpression of TPX2 may be another route to stabilizing AurA in cancers, contributing to excess AurA activity after 20q amplification, since *AURKA* and *TPX2* are both located on the long arm of chromosome 20 (68).

In the following sections of this review, we will discuss factors that determine, or influence, the ubiquitin-mediated regulation of Aurora kinase levels in the cell.

THE KINETICS OF AurA AND AurB DEGRADATION AT MITOTIC EXIT

In mammalian cells, anaphase substrates of the APC/C fall into groups that show distinct kinetics of degradation when measured in single cell assays *in vivo*. These kinetics are determined by the multilayered complexity of the UPS, which includes post-translational modifications (PTMs) on substrates (and on the APC/C) and other characteristics of substrate interactions with the APC/C that determine the on-rate and the residence time of the substrate (69). The activity of deubiquitinating enzymes (DUBs) and of other ubiquitin modifiers can also influence the degradation of ubiquitinated substrates, and the p97 AAA-ATPase may be required to unfold ubiquitinated substrates to render them accessible for degradation (70). The topology of ubiquitinated substrates undoubtedly influences their interaction with the proteasome, since polyubiquitin chains and an unstructured region that serves as the degradation initiation region need to be in the right proximity to one another for proteasomal proteolysis (71).

In vivo assays of GFP-tagged Aurora kinases report on the timing and kinetics of their degradation, which begins 10 min after anaphase onset (**Figure 2A**) (47). The timing of degradation onset for AurA and AurB is identical by this assay. However, their rates of degradation are very different (**Figure 2B**), explaining the long-standing observation that AurA is removed from the cell well ahead of AurB during mitotic exit (16, 17, 72, 73).

Progress curves for substrate degradation can provide information on the kinetics of the underlying reactions. The progress curve of GFP Venus-tagged AurA is consistent with the idea that distributive (stepwise) ubiquitination of AurA determines the kinetics of disappearance of this substrate (**Figures 2A,C,D**). AurA was previously shown to be a distributive substrate *in vitro*, where building a proteolytic ubiquitin chain requires multiple rounds of substrate–APC/C binding each binding event considered an independent and reversible step (69, 74) (**Figure 2E**). In contrast to the switch-like kinetics of AurA-Venus degradation, AurB-Venus degradation progressed at a rate that was slow but constant – even when AurB-Venus levels were low (**Figures 2A–C**) – with the inference that degradation is governed by a rate-limiting step with low catalytic activity and high affinity of the rate-limiting enzyme for the substrate (75). Since both substrates appear ubiquitinated to the same extent during mitotic exit (47, 76), we propose that this rate-limiting step in AurB degradation occurs post-ubiquitination. We note that both of these progress curves are distinct from those of other substrates we have studied, such as Plk1 and KIFC1 (16, 48), which show first-order (or Michaelis–Menten) kinetics (rate of disappearance dependent on substrate concentration) (**Figures 2C,D**). First-order kinetics is consistent with a model where processive (single-step) substrate ubiquitination would be rate limiting for degradation. It seems therefore that distinct steps in processing of the Aurora kinases underlie their characteristic degradation curves and differential removal from the cell (**Figure 2E**).

HOW ARE AURORA KINASES TARGETED IN MITOTIC EXIT?

Aurora Kinases Are Cdh1-Dependent Substrates of the APC/C

AurA was found early on to be an efficiently degraded substrate of the APC/C (77). Its efficient degradation in *in vitro* assays using extracts from human cells or *Xenopus* oocytes has facilitated identification of substrate-specific determinants of degradation (78–81). The APC/C relies on either of the two coactivators, WD40 repeat factors Cdc20 or Cdh1 (FZR1 in humans). AurA is specifically targeted by Cdh1 *in vitro* (79, 80, 82) and is robustly stabilized by depletion of Cdh1 in various systems (17, 40, 72). Recombinant AurB is not degraded efficiently in the same *in vitro* assays (78), but AurB levels are highly sensitive to Cdh1 in cell-based assays (17, 47, 72, 83).

The specificity of the APC/C for its substrates shifts as cells pass through mitosis. As cells enter anaphase, specificity switches from a relatively restricted pool of substrates to a large one that may number in the hundreds (76, 84). Although the switch from Cdc20 to Cdh1 was originally thought to account for this change in specificity (85), it is now evident that a majority of substrates are efficiently degraded during mitotic exit in the absence of Cdh1, through altered targeting specificity of APC/C–Cdc20 (17, 47, 72, 73, 86). For these substrates, therefore, the requirement for Cdh1 only reveals itself in G1 phase, or in *in vitro* assays, when the APC/C is in a dephosphorylated state that cannot interact with Cdc20. The strict dependence of AurA and AurB targeting

on Cdh1, even when APC/C–Cdc20 is active, marks them out from other APC/C substrates. Only Cdc6 is known to share this specificity (86), and we propose that the shared timing of destruction of these substrates signals the moment of activation of APC/C–Cdh1 in mitotic exit.

The unique specificity of Aurora kinases and Cdc6 is probably determined by the way in which they interact with the APC/C. It is well known that APC/C substrates contain receptor motifs, the so-called degrons, which are recognized and bound by coactivator-associated APC/C (87, 88). Now, structural studies [recently reviewed in Ref. (89)] are able to show the direct binding of coactivators, via their WD40-repeat propellors, to canonical degrons in substrates. However, although Aurora kinases contain such canonical degron motifs (D-boxes and KEN motifs), it is not clear what roles these play, since an additional non-canonical degron, called the "A-box" is also present (79) (**Figure 3**).

Aurora Kinases Contain Multiple Degrons

Canonical Degrons

APC/C substrates are usually characterized by the presence of a D-box ("destruction box," consensus RxxL), first identified in the N-terminus of B-type cyclins (90). However, the D-box alone may not be sufficient for productive binding of most substrates: APC/C–substrate interactions are more likely governed by the weak interactions through multiple degrons categorized as SLiMs (91, 92). The most important of these additional degrons is the KEN motif, first identified in Cdc20, which lacks a D-box (88). The KEN motif binds to a different surface of the coactivator WD40 domain to the D-box, and is prevalent in APC/C substrates (and in 8% of the proteome). Aurora kinases contain conserved D-boxes and a KEN motif (**Figure 3A**). *In vitro* degradation assays identify the functional D-box of Aurora kinases as that conserved in a position close to the C-terminal end of the kinase domain (79, 81–83, 93). However, the orientation of the RxxL within the known structure of the kinase domain (94, 95) raises a question mark over how it could be accessible to the APC/C. Mutation of this motif not only abrogates the destruction of GFP-tagged AurA in cells undergoing mitotic exit but also abolishes the localization of AurA to any mitotic structures, rendering *in vivo* assessment of its role problematic (**Figures 3B,C**).

It is notable that all mitotic interactions of Aurora kinases are acutely sensitive to disruption in the C-terminal region (96, 97). Structural simulations of AurA–TPX2 interaction predict that the cancer-associated somatic mutation S155R in AurA, which prevents interaction with TPX2 (97), increases disorder in the C-terminus (98). Therefore, interaction with binding partners through the C-terminus maintains the overall structure of the kinases. Loss of critical interactions could allow partial unfolding of Aurora kinases prior to targeting of the D-box by the APC/C, explaining the destabilization seen after loss of TPX2 or INCENP (24, 25).

An alternative idea, where the D-box is not assumed to function as a degron, is that the C-terminus of Aurora kinase is required for an intramolecular interaction, such as that proposed for AurA (99), influencing the structure of the N-terminus. The structure of the N-terminus could, in turn, determine the availability of SLiM-type degrons in the N-terminus for targeting by the ubiquitination machinery.

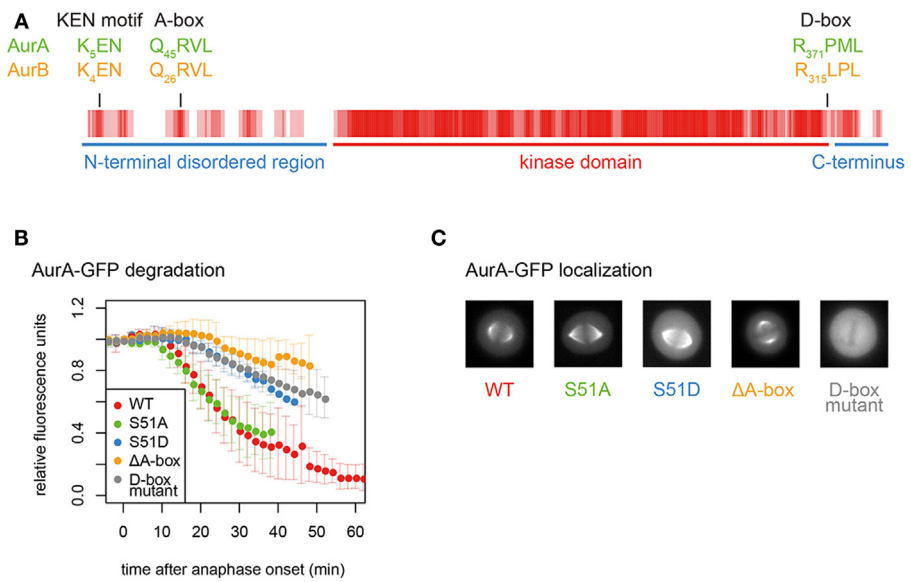

FIGURE 3 | Conserved degrons in Aurora kinases. (A) Aurora kinase degrons are the most conserved motifs outside the kinase domain. The sequence alignment of AurA and AurB was converted to a vector of values corresponding to conservation at each position (from 4 for fully conserved position to 0 for no conservation). Rolling averages of a five-residue window across the whole alignment is presented as a heat map. Therefore, the shade of red indicates residue conservation between the two paralogs. **(B)** Degradation plots for A-box- (including S51-) and D-box-mutated versions of AurA-GFP, as described in Ref. (81). Fluorescence levels measured over time in single cells exiting mitosis are normalized to anaphase onset. ΔA-box = Δ31–66; D-box mutant = R371A, L374A. **(C)** Mitotic localization of mutants analyzed in **(B)**, showing loss of functional localization of the D-box mutant.

Among these SLiMs are the Aurora KEN close to the N-terminus and the "A-box." Despite multiple reports from *in vitro* studies that KEN plays no role in AurA mitotic destruction (79, 80, 82, 93), it contributes to the degradation in cell-based assays of both AurA and AurB (19, 100, 101). However, the AurA K5 within the KEN motif is ubiquitinated during mitotic exit (101), in apparent conflict with function as a degron. Structural or cross-linking studies of KEN interactions will be required to resolve the function of this motif. The A-box appears to qualify as a degron since the A-box-deleted version of AurA localizes correctly in mitosis and is resistant to mitotic exit degradation (**Figures 3B,C**).

The A-Box, a Specific Determinant of Aurora Kinase Destruction

The A-box motif was identified in AurA (residues 31–66) as the sequence required for APC/C–Cdh1-mediated destruction of AurA in mitotic/G1 extracts (79). More recent studies indicate that Q_{45}RVL – conserved in AurB – is sufficient to mediate degron function in both kinases (67, 81, 100).

The A-box is predicted to mediate an atypical degron interaction with APC/C–Cdh1, but the structural basis for this specificity has not been investigated. To our knowledge, the only structurally defined contributor of specificity for Cdh1 is the "A-motif" found in APC/C inhibitor Acm1 in yeast (91, 102). Distinct from the Aurora A-box, the "A-motif" is a 10 amino acid loop, including a key FxLxYE region that interacts with a non-canonical binding site on Cdh1 via a salt bridge and two hydrophobic interactions. Aurora kinases may employ an equivalent strategy in assembling substrate-specific APC/C–coactivator–E2 complexes, as discussed below.

Aurora Kinases Are Ube2S-Dependent Substrates of the APC/C

The APC/C ubiquitinates its targets in conjunction with two E2 enzymes, Ube2C (UbcH10) and Ube2S. Ube2C adds the first, or "priming," ubiquitin, and can generate short chains on substrates, while Ube2S elongates ubiquitin chains through the addition of K11-specific ubiquitin linkages (103, 104).

K48 linkages are the canonical proteasomal degradation signal, while K11 linkages have recently been found to mediate rapid degradation of mitotic substrates (47, 105, 106). The two E2s bind non-competitively to the APC/C (UbcH10 via the RING domain subunit APC11 and Ube2S via APC2), acting together as a highly efficient module for rapid targeting of substrates to the proteasome. The coactivators Cdc20 and Cdh1, as well as participating in substrate recognition, also promote the activity of APC/C through a critical substrate-induced stabilization of E2 binding to the APC/C (92, 107, 108).

Our own work has shown that Aurora kinases are decorated with a mixture of K48- and K11-linked ubiquitin chains during mitotic exit, and that Ube2S is essential both for the K11 linkages and for efficient degradation of these substrates (47). Other substrates are able to receive K11 chains in the absence of Cdh1 (presumably via Cdc20–Ube2S), while in Cdh1-depleted cells, Aurora kinases lose all their K11 chains but are still ubiquitinated with K48 chains in an APC/C-dependent manner, presumably because Ube2C recruitment can still occur (47). In other words, Aurora degradation depends on Cdh1 not for recruitment to the APC/C, but for generating K11 linkages via Ube2S (**Figure 4**).

Why Cdh1 Specificity?

The functional significance – if there is any – of exclusive targeting by Cdh1 is not clear. Cdh1 could be specifying the timing of Aurora kinase destruction with respect to anaphase functions. It has been shown that APC/C–Cdh1 assembly depends on prior anaphase Plk1 destruction (109), thus the exclusive targeting of Aurora kinases by Cdh1 imposes strict order on the destruction of these substrates; Plk1 ahead of AurA (16). In the case of the replication factor Cdc6, which shows identical coactivator specificity and degradation timing to AurA, it is suggested that delayed degradation with respect to the licensing inhibitor geminin, a substrate of APC/C–Cdc20, creates a short but clearly defined window of opportunity for replication licensing during mitosis (86). Similarly, there may be an event in mitotic exit that requires Aurora kinase activity in the absence of Plk1 or some other Cdc20 substrate.

An alternative explanation for the Cdh1 specificity is suggested by the progress curve of AurA degradation (**Figure 2**) (47). The "switch-like" kinetics imparts robustness to the destruction of this substrate once a cell is committed to mitotic exit (activation of Cdh1) and may depend on low processivity arising from weak Cdh1-substrate interactions. Finally, specificity for Cdh1 may introduce possibilities for modulating the degradation of substrates via chain editing – for example, the DUB USP37 interacts with APC/C–Cdh1 to modulate K11 linkages on at least one substrate (110).

REGULATION OF AURORA KINASE DEGRADATION

Posttranslational Modification of Aurora Kinases

Recent advances in proteomics have not only started to reveal the identity of *in vivo* ubiquitination sites (111), but also the complexity of PTMs that can modify the fate of target proteins. Tens of thousands of ubiquitinated lysines are known, although mostly these lack functional annotations. We find it interesting that only four endogenously ubiquitinated sites have been found for AurA but a large number for AurB (18 out of 22 lysines). Many of these ubiquitination sites are likely to serve non-proteolytic functions, by creating or disrupting interfaces with other partners. For example, CUL3-KHLH9/13/21-dependent ubiquitination is required for the correct localization of AurB in anaphase (112, 113). We have found it necessary to disrupt multiple lysines in the N-terminus of AurB to significantly disrupt AurB degradation in mitotic exit (Mingwei Min, Catherine Lindon, unpublished data), suggesting that several or all of these ubiquitinated lysines could carry chains that contribute to processing of AurB at the proteasome. The same is not true for AurA, which seems to rely strongly on its most N-terminal lysine, K5 (101) for mitotic exit degradation. This difference in lysine usage could explain the differential

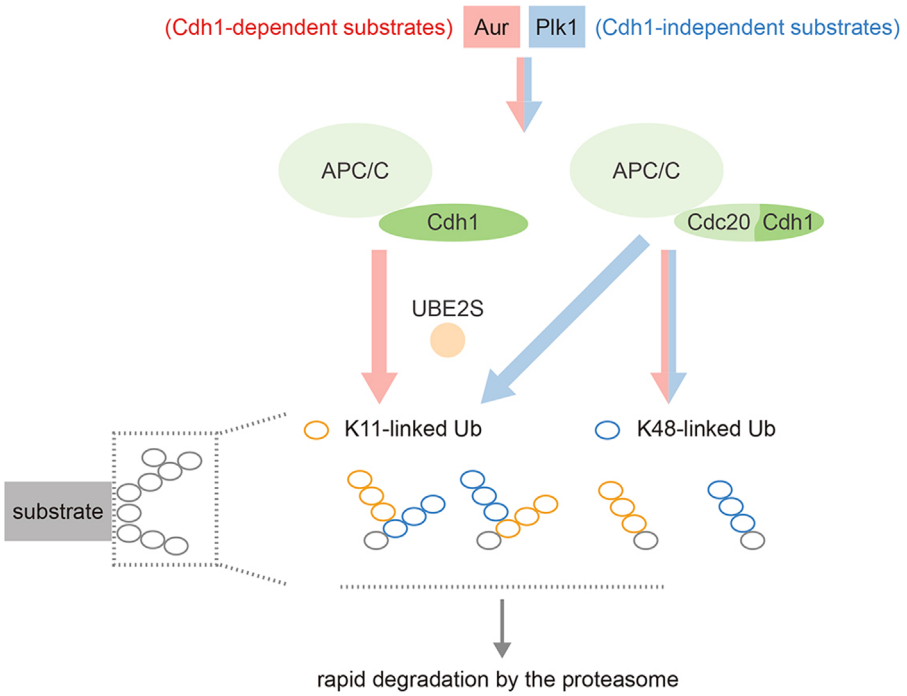

FIGURE 4 | Schematic illustrating the complexity of APC/C substrate specificity after anaphase onset. Most substrates can be targeted by either APC/C–Cdc20 or APC/C–Cdh1, working with both Ube2C and Ube2S (blue stream). Cdh1-only substrates, although they can still bind Cdc20, show restricted targeting by the APC/C–Ube2S that depends on Cdh1 (red stream). APC/C–Ube2S is required to generate the K11 linkages for rapid degradation at the proteasome.

degradation kinetics that we measure for the two substrates, for example, if removal of ubiquitin chains at the proteasome prior to proteolysis is slow, as has been suggested (114).

Lysines can be subject to several PTMs beyond ubiquitination. Functionally important sumoylation of both Aurora kinases has been reported to occur on conserved lysines that are also reportedly ubiquitinated (in humans, AurA K258 and AurB K202), but it is not known how this might have impact on potential ubiquitination at these sites (115–117). The deacetylase SIRT2 has been found to strongly regulate both AurA and AurB levels *in vivo* (proposed to explain the high rate of tumorigenesis in SIRT2$^{-/-}$ mice) (118). Investigation of the underlying mechanism was unable to detect acetylation on AurA lysine residues, but found that acetylation of APC/C coactivators interferes with their function. Aurora-specific recruitment of SIRT2 could therefore act to promote Aurora degradation, either directly or indirectly. We note that recent work showing acetylation on ubiquitin as a potential route to switches in chain specificity, or between mono- and poly-ubiquitinated states, could put deacetylases center stage as regulators of protein degradation dynamics (119).

Finally, proteomics approaches have identified several functional phosphorylation sites on AurA (most of them autophosphorylation sites), reviewed recently elsewhere (4). The most interesting from the point of view of Aurora stability is phosphorylation on the serine immediately downstream of the QRVL motif (S51 in human AurA), which appears to regulate the degron function of the A-box since phosphomimic mutation of this residue (S51D) stabilizes AurA in mitotic exit as efficiently as removal of the A-box (67, 79, 81, 120) (**Figure 3B**). This PTM has therefore been proposed to control the degradation of AurA at the end of mitosis (79, 81, 120). However, replacement of S51 with a non-phosphorylatable residue (S51A) does not alter the timing of degradation of AurA during mitotic exit (**Figure 3B**). Dephosphorylation on this residue would therefore be a permissive state, rather than the trigger of AurA destruction.

The serine residue S4, adjacent to the mitotic exit-specific ubiquitin acceptor lysine K5, is also phosphorylated *in vivo* (121) and phosphomimic replacement increases ubiquitination efficiency on the neighboring lysine (101).

Interactors of Aurora Kinases

AurA has multiple interactors, many of which, like TPX2 (25), are reported to modulate AurA levels through ubiquitin-dependent and -independent pathways. There is limited information about modulators of AurB stability, probably reflecting that AurB levels are effectively suppressed in interphase. Examples of interactors that influence AurA stability are Nedd9/HEF1, Pleckstrin-homology-like domain protein PHDLA1, PUM2, LIMK2, and FAF1 (122–126), and in many cases, this stabilization occurs through competition for access to regions of AurA usually targeted by the UPS.

However, the switch-like function of the pS51 PTM in stabilizing AurA may provide another route to modifying AurA turnover: recent studies by Erica Golemis and colleagues show that AurA can be activated through Ca^{2+}-induced binding of Calmodulin (CaM) to the A-box region, and that CaM binding depends on the presence of serine residues, including S51, that are phosphorylated under the same conditions (127, 128). CaM binding to pS51 can be predicted therefore to stabilize AurA by blocking access to the A-box region. In this model of AurA regulation, autophosphorylation of AurA on S51 connects activity and stability and allows for functionally relevant stabilization of active forms of the kinase through Ca^{2+}-mediated signaling. There are reports that CaM also binds to AurB (127, 129), although in this case, it is proposed that AurB is stabilized through competition for FBXL2 access to a region that does not include the A-box (129).

OTHER UPS COMPONENTS TARGETING AURORA KINASE

Although the APC/C appears to be the major E3 regulating Aurora kinase levels *in vivo* and destroys most of the detectable Aurora kinase in cells that exit mitosis, a small pool of AurA is thought to remain to fulfill interphase functions, either protected from APC/C-mediated destruction (perhaps through activity of a DUB, or through sequestering in the cytoplasm) or a newly synthesized pool as cells return to interphase. So, are there UPS components that turn over Aurora kinases in interphase cells? Candidate E3 ubiquitin ligases are CHFR, shown to target AurA both *in vitro* and *in vivo* (130), the BRCA1-associated BARD1 that interacts with AurB (131), and SCF complexes containing a number of reported F-box proteins. FBXW7, FBXL7, and FBXL2 are all reported to target Aurora kinases (53, 132–135), but it is not clear how well *in vitro* targeting predicts *in vivo* pathways, especially since the effects of overexpressing or depleting F-box proteins on global levels of Aurora kinases are frequently rather modest. It seems likely that small subpopulations are being targeted in each case (for example, FBXL7 localizes to the centrosomes), as part of the complex spatial organization of kinase activity that underlies the multiple and divergent functions of these kinases. Interestingly though, dramatic stabilization of AurA is seen after treatment of cells with GSK3B inhibitor (136). In this study, GSK3B was proposed to promote FBXW7 targeting of AurA through priming a phospho-degron located in the kinase domain. However, phosphorylation of AurA by GSK3B on S283/4 is known to promote autophosphorylation on S342 that is inhibitory to AurA activity (137), making it likely that GSK3B can govern AurA stability indirectly through conformational effects.

Dramatic effects on AurA levels are also reported in response to a factor called AURKAIP1, an AurA-interacting protein that promotes AurA destruction in a ubiquitin-independent manner. AURKAIP1 may direct AurA to the proteasome through an interaction that competes with the ubiquitination machinery, since polyubiquitination is abolished upon overexpression of AURKAIP1 (138). However, AURKAIP1 turns out to be a mitochondrial ribosomal protein (139), such that the physiological relevance of these observations remains to be demonstrated. Finally, another interesting study reported that AurA is a substrate for the ubiquitin conjugating enzyme Ube2N, with which it interacts directly through an N-terminal domain that requires the residue F31. An F31I polymorphism that has lost Ube2N interaction is preferentially amplified in tumors (140), raising the still unanswered question of whether increased stability of AurA F31I could explain its role in colon cancer susceptibility.

CONCLUSION

Aurora activity is a major regulator of the cell cycle, with a separation of functions between paralogous Aurora kinases whose degradation kinetics in vertebrates have apparently evolved hand in hand with their specialization. The distribution of functions of the ancestral Aurora between two more specialized paralogs is a process that phylogenies indicate to have occurred more than once in eukaryotic lineages (12): Aurora kinases A and B in vertebrates, and the two Aurora kinases in flies and worms, arose via independent duplication events, following similar pathways toward specification of function. Such convergent evolution of Auroras suggests positive selection for specialization of two pools of Aurora kinase. While the kinase activity of the paralogs remains conserved, the regulatory modules including short linear motifs in their disordered regions, have largely diverged. We suggest this could be linked to the importance of differentially regulating pools of Aurora kinase activity in time. Although not well studied in other species, differential proteolysis is a feature of human Aurora kinases that strictly depends on these divergent terminal regions. While different interactors can achieve spatial regulation of Aurora kinase activity, differential proteolysis adds complexity to the control of Aurora kinase activity in a temporal domain.

AUTHOR CONTRIBUTIONS

All authors contributed to writing the review and preparing figures.

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REVIEW
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Possible role of Aurora-C in meiosis

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The meiotic generation of haploid gametes with equal contents of genetic material is important for sexual reproduction in mammals. Errors in the transmission of chromosomes during meiosis may lead to aneuploidy, which is the leading cause of miscarriage and congenital birth defects in humans. The Aurora kinases, which include Aurora-A, Aurora-B, and Aurora-C, are highly conserved serine–threonine kinases that play essential roles in centrosome function, chromosome segregation, and cytokinesis during mitosis and meiosis. While Aurora-A and Aurora-B have been extensively studied in mitosis, the role of Aurora-C in meiosis is only now starting to be revealed. For example, the perturbation of Aurora-C kinase activity by microinjection of Aurora-C-kinase-dead mutant mRNAs into mouse oocytes induced multiple defects, including chromosome misalignment, abnormal kinetochore–microtubule attachment, premature chromosome segregation, and failure of cytokinesis during meiotic division. However, the analysis of such defects is complicated by the possibility that Aurora-B may be present in mammalian germ cells. Interestingly, a homozygous mutation of Aurora-C in humans leads to the production of large-headed polyploid spermatozoa and causes male infertility, but homozygous females are fertile. Mouse studies regarding the roles of Aurora-B and Aurora-C in female meiotic divisions have yielded inconsistent results, and it has proven difficult to explain why homozygous human females have no significant clinical phenotype. In this review, we will discuss the controversial status of Aurora-B in oocytes and the possible role of Aurora-C during meiotic division.

Keywords: meiosis, oocyte, spermatocyte, aurora kinase, mitosis, polyploidy, male infertility, aneuploidy

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Introduction

An essential process during the sexual reproduction of mammals is the production of haploid gametes from diploid precursors. This is done via meiosis, which consists of a single round of DNA duplication and two rounds of cell division that are called meiosis I (MI) and meiosis II (MII). Homologous chromosomes are segregated in MI, while sister chromatids are separated in MII via a process similar to that seen during mitosis (1, 2). Failures in chromosome segregation at meiosis result in aneuploidy, which is a major cause of miscarriages and birth defects in humans. However, the mechanisms underlying such failures are not completely understood (3). The Aurora kinases belong to a family of serine/threonine kinases that are pivotal in the regulation of cell division processes, including mitosis (4, 5) and meiosis (6–8). There are three Aurora kinases in mammals: Aurora-A and Aurora-B are ubiquitously expressed, and their functional roles in mitosis have been extensively studied (9–11); whereas Aurora-C is mainly restricted to germ cells (12), and is beginning to be functionally studied in meiosis. It is interesting to note that these three kinases share sequence homology in their central catalytic kinase domains, but differ widely in their N- and C-terminal

sequences (12). Mouse Aurora-B and Aurora-C share 77.6% amino acid sequence identity in their catalytic domains, while Aurora-A and Aurora-C share only 66.3% sequence identity in this region, suggesting that there may be a close functional link between Aurora-B and -C (12).

Aurora-C (also called AIE1/AIE2/STK13) was first identified in the Tang lab, in a screening for kinases expressed in sperm and eggs (12), and also independently by Bernard et al. in a homologous kinase screening in a human placental cDNA library (13). Aurora-A and -B are ubiquitously expressed in many tissues, particularly in actively dividing cells. In contrast, Aurora-C is predominantly expressed in the testis (12, 13) and is mainly restricted to meiotically active germ cells, including spermatocytes (14) and oocytes (6). Aurora-C was reported to be overexpressed in a variety of human cancer cell lines (15, 16) and ectopic overexpression of Aurora-C can also induce cell transformation and tumor formation (17). However, its expression in tumor cells and normal somatic tissues is still the matter of some debate (14, 18). Aurora-B is a member of the chromosomal passenger complex (CPC), which localizes to the centromeres/kinetochores from prophase to metaphase and to the central spindle and midbody during cytokinesis (19, 20). In contrast, endogenous Aurora-C protein has never been detected in normal somatic cells by immunofluorescence or Western blot analyses using fully validated antibodies (6, 14). Instead, ectopically expressed tagged Aurora-C has been detected in transfected cells, where it showed a localization pattern similar to that of Aurora-B (21–23). The role of Aurora-B in meiotic chromosome orientation during meiosis has recently been reviewed by Watanabe (1). In this review, we will focus on the possible role of Aurora-C during male and female meiotic divisions.

Aurora-C in Mouse Spermatocytes: Subcellular Localization, Transcriptional Regulation, and Functional Implications

The subcellular localization of endogenous Aurora-C during male meiotic division had been carefully examined by confocal immunofluorescence microscopy in mouse spermatocytes (14). In germ cells, the meiotic prophase consists of five sequential stages: leptotene, zygotene, pachytene, diplotene, and diakinesis. Aurora-C was first detected at the centromeric regions in early diplotene spermatocytes, after which it was found to spread along the chromosomal arms of sister chromatids during diakinesis. Upon the transition from diakinesis to MI, Aurora-C gradually dissociates from the chromosome arms and becomes concentrated at the centromeres near the kinetochores. Thereafter, it relocalizes to the spindle midzone and midbody during the anaphase I/telophase I and anaphase II/telophase II transitions, respectively (**Figure 1**) (14). A similar localization pattern was reported for Aurora-B in mouse spermatocytes (14, 24). However, while Aurora-B was detected in mitotic spermatogonia, Aurora-C was not, suggesting that Aurora-C may play a unique role in male meiotic division (14).

The finding that Aurora-C and -B co-localize during male meiotic divisions raised several interesting questions: (i) how are Aurora-C/-B recruited to the appropriate positions to execute their meiotic functions during spermatogenesis? (ii) Do Aurora-C/-B play similar or different roles during male meiotic divisions? (iii) Since Aurora-C is mainly restricted in germ cells, how is Aurora-C regulated during spermatogenesis?

In somatic cells, Aurora-B is a member of the CPC along with several non-enzymatic subunits, including INCENP, survivin, and Borealin; together, the members of this complex contribute to regulation of chromosome segregation, microtubule–kinetochore attachments, and cytokinesis (19, 25). INCENP contains a conserved C-terminal IN-box that binds Aurora-B (26) and an N-terminal region that targets to centromeres (27).

Interestingly, INCENP can be detected in meiotic cells prior to the appearances of Aurora-B and -C (14, 24). It is first found at the central element (CE) of the synaptonemal complex (SC), from the zygotene to late pachytene stages (24). It then moves to heterochromatic chromocenters (14, 24) and co-localizes with Aurora-B and -C at the diplotene stage (14). Immunoprecipitation analyses showed that INCENP can form distinct complexes with either Aurora-C (INCENP/Aurora-C) or Aurora-B (INCENP/Aurora-B) in the testis (14). Together, these findings strongly support a model, in which INCENP recruits Aurora-C and -B to their appropriate locations and activates them to execute their meiotic functions in spermatocytes (14). Consistent with this notion, INCENP was reported to bind (21, 22) and activate Aurora-C (21) in somatic cells, and ectopically expressed Aurora-C was found to associate with survivin (28) and borealin (29). However, the functional linkage of these proteins during meiotic divisions has not yet been fully resolved. Recent studies have shown that BUB1, shugoshin proteins, and haspin kinase are also required for targeting Aurora-B to the centromeres of meiotic chromosomes (30–34). It will be interesting to test whether these proteins are also required for Aurora-C targeting to the centromeres in the future.

What is the role of Aurora-B and -C during male meiotic divisions? In somatic cell mitosis, Aurora-B and Polo-like kinase 1 (Plk1) phosphorylate the cohesion complexes to promote their dissociation from the chromosome arms (35–37). Interestingly, during meiosis, some SC components (e.g., SCP2 and SCP3) and cohesion subunits (e.g., SMC1b and SMC3, but not REC8) are gradually released from the chromosome arms and accumulate at the centromeres during the prophase I to metaphase I transition (38, 39). In accordance with this finding, Aurora-C was reported to be dissociated from the chromosome arms and concentrated at the centromeres during the diakinesis–metaphase I transition (14). Together, this seems to suggest that Aurora-C might regulate the release of cohesion subunits and SC components from the chromosome arms during MI. Future work is needed to test this possibility.

To investigate the role of Aurora-B/-C in spermatogenesis, Kimmins et al. (40) generated transgenic mice in which a pachytene-specific promoter drove the expression of an inactive Aurora-B mutant, and produced *Aurora-C* knockout mice by homologous recombination. Expression of the inactive Aurora-B dominant-negative (DN) mutant severely impaired spermatogenesis, resulting in abnormal spermatocytes, increased apoptosis, and spermatogenic arrest. The *Aurora-C* null mice were viable and had normal testis weights, sperm counts, and meiotic

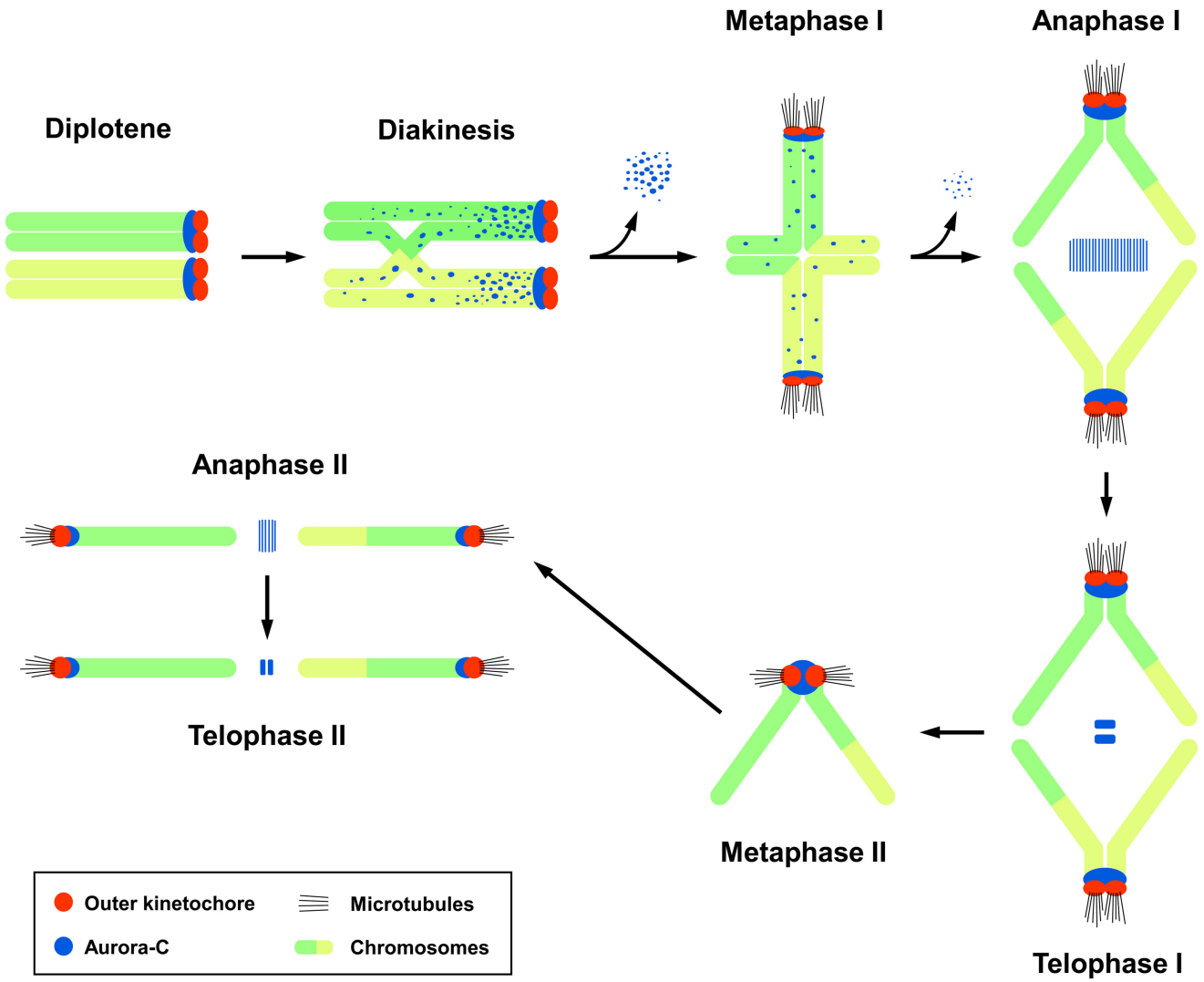

FIGURE 1 | The subcellular localization of Aurora-C during male mouse meiosis. Aurora-C is labeled dark blue and outer kinetochores are labeled red. Chromosomes are labeled green/yellow. Figure modified from Ref. (14). Aurora-B shows a similar localization pattern as that of Aurora-C (24). Aurora-C signals were gradually lost from the chromosome arms and accumulated at the centromeres during the diakinesis-to-metaphase I transition (14). Currently, it is not clear whether Aurora-B also targets to the chromosome arms in diakinesis chromosomes. INCENP was first detected at the zygotene (24), prior to the appearance of Aurora-B and -C, and co-localized with Aurora-B (24) and Aurora-C at a later diplotene stage (14). INCENP has been implicated to recruit Aurora-C (14) and Aurora-B (24) to meiotic chromosomes.

progression, but some of the mutant males were sterile and had sperm abnormalities, including heterogeneous chromatin condensation, loose acrosomes, and blunted heads (40). As Aurora-B (24) and Aurora-C (14) co-localize and associate with INCENP, it has proven difficult to differentiate their roles in spermatogenesis. Furthermore, it is unclear why *Aurora-C* null mice show only minor sperm-related alterations. Previous reports have shown that ectopic expression of an Aurora-C kinase-dead mutant disrupts the association of INCENP with Aurora-B (22) and that Aurora-C can complement the function of Aurora-B Kinase in somatic cells (21, 23, 41). Thus, it is possible that endogenous Aurora-B could compensate for the function of Aurora-C in the *Aurora-C* null mice and that ectopic expression of the Aurora-B DN mutant could non-specifically block the function of endogenous Aurora-C in *Aurora-B* mutant mice. Alternatively, studies have suggested that multiple tandem copies of the *Aurora-C* gene (42) or a potential "functional pseudogene" in the mouse genome may alleviate the spermatogenic effects in the *Aurora-C* null mice. Thus, why do mammals require both Aurora-C and -B kinases in spermatocytes? Do they play overlapping or differential roles during male meiotic divisions? These questions remain open in the context of mammalian spermatocytes.

Finally, the transcriptional regulation of Aurora-C during spermatogenesis is poorly understood. Our group isolated the cDNA clones encoding human TZFP (testis zinc finger protein) and mouse Tzfp, which are predominantly expressed in testis (43, 44). Human TZFP and mouse Tzfp contain a conserved N-terminal BTB (bric-a-brac, tramtrack, broad complex)/POZ (poxvirus,

zinc finger) domain and three C-terminal C2H2 zinc fingers (43, 44). Interestingly, the zinc finger domain of TZFP/Tzfp is closely related to the promyelocytic leukemia zinc finger (PLZF) protein, a known DNA-binding transcriptional repressor (45). Biochemical studies demonstrated that the C-terminal zinc finger domain of Tzfp directly binds to the TGTACAGTGT motif (designated as the Tzfp binding site, or tbs), located in the upstream flanking sequence of the *Aurora-C/Aie1* gene (44). These studies also showed that the N-terminal BTB/POZ domain has repressor activity, suggesting that Tzfp may negatively regulate *Aurora-C* gene expression in spermatocytes (44). Consistent with this notion, Tzfp is highly expressed in spermatocytes at the pachytene stage in MI, and *Tzfp*-knockout mice show downregulation of *Aurora-C/Aie1* expression (46).

Aurora-C/-B in Mouse Oocytes: Subcellular Localization and Potential Functions during Female Meiotic Divisions

The localization of endogenous Aurora-C has been examined in detail during the various stages of meiotic division in mouse oocytes (6). Aurora-C was detected at the chromosome axes and centromeres in prometaphase I–metaphase I, in which Aurora-C was also phosphorylated at Thr171 (**Figure 2**) (6). During the anaphase I–telophase I transition, Aurora-C was dephosphorylated and relocalized to the midzone and midbody (**Figure 2**) (6), and thus shows a pattern similar to that reported in spermatocytes (14). Interestingly, protein kinase A (PKA) can phosphorylate recombinant Aurora-C/Aie1 protein *in vitro* at Thr171 (47), yet its physiological meaning is not clear. Unexpectedly, no endogenous Aurora-B protein was detected on the meiotic chromosomes of mouse oocytes when assessed by immunofluorescence staining with the same antibody that had successfully detected Aurora-B in spermatocytes (6) nor was it detected in experiments using other antibodies and fixation conditions (48). In contrast, Balboula and Schindler (7) detected endogenous Aurora-B at the nuclei of prophase-arrested oocytes and the meiotic spindle at metaphase I and metaphase II. This apparent discrepancy may reflect the specificities of the utilized different antibodies or other, yet unknown factors.

In experiments using exogenous proteins, GFP-Aurora-B expressed in injected oocytes was clearly detected at the centromeres/kinetochores at metaphase I (6, 48, 49, 51) and at the spindle midzone and midbody during the anaphase I–telophase I transition (6, 48, 51), thereby showing a pattern similar to that of endogenous Aurora-C (6). Furthermore, it was reported that *Aurora-C* mRNA is recruited for translation more efficiently than the *Aurora-B* mRNA, and that exogenously expressed Aurora-B protein is not stable during meiosis (49). Thus, despite the abundance of the mRNAs for Aurora-B and Aurora-C in mouse oocytes (6, 49) and the high-level expression of the Aurora-C protein in both male and female mouse germ cells (6, 14), little or no Aurora-B protein appears to be expressed in mouse oocytes. This interesting observation suggests that the translation of Aurora-B protein level is differentially regulated in female germ cells.

The role of Aurora-C in oocytes has recently been investigated using a number of approaches, including exogenously expressed Aurora-C kinase-dead or gatekeeper mutants (6, 7), treatment with small molecule inhibitors (ZM447439 and AZD1152) of Aurora kinases (6, 48, 51, 52), siRNA-mediated knockdown (51), and the generation of *Aurora-C* knockout (*Aurkc*$^{-/-}$) mice (7, 49, 53). Yang et al. (6) first reported that exogenous expression of kinase-dead Aurora-C mutant (T171A, T175A, designated Aurora-C-KD) in mouse oocytes significantly inhibited endogenous Aurora-C activity and produced multiple defects, including chromosome misalignment, abnormal kinetochore–microtubule (K-MT) attachment, premature chromosome segregation, and failure of cytokinesis in MI. This phenotype was partially recapitulated in oocytes injected with an INCENP-targeting siRNA (51), in an INCNEP-delIN deletion mutant that lacked the Aurora-C-binding motif (6), and in oocytes treated with high doses of small molecule inhibitors of Aurora-B (ZM447439 and AZD1152), that are also likely to inhibit Aurora-C (6, 51, 52). Unexpectedly, *Aurkc*$^{-/-}$ knockout mice were found to be subfertile (49). The overall percentage of chromosome misalignment in MI oocytes of *Aurkc*$^{-/-}$ mice was not strikingly different from that of wild-type controls, but a portion of the oocytes in knockout mice arrested in MI and displayed abnormally aligned chromosomes (49). Recently, Balboula and Schindler (7) developed an ATP-binding-pocket-Aurora-C mutant (L93A, gatekeeper mutant) that appears to selectively disrupt the function of Aurora-C, but not Aurora-B, during female meiotic divisions, and microinjected this mutant into mouse oocytes. Their observations suggested that the specific loss of Aurora-C function caused chromosome misalignment and failure to correct erroneous K–MT attachments (7), which is similar to the deficits observed in oocytes expressing the Aurora-C kinase-dead mutant (T171A/T175A) (6). Meanwhile, the process of cytokinesis in oocytes appears to be regulated by either the Aurora-B–CPC complex or by the activities of both Aurora-B and Aurora-C (7).

In sum, there is currently no suitable model that encompasses all of the reported roles of Aurora-C during female meiotic divisions. The efforts to generate such a consensus have been complicated by the possible functional compensation of Aurora-B in oocytes (7, 48, 49, 51, 54), the lack of selectivity and specificity among the known small molecule inhibitors (6, 51, 52), problems with the efficiency of siRNA knockdown (51), and the possible presences of multiple tandem copies of the *Aurora-C* gene (42) and/or a potential "functional pseudogene" in the mouse genome. Given these limitations, however, the speculated roles of Aurora-C and -B during female meiotic divisions are summarized in **Figure 2**.

Aurora-C/-B in Human Germ Cells and Preimplantation Embryos: Subcellular Localization and Aurora-C-Deficient Human Patients

Recently, Santos et al. (50) reported the localizations and mRNA expression levels of endogenous Aurora-B and Aurora-C in human germ cells and preimplantation embryos developed from tri-pronuclear (3PN) zygotes. They observed the signal corresponding to Aurora-C in the region surrounding the centromeres

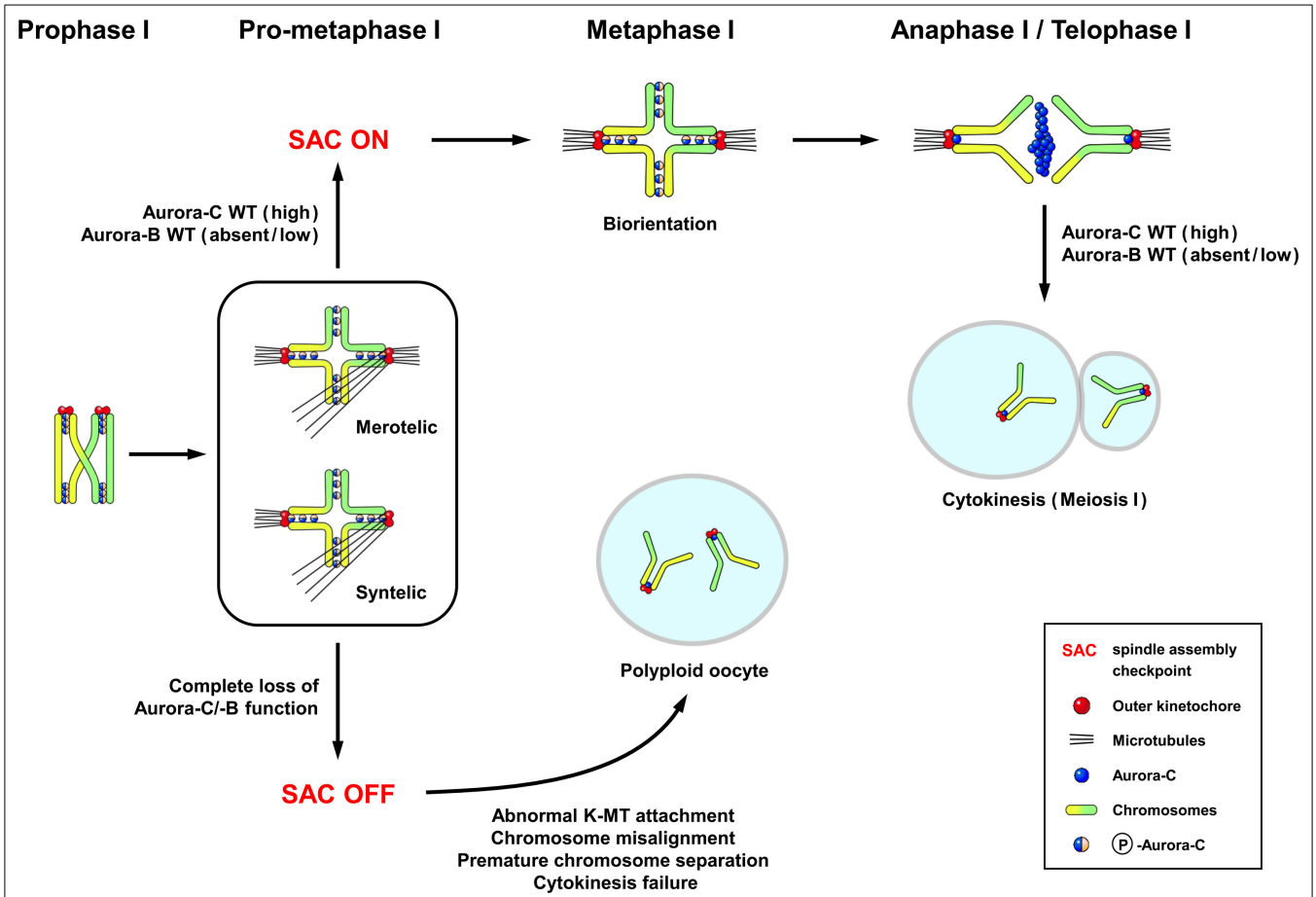

FIGURE 2 | The subcellular localization of Aurora-C and its possible functions in female mouse meiosis I. The localization pattern of Aurora-C in oocytes (6) is similar to that reported in spermatocytes (14). Aurora-C is phosphorylated at Thr171 and located at the chromosome axes and centromeres during late prophase–metaphase I. Aurora-C is dephosphorylated and relocalized to the midzone and midbody during anaphase I–telophase I transition (6). Endogenous Aurora-B appears to be either undetectable (6) or present at low levels in mouse (49) and human oocytes (50). Complete loss of both Aurora-C and Aurora-B activities by ectopic expression of Aurora-C kinase-dead mutant caused more severe effects, including chromosome misalignment, aberrant kinetochore–microtubule (K-MT) attachments, premature chromosome segregation, and cytokinesis failure in meiosis I, resulted in producing polyploid oocytes (6, 7). Figure modified from Ref. (6).

in human MI and MII oocytes. This was consistent with the localization pattern described in mouse oocytes (6). Human Aurora-C first appeared at the pericentric heterochromatin in pachytene spermatocytes (50), whereas mouse Aurora-C was first detected at the diplotene stage (6). In contrast, endogenous Aurora-B was hardly detected in human oocytes at MI (50).

In preimplantation embryos, Aurora-C appears to be the major Aurora kinase expressed during the first three embryonic cell cycles, where it can be visualized on prometaphase chromosomes in zygotes and two- and four-cell-stage human embryos. The endogenous Aurora-B protein was expressed at low-to-undetectable levels during these embryonic stages, but increased significantly after the eight-cell stage. It is interesting to note that the expression of Aurora-C occurs earlier, and is completely replaced by Aurora-B at the blastocyst stage of human embryonic development. These findings prompted the authors to hypothesize that Aurora-C could be the main enzymatic component of the CPC, and thus plays a specific role during human female meiosis and preimplantation embryo development (50). However, it is not yet clear whether its deficiency is linked to a high aneuploidy rate in human preimplantation embryos.

Recently, three naturally occurring mutations in the human *Aurora-C* kinase gene were reported to be associated with male infertility: c.144delC, which deletes a cytosine in exon 3 (8); c.686G > A, which is a missense mutation in exon 6 (p.Cys229Tyr) (55); and c.436-2A.G, which is a splicing site mutation that leads to the skipping of exon 5 (56). Individual males carrying homo- or hetero-allelic combinations of null or strong loss-of-function Aurora-C mutations frequently produce polyploidy and multi-flagellar spermatozoa that are unsuitable for fertilization. Males homozygous for c.144delC had no obvious physiological or anatomical defects beyond sperm abnormalities, suggesting that Aurora-C is not essential for somatic cell division (55). Moreover, females carrying the same homozygous mutation (c.144delC) were fertile, suggesting that Aurora-C may be dispensable for meiotic divisions in the human female (55).

The question of how the large-headed multi-flagellar polyploid spermatozoa are generated in humans cannot be answered using

the *Aurkc*$^{-/-}$ knockout mice. However, speculations can be made. One possible explanation is that Aurora-C plays a critical role in cytokinesis during spermatogenesis. Indeed, mouse oocytes injected with Aurora-C-kinase-dead mRNAs showed failure in the cytokinesis of MI (6). This resulted in the production of large polyploid mouse oocytes, which could be compared to the polyploid spermatocytes found in Aurora-C-deficient humans. However, we cannot yet explain why Aurora-C-deficient human females are fertile and do not have polyploid oocytes.

Conclusion

In mouse spermatocytes, both Aurora-B (24) and Aurora-C (14) proteins are present at relatively high levels and show a similar localization pattern (**Figure 1**). Both are also likely to be recruited to meiotic chromosomes by INCENP (14, 24). The functional differences in these proteins during male meiotic divisions remain largely unknown. In females, endogenous Aurora-B is either undetectable (6) or present at low levels in mouse (49) and human oocytes (50). Here, Aurora-C appears to be the major enzymatic component of the CPC, and thus may play a specific role during female meiotic divisions (6, 49–51). The differential roles of Aurora-B and Aurora-C during female meiosis have been addressed by a number of different approaches, but no conclusive answer has yet been obtained. Furthermore, it is difficult to use the results obtained from mouse studies to interpret the clinical phenotypes in human Aurora-C-deficient subjects. For example, microinjection of Aurora-C-kinase-dead mRNAs into mouse oocytes caused failure of cytokinesis in MI and the production of large polyploid oocytes (6), whereas a homozygous Aurora-C mutation in human affects male (but not female) germ cells. This discrepancy could reflect species-specific differences, and further studies are needed to resolve the differential roles of Aurora-B and Aurora-C during meiotic divisions in mouse and human germ cells.

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Functions of Aurora kinase C in meiosis and cancer

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The mammalian genome encodes three Aurora kinase protein family members: A, B, and C. While Aurora kinase A (AURKA) and B (AURKB) are found in cells throughout the body, significant protein levels of Aurora kinase C (AURKC) are limited to cells that undergo meiosis (sperm and oocyte). Despite its discovery nearly 20 years ago, we know little about the function of AURKC compared to that of the other 2 Aurora kinases. This lack of understanding can be attributed to the high sequence homology between AURKB and AURKC preventing the use of standard approaches to understand non-overlapping and meiosis I (MI)-specific functions of the two kinases. Recent evidence has revealed distinct functions of AURKC in meiosis and may aid in our understanding of why chromosome segregation during MI often goes awry in oocytes. Many cancers aberrantly express AURKC, but because we do not fully understand AURKC function in its normal cellular context, it is difficult to predict the biological significance of this expression on the disease. Here, we consolidate and update what is known about AURKC signaling in meiotic cells to better understand why it has oncogenic potential.

Keywords: meiosis, Aurora kinase, cancer, fertility

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Discovery and Genomic Features

Three laboratories independently discovered *AURKC* and reported high transcript levels in testes and oocytes (Gopalan et al., 1997; Bernard et al., 1998; Tseng et al., 1998). A subsequent study reported low expression of *AURKC* in some normal somatic cells including skeletal muscle, placenta, lung and bladder (Yan et al., 2005b) although germ cell expression is much higher (49 times) (Assou et al., 2006). In addition, Kimura et al. (1999) found elevated levels of AURKC in breast, cervical, and liver cancer cells lines.

AURKC is a member of the conserved serine/threonine Aurora kinase family. These kinases are related to *Increase-in-ploidy1* in budding yeast and *Aurora* in *Drosophila*, both of which regulate spindle formation and chromosome segregation (Francisco and Chan, 1994; Glover et al., 1995). Yeast contains one Aurora kinase (Petersen et al., 2001), while *Drosophila, C. elegans* and *Xenopus* express two (Roghi et al., 1998) generated from gene duplication in cold-blooded vertebrates (Brown et al., 2004). The mammalian genome encodes three Aurora kinases. *AURKC* is located on human Chromosome 19 [19q13.43 (Kimura et al., 1999)] and *Aurkc* on mouse Chromosome 7 A2-A3 (Gopalan et al., 1997). Human AURKC shares 82.1 and 68.8% amino acid identity with mouse AURKC in the kinase and N-terminal domains, respectively however only 26.7% identity in the C-terminal domain suggesting species-specific differences (Tseng et al., 1998).

Abbreviations: APC/C, Anaphase promoting complex/cyclosome; AURKA, Aurora kinase A; AURKB, Aurora kinase B; CPC, Chromosomal passenger complex; ICA, Interchromatid axis; K-MT, Kinetochore microtubule; MI, Meiosis I; MII, Meiosis II; SAC, Spindle assembly checkpoint; TACC1, Transforming acidic coiled-coil 1.

Alternative splicing results in three protein variants of AURKC (Bernard et al., 1998; Tseng et al., 1998; Yan et al., 2005b) (**Figure 1A**). Variants 2 and 3 lack amino acid residues in the N-terminus that do not appear to regulate localization (Fellmeth et al., 2015). While all variants are catalytically active, variant 1 is better at phosphorylating targets in oocytes suggesting the N-terminus positively regulates activity. Human oocytes contain all three variants while only one or two variants are measured in sperm (Fellmeth et al., 2015).

At the protein level, AURKC shares sequence homology with AURKA (60% identical) and AURKB (75% identical) in the kinase domain (Quintas-Cardama et al., 2007). Autophosphorylation of a threonine contained within the activation loop (T-loop) activates the kinases (**Figure 1B**) (Goldenson and Crispino, 2015). AURKC lacks the N-terminal domain found in AURKA and B (Gopalan et al., 1997; Kimura et al., 1999) containing the KEN (KENXXX) and D-box activating domain (DAD/A-box, QRVL) motifs suggesting that it is differentially regulated. The anaphase promoting complex/cyclosome (APC/C) recognizes these sequences and marks the protein for degradation (Nguyen et al., 2005). AURKB and AURKC do contain four D-box motifs (RXXL), which can be recognized by the APC/C, however their regulatory function is unknown (Nguyen et al., 2005; Stewart and Fang, 2005; Schindler et al., 2012).

AURKC Signaling in Sperm

Spatiotemporal Regulation

Localization of AURKC in spermatocytes is dynamic and linked to its function. Mouse spermatocytes express measurable levels of AURKC protein at centromeres in the diplotene stage of prophase (Tang et al., 2006) followed by localization at centromeres and along chromosome arms during diakinesis (Tang et al., 2006). Next, AURKC translocates to the spindle midzone at anaphase I and the midbody at telophase I. AURKC follows the same distribution pattern through meiosis II (MII) (Tang et al., 2006) then dissociates from centromeres (Tang et al., 2006). Human spermatocytes exhibit the same localization pattern of AURKC (Avo Santos et al., 2011). AURKC co-localizes with AURKB and immunoprecipitates with INCENP in spermatocytes suggesting that it is a member of the meiotic chromosomal passenger complex (CPC) (Tang et al., 2006) that regulates chromosome alignment and condensation, kinetochore-microtubule attachments (K-MT) and cytokinesis (Sharif et al., 2010; Yang et al., 2010; Balboula and Schindler, 2014).

Expression Levels

Aurkc expression is also regulated in a stage-specific manner (Kimmins et al., 2007). *In situ* hybridization revealed positive expression in some seminiferous tubules from mice with meiotic cells in prophase (4C) having the highest levels (Tang et al., 2006). *Aurkc* transcript first appears in the testes of mice 14 days after birth (Hu et al., 2000). mRNA levels increase and plateau at day 21 before decreasing at day 28, but mRNA is still observed at day 42 (Hu et al., 2000).

Male Fertility

Male $Aurkc^{-/-}$ mice are viable with normal testis weight and sperm counts but are subfertile (Kimmins et al., 2007). This subfertility is attributed to blunted sperm heads, defects in chromatin condensation and acrosome detachment (Kimmins et al., 2007). In humans, AURKC is essential for male fertility. Current studies indicate that mutations in AURKC are the most frequent genetic cause of macrozoospermia (Ounis et al., 2015), a condition where ∼100% of a patient's sperm have large, misshapen heads. These sperm have multiple flagella (Dieterich et al., 2009) due to a meiotic arrest in MI (Dieterich et al., 2007) suggesting AURKC is critical for cytokinesis.

A genome-wide microsatellite scan of 10 affected men from the Rabat region of Morocco revealed cysteine deletion in exon 3 of *AURKC* (c.144delC, also called L49W) (**Figure 1A**). The mutation induces a frameshift leading to premature termination of translation and truncated protein (Dieterich et al., 2007). A subsequent study found that the mutation, which also induces non-sense mediated mRNA decay (Ben Khelifa et al., 2011), occurs at a rate of 1 in 50 in the Maghrebian population (Dieterich et al., 2009) suggesting a selective advantage for harboring this allele. Heterozygous mutations of c.144delC combined with C229Y, Y248X (Dieterich et al., 2009) or c.436-2A>G required for proper slicing (Ben Khelifa et al., 2011) produced a similar phenotype. Few morphologically "normal" sperm can be isolated from these men and used for intracytoplasmic injection into eggs. However, euploid embryos were never generated indicating that sperm from men with AURKC mutations cannot be used in the *in vitro* fertilization clinic (Ben Khelifa et al., 2011; El Kerch et al., 2011).

Interestingly, women homozygous for c.144delC are not sterile indicating a sexually dimorphic role of AURKC. But the small sample size ($n = 2$) of the study limits the impact of this finding (Dieterich et al., 2009).

AURKC Signaling in Oocytes

Spatiotemporal Regulation

Mammalian oocytes display dynamic localization of AURKC. AURKC localizes to centromeres and along chromosome arms during prometaphase and metaphase I before concentrating at the midzone and midbody during anaphase I and telophase I, respectively (Uzbekova et al., 2008; Avo Santos et al., 2011). AURKC's localization at the interchromatid axis (ICA) of bivalents at metaphase of MI is regulated by haspin in mouse oocytes (Nguyen et al., 2014) and distinguishes the kinase from AURKB that is found on the spindle. Therefore AURKC localization in sperm and oocytes is identical.

Expression Levels

In oocytes, *Aurkc* expression is also regulated temporally. The relative mRNA level of *Aurkc* in prophase I-arrested mouse oocytes is similar compared to mRNA levels of *Aurkb* but 9–20 fold less than *Aurka* (Shuda et al., 2009; Schindler et al., 2012). Oocytes that are competent to complete meiosis are transcriptionally silent. This silence persists until zygotic genome activation. To ensure plentiful protein stores, oocytes

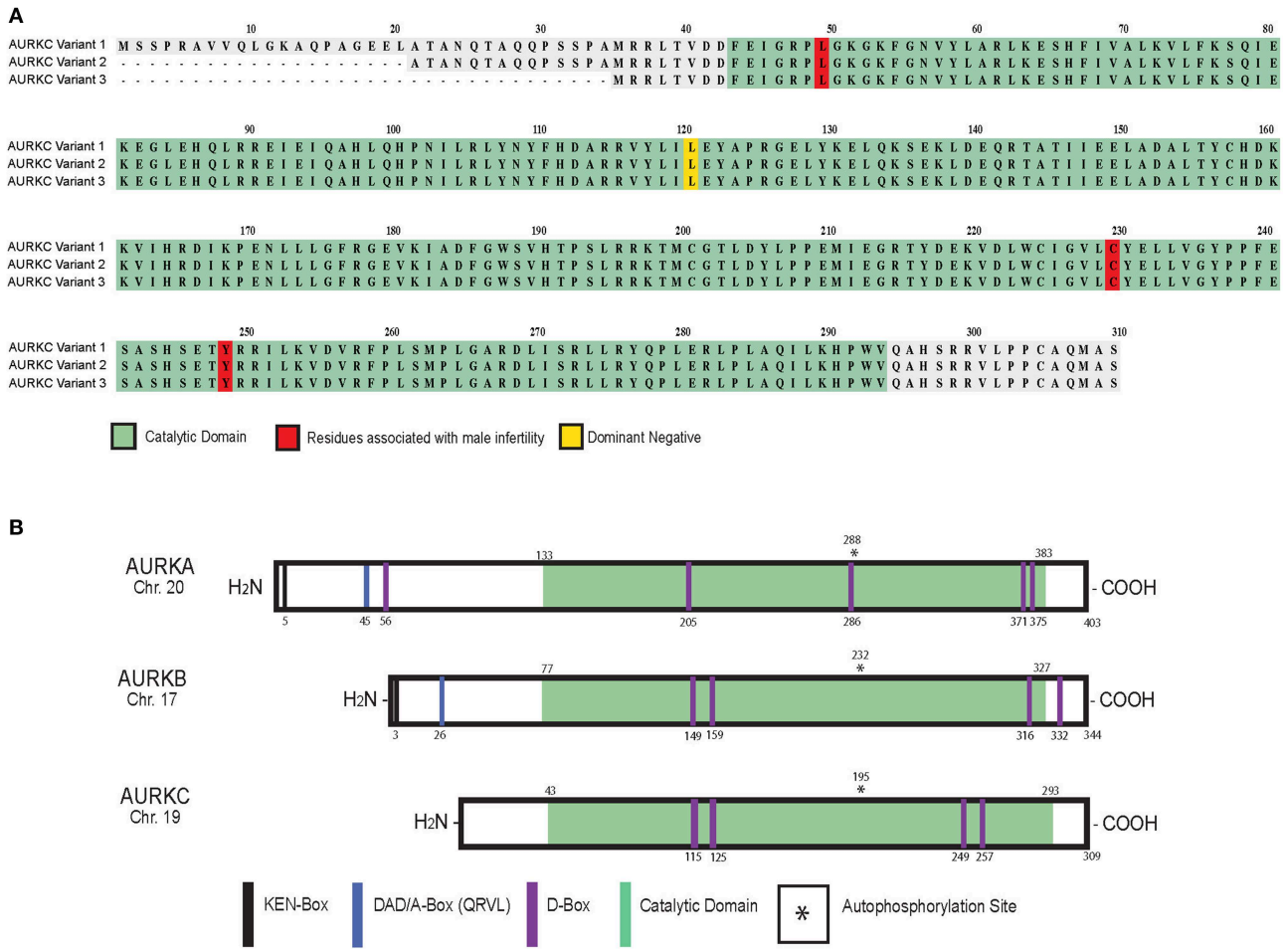

FIGURE 1 | AURKC variants and Aurora kinase family members in mammals. Schematic of human AURKC variants **(A)** and Aurora kinase isoforms **(B)** with key domains and residues identified.

recruit maternal messages for translation during MI through a cytoplasmic polyadenylation element in the 3′ untranslated region of genes. *Aurkc* contains this element and is recruited (Schindler et al., 2012). Therefore although *Aurkc* mRNA levels drop to undetectable levels in blastocysts, (Avo Santos et al., 2011; Schindler et al., 2012) protein remains in the embryo until AURKB becomes the predominant CPC kinase (Fernandez-Miranda et al., 2011; Schindler et al., 2012).

Female Fertility

Female $Aurkc^{-/-}$ mice survive but are subfertile due to meiotic abnormalities and compromised embryonic development (Schindler et al., 2012). Oocytes from $Aurkc^{-/-}$ mice often contain misaligned chromosomes and arrest at MI. Some oocytes do undergo cytokinesis and extrude a polar body but are delayed. In addition, fewer one-cell embryos from $Aurkc^{-/-}$ mice reach the two-cell stage due to cytokinesis failure, and this phenotype worsens during development (Schindler et al., 2012).

While overexpression of AURKB can rescue MI arrest and cytokinesis failure (Schindler et al., 2012) endogenous levels of AURKC are sufficient for preimplantation embryonic development $Aurkb^{-/-}$ embryos (Fernandez-Miranda et al., 2011). These phenotypic data combined with the instability of AURKB and recruitment of *Aurkc* messages during MI (Schindler et al., 2012) drove the conclusions that mouse oocytes require AURKC because AURKB levels are insufficient to ensure completion of meiosis and embryonic mitoses. Importantly, wild-type mouse oocytes expressing a dominant-negative allele of AURKC that does not inhibit AURKB (AURKC-LA) (L93A in mouse [variant 2]; L120A in human [variant 1]) are aneuploid (Balboula and Schindler, 2014). These data indicate that when a non-functional AURKC protein is bound in the CPC, AURKB cannot compete for binding to support meiosis. We anticipate that as more genomes are sequenced, mutations in AURKC that alter activity in the CPC will be correlated with female infertility.

Overlapping AURKB and AURKC Function

The CPC regulates the spindle assembly checkpoint (SAC), cytokinesis and correction of K-MT attachments. AURKC

specific inhibition (AURKC-LA) does not alter the localization of SAC component BUB1 in oocytes suggesting that both AURKB and AURKC regulate SAC activation in meiosis (Balboula and Schindler, 2014). Only after microinjection of the dominant negative form of *Aurkc* (*Aurkc-DN*) (T171A/T175A, variant 2), which disrupts the function of both AURKs, is BUB1 localization altered and SAC non-functional (Yang et al., 2010; Balboula and Schindler, 2014). AURKB and C also share regulation of cytokinesis. AURKC-LA-expressing oocytes that complete MI extrude a polar body, while AURKC-DN oocytes retract polar bodies (Kimura et al., 1999; Balboula and Schindler, 2014). In contrast, similar levels of incorrect K-MT attachments (Balboula and Schindler, 2014) are observed in AURKC-LA and AURKC-DN oocytes suggesting AURKC, particularly at the ICA (Nguyen et al., 2014), is the primary CPC kinase to correct attachments (Balboula and Schindler, 2014). In mitotic cells the CPC preferentially binds AURKB (Sasai et al., 2004), but increased translation of AURKC during MI is consistent with AURKC being the preferred catalytic component of the CPC in oocytes (Assou et al., 2006).

AURKB and AURKC share a consensus phosphorylation motif (R-X-S/T-Φ, Φ represents any hydrophobic residue except P)(Alexander et al., 2011) and therefore phosphorylate many of the same substrates. These kinases can bind the "IN box" of INCENP (Tang et al., 2006; Ben Khelifa et al., 2011) leading to autophosphorylation and kinase activation (Li et al., 2004). AURKC binds the other CPC components (Survivin and Borealin) when overexpressed in mitotic cells (Sasai et al., 2004; Chen et al., 2005; Yan et al., 2005a; Slattery et al., 2008, 2009) and phosphorylates histone H3 at S10 in meiotic and mitotic cells (Li et al., 2004; Avo Santos et al., 2011), which may play a role in chromosome condensation (Swain et al., 2008). In addition both AURKB and AURKC phosphorylate Centromere protein A in mitotic cells (Sasai et al., 2004; Slattery et al., 2008), which is required for the recruitment of kinetochore proteins, chromosome segregation and cell cycle progression. Future investigations need to evaluate whether other known downstream targets of AURKB, such as Hec1 (Zhu et al., 2013) and the MAPK pathway (Xu et al., 2012), are also targeted by AURKC.

Unique AURKC Functions

Although AURKB and AURKC often exhibit conserved function, they cannot fully compensate for the loss of one another (Kimmins et al., 2007; Fernandez-Miranda et al., 2011; Schindler et al., 2012; Balboula and Schindler, 2014) indicating that non-overlapping roles exist. Evidence of these differences can be seen in the divergent phenotypes of knockout mice. *Aurkb*$^{-/-}$ die at the blastocyst embryonic stage while *Aurkc*$^{-/-}$ knockouts are viable (Kimmins et al., 2007; Schindler et al., 2012). Transgenic mice expressing a dominant negative AURKB driven by the male-specific β-4-galactosyltransferase promoter exhibit severe disruption in spermatogenesis with reduced sperm counts, reduced testis size and disorganized spermatogenic staging. 48% of these mice are sterile and cytokinesis failure is observed (Kimmins et al., 2007). This inability of AURKC to physiologically compensate for AURKB absence in sperm suggests that AURKB has specific functions in mouse spermatogenesis (Kimmins et al., 2007) although the dominant negative allele used may also affect the function of AURKC.

The non-overlapping functions of AURKB and C have also been demonstrated in experiments with oocytes, consistent with their spatial separation (Balboula and Schindler, 2014). Overexpression of AURKC causes arrest in MI due to cytokinesis failure (Sharif et al., 2010). Securin levels decrease (a sign of APC/C activation) and activated separase triggers homologous chromosome separation (Sharif et al., 2010). This phenotype differs from AURKB-overexpressing oocytes, which fail to activate the APC/C and stabilize securin and have unresolved chiasmata (Sharif et al., 2010). These data indicate that AURKC plays a role in cell cycle progression while AURKB acts to maintain the SAC. Another indication of unique activity: overexpression of AURKB, and not AURKC, can rescue the misaligned and slowed progression phenotype of ZM447439-treated oocytes (Shuda et al., 2009). These data suggest that high levels of AURKB can displace AURKC from the CPC, that AURKB has a non-CPC function, or that AURKB-CPC has a chromosome-independent function. Future studies are critical to decipher other AURKC specific functions.

Expression of Meiotic Genes in Cancer

Meiomitosis is the expression of meiosis-specific proteins in mitotic cells (Grichnik, 2008) and can negatively impact genetic stability. Meiotic proteins, or cancer testis antigens (CTA), are used as diagnostic and prognostic indicators (Fratta et al., 2011; Rosa et al., 2012) in skin, bladder, lung and ovarian tumors. Upregulation of CPC components, including AURKC, occurs in cancer cells (Yan et al., 2005a) and may correlate with clinical characteristics in primary colorectal cancers (Takahashi et al., 2000; Lin et al., 2014). It is unclear if CTA expression is the initiating oncogenic event or a downstream consequence of transformation (Rosa et al., 2012), but could indicate that cancer cells use meiotic divisions (i.e. separating homologs) for growth and survival advantages (Ianzini et al., 2009). These proteins represent desirable diagnostic biomarkers for tumor subtype and ideal candidates for targeted therapeutics because their expression is limited to germ cells, thereby minimizing side effects.

AURKC Signaling in Cancer Cells

AURKC is oncogenic because its overexpression transforms NIH 3T3 cells into tumors (Khan et al., 2011). AURKC is overexpressed in many cancer cell lines, including NB1RGB, MDA-MB-453, HEPG2, HeLa, and HuH7 (Kimura et al., 1999), and in cancer of the reproductive tract (Tsou et al., 2011). Overexpression increases cellular proliferation and migration and enhances xenograft tumor growth (Tsou et al., 2011). Kinase-dead AURKC decreases proliferation of HeLa cells while expression of the constitutively active AURKC (Spengler, 2007a; Khan et al., 2012) leads to more aggressive tumors (Khan et al., 2011; Tsou et al., 2011). Other carcinogenic genes are

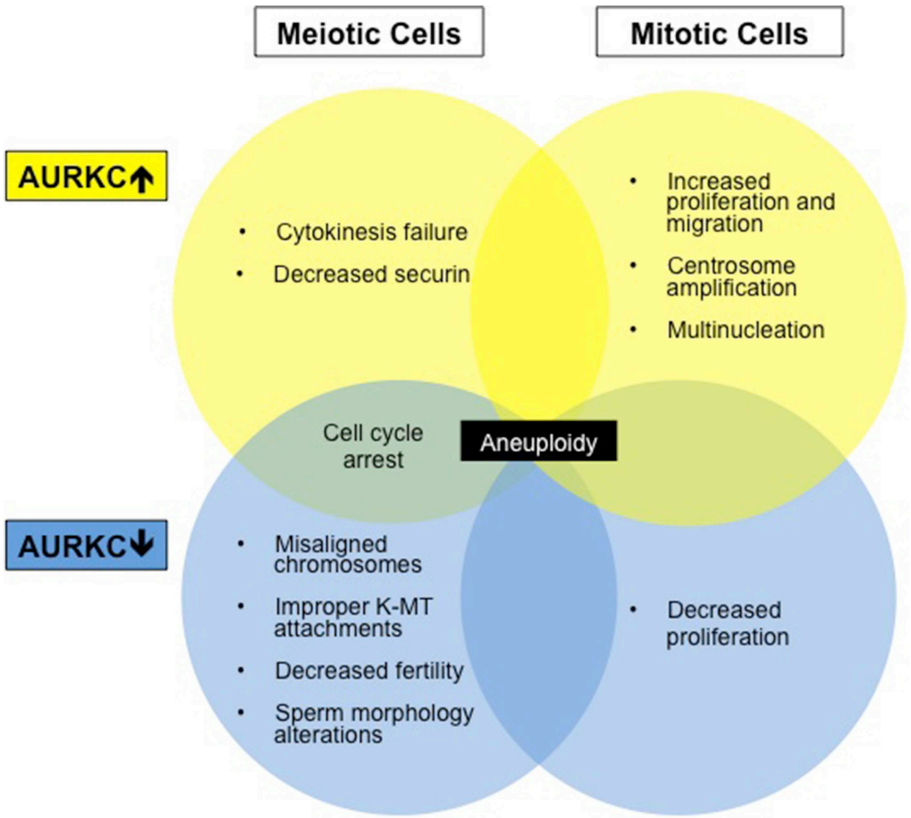

FIGURE 2 | Aberrations in AURKC levels results in altered cell phenotypes. Diagram summarizing cell phenotypes observed when AURKC expression is disrupted in mitotic and meiotic cells.

also located in the telomeric region of human Chromosome 19 (Bernard et al., 1998), a genomic region susceptible to translocations and deletions (Bernard et al., 1998; Kimura et al., 1999). Although some plasticity exists between the Aurora kinase family members allowing for functional compensation; some roles are kinase specific and maintaining the correct balance is necessary for genomic stability (**Figure 2**).

The functional significance of AURKC expression in cancer cells is unknown but may relate to centrosome regulation. Overexpression of AURKC in mitotic cells leads to centrosome amplification and multinucleation (Khan et al., 2011), a hallmark of cancer. Extra centrosomes are associated with the formation of multipolar spindles. Multipolar spindle formation usually leads to cell death however centrosome clustering appears to support cancer cell survival and frequently leads to chromosome segregation defects (Marthiens et al., 2012). AURKC localizes to centrosomes with AURKA during interphase (Takahashi et al., 2000; Dutertre et al., 2005) and may play a role in centrosome clustering. Many new cancer therapies are aimed at declustering centrosomes (Pannu et al., 2014) which forces cancer cells to form a multipolar spindle and induces cell death. AURKC inhibition may alter this clustering pathway.

AURKC interactions with other proteins linked to cancer may also explain its oncogenic role. AURKC, as well as AURKA and AURKB, phosphorylate the transforming acidic coiled-coil 1 protein (TACC1) (Gabillard et al., 2011). Overexpression of *TACC1* drives cell transformation (Cully et al., 2005) and serves as a prognostic marker of endocrine therapy resistance in breast cancer (Ghayad et al., 2009). AURKC also phosphorylates TRF2, a protein involved in telomere length regulation (Spengler, 2007b). Decreased telomere length predisposes individuals to cancer (Shammas, 2011) and negatively impacts fertility (Spengler, 2007b). In addition, tumor necrosis factor alpha induces increased AURKC expression through the inflammation response factor CEBPD in HeLa cells (Wu et al., 2011). Ongoing studies of normal AURKC functions in meiotic cells are critical to improving our understanding of the role of aberrant expression in cancer.

Small Molecule Inhibitors

More than 70 clinical trials have been conducted on Aurora kinase inhibitors. First generation inhibitors failed due to low efficacy and high toxicity (Goldenson and Crispino, 2015) however second-generation inhibitors are more sub-type specific which may alleviate side effects. SNS-314 is a pan-Aurora kinase inhibitor (Oslob et al., 2008) with AURKA, B and C IC50 values of 9, 31, and 3 nM, respectively (Kollareddy et al., 2012). This ATP-competitive inhibitor can inhibit proliferation of anaplastic thyroid cancer cells *in vitro* (Baldini et al., 2012) and inhibit tumor growth of colon cancer xenografts (Arbitrario et al., 2010). A phase I clinical trial on advanced solid tumors

showed modest results of SNS-314 treatment alone (Robert et al., 2008), however sequential administration of SNS-314 and chemotherapy docetaxel exhibited synergistic anti-proliferative effects (VanderPorten et al., 2009). AMG-900 inhibits AURKC with a 1nM IC50 (Payton et al., 2010). The compound induces apoptosis in a diverse set of cancer cell lines *in vitro* and inhibits tumor growth *in vivo* (Payton et al., 2010) AMG-900 inhibits colony formation of multidrug resistant cell lines (Payton et al., 2010; Bush et al., 2013) and shows additive effects when combined with histone deacetylase inhibitors (Paller et al., 2014). Two Phase I clinical trials are being conducted on advanced solid tumors and acute leukemias (Kollareddy et al., 2012).

Conclusion

Many advances have been made regarding our knowledge of AURKC as a regulator of chromosome segregation, but many questions remain. Does AURKC have unique, MI-specific substrates and do they differ between sperm and oocyte? What cofactors are needed for full AURKC activation? Does AURKC function outside of the CPC? Does AURKC drive meiotic events when expressed in mitotic cells giving rise to tumors? Not until we have a complete understanding of the function and substrates of AURKC in meiotic cells can we begin to understand the significance of its expression in cancer cells. However, once these and other meiomitotic protein studies are complete, this class of proteins represent a promising diagnostic and therapeutic cancer target.

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REVIEW
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The non-canonical role of Aurora-A in DNA replication

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Aurora-A is a well-known mitotic kinase that regulates mitotic entry, spindle formation, and chromosome maturation as a canonical role. During mitosis, Aurora-A protein is stabilized by its phosphorylation at Ser51 via blocking anaphase-promoting complex/cyclosome-mediated proteolysis. Importantly, overexpression and/or hyperactivation of Aurora-A is involved in tumorigenesis via aneuploidy and genomic instability. Recently, the novel function of Aurora-A for DNA replication has been revealed. In mammalian cells, DNA replication is strictly regulated for preventing over-replication. Pre-replication complex (pre-RC) formation is required for DNA replication as an initiation step occurring at the origin of replication. The timing of pre-RC formation depends on the protein level of geminin, which is controlled by the ubiquitin–proteasome pathway. Aurora-A phosphorylates geminin to prevent its ubiquitin-mediated proteolysis at the mitotic phase to ensure proper pre-RC formation and ensuing DNA replication. In this review, we introduce the novel non-canonical role of Aurora-A in DNA replication.

Keywords: Aurora-A, geminin, DNA replication, pre-RC, ubiquitin, proteasome, degradation

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Introduction

Cyclin-dependent kinases (CDKs) acquire catalytic activity by forming complexes with the cyclins and promote cell cycle progression via phosphorylation of crucial target proteins (1). In mitosis, other kinases such as Aurora-A, Aurora-B, and Aurora-C tightly regulate drastic and rapid morphological changes (2). Aurora-A, the serine/threonine kinase, is essential for several events during mitosis including entry of mitosis, duplication of centrosome, spindle formation, segregation of chromosome, and cytokinesis (3). Aurora-A protein expression peaks during mitosis and decreases at G_1 phase in mammalian cells (4). Expression of Aurora-A protein is reduced in late mitosis as a consequence of ubiquitin-mediated proteolysis by anaphase-promoting complex/cyclosome (APC/C) and its co-activator Cdh1 (5–7). It is well known that protein level of various cell cycle regulators is regulated by the ubiquitin–proteasome system (UPS) for proper regulation of cell cycle (1, 8). Aurora-A protein is ubiquitylated via recognition of destruction box (D-box) in the C-terminal by Cdh1 (5) and an additional A-box/DAD motif (9, 10). Furthermore, Ser53 (equivalent to Ser51 in human Aurora-A) of the A-box is phosphorylated during mitosis and this phosphorylation is important for the protein stabilization of *Xenopus* and human Aurora-A (4, 11, 12).

DNA replication is strictly restricted to occur only once per cell cycle in eukaryotes. To prevent over-replication, replication origins are restricted to activate only once per cell cycle by a mechanism called "licensing." The assembly of the pre-replication complex (pre-RC) mediates licensing at the origins of replication (13, 14). The assembly of the pre-RC at replication origins can only occur from late mitosis to early G_1 with low CDK activity and high activity of APC/C (13, 14). Once pre-RC complexes are assembled, origins are licensed for replication in the ensuing S phase. Geminin is

known as a repressor of re-replication and directly binds to chromatin licensing and DNA replication factor 1 (Cdt1) to prevent pre-RC formation (15). Recently, we found that Aurora-A phosphorylates geminin to prevent its ubiquitin-mediated proteolysis at the mitotic phase to ensure proper pre-RC formation and ensuing DNA replication. In this review, we introduce the novel non-canonical role of Aurora-A in DNA replication, notably its initiation process called "licensing."

Ubiquitin–Proteasome Pathway

The UPS marks proteins for destruction by attaching a polyubiquitin chain and subsequently degrading these proteins via the activity of a multicatalytic enzyme, 26S proteasome (8). Ubiquitin in its monomeric form is a small protein that contains only 76 amino acids. Attachment of a polyubiquitin chain to a substrate requires the concerted action of three enzymes, E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) (8). E1 forms a high-energy thioester bond with ubiquitin in an ATP-dependent reaction, and then the ubiquitin molecule is transferred from E1 to E2. E3 is classified into two distinct classes based on the homology domain: HECT and RING domains. The HECT-type E3s form covalent linkages with ubiquitin from E2 by using a conserved cysteine and subsequently transfer ubiquitin to substrates. On the contrary, the RING-type E3s function as adaptors to facilitate the positioning and transfer of ubiquitin from E2 directly onto the substrate (16). A number of E3s have been found to physically bind to the substrate. Both E2 and E3 proteins exist as large families and the substrate specificity is thought to be defined by different combinations of E2s with different E3 proteins. The human genome encodes only two E1s and less than 40 E2s. Moreover, more than 600 different E3 ligases have been identified in the human genome, allowing for tremendous diversity in substrates (17).

Cell Cycle Control by APC/C Ubiquitin Ligase

The specific, rapid, and timely proteolysis of cell cycle regulators by the UPS represents an important mechanism that ensures proper progression via the cell division cycle in a unidirectional and irreversible manner. The proteolysis of many core components of the cell cycle machinery is controlled by two major classes of ubiquitin ligases, the SCF complex and the APC/C complex, which are RING-type E3s. SCF complexes represent an evolutionarily conserved class of E3 enzymes containing four subunits: *S*kp1, *C*ul1, one of many *F*-box proteins, and Roc1/Rbx1 (18). APC/C is composed of at least a dozen different subunits, namely APC1, APC2, Cdc27/APC3, APC4 APC5, Cdc16/APC6, APC7, Cdc23/APC8, Doc1/APC10, APC11, CDC26, and APC13, but it can only ubiquitylate substrates with the help of a co-activator protein (19). In mammalian cells, APC/C activity is regulated by its binding with the co-activator proteins Cdc20 and Cdh1 during specific periods of the cell cycle (19) (**Figure 1**). All of these proteins are characterized by the presence of sequence elements, known as the C-box and the IR-tail, which mediate their binding to APC/C (20–22). Cdc20 and Cdh1 contain a C-terminal WD40 domain that is predicted to fold into a propeller-like structure and that is believed to recognize APC/C substrates by interacting with specific recognition elements in these substrates called D-box (RxxL) and KEN-box (KEN) (23–25). In addition to D-box and KEN-box, other motifs, including A-box (RxLxPSN), CRY-box (CRYxPS), GxEN-box (GxEN), Spo13 D-box (LxExxN), and O-box (unknown sequence), are also recognized by APC/C (10, 11, 26–29). The APC/C^{Cdc20} complex is necessary for progression through mitosis and it facilitates the exit from mitosis by inactivating CDK1, and the APC/C^{Cdh1} complex helps to maintain low CDK activity and the G_0/G_1 state (19, 30) (**Figure 1**). The APC/C^{Cdh1} and APC/C^{Cdc20} complexes target distinctive specific

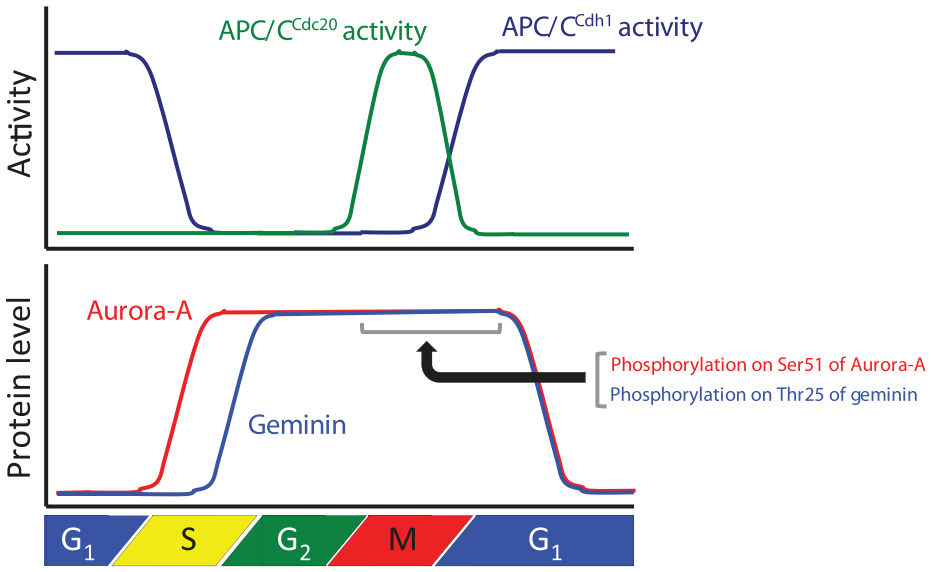

FIGURE 1 | Relationships between the protein levels of Aurora-A and geminin and anaphase-promoting complex/cyclosome (APC/C) activity during cell cycle progression. The graph shows APC/C^{Cdc20} and APC/C^{Cdh1} activities and the protein levels of Aurora-A and geminin during cell cycle progression.

substrates. Although several recent studies have indicated that both co-activators and APC/C have important roles in substrate recognition, the mechanism by which APC/C recognizes its substrates is unclear. As inappropriate activation of APC/C could cause fatal errors in cell cycle progression, protein degradation via APC/C activation is tightly controlled. APC/C activation is also regulated by APC/C inhibiting proteins, such as mitotic arrest-deficient 2 (Mad2), budding uninhibited by benzimidazole-related 1 (BubR1), budding uninhibited by benzimidazole 1 (Bub1), and early mitotic inhibitor 1 and 2 (Emi1 and Emi2) (19). However, it is also unclear how the timing of degradation of numerous APC/C substrates is regulated. Indeed, substrates are not degraded at the same time by APC/C in spite of activation of APC/C during mitosis. It is unclear why the timing of the ubiquitylation of substrates is different. It was recently demonstrated that (i) phosphorylation and acetylation interfere with ubiquitylation of substrates by APC/C (4, 31–33), (ii) intrinsic regulation of APC/C by substrate ordering is attributable to kinetic differences in the ubiquitylation process (34), and (iii) ubiquitylation of the substrate is inhibited by the binding protein of APC/C (35). Thus, the timing of the ubiquitylation by APC/C may be regulated by protein modification, the processing of ubiquitylation, and binding by an inhibitor.

Aurora-A Kinase

Aurora-A is one of the Aurora kinases (Aurora-A, Aurora-B, and Aurora-C), which are highly conserved serine/threonine kinases (36). Aurora-A plays an important role in chromosomal alignment and segregation during mitosis and meiosis (36). Indeed, Aurora-A phosphorylates a large number of substrates, including p53, polo-like kinase-1 (PLK1), CDC25B, BRCA1, centrin, LATS2, GEF-H1, TACC3, NDEL1, HDAC6, Ski, HURP, PP1, TPX2, Eg5, histone H3, CENP-A, CENP-E, CEP192, CEP192, CPEB, LIMK1, LIMK2, SRC, RalA, AKT, and PC2 (37). Aurora-A-mediated phosphorylation of substrates contributes to the activation of kinase activity, protein degradation, protein stabilization, targeting of the centrosome, maturation and separation of centrosome, translocation, and negative regulation of protein function (37). For example, phosphorylation of p53 is involved in its protein degradation (38). Aurora-A activates Plk-1 in G_2 phase via the direct phosphorylation of Thr210 (39). Phosphorylation of LATS2, NDEL1, and TACC3 promotes centrosome maturation (40–42). Aurora-A shares high homology between species and it is evolutionarily ancient, with Aurora-A sharing 82% sequence identity between the human and rodent genes. Aurora-A contains a key threonine, the T-loop residue Thr288, within its kinase domain, and Thr 288 is phosphorylated to allow for kinase activity via autophosphorylation (9, 43, 44). The expression level of Aurora-A mRNA and protein is controlled in a cell cycle-dependent manner. Expression of Aurora-A mRNA peaks at G_2/M, with protein expression peaking slightly later (45, 46). The promoter of Aurora-A contains specific sequences required for transcription in G_2 phase (46–48). Expression of Aurora-A protein peaks during mitosis and decreases in G_1 phase as a consequence of ubiquitylation by APC/C^{Cdh1} (4–7) (**Figure 1**).

The APC/C^{Cdh1} ubiquitin ligase complex recognizes its substrates with either D-Box and/or KEN-box motifs (19, 24, 25). Although Aurora-A has four D-Box motifs and one KEN-box motif, the one of four D-box (D-box at C-terminal) and N-terminal A-box (^{47}RxLxPSN^{52}) are required for the ubiquitylation of human Aurora-A protein (4, 5, 9, 10). Moreover, *Xenopus* Ser53 (or Ser51 in humans) within the A-box is phosphorylated during mitosis, and this phosphorylation is essential for mitotic-specific stabilization (4, 11, 12) (**Figure 2**). Similarly as Aurora-A regulation via phosphorylation, CDC6 protein is protected from APC/C^{Cdh1}-mediated degradation by virtue of its phosphorylation (31). The phosphorylation sites of CDC6 by cyclin E/CDK2

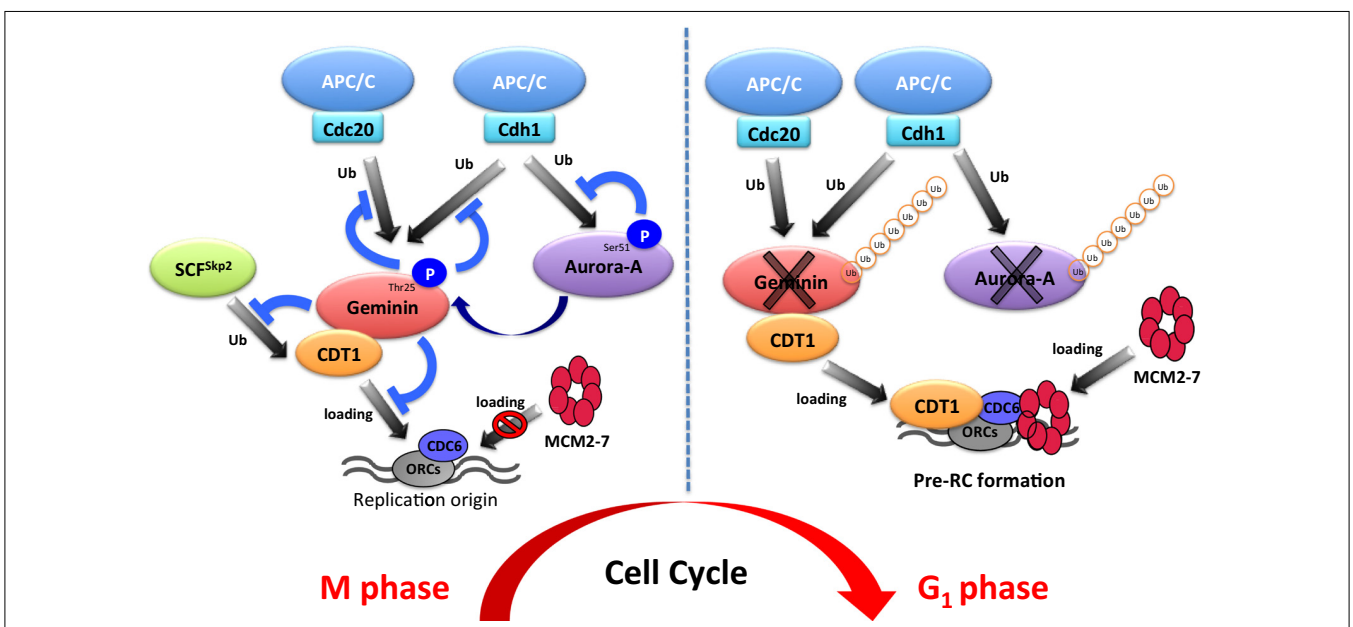

FIGURE 2 | Schematic model of DNA replication via the aurora-A–geminin–CDT1 axis.

are located directly adjacent to the D-box, therefore preventing the recognition of CDC6 by APC/C^{Cdh1}. In the case of human Aurora-A protein, Ser51 is located far from the D-box, but Ser51 is located in the A-box. However, phosphorylated Aurora-A at Ser51 can bind to Cdh1 (4). The mechanism by which Aurora-A degradation is prevented by phosphorylation on Ser51 is unclear. Other regulators, such as Cdc4/Fbxw7, checkpoint with forkhead and ring finger domain (Chfr), and Aurora-A–interacting protein 1 (AIP), are involved in degradation of Aurora-A protein (49–51).

It is well known that overexpression of Aurora-A protein is frequently observed in various human cancers, and that aneuploidy, centrosome amplification, and tumorigenic transformation are induced by its overexpression in cultured human and rodent cells (3, 45, 52). Indeed, *Aurora-A* is mapped to chromosome 20q13.2, a region commonly amplified in human cancers (45, 52, 53). Therefore, Aurora-A overexpression is believed to be caused by gene amplification or transcriptional activation. However, a previous report illustrated that *Aurora-A* amplification was detected in only 3% of cases, but overexpression of Aurora-A mRNA and protein was observed in more than 60% of cases in hepatocellular carcinomas (54). Similar discrepancies between gene amplification and protein overexpression rates of Aurora-A are reported in other types of cancers, including head and neck, breast, gastric, and ovarian (4, 52, 55, 56). Interestingly, constitutive phosphorylation of Ser51 is observed in head and neck cancer cells with overexpression of Aurora-A protein. As Ser51 phosphorylation inhibits APC/C^{Cdh1}-mediated degradation, it is possible that constitutive phosphorylation on Ser51 may induce protein stabilization and its consequent accumulation in cancer cells that exhibit overexpression of Aurora-A protein (4). Importantly, Aurora-A overexpression is considered to promote tumorigenesis via disruption of maintenance of the normal centrosome or chromosome number (3, 57, 58).

DNA Replication and Pre-RC Formation

The ability of a eukaryotic cell to precisely and accurately replicate its DNA is crucial to maintain genome stability. Eukaryotic chromosomes need to be replicated by numerous replication forks that are initiated from replication origins spaced throughout the genome because of the sizes of the chromosomes. Therefore, eukaryotic cells are continually exposed to a risk of over-replication. As described previously, licensing is restricted to occur only once per cell cycle to prevent over-replication. Licensing is the assembly of the pre-RC on replication origins (13, 14). Pre-RC is composed of the origin recognition complex (ORC), cell division cycle 6 (Cdc6), CDT1, and the mini-chromosome maintenance (MCM) proteins (59). Cdc6 and CDT1 are loaded onto replication origins in an ORC-dependent manner during late M and early G_1 phase, after which they subsequently recruit MCM proteins to the origins. Pre-RC formation occurs from late mitosis to early G_1. The pre-RC is a protein complex composed of ORC, CDC6, CDT1, and MCM2–7, known as putative DNA helicase (13, 14). During late M and early G_1, CDC6 and CDT1 bind to replication origins and subsequently induce the recruitment of MCMs to the origins (13, 14). Pre-RC formation is needed for replication in the subsequent S phase. Therefore, it is necessary to prevent re-assembly of the pre-RC during S, G_2, and M phase. Two major inhibitory pathways exist to prevent pre-RC re-assembly, namely CDK1- and CDK2-based pathways. CDK1 inactivation during G_2 phase induces re-replication through re-assembly of MCMs (60). Consistently, silencing of cyclin A, but not cyclin B, causes re-replication in *Drosophila* cells (61). Taken together, CDKs suppress re-replication by preventing pre-RC re-assembly. To explain this phenomenon, multiple mechanisms are considered in S and G_2 phases. For example, CDT1 and ORC1 are phosphorylated by CDKs, resulting in their degradation in an SCF^{Skp2}-dependent manner (62–65). Additionally, CDKs phosphorylate CDC6 and induce its nuclear export in mammalian cells (66–68). Another pathway involves geminin, known as an inhibitor of DNA replication. Geminin functionally inhibits pre-RC re-assembly through direct binding to CDT1 during S, G_2, and M phases, which ensures genome stability and prevents aneuploidy (15). Indeed, ectopic overexpression of geminin suppresses pre-RC formation and subsequently blocks DNA replication (69). In addition, geminin knockdown in mammalian cells induces re-replication (70, 71), indicating that geminin has critical roles in the regulation of replication. Although it seemingly sounds contradictory, geminin stabilizes CDT1 protein during mitosis via preventing its ubiquitin-mediated proteolysis (69). Furthermore, the mitotic depletion of geminin induces CDT1 downregulation and prevents MCM loading in the ensuing G_1 phase (69, 72). Thereby, the negative and positive roles of geminin are essential for pre-RC formation, indicating that the protein level of geminin must be strictly controlled for proper DNA replication.

Involvement of Aurora-A in Pre-RC Formation

To ensure pre-RC assembly during late mitosis and early G_1 phase, cell cycle-dependent degradation of geminin is caused by the UPS (73). The geminin protein level oscillates during the cell cycle via APC/C-mediated ubiquitylation (69, 73) (**Figure 1**). Recent analyses at the single-cell level by time-lapse fluorescence microscopy analysis revealed that geminin degradation takes place following cyclin B degradation in late anaphase (74). Although Geminin is a substrate of APC/C, geminin is stable even in mitosis in spite of active APC/C. Indeed, geminin is phosphorylated by Aurora-A on Thr25 to prevent its APC/C-dependent proteolysis in mitosis (69) (**Figure 1**). Geminin contains the consensus sequences (R-X-S/T-L/V) recognized by Aurora-A as observed in amino acids 23–26 (*RRTL*) within the D-box of geminin (69). Interestingly, immunoprecipitation analysis revealed that both HA-tagged Cdh1 and HA-tagged Cdc20 interacted with wild-type geminin and Thr25 phospho-defective mutant ($geminin^{T25A}$) but not with Thr25 phospho-mimicking mutant ($geminin^{T25D}$), indicating that the inability of $geminin^{T25D}$ to interact with APC/C^{Cdh1} and APC/C^{Cdc20} may explain its resistance to APC/C-dependent proteolysis (69). In general, distinct substrates are specifically recognized by APC/C complex and are tightly degraded to adjust the critical timing (19). In fact, all of substrates of APC/C are not degraded at same time even though APC/C is active. Phosphorylation in CDC6, Aurora-A, and Skp2 as well as geminin protects from APC/C-mediated ubiquitylation

(4, 31, 32). In particular, phosphorylation in CDC6, Skp2, or geminin interferes with the binding of APC/C^{Cdh1} (31, 32, 69). We previously have shown that the phosphorylation of human Aurora-A on Ser51 interferes with its ubiquitylation by APC^{Cdh1}. Interestingly, constitutive phosphorylation on Ser51 is well correlated with protein overexpression and stabilization in cancer cells (4). As geminin is frequently overexpressed in certain types of human cancer (75, 76), it is interesting to examine if constitutive phosphorylation at Thr25 induces its protein overexpression in cancer. Importantly, stabilized geminin during mitosis ensures pre-RC formation via protecting CDT1 ubiquitylation by SCF^{Skp2} (69). Aurora-A–geminin–CDT1 axis regulates proper DNA replication (**Figure 2**).

Conclusion

Aurora-A is a well-known mitotic kinase that regulates mitotic entry, spindle formation, and chromosome maturation as a canonical role. In this review, we shed light on a novel function of Aurora-A for regulating DNA replication via proper formation of the pre-RC. Indeed, Aurora-A phosphorylates geminin to prevent APC/C-mediated proteolysis in mitosis. To ensure pre-RC formation, stabilized mitotic geminin protects CDT1 from SCF^{Skp2}-dependent proteolysis. This novel mechanism controlled by the Aurora-A–geminin–CDT1 axis is essential for proper regulation of DNA replication (**Figure 2**). Emi1 was identified as a factor inhibiting the function of APC/C^{Cdh1} and it is degraded by $SCF^{\beta Trcp}$ at early M phase (77–80). It was recently revealed that Emi1 silencing prevents the transition from S to G_2 phase by downregulating geminin via APC/C activation (81, 82). Therefore, the protein level of geminin is also regulated by the Emi1-mediated inhibition of APC/C^{Cdh1} activity. During cell cycle progression, strict regulation of the amount of geminin protein is essential for proper DNA replication. The protein level of geminin is strictly determined by Emi1- and Aurora-A-mediated protection from ubiquitylation by APC/C.

A series of periodic kinase reactions by CDKs promote cell cycle progression and the fidelity of cell division is dependent on the accumulation and ordered destruction of critical protein regulators (1). Thus, the UPS contributes to the precise regulation of the cell cycle. The UPS also contributes to the precise regulation of DNA replication via the Aurora-A–geminin–CDT1 axis (**Figure 2**). Interestingly, Aurora-A protein is also ubiquitylated by APC/C^{Cdh1}. It is well known that overexpression and/or hyperactivation of Aurora-A is involved in tumorigenesis via aneuploidy and genomic instability (3). Moreover, Aurora-A is frequently overexpressed in various cancers (3, 43, 52–54). As DNA replication is strictly regulated to prevent over-replication in mammalian cells, disruption of this mechanism may be involved in Aurora-A-mediated tumorigenesis. We suggest that deregulation of DNA replication via Aurora-A–geminin–CDT1 axis can be used as a potential diagnostic and therapeutic target in cancer.

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Unconventional functions of mitotic kinases in kidney tumorigenesis

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Human tumors exhibit a variety of genetic alterations, including point mutations, translocations, gene amplifications and deletions, as well as aneuploid chromosome numbers. For carcinomas, aneuploidy is associated with poor patient outcome for a large variety of tumor types, including breast, colon, and renal cell carcinoma. The Renal cell carcinoma (RCC) is a heterogeneous carcinoma consisting of different histologic types. The clear renal cell carcinoma (ccRCC) is the most common subtype and represents 85% of the RCC. Central to the biology of the ccRCC is the loss of function of the Von Hippel–Lindau gene, but is also associated with genetic instability that could be caused by abrogation of the cell cycle mitotic spindle checkpoint and may involve the Aurora kinases, which regulate centrosome maturation. Aneuploidy can also result from the loss of cell–cell adhesion and apical–basal cell polarity that also may be regulated by the mitotic kinases (polo-like kinase 1, casein kinase 2, doublecortin-like kinase 1, and Aurora kinases). In this review, we describe the "non-mitotic" unconventional functions of these kinases in renal tumorigenesis.

Keywords: mitotic kinases, non-mitotic functions, Aurora-A kinase, kidney, tumorigenesis

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INTRODUCTION

Renal cell carcinoma (RCC) represents approximately 3.8% of adult malignancies and 90–95% of kidney neoplasms. The most common histological RCC subtype is the clear cell carcinoma (ccRCC), which accounts for 85% of cases. At least 80% of ccRCCs have deletions or translocations involving the short-arm of chromosome 3, which contains the von Hippel–Lindau (VHL) gene at 3p25. Clear cell carcinoma shows highly consistent chromosomal aberrations involving loss of the short-arm of chromosome 3, a partial or complete trisomy 5q, and abnormalities of 6q, 8p, 9p, 10q, 11q, 12q, and 14q. The second most common cytogenetic abnormality associated with ccRCC is a gain of chromosome 5q6; however, very little is known about its effects. Mitotic errors and misregulation of cell-cycle process are considered to be an important characteristic of kidney cancer. Successful cancer therapies depend mainly on the recognition of physiologic targets that are primarily involved in the regulatory mechanism of cell-cycle progression (1). The members of serine/threonine kinases, such as cyclin-dependent kinases, polo-like kinases, and aurora kinases, are the well studied families that coordinate the mitosis sequence (2). Many studies are generally focused on the role of mitotic functions of these kinases and efforts have been put up to use targets for generation of new anti-cancer drugs (3). However other non-mitotic functions of these kinases have been identified as diverse as control of the resorption of the cilia, the cell differentiation, and the cell polarity control in interphase cells.

Kidney Cancer and (loss of) Cell Polarity

Carcinomas are frequently characterized by loss of cell differentiation and excess of cell proliferation. They are also characterized by a loss of cell polarity, which includes polarity complexes and adhesion complexes proteins dysfunction (4–6). Loss of cell polarity induces cancer development by deregulating different signaling pathways (7). Ultimate loss of the epithelial phenotype may contribute to epithelial-to-mesenchymal transition (EMT) and metastasis. Cell polarity is defined as asymmetry in functional organization of the cell. It is required for the formation and maintenance of functional epithelia. Epithelial cells are highly polarized (i.e., asymmetric distribution of lipids, proteins, RNA, organelles) and are tightly connected by specialized intercellular junctions. In epithelia, maintenance of apico-basal (AB) polarity is crucial for preserving epithelial integrity and depends on cell polarity complexes and cell junctions. Cell polarity is mostly driven by complexes composed of adherens junctional complexes (including cadherin and catenin) and tight junctional complexes that contain zonula occludens (ZO) proteins, occludin, claudin, and junctional adhesion molecules (JAM). These cell polarity complexes have antagonistic roles in regulating the specific distribution of key molecules. The Scribble complex (Scrib, Dlg, and Lgl2) is present in the baso-lateral region of the cell and has antagonistic roles with the apical complexes, Par (Par3, Par6, Cdc42, atypical protein kinase C) and Crumbs (Crb3, Pals1, and PatJ). Other conserved Par proteins such as Lkb1 (Par4) and the Par1 kinase regulate cell polarity (8).

In the kidney, the epithelium is a simple tube-based structure with the apical membrane facing the lumen whereas the basolateral membrane interacts with the matrix. In kidney tubes, as well as for podocytes, the establishment and maintenance of the epithelium require the presence of the Par, Crumbs and Scribble complexes (9). AB polarization of the renal epithelium is crucial for the appropriate function of the normal kidney in waste products and extra water removal and electrolyte balance. Disruption of cell polarity in kidney is involved in renal pathologies such as acute renal failure, which affects tight junction function (10) and polycystic kidney disease (PKD) in which polarity factors are frequently mislocalized (11). Polarity complexes expression and functions are frequently deregulated in cancer. Implication of polarity complex proteins as oncogene or tumor suppressor in tumorigenesis depends on the kind of alteration of their expression. It is also dependent on the origin of the epithelium (12). For instance, Par3 expression is lost in glioblastoma, esophageal squamous carcinoma, breast, lung, head, and neck cancers (13–16) but is overexpressed in some RCC (17) or in some severe hepatocellular carcinoma (18). Thus both up- and down-regulation of critical cell polarity proteins may be associated with tumorigenesis. Consistent with this notion, expression and functional studies in different skin tumors showed either oncogenic or tumor-suppressive functions of Par3 depending on the cellular context (19).

It has been widely admitted that ccRCC originates from proximal epithelial tubular cells. This is based on specific cellular marker expression (20, 21) or genetic expression pattern (22). However, epithelial cells of other nephron segments may also promote some cases of ccRCC. Interestingly, based on studies in VHL patients, ccRCC may also arise from VHL-null epithelial cells, which show markers of dedifferentiation (23). In colon adenocarcinoma, it has been reported that both expression levels and subcellular localization of the Scribble polarity complex component Dlg could be deregulated. However, in ccRCC, Dlg expression level is unaltered compared to normal kidney epithelial cells, but the protein is mislocalized in a granular distribution in clear cells. It was proposed that altered localization of Dlg may contribute to cell transformation and promotes high migration ability (24).

In ccRCC, the polarity protein Par3 overexpression is correlated with a poor prognosis (17). The localization of Par3 at both the plasma membrane and in the cytoplasm was associated with worse clinical factors in a cohort of 101 ccRCC patients (25). Furthermore, Dugay et al. showed that Par3 up-regulation was associated with cytoskeleton defects and increased cell migration capacity. This was restored by Par3 down-regulation whereas other Par complex component expression levels remained unchanged (17). Crumbs3 (Crb3) is involved in establishment of AB polarity and formation of tight junctions. Knock-out mice showed strong defects in epithelial AB polarity and thus in establishment and maintenance of epithelial cells. In contrast to Par3, loss of Crb3 expression induces tumorigenesis in kidney cells. This effect was associated with several characteristics of cell polarity disruption. This phenotype was reversed upon restoration of Crb3 expression (26, 27). Accordingly, loss of Crb3 is associated with a shorter overall survival in ccRCC (28). Altogether, polarity factors mislocalization and/or their deregulated expression are associated with tumor progression. This may be due to altered epithelia organization involving cell–cell contact disruption, a higher ability to transform phenotype (EMT) and for cell migration, and/or increased cell proliferation signaling.

Kidney Cancer and EMT

The EMT is known for more than a decade to participate in tumor progression and metastasis formation in many carcinomas. This process by which epithelial cells acquire a mesenchymal phenotype starts with loss of cell–cell adhesion and polarity and leads to increased cell motility and invasion. At the molecular level, EMT is characterized by the alteration of some gene expression profiles resulting in down-regulation of epithelial markers (such as E-cadherin, ZO-1, and cytokeratins), and up-regulation of mesenchymal markers, such as vimentin (29).

The implication of EMT in clear cell renal cell carcinoma (ccRCC) progression and invasiveness has been established within the last 5 years, especially when investigators were searching for new biomarkers associated with clinical outcome. While comparing mRNA levels of 46 EMT-related genes between RCC and healthy kidney samples, Chen and colleagues showed that low vimentin, CXCR4, fibronectin, and TWIST1 transcript levels are correlated with a better outcome whereas overexpressed vimentin and CXCR4 constitute independent markers for poor prognosis in RCC patients (30). Likewise, using an

immunohistochemical approach on a cohort of 122 RCC patients, protein expression levels of vimentin, TWIST, E-cadherin, and clusterin were identified as predictors of disease recurrence (31). Overexpression of zinc-finger transcription factors SNAI1/Snail or ZEB2/SIP1, well-known EMT inducers acting as repressors of E-cadherin transcription, has also been associated with poor prognosis in RCC patients (32, 33). It could result at least in part from the loss or a decreased expression of NDRG2 and FOXO3a observed in high grade or metastatic RCC tumors, respectively (34, 35). Several mechanisms have been proposed to explain how EMT is induced and/or sustained during kidney tumorigenesis.

As a consequence of the functional inactivation of VHL very often observed in ccRCC, accumulation of Hypoxia-induced transcription factors HIF1 and/or 2α leads to the transcriptional activation of many HIF target genes. Among them, erythropoietin (EPO) may stimulate EMT in RCC *via* the PI3K/Akt/mTOR pathway (36) in agreement with the observations that the mTOR inhibitor Everolimus was able to slow down RCC tumor growth and to reverse EMT phenotype in a mouse xenograft model (37). At least two proinflammatory cytokines, tumor necrosis factor alpha (TNFα) and interleukin 15 (IL-15), have been reported to play a role in EMT induction in RCC. Serum levels of TNFα are significantly increased in RCC patients as well as secretion by tumor cells and it was demonstrated that TNFα promotes EMT in RCC by decreasing E-cadherin expression and increasing vimentin expression and MMP9 activity (38, 39). This TNFα effect is mediated through the inhibition of GSK3β in a NFκB-independent manner (39, 40). In contrast, IL-15 production is not altered in RCC but the IL-15 signaling pathway is profoundly modified because of the expression of a particular transmembrane IL-15 form and the defective expression of CD132 (γc chain of the IL-2 receptor family) and JAK3 (41), which both favor EMT through down-regulation of E-cadherin expression (42).

Kidney Cancer and Angiogenesis

To proliferate, cancer cells require a continuous supply of nutrients and oxygen. This supply is function of the distance between tumor vessels and cancer cells, leading to intratumoral hypoxia heterogeneity (43). To overcome this phenomenon, angiogenesis is up-regulated in most cancers, due to an overproduction of angiogenic stimulators and the consequent unbalanced proportion of activators (such as VEGF, MMPs, FGF, HGF…) and inhibitors (such as thrombospondins, endostatin, angiostatin…) in favor of the hyperactive tumor vasculature development (44). Therefore, tumor vessels are disorganized, tortuous, and mal-shaped with fewer mural pericytes (45). Moreover, proliferating cancer cells can also exert a pressure on intratumoral blood and lymphatic vessels (46) leading to an impaired blood perfusion. These vascular abnormalities lead to a hypoxic, acidic, and hypertensive tumor microenvironment. Adaptation to hypoxia at the cellular level is mainly regulated by the Hypoxia Inducible Factors (HIFs).

In ccRCC, the HIF system is up-regulated both by micro-environmental hypoxia and a genetic event, the VHL inactivation, that lead to HIF-α stability. Indeed, the *VHL* gene is deleted, mutated or hypermethylated in ~90% of the cases, leading to the absence or to the expression of a non-functional pVHL protein (47). HIFs, the main targets of pVHL, are transcription factors responsible for numerous hypoxia responses by promoting expression of genes involved in the cellular adaptation to hypoxia. In normoxia, the oxygen-dependent prolyl hydroxylated domain containing proteins (PHDs) specifically hydroxylate HIF-α in its N-terminal transactivation domain (NTAD) (48, 49), allowing its interaction with pVHL, the substrate recognition subunit of an E3 ubiquitin ligase complex, and its subsequent degradation by the 26S proteasome (50). HIF-α can also be hydroxylated in an oxygen-dependent manner on its C-terminal transactivation domain (CTAD) by the Factor Inhibiting HIF (FIH-1). This hydroxylation prevents the recruitment of the transcriptional co-activators CBP and p300. Thus, under low oxygen conditions or in absence of a functional pVHL, HIF-α is stabilized and can dimerize with the stable HIF-β, and this heterodimer transcriptionally activates up to 200 genes involved in cell growth, glucose metabolism, angiogenesis, apoptosis, pH regulation… One of the most described targets of HIFs is the vascular endothelial growth factor (VEGF). It is overexpressed at the mRNA and protein levels in ccRCC compared to normal kidney tissues (51, 52). Endothelial cells but also RCC cells express the VEGF Receptor (VEGFR-2), inducing increased tumor angiogenesis. VEGF and its receptor constituted the main targets for metastatic RCC treatments such as Sunitinib, Sorafenib, Pazopanib, and Bevacizumab (53–55). However, most of the ccRCC patients develop resistance to these VEGF inhibitors. mTOR inhibitors (Temsirolimus and Everolimus) were also used for RCC treatment by acting downstream of the VEGF receptor through HIF down-regulation since mTORC1 drives HIF-1α synthesis (56).

The HIFα family is composed of three different members (HIF-1α, HIF-2α, and HIF-3α). HIF-1α is ubiquitously expressed whereas HIF-2α expression is more restricted, but both isoforms are co-expressed in numerous cell types. HIF-1α and HIF-2α have common and also unique targets and are thought to have overlapping functions, but also divergent outcomes in tumorigenesis. In fact, HIF-1α and HIF-2α were first considered as essential for ccRCC progression but several studies tend toward an oncogenic role for HIF-2α in ccRCC and a tumor suppressor function for HIF-1α (23, 57, 58). Moreover, most of the $VHL^{-/-}$ ccRCC cell lines do not express HIF-1α whereas they all express HIF-2α, suggesting a selective pressure to maintain HIF-2α expression.

Kidney Cancer and Ciliogenesis

Kidney epithelial cells have developed primary cilia that extend into the tubular lumen. This includes cells in the parietal layer of Bowman's capsule, proximal tubules, the loop of Henle, and the collecting duct. Cilia are present on almost all cells lining the nephron, with the exception of intercalated cells (59).

In the kidney, the cilium serves as a flow sensor in the kidney tubules lumen, with flow-induced ciliary bending causing a transient increase in intracellular calcium (60). Polycystins (PC) 1 and 2, gene products of PKD1 and PKD2, are large multi-pass transmembrane proteins of the transporter receptor potential channel (TRPC) family of calcium transporters. Mutations of PKD1 and PKD2 induce cyst formation in the kidney, and cyst

formation in part arises because of derestricted cell proliferation. Thus PC1 and PC2 at least indirectly participate in cell-cycle control. Signaling downstream of PC1 and PC2 is quite complex. In normal cells, these proteins negatively regulate the cAMP and Raf–MEK–ERK signaling pathways (61), both of which being hyper activated in renal epithelial cysts (62).

The primary cilium of the kidney epithelium mediates sensation of mechanical signals produced by apical fluid shear stress and its transduction into an intracellular Ca^{2+} signaling response (63, 64). In cell culture, deflection of the cilia axoneme initiates a transient increase in the level of intracellular Ca^{2+} resulting from Ca^{2+} entry through a channel, possibly PC1 and PC2, located in the cilium. This is supported by the fact that the Ca^{2+} influx generated by fluid flow is abolished in cell lines lacking polycystin-1 (65) and by the loss of fluid flow-mediated calcium signaling in the embryo.

Accordingly, defects in assembly or function of primary cilia lead to a plethora of developmental disorders and pathological conditions known as ciliopathies (66). Cystic kidney disorders are one of the leading causes of end-stage renal disease. Several proteins implicated in the pathogenesis of PKD localize to cilia. In the growth of the renal normal tubule, the mitotic spindle of dividing cells aligns along the axis of the nephron. However, in cells with mutations within *Pkhd1* (encoding fibrocystin) as well as *Hnf-1* genes, the spindle fails to correctly orient inducing abnormal cell division (67).

An important feature of ciliary signaling is the continuous interaction with regulatory signaling molecules at the ciliary base, i.e., the centrosomal region, which may coordinate the crosstalk between separate ciliary signaling pathways to activate specific cellular targets and gene arrays for specified cellular or tissue responses (67, 68).

The VHL disease is caused by germline mutation in the *vhl* tumor suppressor gene. One of the major clinical manifestations of the disease includes kidney tumor. Around 80% of the VHL patients developed renal cysts. pVHL localized to the cilia. Ectopic expression of VHL gene in renal clear cell carcinoma cell lines restored cilia formation, implying that pVHL might directly support ciliogenesis (69). Inactivation of VHL and GSK-3b was required to allow loss of cilia based on cooperative function of these proteins in ciliary maintenance. pVHL also regulates microtubule orientation during ciliogenesis and interacts with the Par3–Par6-atypical PKC complex, which supports ciliogenesis (70). Moreover, pVHL associates with kinesin 2, allowing pVHL to influence microtubule dynamics in support of cilia (71, 72).

Genetic Instability and Kidney Cancer

A large proportion of tumors are aneuploid and this abnormal number of chromosomes is thought to contribute to tumorigenesis. It is associated with poor prognosis, multidrug resistance, and increased capacity to metastasis. Genomic instability may be the result of several mitotic defects (73–75).

During mitosis, the formation of a bipolar mitotic spindle is essential for proper chromosome segregation. The chromosome segregation mediated by the anaphase-promoting complex/cyclosome (APC/C) is controlled by the spindle assembly checkpoint (SAC). This checkpoint arrests cell-cycle progression by modulating the activity of the mitotic kinase CDK1 until all chromosomes are properly attached to the mitotic spindle (76, 77). The chromosomal passenger complex (CPC), composed of Aurora-B, INCENP, Borealin, and Survivin, plays also a critical role at different stages of mitosis and cytokinesis by recruiting condensin complex in mitotic entry (78, 79) and by activating SAC in metaphase if chromosomes are not properly attached to the spindle. Its relocalisation from anaphase chromosomes to cell equator promotes mitotic exit and cytokinesis (80). Hence, deregulated CDK1 activation, weakened mitotic checkpoint, defective chromatid cohesion or condensation, may contribute to genomic instability. Indeed several alterations in mitotic genes have been reported in human cancer with chromosome instability (81). The duplication and maturation of centrosome is a critical step for the formation of a bipolar mitotic spindle. Thus, centrosome amplification, enrichment for centrosome components [such as CDK1, NEK2, Aurora-A, Aurora-B, or Polo-like kinase 1 (Plk1)] or merotelic attachment of chromosomes to the mitotic spindle may promote abnormal cell division and aneuploidy (77, 82, 83).

A recent study on VHL showed that this protein is a controller of mitotic fidelity *in vivo* (84). VHL is localized to the mitotic spindle. The loss of function of pVHL *in cellulo* and *in vivo* provokes spindle misorientation as a result of unstable astral MT and chromosome instability due to SAC impairment (85, 86). This aneuploidy phenotype in VHL-deficient renal carcinoma cell lines is suppressed by ectopic expression of Mad2, a regulator of APC/C (85) or inhibition of miR-28-5p, a key regulator of Mad2 protein translation upregulated in a variety of cancers (84, 87). This result suggests that low level of Mad2 is linked to chromosomal instability (CIN) in VHL-associated kidney cancers (86).

It has been observed that the VHL loss of function is not sufficient to explain tumor formation in kidney. Recently, Albers et al. have shown that secondary genetic alterations of p53 can cooperate with loss of pVHL to induce tumors in mice (88). Detailed cytogenetic analysis of tumor at different stages and gene expression analysis are necessary to understand tumor development and the molecular basis that contribute to aneuploidy.

Non-Mitotic Roles of Mitotic Kinases in Kidney Cancer

Polo-Like Kinase 1

Polo-like kinase 1 is the best characterized member of the Plks family (89). This serine/threonine kinase is known to regulate multiple stages of mitosis. Its expression is cell-cycle regulated since it increases from late S phase to mitosis, and its degradation mediated by APC/C starts in late mitosis. In interphase, Plk1 is expressed in the cytoplasm and at the centrosomes whereas in mitosis, it localizes to the centrosomes in prophase, then becomes enriched at kinetochores during prometaphase and metaphase, and in late mitosis a fraction of Plk1 is found at the spindle midzone (90). Plk1 is not only involved in the assembly of a bipolar spindle, centrosomes duplication and maturation, DNA replication, and DNA damage checkpoint, but also in the control of the G2/M transition. Several studies demonstrate its role not only in sister chromatid dissociation but also in mitotic exit and cytokinesis [reviewed in Ref. (91, 92)].

Since Plk1 is a key regulator of the cell division, it evidently appeared as an interesting target for anti-mitotic chemotherapeutic drugs (93). Small-molecule inhibitors of Plk1 activity have been developed. Several phase II trials were performed in solid tumors using for instance BI2536, a Plk1 inhibitor, but it exhibited a limited anti-tumoral activity, suggesting that more favorable pharmacological derivates are required (94). Despite these non-conclusive trials, Plk1 remains a promising target since it is overexpressed in a number of human tumors: esophageal squamous cell carcinoma (95), hepatocellular carcinoma (96), bladder carcinoma (97), thyroid carcinoma (98, 99), colorectal cancer (100), pancreatic cancer (101), prostate cancer (102), melanoma (103), breast cancer (104), and ovarian cancer (105). Its overexpression is often correlated with tumor grade, sometimes with metastatic disease (103), and proposed as prognosis factor. Interestingly, a gene expression profiling performed on ccRCC patient primary tumors identified Plk1 as significantly correlated with disease malignancy (106). Plk1 is overexpressed at the mRNA and protein level in RCC patient tissues (107) and this overexpression is correlated with the tumor grade and metastases. Moreover, Plk1 knock-down by siRNA strategy or small-molecule inhibitor decreased ccRCC cell proliferation *in vitro* by G2/M blockade (106) and invasion properties (107). To date, no clinical trial was performed using Plk1 inhibitors alone or in combination with other drugs in RCC patients. Intratumoral injections of Plk1 inhibitor in ccRCC xenograft nude mice induced a tumor volume decrease indicating that a sustained inhibition of Plk1 function may inhibit ccRCC tumor growth *in vivo* (106). Intriguingly, another *in vivo* study reported that a liposomal anti-Plk1 siRNA delivery system failed to inhibit tumor growth in a mouse xenograft RCC model (108).

Non-mitotic functions were recently attributed to Plk1, including a role in cilia disassembly. Indeed, an overexpression of Plk1 dramatically reduces the length and the percentage of primary cilia whereas the depletion of Plk1 or the inhibition of its kinase activity induces a delay in cilia disassembly (109). Even if Plk1 is not required for proper ciliogenesis (110), Lee and colleagues identified a new primary cilia disassembly pathway mediated by Wnt5a, CK1ϵ, Dvl2, and Plk1 (111). Under growth stimulation, the non-canonical ligand of the Wnt pathway, Wnt5a, enhances the Plk1 bound to Dvl-2, formerly phosphorylated by CK1ϵ. This interaction stabilizes HEF1 and the HEF1/AurA complex, leading to the HEF1/AurA dependent primary cilia disassembly. Another study has demonstrated that Plk1 is recruited to the pericentriolar matrix by PCM1, a centriolar satellite protein (109). This interaction requires prior phosphorylation of PCM1 by CDK1. Then, Plk1 is activated by Aurora-A and promotes primary cilia disassembly. Moreover, KIF2A, a member of the kinesin-13 protein family with only an ATP-dependent microtubule depolymerization activity, is phosphorylated by Plk1 at the level of subdistal appendages of the mother centriole (112). This event enhances MT depolymerization to disassemble primary cilia. KIF24, another kinesin-13 protein, was previously described to suppress inappropriate ciliogenesis in proliferating cells by stabilizing CP110 (113). In quiescent cells, KIF2A and KIF24 are both ubiquitinated by APC/C, which may prevent a premature initiation of cilia disassembly (112). Interestingly, this Plk1–KIF2A pathway was found constitutively active and described as involved in defective ciliogenesis in a ciliopathy named premature chromatid separation (PCS) syndrome (114). Of note, Plk1 was also associated to another ciliopathy, the nephronophthisis (NPH), a cystic kidney disease. Indeed, Plk1 colocalizes at the transition zone of the cilia with nephrocystin 1 (NPHP1), a scaffold protein of the NPH protein complex frequently mutated in NPH patients. Plk1 phosphorylates NPHP1, leading to cilia disassembly (115). The function of this phosphorylation and its link with cilia disassembly remain to be determined, but this study highlighted another signaling role for Plk1 in cilia disassembly at the transition zone. Further investigations are needed to study whether the non-mitotic function of Plk1 in cilia disassembly is involved in ccRCC development since this pathology is strikingly linked to cystic lesions and thus cilia defects.

Casein Kinase 2

Casein kinase 2 (CK2) is a ubiquitously expressed and much conserved serine/threonine protein kinase, which is composed of two catalytic α- (or α′-) and two regulatory β-subunits. This constitutively active kinase is involved in cell proliferation and survival; it exerts pleiotropic effects throughout cell-cycle progression and notably during mitosis, when CK2α is transiently hyperphosphorylated (116). During the G2-M transition, it first plays a role in chromatin condensation by phosphorylating DNA topoisomerase 2α (117). It also participates in the activation of M-phase promoting factor, CDK1-cyclin B, both by phosphorylating/activating the phosphatase CDC25B and by facilitating PLK1-mediated Wee1 inhibition (118, 119). CK2 is then located on the mitotic apparatus in a Pin-1-dependent manner where it shares with Plk1 the microtubule plus-end-tracking protein CLIP170 as a substrate, the phosphorylation of which regulates the timely microtubule-kinetochore attachment and contributes to proper chromosome alignment at metaphase (120). Together with the Aurora-B mitotic kinase, CK2 is later involved in spindle elongation and chromosome segregation during anaphase in yeast and interestingly, loss of CK2 activity has been shown to activate SAC (121).

Casein kinase 2 has been shown to be overexpressed, and its activity increased, in ccRCC compared to healthy kidney (122), as reported in many other cancer types. Such deregulation of CK2 activity during mitosis may be sufficient to cause mitotic chromosome instability and the ensuing aneuploidy that have been established to promote tumorigenesis. There is however evidence from several reports regarding solid tumors to suggest that overexpression of CK2 could also influence kidney tumor development through non-mitotic mechanisms. First, CK2 could exacerbate angiogenesis in hypoxic renal tumors since the potent and selective small molecule CK2 inhibitor CX-4945 (also called silmitasertib) has been shown to exert anti-tumor activity through inhibition of angiogenesis in breast cancer (123). This proangiogenic effect of CK2 has been recently discussed [see for review in Ref. (124)]; it is likely the result of several possible actions of the kinase in the signaling cascade leading to hypoxia-mediated transcription of HIF target genes (see **Figure 1** for details).

Proangiogenic effect of overexpressed CK2 could additionally be mediated by local overproduction, by tumoral and peripheral blood mononuclear cells, of prostaglandin E2 (PGE2), the most predominant and biologically active eicosanoid produced

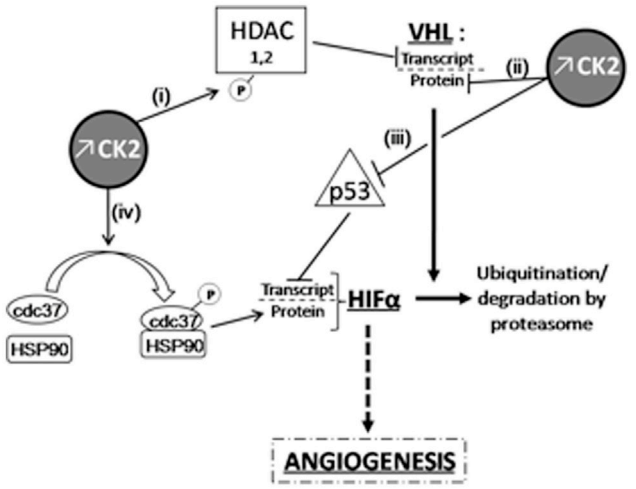

FIGURE 1 | Overexpressed CK2 induces HIFα accumulation through VHL-dependent or independent mechanisms. CK2 can down-regulate VHL expression at the transcriptional level (i) by phosphorylating HDACs (125) as well as at post-translational level and (ii) by destabilizing phosphorylation in the NH_2-terminal acidic domain of VHL (126). In the latter case, CK2-induced destabilization of VHL also results in p53 inactivation, which can no longer inhibit HIFα transcription (iii); (126, 127). Finally, (iv) cdc37 phosphorylation by CK2 allows HSP90/cdc37 dimer formation and the subsequent interaction of this complex with HIF-1α, which is essential for its cellular stability (128, 129).

by cyclooxygenase 2 (COX-2); this enzyme is indeed often upregulated in ccRCC, likely through a CK2-induced Wnt/β-catenin pathway and its expression is correlated with prognosis [see for review in Ref. (130)].

Besides, overexpression of COX2 is also associated with tumor cell migration and invasion as well as the PGE2 receptor EP4, the expression of which is strongly correlated with ccRCC tumor stage and aggressiveness and the presence of metastases (130) but there is to date no such available data connecting CK2 expression levels and tumor progression. A few recent studies on other solid tumors (lung and colon) nevertheless suggest that CK2 could also be involved in the EMT: CK2α is overexpressed in colorectal cancer compared to adenoma or healthy colorectal tissue and its silencing by RNA interference, while slowing down tumor cell proliferation also inhibited cell motility and invasion. At the molecular level, CK2α knock-down was able to reverse the EMT process by decreasing vimentin expression and upregulating E-cadherin (131). Similar observations were reported in A549 lung adenocarcinoma cells in which CK2 inhibition with CX-4945 strongly decreased cell migration and invasion at least through the attenuation of PI3K/AKT and ERK signaling pathways and blocked TGF-β-induced EMT (132, 133). Further investigation is worth pursuing in ccRCC to reveal such implication of overexpressed CK2 in EMT process and in the regulation of tumoral cell migration and invasion.

Doublecortin-Like Kinase 1

Doublecortin-Like Kinase 1 (DCLK1) was first isolated in *Caenorhabditis elegans* as the product of the zyg-8 gene. Zyg-8 loss-of-function alleles were isolated in a visual screen for mutants deficient in spindle positioning in the *C. elegans* one-cell stage embryo. The phenotype was due to shortening of astral MT during anaphase leading to a defect in spindle-cortex interaction. ZYG-8 is a microtubule-associated protein (MAP), which promotes MT stabilization. ZYG-8 encodes a protein kinase that harbors a catalytic kinase domain and a domain similar to human Doublecortin (DCX), a MAP, which stimulates MT polymerization. Both domains are required for its function in *C. elegans* where ZYG-8 localizes to MT. ZYG-8 kinase also binds to MT *in vitro* and *in vivo* through its DCX domain in mammalian cells. ZYG-8 function is required to maintain spindle architecture during anaphase and influences spindle positioning and thus the proper progression of mitosis (134). Later, it was shown that DCLK is a MT-associated kinase, which regulates spindle formation in neuronal cells. DCLK overexpression induced large monopolar spindles and a prometaphase arrest in mitotic neuronal cells. On the other hand, DCLK silencing perturbed mitotic spindle organization with shorter and thinner MT also promoting prometaphase arrest (135). Thus, in metazoans, DCLK1 has a conserved mitotic function in regulating spindle formation.

Doublecortin-Like Kinase 1 was identified as a marker of tumor stem cells (TSC) for instance in pancreatic and colorectal cancers (136) and is upregulated in many other solid tumors. It has been recently suggested that DCLK1 may constitute a potential relevant diagnostic and prognostic marker of circulating cancer cells [e.g., pancreatic adenocarcinoma (137)]. RCC cells share many characteristics with TSC, including an EMT phenotype. Interestingly, DCLK1 is epigenetically dysregulated and overexpressed in more than 90% of RCC tumors. Increased expression of DCLK1 correlates with stages II and III tumors. Expression of DCLK1 was correlated with the EMT phenotype in RCC. Consistently, silencing of DCLK1 in renal Caki-2 cell line promoted EMT-specific transcription factors down-regulation (SNAI1/SNAI2, TWIST1, and ZEB1) and reduced migration and invasion capacities. A decreased adhesive phenotype was also observed and correlated with a decrease in the expression of the focal adhesion regulator PTK2 (FAK). DCLK1 may regulate migration and invasion through the maintenance of focal adhesion (138). Whether MT and F-actin binding of DCLK1 is involved in its adhesion function remains to be investigated. Interestingly, it has been recently suggested that phosphorylation of FAK by the sphingosine kinase-1 may promote renal cell invasion (139). Thus DCLK1 may contribute to the metastatic process and targeting this kinase should be considered as part of an anti-cancer therapy (140).

Aurora-A Kinase

Aurora-A is a centrosomally localized cell-cycle regulatory serine/threonine kinase that activates the cyclin B1-Cdk1 mitotic kinase and coordinates formation of a bipolar spindle and nuclear envelope breakdown in M phase. The serine/threonine kinase Aurora-A localizes on duplicated centrosomes from the end of S phase to the beginning of the following G1 and is essential for mitotic entry, centrosome duplication, spindle formation, chromosome segregation, and cytokinesis (141). Aurora-A is activated by phosphorylation from the end of S phase until the next G1 when

the kinase is ubiquitinated and degraded by the proteasome in a Cdh1-dependent manner (142, 143). The major roles of Aurora-A kinase have been widely described in the centrosome separation and spindle formation. Aurora-A is activated mainly by Ajuba (144); TPX2, Bora, as well as protein phosphatase inhibitor-2 (145, 146) and the focal scaffolding proteins HEF1 and NEDD9 (72). Phosphorylation of Aurora-A on T288 residue within the activation loop of the catalytic domain results in the activation of the protein. Aurora-A has multiple other phosphorylation sites modulating its mitotic and non-mitotic activities (S51, S53/S54, S66/S67, S89, S98, and S342 residues) (147). The relevance of the different phosphorylation sites is not actually well known.

The human *Aurora-A* gene resides at chromosome 20q13.2, a region that is commonly amplified in primary breast tumors, colorectal cancers, and other cancer cell lines, including breast, ovarian, colon, prostate, neuroblastoma, and cervical cell lines (148). Aurora-A abnormalities have been reported in a variety of malignant tumors correlated with an *Aurora-A* gene amplification or upregulation of Aurora-A expression in tumor tissues compared with normal tissues (149). Controversial studies suggested that Aurora-A abnormalities were positively correlated with aggressive tumor behavior invasion, and nodal metastasis, but some studies showed no correlation or an inverse correlation (150). The oncogenic effects of Aurora-A was attributed to its interaction with several important cellular proteins, including protein phosphatase 1, target protein for xklp2, HEF1, p53, CENP-A, Ajuba, and transforming acidic coiled-coil (141).

Even though the expression of the kinase from S to M phase and the localization of Aurora-A onto the centrosome and the spindle pole have been widely described, it becomes more and more evident that Aurora-A is present in all phases of the cell cycle and might fully participate in other functions that mitotic ones. Several recent studies have described non-mitotic functions of Aurora-A in cellular calcium signaling, cilia resorption or cytoskeleton organization. The presence of diffuse Aurora-A staining in the cytosol, the Golgi and perinuclear region hinted that it had a possible role unrelated to mitosis (151).

Previous studies have shown a potential role of Aurora-A in metastasis and that Aurora-A ectopic expression induced a robust increase in cell migration through its effect on tubulin polymerization (147, 152). The mechanism by which the kinase is involved in the process of mobility, migration, and invasion is not completely defined but it has been suggested a role of RAS, AKT, RALa, and MAPKs (153). Aurora-A has been shown to be implicated in cell migration along with SRC and FAK and, unexpectedly, is regulated by a lipase, phospholipase D2 (PLD2). Phosphatidic acid is able to bind and activate Aurora-A causing rapid tubulin polymerization and leading to an enhanced cell migration (154). The action of Aurora-A on cell mobility has also been investigated through a group of proteins involved in the focal adhesion as NEDD9, SRC and FAK. The effect of Aurora-A on cell migration is augmented in the presence of SRC and, in return, Aurora-A also activates FAK. Cell migration is also physically mediated by actin cytoskeleton and is initiated by the protrusion of the cell membrane (155). Overexpression of Aurora-A regulates actin reorganization, leading to free barbed end formation. Cofilin has emerged as an essential player for the localized formation of the barbed ends, which act as sites for new local actin polymerization, thus determining the direction of cell protrusion and movement (156). A significant correlation between Aurora-A expression and cofilin dephosphorylation was described in the immunohistochemical analysis of clinical breast cancer specimens, supporting a novel signaling mechanism by which Aurora-A indirectly induced cofilin dephosphorylation and actin reorganization, thus promoting mammary cell movement and breast cancer metastasis (157). Most importantly, Aurora-A was demonstrated to enhance EMT and invasiveness *via* activation of MAPK signaling pathway. A novel oncogenic cross-talk between Raf/MAPK and Aurora-A signaling pathways has been established in the development of EMT, stemness, and tumor progression in ERα breast cancer cells. The constitutive activation of MAPK signaling pathway during tumor growth leads to the stabilization and accumulation of Aurora-A which induces the EMT (158). Aurora-A inhibition by VX-680 induced a significant suppression of cell invasion ability, as well as reversed its EMT behavior by reducing membrane expression of epithelial markers E-cadherin and β-catenin in cervical CN2 cells (152, 159).

Recent studies have shown more diverse, non-mitotic functions of Aurora-A orchestrating remodeling of the microtubular cytoskeleton during neurite extension (160) but also regulating protrusion and resorption of cellular cilia participating in cellular calcium signaling (161, 162). Aurora was shown to localize at the basal body of the cilium and its activation was shown to participate to the ciliary resorption by promoting histone deacetylase-dependent tubulin depolymerization of the ciliary axoneme (161).

The serum growth factors were also shown to induce Aurora-A activation at the basal body of the cell cilium in non-cycling G0/G1 cells causing Aurora-A and NEDD9-dependent cilia resorption (161). HEF1/Cas-L/NEDD9 is a component of focal adhesions that has a prominent role in inducing metastasis and that co-localizes with Aurora-A at the centrosome, thereby enhancing the effect of Aurora-A on the resorption of the cilium. After cilia resorption, Aurora-A ceases to be active (as judged by kinase activity) and will probably be reactivated for mitotic function as soon as the cell enters the cell cycle. By microinjecting active Aurora-A into cells, the cilium disappeared, leading to the conclusion that active Aurora-A is necessary and sufficient to induce cilium resorption (163). Loss of cilia associated with high level Aurora-A expression would indirectly impact the functionality of the cilia-dependent and cancer-relevant signaling cascades, such as those involving Hedgehog (164).

Pathological conditions of the kidney include renal cell carcinoma, in which elevated Aurora-A expression has (often) been reported (165) as well as its partner NEDD9 (72, 166, 167). Aurora-A pathway is induced through HIF (hypoxia-inducible factor-1) in ccRCC cells and has significant impact on two relevant features of *VHL*-defective cells: the suppression of primary cilia that *in vivo* can lead to premalignant cysts and the increased motility that can lead to metastasis (168). However, the underlying mechanism by which VHL loss increases Aurora-A levels has not been clearly elucidated, although it has been suggested that HIF-1α mediates increased Aurora-A expression in VHL-null cells. Dere et al suggested that Aurora-A expression

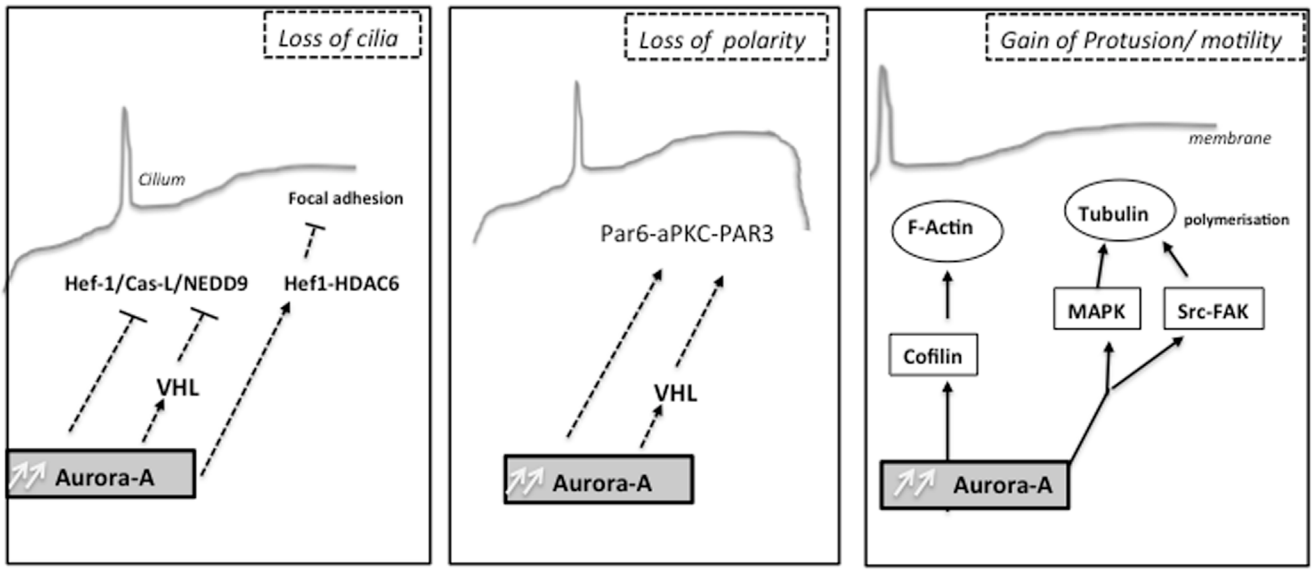

FIGURE 2 | Non-mitotic functions of the kinase Aurora-A: different biological processes as cilia resorption, cell polarity or cell migration are influenced by Aurora-A upstream mitosis, which can later affect chromosome segregation.

is driven by β-catenin transcription in the VHL null cells and that the level of Aurora-A was not modified by Hif1-α (169).

Clear cell renal cell carcinoma is also characterized by VHL inactivation and more recent data indicate that VHL interacts with primary cilia in renal epithelial cells. A hallmark of ccRCC is loss of the primary cilium. Loss of this key organelle in ccRCC is caused by loss of VHL and associated with increased Aurora-A and histone deacetylase 6 (HDAC6) activities, which drive disassembly of the primary cilium. Aurora-A is typically described as solely localized to the centrosome or centrosomally derived ciliary basal body and otherwise hard to detect in non-cycling normal mammalian cells. Evidence that HEF1/Aurora-A/HDAC6 signaling axis governs the resorption of cilia in addition to the previously defined roles for these proteins suggests a novel molecular mechanism to explain some cancer-associated ciliary loss. The focal adhesion protein HEF1 initially interacts with Aurora-A in G2 prior to the kinase activation. As the cell progresses throughout the cell cycle, focal adhesion disassembly releases a pool of HEF1, which promotes Aurora-A activation. Overexpression of HEF1 and Aurora-A promotes cytokinetic failure, and contributes to genomic instability. The protein association may interfere with a normal cellular interconversion between cilia and centrosome and contributes to cell cycle by staging critical signaling complexes that govern emergence from quiescence to S phase, and initiation of M phase.

Interestingly, formation of renal cysts is very strongly linked to defects in planar cell polarity control (170, 171). Aurora-A has also been found to directly phosphorylate Par-6, which together with atypical PKC and Par-3 regulate asymmetric cell division and cell polarity (172) and the changes in Ca^{2+} signaling induced by autosomal-dominant PKD (ADPKD)-associated mutations in the *PKD1* and *PKD2* genes (173–175). Low concentrations of drugs that inhibit Aurora-A activity raise basal intracellular Ca^{2+} levels in renal cells and PC2-dependent Ca^{2+} release. It has been also demonstrated that Aurora-A directly binds and phosphorylates PC2, and consequently may provide a mechanism by which Aurora-A inhibition limits PC2 Ca^{2+} channel activity. Moreover, the release of Ca^{2+} from the ER to the cytoplasm transiently activated Aurora-A, based on induced direct Ca^{2+}-calmodulin (CaM) binding to Aurora-A. The non-mitotic activities of Aurora-A likely contribute to deregulation of growth in tumor cells overexpressing Aurora-A (**Figure 2**).

CONCLUSION

Clear cell renal cell carcinoma is the predominant type of kidney cancer. ccRCC develops in the renal proximal tube and is linked to biallelic inactivation of the VHL tumor suppressor gene. Mitotic kinases defects mostly lead to aneuploid tumors and the sustained overexpression and activity of various mitotic kinases, including Aurora-A, Polo-like (Plk1), CK2, DCLK in diverse human tumors strongly indicate that these entities are intimately involved in the development of errors in chromosome segregation. Nevertheless, the non-mitotic functions of these kinases involved in the process of ciliogenesis, hypoxia, the EMT as well as the cell polarity likely play an important role in the process of tumorigenesis in kidney cancer as they also lead to genetic instability.

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REVIEW
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Aurora Kinase Inhibitors: Current Status and Outlook

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The Aurora kinase family comprises of cell cycle-regulated serine/threonine kinases important for mitosis. Their activity and protein expression are cell cycle regulated, peaking during mitosis to orchestrate important mitotic processes including centrosome maturation, chromosome alignment, chromosome segregation, and cytokinesis. In humans, the Aurora kinase family consists of three members; Aurora-A, Aurora-B, and Aurora-C, which each share a conserved C-terminal catalytic domain but differ in their sub-cellular localization, substrate specificity, and function during mitosis. In addition, Aurora-A and Aurora-B have been found to be overexpressed in a wide variety of human tumors. These observations led to a number of programs among academic and pharmaceutical organizations to discovering small molecule Aurora kinase inhibitors as anti-cancer drugs. This review will summarize the known Aurora kinase inhibitors currently in the clinic, and discuss the current and future directions.

Keywords: aurora kinase, small molecules, hematologic diseases, kinase inhibitors, neuroblastoma

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INTRODUCTION

The Aurora kinases are a family of highly conserved serine/threonine kinases that are important for faithful transition through mitosis (1–3). The gene for *Aurora-A*, maps to chromosome region 20q13.2, a region that has been found amplified in different human cancers. Aurora-A plays an important role in centrosome maturation, spindle assembly, meiotic maturation, and metaphase I spindle orientation [**Figure 1** (2)]. Aurora-A function is regulated by degradation, phosphorylation, and dephosphorylation, with its kinase activity dependent upon phosphorylation of threonine 288 (Thr288) in the activation loop (2). Selective inhibition of Aurora-A results in inhibition of autophosphorylation of Aurora-A at Thr288, monopolar spindles, and G2-M arrest (4, 5). The *Aurora-B* gene maps to chromosome region 17p13.1 (1, 6) and this kinase forms part of the chromosomal passenger complex (CPC) with three non-enzymatic subunits: inner centromere protein (INCENP), Survivin, and Borealin [**Figure 1** (7)]. The highly dynamic CPC is critical for chromosome condensation, chromosome orientation on the mitotic spindle, through correcting chromosome-microtubule attachment errors, and the spindle-assembly checkpoint (SAC), as well as the final stages of cytokinesis (8–11). Aurora-C expression has been reported in testis, thyroid, and placenta and in meiotically dividing gametes (2, 12–15). Recently, it was demonstrated that overexpression of Aurora-C induces abnormal cell division resulting in centrosome amplification and multinucleation in cells. NIH3T3 mouse fibroblasts overexpressing Aurora-C induced tumor formation in nude mice, demonstrating its oncogenic activity (16).

Aurora-A was first described in human cancer cell lines but has subsequently been found to be overexpressed in a broad range of human tumors, including primary colorectal carcinoma, gliomas,

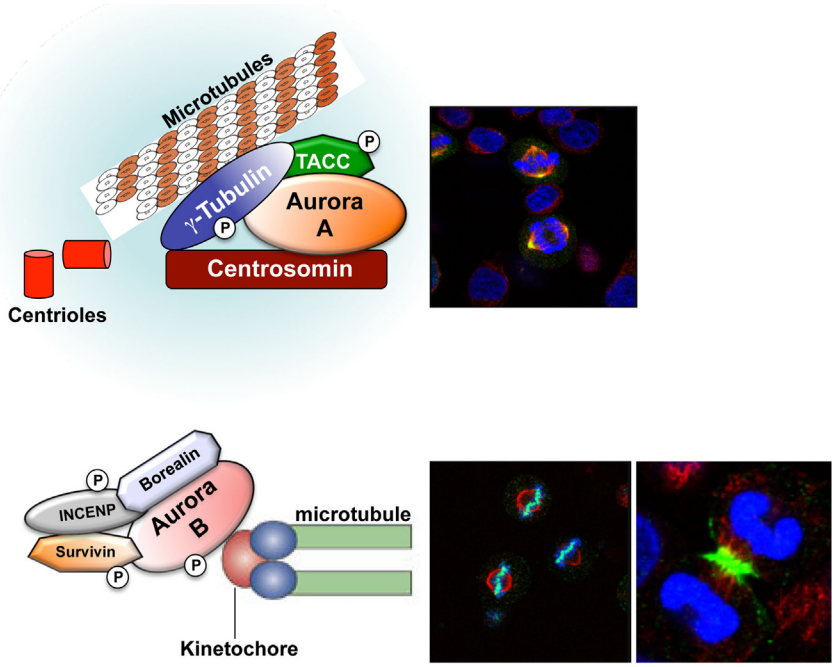


FIGURE 1 | Aurora-A and Aurora-B localization. Aurora-A (yellow) localizes at the centrosome during interphase and at the mitotic poles and the adjacent spindle microtubules during mitosis. Aurora-B (green) localizes at the chromosomes in prophase, the centromere in prometaphase and metaphase, and the central mitotic spindle in anaphase.

breast, ovarian, and pancreatic cancers (1, 17–20). Aurora-B is also overexpressed in human tumors such as gliomas, thyroid carcinoma, seminoma, and colon cancer (21–23).

The overexpression of the Aurora kinases and their association with genetic instability and aneuploidy in tumors suggests that a wide range of cancers could respond therapeutically to inhibitors of the Aurora kinases (24). Over the past decade, many pharmaceutical companies and academic institutions have reported the development of Aurora kinase inhibitors. Both Aurora-A and Aurora-B inhibitors induce cell death. However, they induce apoptosis through distinct mechanisms **Figures 2A,B** (25). Aurora-A inhibition induces defects in mitotic spindle assembly, which causes a transient spindle checkpoint-dependent mitotic arrest. This cell cycle arrest is not maintained, and subsequently, Aurora-A inhibited cells exit from mitosis leading to apoptosis, either by induction of a G1 arrest, followed by apoptosis, or by a p53-independent mechanism (25). In contrast, inhibition of Aurora-B also interferes with normal chromosome alignment during mitosis and overrides the mitotic spindle checkpoint causing polyploidy, failure of cytokinesis and endoreduplication followed by cell death at time more than 48 h (4, 5, 25). In a completely different direction, mechanistic studies have shown that Aurora-A inhibition also leads to MYCN degradation. The MYC family member MYCN, which is associated with amplification in the childhood tumor neuroblastoma, is stabilized by Aurora-A in a kinase-independent fashion, but involves a direct protein–protein interaction (26, 27). In this interaction, Aurora-A functions to sequester MYCN away from ubiquitin-mediated proteolytic degradation.

In this review, we will first present inhibitors of Aurora kinase family that have been recently assessed in clinical studies, with a focus on hematologic malignancies. Subsequently, we will discuss in more detail the areas in which inhibitors of Aurora kinases have shown greater promise, and we will highlight recent advances in the understanding of Aurora kinases biology that could be exploited with small-molecule modulators, bringing greater benefit to patients.

INHIBITORS OF AURORA KINASES IN CLINICAL TRIALS

Barasertib (AZD1152)

Barasertib (AZD1152, **Table 1**) is a phosphate-based prodrug that is rapidly converted into barasertib-hQPA (**Table 1**) *in vivo* (28, 29). Barasertib-hQPA selectively inhibits the Aurora-B kinase (Aurora B-INCENP $K_i < 0.001$ μM) over Aurora-A $K_i = 1.4$ μM (28). In clinical studies, barasertib has been evaluated in patients with solid malignant tumors (30), advanced solid tumors (31), and hematologic cancers (32–35). The pharmacokinetic profiles of barasertib and barasertib-hQPA were investigated in a small phase I study in patients with newly diagnosed, relapsed or refractory acute myeloid leukemia AML (29), and in this study, one patient achieved a complete response. In a phase 1/2 study to investigate the safety, efficacy, and pharmacokinetics of barasertib in patients with advanced AML, barasertib was administered as a continuous 7-day infusion every 21 days and the MTD was established as 1200 mg (32). In part B of this study, 32 patients were

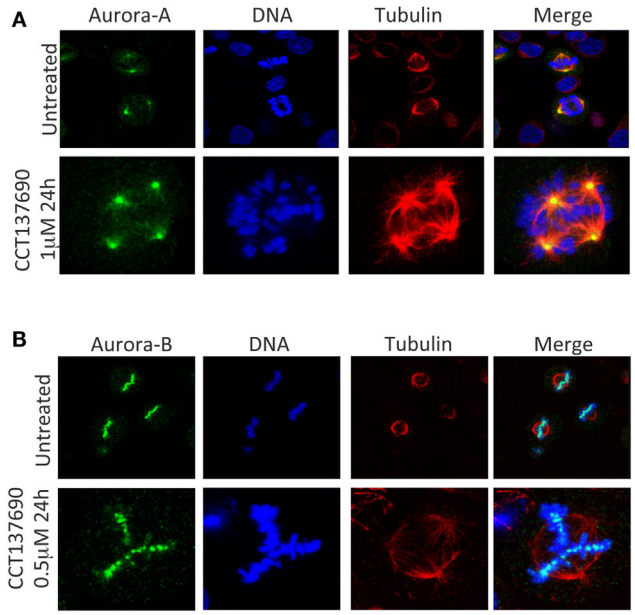
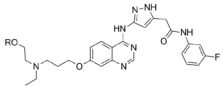
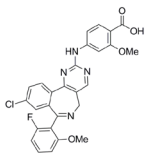
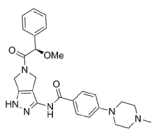
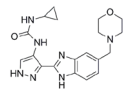
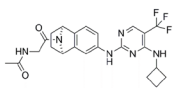
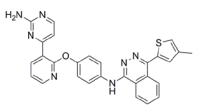


FIGURE 2 | Aurora-A and Aurora-B inhibition induces mitotic aberrations. **(A)** Aurora-A inhibition induces temporarily a mitotic arrest, cells exit from mitosis with multipolar and monopolar spindle formation leading to apoptosis. **(B)** Aurora-B inhibition induces endoredublication, chromosome misalignments, and polyploidy.

TABLE 1 | Aurora kinase inhibitors in clinical trials.

Compound	Structure	Aurora inhibition	Reference
R = Barasertib (AZD1152) R = H, Barasertib-hQPA		Aurora-A *Ki* = 1.4 μM Aurora-B *Ki* <0.001 μM; for barasertib-hQPA	(28–35)
Alisertib (MLN8237)		Aurora-A IC_{50} = 1.2 nM Aurora-B IC_{50} = 396.5 nM	(36–47)
Danusertib (PHA-739358)		Aurora-A IC_{50} = 13 nM Aurora-B IC_{50} = 79 nM	(48–56)
AT9283		Aurora-A: 52% inhibition at 3 nM Aurora-B: 58% inhibition at 3 nM	(57–64)
PF-03814735		Aurora-A IC_{50} = 5 nM Aurora-B IC_{50} = 0.8 nM	(65, 66)
AMG 900		Aurora-A IC_{50} = 5 nM Aurora-B IC_{50} = 4 nM	(67–70)

treated with barasertib at 1200 mg. The most frequently observed grade ≥3 adverse events were febrile neutropenia and stomatitis/mucosal inflammation (32). In this investigation, treatment with barasertib resulted in an overall hematologic response rate of 25% with a manageable toxicity profile (32). A phase I study to investigate the safety, pharmacokinetics, and efficacy of barasertib in advanced AML was also undertaken in Japanese patients (33). Patients received barasertib as a continuous 7-day i.v. infusion every 21 days with no dose-limiting toxicities reported at doses up to and including 1200 mg, a dose that had been established as the MTD in a study by Lowenberg et al. (32). Febrile neutropenia, neutropenia, leukopenia, and thrombocytopenia were reported as the most frequent grade ≥3 adverse events (33), and in this investigation, an overall hematologic response rate of 19% was in line to response rates observed by Lowenberg et al. (32).

The safety and tolerability of barasertib has also been assessed in combination with low-dose cytosine arabinoside (LDAC) in elderly patients with newly diagnosed AML, considered unfit for intensive induction chemotherapy (34). In this study, patients received barasertib (7-day continuous intravenous infusion from day 1 to 7) and LDAC 20 mg (subcutaneously twice daily from day 1 to 10) of 28-day treatment cycles. This combination showed acceptable tolerability at doses up to and including 1000 mg of barasertib, which was established as the MTD; the most common grade ≥3 adverse event was febrile neutropenia. In this investigation, the combination of barasertib and LDAC resulted in an overall response rate of 45% (34).

The efficacy, safety and tolerability of barasertib *versus* LDAC in elderly patients with AML were also investigated in a randomized Phase II study (35). In this study, patients were randomized 2:1 to be administered either barasertib 1200 mg (as a 7-day continuous intravenous infusion) or LDAC 20 mg (subcutaneously twice daily for 10 days) in 28-days cycles. A higher objective complete response rate was reported with barasertib treatment: 35.4% compared with 11.5% observed with the LDAC treatment. The median overall survival for patients who received the barasertib treatment was longer relative to that observed with the LDAC treatment (35). Barasertib had a more toxic safety profile compared with that of LDAC treatment, the most commonly observed adverse events were stomatitis (71% in barasertib group versus 15% in LDAC group) and febrile neutropenia [67% in barasertib group versus 19% in LDAC group; (35)].

Alisertib (MLN8237)

Alisertib (MLN8237; **Table 1**) is a selective inhibitor of the Aurora-A kinase, with an IC_{50} against Aurora-A of 1.2 and 396.5 nM against Aurora-B (36, 37). Alisertib has been extensively characterized using *in vitro* and *in vivo* preclinical models. It displays antiproliferative activity in a wide range of human tumor cell lines including lung, prostate, ovarian, and lymphoma cells (36). In relation to pediatric cancers, MLN8237 was active against neuroblastoma and Ewing sarcoma cell lines and efficacious in neuroblastoma and acute lymphoblastic leukemia (ALL)

xenograft models (38). *In vitro*, alisertib also disrupts the viability of AML cell lines and primary AML cells (39) and significantly increases the *in vitro* and *in vivo* efficacy of nilotinib (40).

Given its broad spectrum of activity in preclinical models, alisertib has been evaluated in Phase I and II clinical trials in patients with advanced solid tumors (41–43), in children with refractory/recurrent solid tumors (44) and in hematologic malignancies. In a Phase I study of MLN8237 in relapsed/refractory multiple myeloma, Non-Hodgkin lymphoma, and chronic lymphocytic leukemia, patients received alisertib orally as either powder-in-capsule (PIC) or enteric-coated tablet (ECT) formulation (45). The most commonly reported grade ≥3 adverse events were neutropenia, thrombocytopenia, anemia, and leukopenia. In this study, partial responses were observed in 6 patients (13%), and 13 patients (28%) had stable disease for 1.9–11 months (45). The recommended phase II dose of alisertib (ECT formulation) was 50 mg twice daily for 7 days in 21-day cycles [i.e., a 7-day treatment followed by a 14-day recovery period; (45)].

Alisertib was evaluated in a Phase II study in patients with relapsed and refractory aggressive B- and T-Cell Non-Hodgkin lymphomas, and in this investigation, alisertib was administered orally at 50 mg, twice daily for 7 days in 21-day cycles (46). The most frequently observed grade ≥3 adverse events were neutropenia, leukopenia, anemia, and thrombocytopenia. In this study, the overall response rate for all treated patients was 27% (46). In an exploratory Phase II study, alisertib was assessed in AML and myelodysplastic syndrome [MDS; (47)]. In this investigation, alisertib was administered at 50 mg twice daily for 7 days in 21-day cycles and shown a modest single-agent antileukemic activity. In AML patients, a 17% overall response rate was observed with an additional seventeen patients (49%) having stable disease (47), but no responses were reported in MDS patients. The most commonly observed grade ≥3 adverse events were febrile neutropenia, anemia, thrombocytopenia, neutropenia, and fatigue (47).

Danusertib (PHA-739358)

Danusertib (PHA-739358, **Table 1**) is a potent inhibitor of all three Aurora kinase isoforms (Aurora-A $IC_{50} = 13$ nM, Aurora-B $IC_{50} = 79$ nM, Aurora-C $IC_{50} = 61$ nM) (48, 49). In addition to the Aurora kinases, PHA-739358 has inhibitory activity against a number of other kinases with relevance as anticancer targets such as ABL, RET, and TRK-A (48, 49). PHA-739358 inhibits both the wild-type and mutant ABL kinase isoforms with clinical relevance, in particular ABL (T315I) which is one of the most common mutations found in imatinib-resistant patients (50).

In the clinical setting, danusertib has been assessed in patients with advanced or metastatic solid tumors (51, 52). In these Phase I and II studies, danusertib was generally well tolerated, neutropenia being one of the most commonly observed hematologic toxicities, but showed only marginal antitumor activity in patients with common advanced solid tumors who had failed systemic therapy (52). In addition, danusertib showed only minimal efficacy when it was assessed in a randomized Phase II study in patients with metastatic castration-resistant prostate cancer after docetaxel failure (53). In another Phase I study, danusertib was administered as 24-h infusion every 14 days with and without granulocyte colony-stimulating factor (G-CSF) and assessed in patients with advanced solid tumors (54). PHA-739358 has also been investigated in hematologic malignancies, including a phase II study in patients with chronic myeloid leukemia (CML) relapsing on imatinib or other targeted therapies (55). In this study, two complete hematologic responses were reported in patients that both carrying the T315I BCR–ABL mutation. Patients received danusertib by a once-weekly 6-h infusion, which was reportedly well tolerated (55). Danusertib was also assessed in phase I study in adult patients with accelerated or blastic phase CML and philadelphia chromosome-positive ALL resistant or intolerant to imatinib and/or other second generation ABL kinase inhibitors (56). In this investigation, danusertib was administered by 3-h IV infusion, daily for either 7 consecutive days in a 14-day cycle (schedule A) or 14 consecutive days in a 21-day cycle (schedule B). The most frequently reported grade 3–4 adverse events were anemia, diarrhea, and febrile neutropenia. Four (20%) of the twenty evaluable patients responded to treatment, all on schedule A treatment and carrying the T315I BCR-ABL mutation (56). Overall in this study, danusertib has shown an acceptable toxicity profile and also promising activity in patients with advanced hematologic malignancies resistant to imatinib and/or other second generation ABL kinase inhibitors (56).

AT9283

AT9283 (**Table 1**) is a small-molecule multitargeted kinase inhibitor with potent Aurora kinase activity [Aurora-A: 52% inhibition at 3 nM; Aurora-B: 58% inhibition at 3 nM; (57)]. Other kinases inhibited by AT9283, with relevance as anticancer targets include JAK2, FLT-3, and ABL(T315I) (57). AT9283 showed potent antiproliferative activity against imatinib-resistant BCR-ABL^+ cells including those carrying the ABL(T315I) mutation (58). AT9283 displayed potent inhibitory activity against a range of human solid tumor cell lines (59), and antiproliferative activity against a panel of human aggressive B-(non-Hodgkin lymphoma) B-NHL cell lines (60).

AT9283 was assessed in Phase I clinical trials in patients with advanced solid malignancies (61), advanced malignancies [administered as a weekly 24-h infusion; (62)] and in children and adolescents with solid tumors (63). A Phase I and pharmacodynamic study of AT9283 in patients with relapsed/refractory leukemia or myelofibrosis was also reported (64). In the initial part of this study, AT9283 was administered as a 72-h continuous infusion every 21 days; and in the second part of the investigation, infusion duration was increased sequentially (in 24-h increments) to 96 and 120 h. The MTD for a 72-h infusion was established as 108 $mg/m^2/d$, and dose-limiting toxicities included myocardial infarction, hypertension, cardiomyopathy, tumor lysis syndrome, and pneumonia (64). In approximately one-third of patients with relapsed/refractory AML, bone marrow blasts decreased by at least 38% after treatment. However, these reductions in blasts were transient and no objective responses were achieved (64).

PF-03814735

PF-03814735 (**Table 1**) is a potent, orally bioavailable inhibitor of both Aurora-A and Aurora-B kinases (Aurora-A $IC_{50} = 5$ nM, Aurora-B $IC_{50} = 0.8$ nM); and also inhibits several other kinases

(e.g., FLT3, JAK2, TrkB, RET, MST3) by ≥90% at a compound concentration of 100 nM (65). PF-03814735 exhibited antiproliferative activity against a range of human tumor cell lines such as HCT-116, HL-60, A549, and H125 (65). In a Phase I clinical study, PF-03814735 was assessed in patients with advanced solid tumors (66). PF-03814735 was administered orally, once daily, as a single agent dosing at days 1–5 or 1–10 of 21-day cycles. Dose limiting toxicities included febrile neutropenia and increased levels of aspartate amino transferase. It was generally well tolerated with a clinically manageable adverse events profile but limited antitumor activity was reported with 19 patients achieving stable disease (66).

AMG 900

AMG 900 (**Table 1**) is an orally bioavailable, potent and selective pan-Aurora kinase inhibitor (67–70). It inhibits Aurora-A, -B, and -C with IC_{50} values of 5, 4, and 1 nM respectively, and in cells shows a phenotype consistent with Aurora-B inhibition (67). AMG 900 displayed potent antiproliferative activity against a range of human tumor cells including cells lines resistant to paclitaxel and the Aurora kinase inhibitors AZD1152, MK-0457, and PHA739358 (67). Notably, AMG 900 was consistently potent against tumor cells irrespective of P-gp or BCRP status (67). Consistent with the *in vitro* findings, AMG 900 inhibited the growth of multiple human tumor xenograft models [e.g., MDA-MB-231, HCT116, NCI-H460-PTX (MDR), MES-SA, MES-SA-Dx5 (MDR)] *in vivo*, using either intermittent or continuous dosing schedules (67). The activity of AMG 900 as a single agent and in combination with paclitaxel or ixabepilone in multidrug-resistant TNBC (triple negative breast cancer) cell lines was also investigated (70). AMG 900 potently inhibited the growth of P-gp-expressing TNBC cell lines. In combination with paclitaxel or ixabepilone, AMG 900 enhanced the antiproliferative activity of these microtubule-targeting agents in TNBC cells *in vitro* and in human tumor xenograft models *in vivo* (70). AMG 900 is currently being evaluated in Phase I clinical trials in adult patients with advanced solid and hematologic cancers (68).

Aurora Kinase Inhibitors in Hematologic Malignancies

The most profound explanation for the limited response of the Aurora kinase inhibitor's in solid tumors in a clinical setting is possibly the need for drug exposures through a number of cell cycles (for the Aurora-B and pan Aurora inhibitors) or for a prolonged time in mitosis (for the Aurora-A inhibitors), to induce their maximum effect in tumor cells before severe toxic effects such as neutropenia appear. The clinical evaluation of Aurora kinase inhibitors, as discussed earlier, indicated toxicities that are consistent with the mechanism of action for this class of compounds. In clinical setting, efficacy against solid tumors is limited, but overall, Aurora kinase inhibitors showed greater promise against hematologic malignancies. The observed significant response in patients with hematologic cancers may be associated with their proliferation rate as well as with secondary pharmacology exhibited by some of these inhibitors which is related to the disease (**Figure 3A**).

AML is a heterogeneous class of leukemia, with prognosis predicted by a number of genetic and molecular abnormalities. Mutations of the fms-like tyrosine kinase 3 (*FLT3*) gene are one of the best characterized genetic alterations, which is frequently mutated in AML. These mutations can consist of internal tandem duplication (ITD) of the juxtamembrane domain coding region, or point mutations of the tyrosine kinase domain (TKD). Both FLT3-ITD and FLT3-TKD mutations result in ligand-independent proliferation and are associated with a poor prognosis in adults and children (71).

AZD1152 was the first selective Aurora-B inhibitor that was evaluated in pre-clinical and clinical studies. AZD1152 has demonstrated impressive efficacy in animal models using human tumor xenografts of AML cell lines with wild type and mutant FLT3 (72–74). Aurora-B inhibition was demonstrated, using phosphorylation of histone H3 (a known substrate of Aurora-B), as a biomarker for target engagement *in vitro* and *in vivo*. Polyploidy was the predominant phenotype associated with Aurora-B inhibition due to cytokinesis failure and endoreduplication. It has been also shown that a secondary target of AZD1152 is FLT3, resulting in higher sensitivity of FLT3-mutated cell lines MV4-11 and MOLM-13 to AZD1152 inhibition, compared to FLT3-WT cell lines (75). Based on these data, AZD1152 entered a number of clinical trials in patients with solid tumors or hematologic malignancies, including AML, as described previously in this review.

Although FLT3 kinase has attracted a great interest in recent years as a target for AML treatment, the clinical impact of early FLT3 inhibitors has been limited when used as single agents, due to acquired resistance (76). Newer FLT3 inhibitors with improved selectivity, pharmacokinetic and pharmacodynamic properties may have improved single-agent efficacy (77), but clinical resistance, including acquired secondary mutations in the FLT3-TKD, is emerging. However, FLT3 inhibitors exhibiting secondary kinase inhibition pharmacology showed promise in overcoming this resistance. For example, it was demonstrated that a human FLT3-ITD+ AML cell line harboring a secondary D835Y mutation, has high relative resistance to the FLT3 inhibitors AC220, MLN518, and Sorafenib, but not to CCT137690, a dual FLT3-Aurora kinase inhibitor (78). CCT241736, an advanced analog of CCT137690, is a preclinical development candidate for the treatment of human malignancies, and in particular AML in adults and children (79). CCT241736 is an orally bioavailable dual FLT3/Aurora kinase inhibitor that also inhibits clinically relevant FLT3-resistant mutants including FLT3-ITD and FLT3 [D835Y; (79)]. CCT241736 significantly inhibited the growth of MV4-11 human FLT3-ITD positive AML tumor xenografts *in vivo*, with biomarker modulation and free drug exposure consistent with dual FLT3 and Aurora kinase target inhibition (79).

Aurora Inhibitors in Neuroblastoma

Neuroblastoma is the most common extra-cranial solid tumor of childhood, accounting for approximately 10% of pediatric tumors, which affects more than ten thousand children worldwide each year. Stage 4 neuroblastoma represents approximately 50% of cases with metastatic dissemination at diagnosis and its prognosis is poor. Therefore, novel therapeutic strategies are urgently needed to improve the prognosis of neuroblastoma

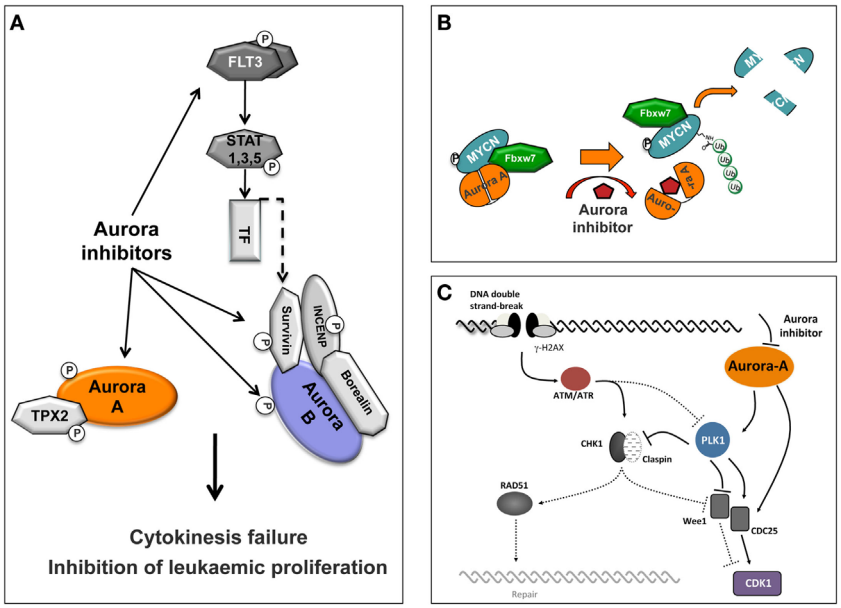


FIGURE 3 | Schematic representation of Aurora inhibitor-targeted pathways. **(A)** Selective inhibition of Aurora-A or Aurora-B or dual specificity Aurora/FLT3 compounds induces cytokinesis failure and inhibition of leukemic proliferation. **(B)** Conformational changes of Aurora-A by a compound induce Aurora-A-FBXW7-MYCN complex disassembly and proteolytic degradation of MYCN. **(C)** Aurora-A is involved in DNA repair pathways and Aurora-A inhibitors confer increased cellular sensitivity to cell death.

patients. Amplification of the MYCN gene is associated with an aggressive form of neuroblastoma that results in a particularly poor clinical outcome (80). Knockout of MYCN protein by targeting with siRNA, or alternatively, destabilizing the protein using an inhibitor of the upstream PI3K signaling pathway, has been shown as an effective preclinical therapy for neuroblastoma (81, 82).

Initially, a study using the pan Aurora inhibitor CCT137690 showed that treatment of MYCN-amplified neuroblastoma cell lines inhibits cell proliferation and decreases MYCN protein expression (83). Importantly, in a transgenic mouse model of neuroblastoma (TH-MYCN) that overexpresses MYCN protein and is predisposed to spontaneous neuroblastoma formation, this compound significantly inhibits tumor growth (83). Later, an additional mechanistic study shown that Aurora-A forms a complex with MYCN in MYCN-amplified neuroblastoma cells, which protects MYCN from proteasomal degradation in mitosis (26). This activity was specific for Aurora-A, since neither Aurora-B inhibition nor depletion affected MYCN protein levels. Interestingly, stabilization of MYCN does not require the catalytic activity of Aurora-A (26). Furthermore, MYCN destabilization was not due to cell cycle arrest in G2/M due to inhibition of the catalytic activity of Aurora-A. However, crystallographic evidence showed that the Aurora-A-specific inhibitor MLN8054 (84) induces a DFG-up conformation, disrupting the Aurora-A/MYCN complex leading to MYCN degradation. The conclusions from this study were that disruption of the Aurora-A/MYCN complex promotes degradation of MYCN, mediated by the FBXW7 ubiquitin ligase. Therefore, inhibition of the Aurora kinases may be an effective strategy to treat MYCN-amplified neuroblastoma. In a completely different approach, a recent study provided evidence for an Aurora-A conformation-specific effect on proteolytic degradation of MYCN. CD532, an ATP-mimetic ligand, binds Aurora-A in a DFG-in, inactive conformation, which results in blocking both the kinase-dependent and independent functions of Aurora-A (27). Importantly, CD532 inhibits Aurora-A at low nanomolar concentrations and, in parallel, effects the proteolytic degradation of MYCN proposing an additional strategy to block MYCN in cancer (**Figure 3B**).

Aurora Inhibitors and DNA Damage

A great effort has been focused on investigating different approaches to enhance the effect of Aurora kinase inhibitors in preclinical models and in clinical trials, including investigating the role of Aurora-A in DNA-damage response (DDR). It is known that Aurora-A activity is tightly regulated during the response to genotoxic agents and is important for a normal DDR (85, 86). There is an intricate connection between the DDR and the cell cycle at multiple levels. Once the homologous recombination (HR) machinery is fully active, the cell cycle is normally stalled by the activation of the DNA damage checkpoints. For the G2/M DNA damage checkpoint, the cell cycle arrest is mostly achieved by the regulation of CDC25 phosphatases and WEE1, either by the checkpoint kinases CHK1 and CHK2, or by the ATM/ATR kinases-dependent phosphorylation of

PLK1 (**Figure 3C**). These two pathways converge to maintain an efficient inhibition of CDK1 and hence prevent cell cycle progression. Finally, during recovery from the DNA damage checkpoint, the signal emanating from the mitotic kinase PLK1 becomes dominant and stimulates cell cycle progression. Interestingly, during this late phase of the DDR, but also during unperturbed cell cycle, Aurora-A has been identified as the upstream activator of PLK1 (87, 88).

A recent study has shown that Aurora-A modulates the repair of DNA double-strand breaks [DSBs; (89)]. Aurora-A expression inhibits RAD51 recruitment to DNA DSBs, decreases DSB repair by HR and sensitizes cancer cells to PARP inhibition (89). This impairment of RAD51 function requires inhibition of CHK1 by PLK1. These results identify a novel function of Aurora-A in modulating the response to DNA DSB that likely contributes to carcinogenesis and suggest a novel therapeutic approach to the treatment of cancers overexpressing this protein. The connection of DDR with Aurora kinases triggered a number of trials combining radiotherapy with Aurora kinase inhibitors. AZD1152, the Aurora-B kinase inhibitor, was shown to enhance the effect of ionizing radiation (IR) in three different settings: neoadjuvant (AZD1152 before IR), adjuvant (IR before AZD1152), or concomitant treatments [AZD1152 plus one single IR dose; (90)]. A more pronounced tumor growth delay was observed in the neoadjuvant and adjuvant schedules as compared to the concomitant schedule. However, AZD1152 enhanced the efficacy of IR when concomitant IR was fractionated over several days. Histopathological examination revealed that AZD1152 + IR induced polyploidy, multinucleation, and micronuclei *in vivo*. Caspase inhibition or removal of the pro-apoptotic protein BAX did not ameliorate the long-term cell survival of AZD1152-treated cancer cells. In contrast, a chemical inhibitor of CHK1, CHIR124, sensitized cancer cells to the lethal effect of AZD1152, supporting the contention that AZD1152 mediates radiosensitization *in vivo* by enhancing mitotic catastrophe (90). More recent studies using the Aurora-A selective inhibitor MLN8237 showed the effect of MLN8237 with and without temozolomide or IR, on the proliferation of glioblastoma tumor stem-like cells. It was reported that Aurora-A inhibition by MLN8237 was synergistic with temozolomide and potentiated the effects of IR on colony formation in neurosphere glioblastoma tumor stem-like cells, supporting the potential of Aurora-A inhibitors as primary chemotherapy agents or biologic response modifiers in glioblastoma patients (91).

CONCLUSION AND FUTURE DIRECTIONS

Aurora kinase inhibitors were initially aimed to target solid tumors including ovarian, breast, lung and colon. Despite the wide use of different chemical classes of Aurora kinase inhibitors in clinical trials, a limited efficacy against solid tumors was observed. The most plausible explanation for this result may relate to the proliferation rate of cells in solid tumors being relatively slow. By the time that an Aurora kinase inhibitor had an effect through several cell cycles and mitoses in tumors, the faster proliferating bone marrow cells were severely affected. A break in treatment, to allow the patient to recover, enabled the tumors to continue growing. Regarding compound preclinical evaluation, it appears that for cell cycle inhibitors, particularly selective mitotic inhibitors, the existing pre-clinical efficacy models in which these compounds were evaluated are not predictive of the efficacy outcome in clinical trials. The disappointing outcome against solid tumors in the clinical setting led to a change in strategy with Aurora kinase inhibitors being evaluated in clinical trials against hematologic malignancies due to their higher homogeneity and higher proliferation rates relative to solid tumors. Higher response rates against hematologic malignancies were observed, in particular when the Aurora inhibitor exhibited a secondary anticancer pharmacology; for example, the inhibition of another oncogenic driver of hematologic cancers such as activated FLT3 in AML. In conclusion, simultaneous inhibition of an activated oncogene driver and Aurora kinases using compounds with dual pharmacology or selective Aurora inhibitors in combination with a selective oncogene driver inhibitor may be a strategy to achieve a significantly improved clinical outcome, and also overcome resistance.

AUTHOR NOTE

Spiros Linardopoulos joined The Institute of Cancer Research (ICR) in September 2000 and hold an appointment as Team Leader in both the Breakthrough Breast Cancer Research Centre and the Cancer Research UK, Cancer Therapeutics Unit. Prior to the ICR, he worked at Onyx Pharmaceuticals, Richmond, CA, USA (1997–2000) as a Scientist. Before his move to the USA, he was awarded a Marie Curie Research Fellowship from the European Community as a postdoctoral researcher at the Beatson Institute for Cancer Research, Glasgow, UK (1993–1997). He pursued his graduate studies at the National Hellenic Research Foundation and obtained his PhD in Cellular and Molecular Biology from the University of Athens, Greece (1989–1993).

AUTHOR CONTRIBUTIONS

Dr. VB wrote the "Inhibitors of Aurora kinases in clinical trials" part of the Review. Dr. SL wrote the introduction, the use of Aurora kinase inhibitors in different human cancers, and the conclusions and future direction.

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A Cell Biologist's Field Guide to Aurora Kinase Inhibitors

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Aurora kinases are essential for cell division and are frequently misregulated in human cancers. Based on their potential as cancer therapeutics, a plethora of small molecule Aurora kinase inhibitors have been developed, with a subset having been adopted as tools in cell biology. Here, we fill a gap in the characterization of Aurora kinase inhibitors by using biochemical and cell-based assays to systematically profile a panel of 10 commercially available compounds with reported selectivity for Aurora A (MLN8054, MLN8237, MK-5108, MK-8745, Genentech Aurora Inhibitor 1), Aurora B (Hesperadin, ZM447439, AZD1152-HQPA, GSK1070916), or Aurora A/B (VX-680). We quantify the *in vitro* effect of each inhibitor on the activity of Aurora A alone, as well as Aurora A and Aurora B bound to fragments of their activators, TPX2 and INCENP, respectively. We also report kinome profiling results for a subset of these compounds to highlight potential off-target effects. In a cellular context, we demonstrate that immunofluorescence-based detection of LATS2 and histone H3 phospho-epitopes provides a facile and reliable means to assess potency and specificity of Aurora A versus Aurora B inhibition, and that G2 duration measured in a live imaging assay is a specific readout of Aurora A activity. Our analysis also highlights variation between HeLa, U2OS, and hTERT-RPE1 cells that impacts selective Aurora A inhibition. For Aurora B, all four tested compounds exhibit excellent selectivity and do not significantly inhibit Aurora A at effective doses. For Aurora A, MK-5108 and MK-8745 are significantly more selective than the commonly used inhibitors MLN8054 and MLN8237. A crystal structure of an Aurora A/MK-5108 complex that we determined suggests the chemical basis for this higher specificity. Taken together, our quantitative biochemical and cell-based analyses indicate that AZD1152-HQPA and MK-8745 are the best current tools for selectively inhibiting Aurora B and Aurora A, respectively. However, MK-8745 is not nearly as ideal as AZD1152-HQPA in that it requires high concentrations to achieve full inhibition in a cellular context, indicating a need for more potent Aurora A-selective inhibitors. We conclude with a set of "good practice" guidelines for the use of Aurora inhibitors in cell biology experiments.

Keywords: Aurora kinase inhibitors, AZD1152, ZM447439, Hesperadin, MLN8237, MLN8054, MK-5108, MK-8745

INTRODUCTION

Aurora kinases were discovered in the mid-nineties in *Drosophila* and yeast (1, 2). Whereas yeasts only have one Aurora kinase, metazoans generally have two, named Aurora A and B. Mammals, but not other vertebrates, also have a third family member, Aurora C. Aurora A localizes to centrosomes and spindle microtubules and plays important roles in centrosome maturation, controlling spindle length and bipolarity, asymmetric cell division, and promoting mitotic entry both in unperturbed cells and following DNA damage (3, 4). Aurora B localizes to chromosomes/inner centromeres and the spindle midzone and is implicated in many processes including chromosome condensation, chromosome biorientation on the spindle, and cytokinesis (5–7). Aurora C is expressed in testis (8), where it exhibits tissue-specific functions (9, 10), and in oocytes, where it contributes to early embryonic divisions by providing functions associated with Aurora B in somatic cells (11–14). In addition, Aurora C is aberrantly expressed in cancer cells (15).

Due to their closely related kinase domains (72% identity for the human proteins), Aurora A and B exhibit similar protein substrate preferences *in vitro* (16–19). *In vivo*, their distinct substrate specificities, localization patterns, and functions arise from interactions with specialized binding partners (3, 4). Aurora B is largely found as part of the four-subunit chromosomal passenger complex (CPC) (5–7) whose three other members – INCENP, survivin, and borealin – localize the kinase to the centromere and the anaphase spindle. INCENP also activates Aurora B via a two-step mechanism (20–22). The IN box at the INCENP C-terminus first wraps around the N-terminal lobe of Aurora B, stimulating autophosphorylation of the activation loop residue Thr 232 (23). This event allows Aurora B to phosphorylate serines in the TSS motif adjacent to the IN box, which generates a feedforward loop by further augmenting INCENP's ability to bind and activate Aurora B.

Aurora A has multiple regulators, with the best-studied one being TPX2, which activates the kinase and targets it to spindle microtubules (24–26). Structural studies have shown that the TPX2 N-terminus binds the N-terminal lobe of Aurora A, in a manner distinct from how the INCENP IN box binds Aurora B, facilitating the alignment of residues essential for substrate binding and catalysis (27–29). In biochemical assays, binding of the TPX2 N-terminus increases autophosphorylation of the activation loop residue Thr 288 (28, 30, 31). As in the case of Aurora B, phosphorylation of this threonine [which readily occurs *in vitro* even in the absence of TPX2 or other activators (16)] promotes high levels of kinase activity (16, 28, 32). However, recent studies have unexpectedly revealed that this autophosphorylation event is not essential for TPX2 stimulation of Aurora A kinase activity; fully dephosphorylated Aurora A bound to TPX2 exhibits robust enzymatic activity (28, 32). The relative contributions of TPX2 binding and Thr 288 phosphorylation to different cellular Aurora A functions is an active area of investigation.

Coincident with the delineation of their cellular roles, the Aurora kinases were also found to be amplified/overexpressed in cancer (33, 34). Functional studies of Aurora A revealed a potential role in tumor initiation and growth – increased expression of Aurora A transformed rodent fibroblasts (albeit weakly) and promoted their ability to form tumors *in vivo* (35, 36). In addition, elevated Aurora A activity was shown to confer resistance to taxol-mediated apoptosis in cancer cells (37). The Aurora kinases therefore emerged as attractive drug targets in cancer and became the focus of intense drug discovery efforts (38–41).

At least 30 Aurora kinase inhibitors have been evaluated preclinically or clinically as potential oncology therapeutics (38). The development of these inhibitors has typically involved high throughput biochemical assays using purified proteins, structure-based drug design, cellular biomarker assays (primarily Aurora A Thr 288 phosphorylation and Aurora B-mediated phosphorylation of its canonical substrate, histone H3), cellular proliferation/cytotoxicity assays, and xenograft models in mice (39). The products of the vast majority of these programs have been compounds that potently inhibit all three Aurora kinases (A, B and C), as best exemplified by the first clinically tested Aurora kinase inhibitor, the Vertex/Merck pyrazolo-pyrimidine compound VX-680 (MK-0457, tozasertib; **Figure 1**) (42, 43). However, compounds that exhibit preference for Aurora A or B/C have also been developed. In 2003, two pioneering academic-industrial collaborations described two distinct Aurora B inhibitors: the indolinone Hesperadin and the quinazoline ZM447439 [**Figure 1**; (44, 45)]. The latter compound was further optimized to produce the structurally related pro-drug AZD1152 (barasertib); barasertib is metabolized to the active form AZD1152-HQPA, which lacks the phosphate group present on AZD1152 and is the form typically used in biochemical and cell-based studies (**Figure 1**) (46, 47). In 2007, Millenium (now Takeda) described the first Aurora A-selective inhibitor, the benzazepine MLN8054 (48–51), which, due to central nervous system side effects (52, 53), was replaced as the lead clinical candidate by the derivative MLN8237 (alisertib; **Figure 1**) (49, 54, 55). In parallel, optimization of the VX-680 scaffold by Merck/Banyu/Vertex resulted in the Aurora A-selective inhibitors MK-5108 (VX-689) (56) and MK-8745 (57, 58) (**Figure 1**). More recently, other structurally unrelated Aurora A- and B-selective inhibitors have been described, such as the bisanilinopyrimidine inhibitor Genentech Aurora Inhibitor 1 (optimized to target Aurora A) (59) and the azaindole-based GSK1070916 (optimized to target Aurora B/C) (60–62) (**Figure 1**).

While these compounds were developed with a primary emphasis on therapeutic benefit, they were rapidly adopted by academic investigators as chemical tools for biochemical, structural, and cell biological studies (63). Application of these small molecules has complemented genetic knockdown and immunodepletion approaches because their inhibitory effects exhibit high penetrance/rapid onset and can be readily reversed. Their use has been wide ranging and influential, resulting in a large body of work defining Aurora kinase cellular functions, identifying potential substrates, and elucidating molecular mechanisms of kinase activation (63).

Despite the common use of several Aurora inhibitors by the cell biology community, a systematic comparison of these compounds in quantitative *in vitro* and cellular assays has been lacking.

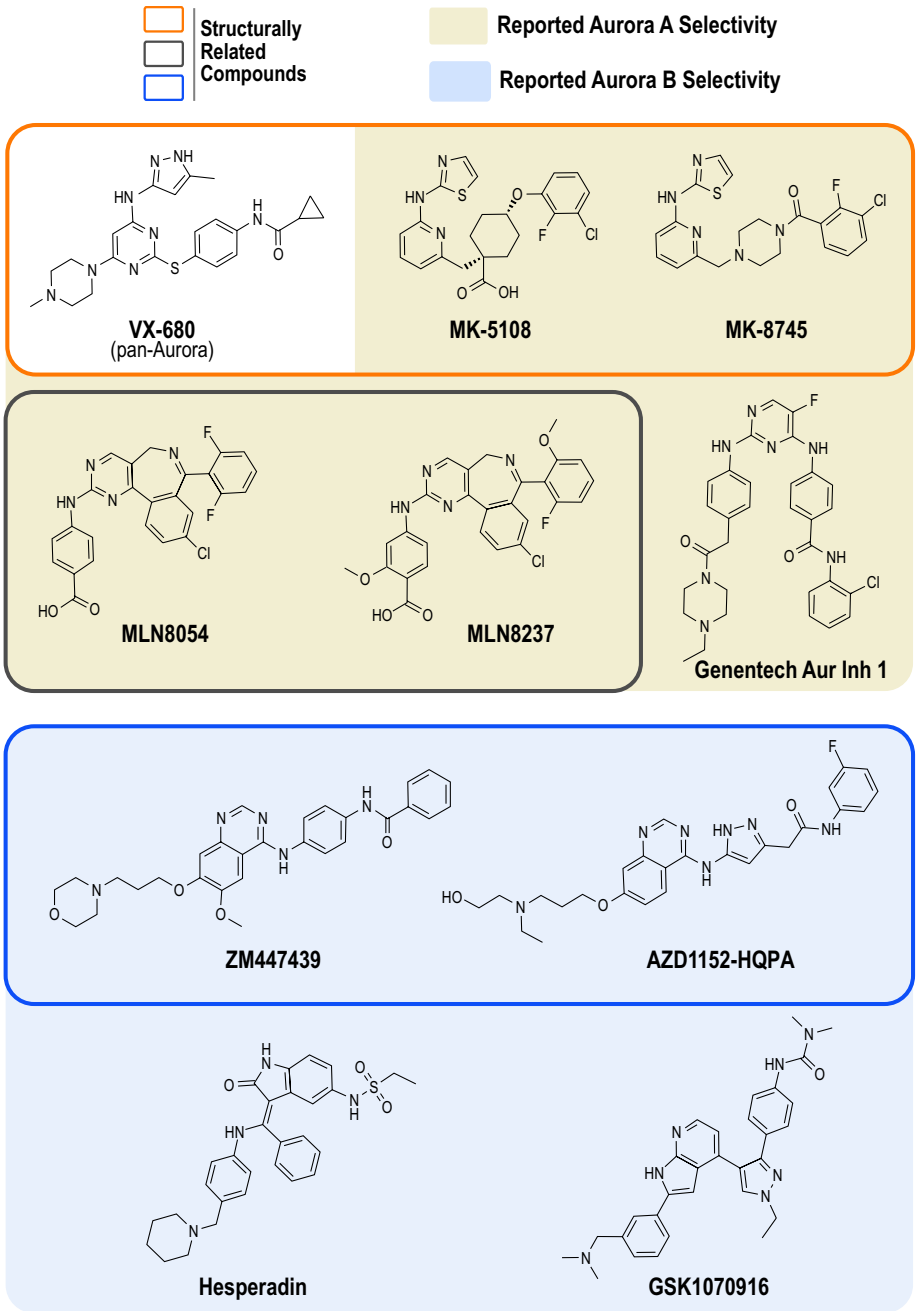

FIGURE 1 | Chemical structures of Aurora kinase inhibitors analyzed in this study. The chemical structures of the 10 commercially available compounds characterized in this study are depicted (see Table S1 in Supplementary Material for suppliers). Outlined boxes group chemically related inhibitors: Pyrazolo-pyrimidine class (Vertex/Merck/Banyu) – MK-5108 and MK-8745 were derived from VX-680 (*orange outlined box*); Benzazepine class (Millenium/Takeda) – MLN8237 was derived from MLN8054 (*gray outlined box*); and Quinazoline class (Astra Zeneca) – AZD1152-HQPA was derived from ZM447439 (*blue outlined box*).

Further, it is presently unclear how the potencies, selectivities, off-target profiles, and cellular efficacies of the most frequently used inhibitors compare to those of more recently described, potentially improved molecules. Here, we fill this gap by profiling the 10 commercially available inhibitors shown in **Figure 1** in biochemical and cell-based assays. Our results highlight significant challenges in the selective inhibition of Aurora A, identify the best compounds for specific and potent targeting of Aurora A and Aurora B, and lead us to present a set of recommendations for the experimental use of these compounds.

RESULTS

Quantitative Biochemical Analysis of Inhibitor Potency and Specificity

We began by analyzing the inhibitory properties of the 10 compounds in **Figure 1** (see Table S1 in Supplementary Material for suppliers) on the *in vitro* activities of full-length human Aurora A, alone or bound to an activating N-terminal peptide fragment of TPX2 (residues 1–43), and full-length human Aurora B bound to a C-terminal fragment of INCENP (residues 783–918, which includes both the IN box and TSS motifs; **Figure 2A**). As the large number of required measurements (3–11 independent sets of triplicate 12–36 point dose–response curves per compound) precluded the use of conventional radiometric substrate phosphorylation assays, we explored several recently developed high throughput methods for measuring kinase catalytic activity in multi-well microplate format (64). To allow comparison of Aurora A and B activity under similar reaction conditions (**Figure 2A**), we ultimately selected a sensitive assay format that monitors ADP production (ADP-Glo™ – see Materials and Methods). Briefly, kinase reactions (with or without inhibitors) were performed in 384-well plates with saturating amounts of a generic peptide substrate containing the Aurora kinase consensus phosphorylation motif (Kemptide – L*RRA*SLG; Aurora kinase consensus *RRXS*/*T*). After a defined incubation period, an enzyme cocktail was added to terminate the reaction and convert any remaining ATP to cyclic AMP. This was followed by a second enzyme cocktail that converted the ADP produced by the kinase reaction to ATP and, in turn, the newly generated ATP to a luminescent signal via luciferase. The resulting luminescence was then quantified using a microplate reader (**Figure 2A**).

Given the high affinities of the inhibitors and the enzyme concentrations required for sufficient signal-to-noise, some experiments were performed under "tight binding" conditions (66). Under these conditions, the total active enzyme concentration ($[E]_t$) matches or exceeds the dissociation constant for the enzyme/inhibitor complex (K_i); therefore, the assumption that the concentration of free inhibitor is equivalent to that added to the reaction is not valid. The K_i was therefore calculated from the measured IC_{50} (concentration for half maximal inhibition) using the equation shown in **Figure 2B** (66–69). Use of this equation assumes that the compounds act through a direct competitive mechanism and requires that the substrate concentration [S], which in this case is [ATP], K_m(ATP), and $[E]_t$ be precisely known. Therefore, for all three enzyme species employed in this analysis, we first measured K_m(ATP) through an ATP titration (**Figure 2C**), and then performed all reactions at [ATP] = K_m(ATP) so that the denominator simplified to two. We also measured $[E]_t$ using inhibitor titrations under conditions where $[E]_t$ and [I] >> K_i, which enables the approximation that $IC_{50} \sim [E]_t/2$ (**Figure 2C**). Example dose–response curves for MK-8745, the measured IC_{50}s, and the resulting K_i values are depicted in **Figure 2D**. K_i values for all 10 inhibitors for Aurora A, Aurora A/TPX2$^{1–43}$ and Aurora B/INCENP$^{783–918}$ are reported in **Table 1**. The K_i values were used to calculate the selectivity ratios of each inhibitor for the three enzyme species (**Table 2**). Because of the extremely slow on-rate of GSK1070916 for Aurora B/INCENP, this K_i could not be accurately measured under our conditions, so the previously described value (61) was used for selectivity analysis. For reference, the published Aurora A/TPX2$^{1–43}$ K_i for GSK1070916 is also presented in **Table 1**.

Consistent with previously reported measurements [Table S2 in Supplementary Material; (42, 70)], the well-characterized pan-Aurora inhibitor VX-680 inhibited both Aurora A and Aurora B/INCENP$^{783–918}$ with essentially identical potencies [(K_i=1.0 nM); **Table 1**]. This compound was therefore included as a reference in the assays for the remaining nine compounds. We note that, based on significant differences in enzyme construct design, sources, purification methods, as well as assay conditions/readouts, it is not straightforward to compare our K_i values to values in the literature (which are, in many cases, wide ranging). Therefore, for all compounds (beyond VX-680), we largely restrict our discussion of prior work to trends in potency and selectivity ratios.

As expected, all of the compounds reported to be Aurora B-selective were extremely potent Aurora B/INCENP$^{783–918}$ inhibitors with a rank order of potency of AZD1152-HQPA > Hesperadin >> GSK1070916 (61) > ZM447439 (**Table 1**) and exhibited a high selectivity (minimum of 30-fold) for Aurora B/INCENP$^{783–918}$ over Aurora A (**Table 2**). Although our mean Aurora B/INCENP$^{783–918}$ K_i value (0.02 nM) (**Table 1**) for AZD1152-HQPA is ~18-fold lower than that previously reported [0.36 nM; Table S2 in Supplementary Material; (46, 47)], this is also the case for the Aurora A K_i values [~16-fold; 84 nM in this study (**Table 1**) versus 1.4 μM from published work (Table S2 in Supplementary Material; (46, 47))]. Thus, the selectivity ratio calculated from our measurements is similar to that which can be derived from prior work (3760-fold versus 3890-fold) (**Table 2** and Table S2 in Supplementary Material).

All of the described Aurora A-selective inhibitors had subnanomolar K_is for Aurora A, with a rank order of potency of MK-5108 > MLN8237 > MK-8745 > MLN8054 > Genentech Aurora Inhibitor I (**Table 1**). MK-5108 exhibited an inhibition constant below what we could accurately measure (≤10 pM). All of these compounds inhibited Aurora B/INCENP$^{783–918}$ less potently than Aurora A, with MK-8745 exhibiting the highest selectivity for Aurora A (1,030-fold) and MLN8054 and MLN8237 the lowest (11- and 27-fold, respectively) (**Table 2**). The selectivity measured for MLN8054 was lower than the published value [Table S2 in Supplementary Material; 43-fold (48)], possibly in part because this previous calculation was based on IC_{50}s, which can be highly dependent upon [ATP], K_m(ATP), and potentially $[E]_t$ (**Figure 2B**). In agreement with this, the K_i-based selectivity ratio we report for MLN8054 (11-fold) (**Table 2**) is close to that described in a structural, biochemical, and mutational analysis of the Aurora A inhibitory properties of MLN8054 (6-fold) (71).

Given the importance of TPX2 as an Aurora A regulator, we also assessed the inhibitory activity of all 10 compounds on the Aurora A/TPX2$^{1–43}$ complex. Excluding Genentech Aurora Inhibitor I and Hesperadin, the presence of TPX2$^{1–43}$ weakened binding by 4- to 8.1-fold (**Tables 1** and **2**). Intriguingly, TPX2$^{1–43}$ increased the affinity of Genentech Aurora Inhibitor I for Aurora A 2.5-fold, whereas Hesperadin binding was unaffected (**Tables 1** and **2**). Decreased Aurora A K_is in the presence of TPX2$^{1–43}$ has

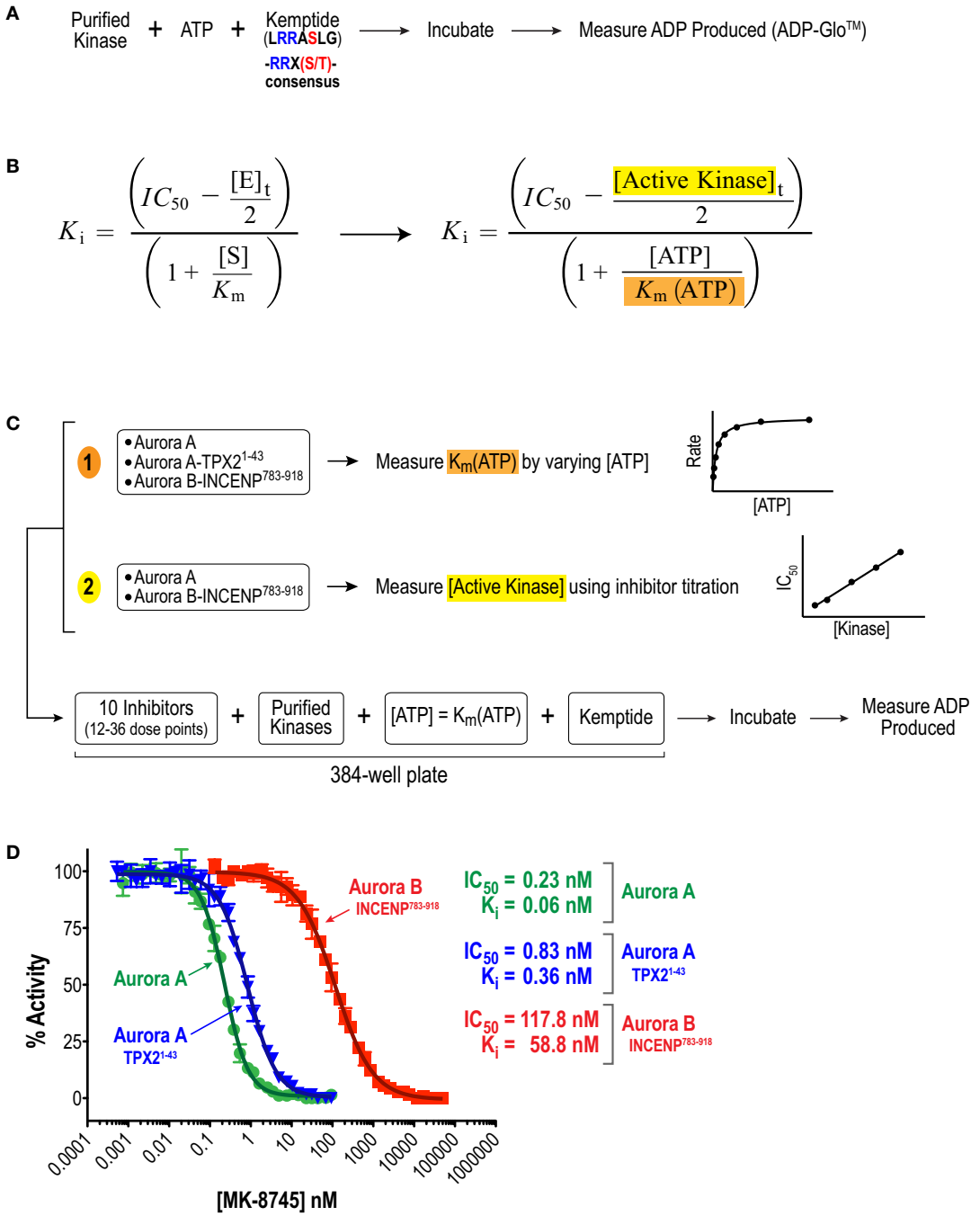

FIGURE 2 | Approach used to quantify inhibitor potency and selectivity *in vitro*. **(A)** Schematic of protocol used to measure kinase activity by quantifying ADP production using the luminescence-based assay technology, ADP-Glo™ (Promega). **(B)** Equation used to convert measured IC_{50} values to K_i values. This equation simplifies to the more familiar Cheng and Prusoff approximation in cases where $K_i >> [E]_t$ (65). **(C)** Schematic of approach used to quantify inhibitor potencies. First, key variables, (1) K_m(ATP) and (2) $[E]_t$ = [Active kinase]$_t$, required for the IC_{50} conversion were measured. Second, a multi-point inhibitor dose titration was performed in 384-well plates under conditions where [ATP] = K_m(ATP) to determine IC_{50} values. **(D)** Example datasets for MK-8745 showing the measured IC_{50}s and the K_is calculated from them using the equation in **(B)**.

been previously reported for VX-680, MK-5108, MLN8054, and MLN8237 [Table S2 in Supplementary Material; (70–72)].

Binding of the TPX2 N-terminus to Aurora A stabilizes a productive conformation of its substrate binding and catalytic elements [including the catalytic lysine (Lys 162), the αC helix which bears the glutamic acid (Glu 181) that interacts with Lys 162, the DFG motif, and the activation loop containing Thr 288] (20, 28). In contrast, inhibitors, such as VX-680, MLN8054, and

TABLE 1 | Biochemical inhibition constants of the Aurora inhibitor panel.

	Aurora A	Aurora A-TPX2$^{1-43}$	Aurora B-INCENP$^{783-918}$
	K_i Mean ± SD (nM)	K_i Mean ± SD (nM)	K_i Mean ± SD (nM)
VX-680	1.03 ± 0.18 (*n*=11)	4.55 ± 0.57 (*n*=9)	1.11 ± 0.20 (*n* = 9)
MK-5108	<0.01[a] (*n* = 4)	0.04 ± 0.008 (*n* = 3)	1.49 ± 0.21 (*n* = 3)
MK-8745	0.06 ± 0.004 (*n* =4)	0.41 ± 0.06 (*n* =3)	66.8 ± 19.6 (*n* =3)
MLN8054	0.15 ± 0.01 (*n* =4)	0.80 ± 0.09 (n = 3)	1.65 ± 0.36 (n = 3)
MLN8237	0.04 ± 0.007 (*n* =3)	0.23 ± 0.02 (*n* = 3)	1.10 ± 0.23 (*n* = 3)
Genentech Aurora Inhibitor 1	0.57 ± 0.06 (*n* = 3)	0.24 ± 0.02 (*n* = 3)	156.2 ± 33.7 (*n* = 3)
ZM447439	55.5 ± 8.2 (*n* = 4)	336.8 ± 50.5 (*n* = 3)	1.83 ± 0.28 (*n* = 3)
AZD1152-HQPA	83.8 ± 14.2 (*n* = 4)	351.9 ± 64.1 (*n*=3)	0.02 ± 0.009 (n = 3)
Hesperadin	1.21 ± 0.14 (n = 3)	1.37 ± 0.12 (*n* = 3)	0.03 ± 0.014 (*n* = 3)
GSK1070916	16.1 ± 1.3 (*n* =3)	130.2 ± 33.2 (*n* =3); 490[b]	0.38[b]

[a]*Upper bound.*
[b]*Values from Ref. (61).*

TABLE 2 | *In vitro* selectivity ratios of the Aurora inhibitor panel (fold difference in potency calculated by dividing K_i values measured for each kinase).

	Aur A versus Aur A-TPX2$^{1-43}$	Aur A versus Aur B-INCENP$^{783-918}$	Aur A-TPX2$^{1-43}$ versus Aur B-INCENP$^{783-918}$	Aur B-INCENP$^{783-918}$ versus Aur A	Aur B-INCENP$^{783-918}$ versus Aur A-TPX2$^{1-43}$
VX-680	4.4	1.1	0.2	0.9	4.1
MK-5108	>4	>149	39.1		
MK-8745	6.3	1030	162		
MLN8054	5.5	11.3	2.1		
MLN8237	5.5	26.8	4.9		
Genentech Aurora Inhibitor 1	0.4	274	654		
ZM447439	6.1			30	184
AZD1152-HQPA	4.2			3759	15779
Hesperadin	1.1			40.2	45.3
GSK1070916	8.1			42.2	343

quinazoline-class compounds, favor distorted inactive conformations of some or all of these elements (59, 71, 73–76). As suggested previously for VX-680 and a quinazoline resembling ZM447439 and AZD1152-HQPA (70), these opposing structural effects likely result in the decreased affinities of the majority of the inhibitors we characterized for the Aurora A/TPX2$^{1-43}$ complex (**Table 2**). Conversely, based on their respective positions in the Aurora A and Aurora B binding pockets, Genentech Aurora Inhibitor I (59) and Hesperadin (20) are predicted to make minimal contact with the active site elements that move upon TPX2$^{1-43}$ binding. This potentially explains the subtle changes in Aurora A K_is for these two compounds in the presence of TPX2$^{1-43}$ (**Table 2**).

From a biochemical selectivity perspective, the K_i shifts driven by TPX2$^{1-43}$ binding have important but different consequences for the Aurora A- and Aurora B-selective compounds. The selectivity ratios of GSK1070916, ZM447439, and AZD1152-HQPA (preference for Aurora B over A) increase to ≥184 in the presence of TPX2$^{1-43}$ (**Table 2**). Conversely, the selectivity ratios of the Aurora A-selective inhibitors diminish significantly, with MLN8054 and MLN8237 exhibiting only two- and fivefold preference, respectively, for Aurora A/TPX2$^{1-43}$ over Aurora B/INCENP$^{783-918}$ (**Table 2**). Given the prevalent use of MLN8054 and MLN8237 as Aurora A-selective tools, these findings motivated us to analyze our inhibitor panel in a battery of cellular assays.

Substrate Phosphorylation-Based Profiling of Aurora Inhibitors in HeLa Cells

The critical parameters influencing inhibitor choice for cell biologists are efficacy and specificity in a cellular context. Thus, we next focused on identifying robust and reproducible cellular readouts for Aurora A and Aurora B kinase activity and employed them to systematically profile inhibitors in dose–response in three cell lines commonly used in cell biological studies: HeLa cervical carcinoma, hTERT-RPE1 retinal pigment epithelial (hereafter referred to as RPE1), and U2OS osteosarcoma cells. Based on previous biochemical studies, it is known that many of the inhibitors we tested can inhibit Aurora C. However, based on our qPCR analysis and previously published work (15), Aurora C mRNA is expressed at low levels in HeLa and RPE1 cells, and only present at ~20% of Aurora B mRNA levels in U2OS cells (Figure S1A in Supplementary Material). Thus, we believe that the biological effects we detect are predominantly, if not exclusively, mediated by Aurora A and B.

As a first approach, we performed immunofluorescence in fixed HeLa cells to detect phospho-epitopes associated with the activity of each kinase. Aurora A has multiple known substrates enriched at centrosomes/mitotic spindles, including the Hippo pathway kinase LATS2 (Ser 83) (77), TACC3 (Ser 558) (78–82), and Aurora A itself (Thr 288) (16, 17). We chose pLATS2(Ser 83) as a cellular readout for Aurora A activity because pilot experiments, guided by a prior study (83), indicated that robust, specific labeling could be obtained using a commercial monoclonal antibody (Clone ST-3B11) targeting this epitope (**Figure 3A**). Aurora B phosphorylates Ser 10 and Ser 28 in the N-terminal tail of histone H3 (84, 85) and reliable antibodies are commercially available for detecting these phospho-epitopes in cells (**Figure 3A**; Table S3 in Supplementary Material). We chose pH3(Ser 28) as the model substrate site because robust labeling could be achieved under fixation conditions compatible with pLATS2(Ser 83) labeling, allowing us to monitor activities of both Aurora A and B in the same cells in 96-well plates. We used RNAi (**Figure 3B**) to confirm that pH3(Ser 28) is sensitive to knockdown of Aurora B but not Aurora A, and that pLATS2(Ser 83) is significantly reduced by knockdown of Aurora A but not Aurora B (**Figures 3B,C**); the partiality of the RNAi likely accounts for the less-than-complete elimination of pLATS2 signal. pH3(Ser 10) behaved similarly to pH3(Ser 28) (Figure S1B in Supplementary Material), as expected (84, 85).

We employed the protocol described in **Figure 3D** to analyze substrate phosphorylation in HeLa cells following treatment with all 10 inhibitors in dose–response. Asynchronous cells were incubated with vehicle (DMSO) or different inhibitor doses for 8 h and then fixed and labeled with a mixture of three antibodies directed against pLATS2(Ser 83), pH3(Ser 28), and MPM2 [which detects mitotic phosphoepitopes; (86)]. While both the anti-pLATS2(Ser 83) and MPM2 antibodies are mouse monoclonals, they are of different IgG subclasses [IgG2b for anti-pLATS2(Ser 83) and IgG1 for MPM2], and can thus be detected with subclass-specific secondary antibodies (Table S3 in Supplementary Material).

In control mitotic cells, pLATS2(Ser 83) is concentrated in foci around the spindle poles (**Figure 3E**; *top row – green arrow*) and pH3(Ser 28) is on the mitotic chromatin (**Figure 3E**; *top row – red arrow)*. Selective kinase inhibition should result in loss of one signal but not the other, as illustrated by the example images for specific conditions in **Figure 3E** (*middle and bottom rows*). Cells were imaged in 4 channels to visualize pLATS2(Ser 83), pH3(Ser 28), MPM2, and DNA (labeled with Hoechst) and mitotic cells were segmented based on their bright MPM2 labeling (**Figure 3E**). Intensity and area thresholds were set to select the pLATS2(Ser 83) foci and the pH3(Ser 28)-labeled chromatin in their respective channels in DMSO-treated control cells and the same thresholds were applied for inhibitor-treated cells. The mean fluorescence intensity per pixel was measured to assess the activities of the kinases targeting these two substrate phosphorylation sites. The results of this analysis for all 10 inhibitors in dose–response are shown in **Figure 4A**.

Two major conclusions emerging from this dataset are:

(1) All four Aurora B-selective inhibitors can be used to specifically and potently inhibit H3(Ser 28) phosphorylation in cells. Consistent with the behavior of these compounds in the enzymatic assays described above, AZD1152-HQPA, Hesperadin, and GSK1070916 are extremely potent, completely eliminating pH3(Ser 28) labeling without affecting pLATS2(Ser 83) labeling at <100 nM concentrations.

(2) The inhibitors designed to target Aurora A require significantly higher concentrations for efficacy and exhibit greater variability with respect to specificity. MK-5108 and MK-8745, two related compounds (**Figure 1**), achieve specific Aurora A inhibition, as demonstrated by loss of pLATS2(Ser 83) labeling without reduction of pH3(Ser 28) labeling. However, both compounds require high micromolar concentrations for full efficacy (**Figure 4A**). In contrast, and consistent with the biochemical data, the commonly used MLN8054 and MLN8237 compounds have narrower specificity windows (10- and 4-fold, respectively), which makes it difficult to fully inhibit Aurora A without affecting Aurora B (**Figure 4A**). This point is illustrated by example images of MLN8054-treated HeLa cells at three different concentrations (**Figure 4B**). With careful optimization, these inhibitors can be employed for selective Aurora A inhibition, especially if the experimental goal is partial Aurora A inhibition. However, based on this dataset, MK-5108 and MK-8745 would be preferred for selectively targeting Aurora A.

Although similar to MK-5108 and MK-8745 in terms of Aurora A specificity, Genentech Aurora Inhibitor 1 led to significantly reduced proliferation and apoptotic cell death in HeLa cells within 24 h of treatment (**Figures 4C,D**; Figure S2A in Supplementary Material). This toxicity, which was also observed in U2OS and RPE1 cells (**Figure 4D**), is most likely due to off-target effects, as it is not observed with MK-5108, MLN8237, or AZD1152-HQPA (**Figure 4D**; Figure S2B in Supplementary Material). Therefore, the narrow window between efficacy and cytotoxicity of Genentech Aurora Inhibitor 1 suggests that it should not be used in routine cell culture experiments for Aurora A inhibition.

Analysis of Inhibitor Efficacy in RPE1 and U2OS Cells Highlights Variation in Potency and Specificity Across Cell Lines

We focused on additional characterization of the four inhibitors designed to target Aurora A that were not cytotoxic (MLN8054, MLN8237, MK-5108, MK-8745; **Figure 4D**; Figure S2B in Supplementary Material), and AZD1152-HQPA and GSK1070916, because they are chemically distinct (**Figure 1**) and the two most potent Aurora B inhibitors in the HeLa substrate phosphorylation assays (**Figure 4A**). As a first step, we analyzed substrate phosphorylation in RPE1 and U2OS cells for these six compounds (**Figures 5A,B**). This analysis revealed that the specificity window for certain inhibitors was significantly narrower in RPE1 and U2OS compared to HeLa cells, as best illustrated by MLN8054 and MLN8237 (compare **Figure 5A** with **Figure 4A**). In addition, inhibitor potency varied up to fourfold across the three cell lines (**Figure 5B**; Figure S2C in Supplementary Material). Regardless of the specific reasons for this variation (discussed below), our results underscore the technical importance of performing a dose–response analysis with the pLATS2(Ser 83) and pH3(Ser

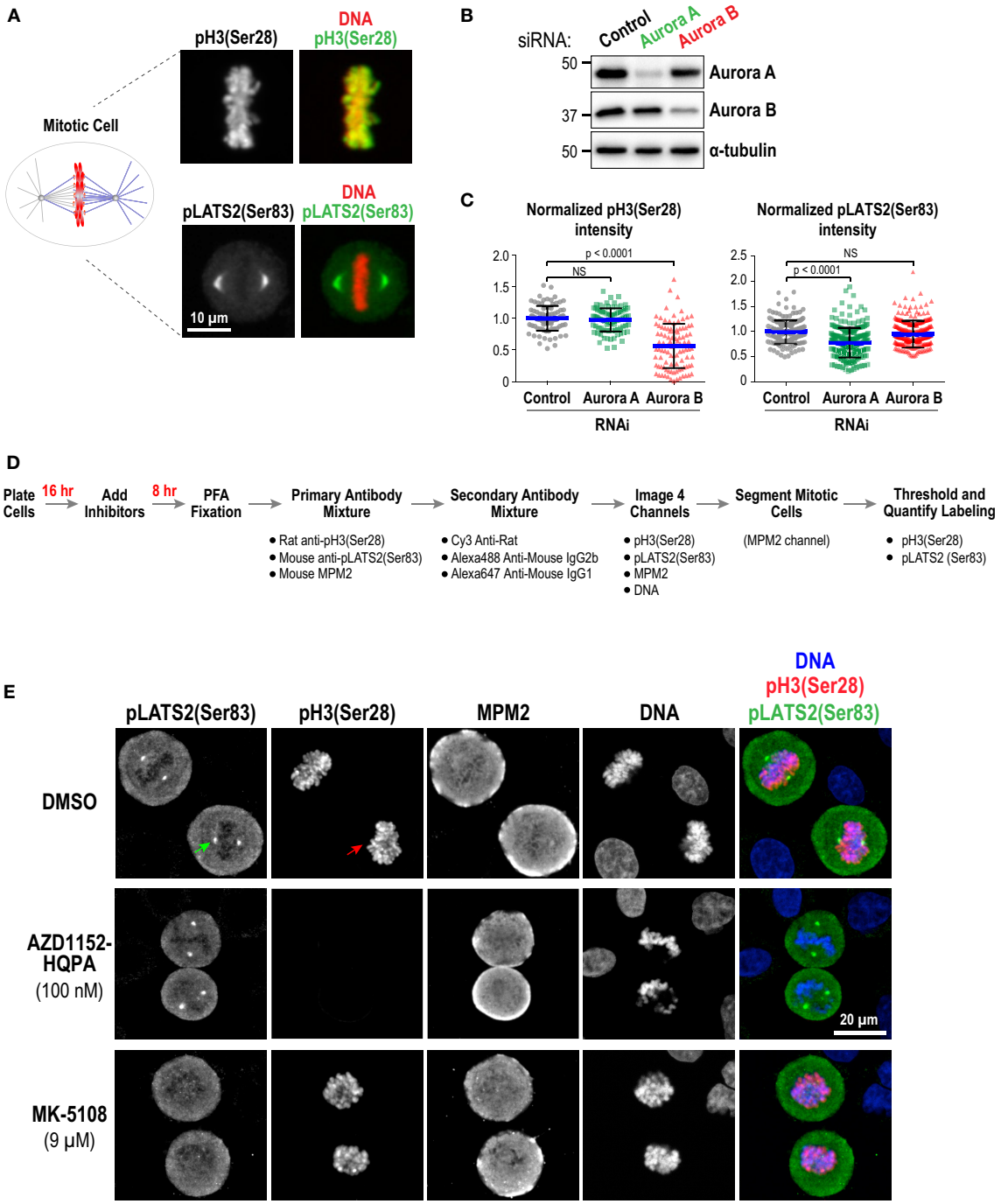

FIGURE 3 | Substrate phosphorylation-based analysis of Aurora inhibitor specificity and potency in a cellular context: assay validation and example images. **(A)** Images of mitotic cells labeled for pH3(Ser 28) (*top panels*) and pLATS2(Ser 83) (*bottom panels*). Merges with DNA (*red*) are shown on the right. **(B)** Immunoblots of HeLa cells following Aurora A or Aurora B RNAi. α-tubulin serves as a loading control. Expression levels of Aurora A and B are reduced by ~90 and ~65%, respectively, under these conditions. **(C)** Quantification of pH3(Ser 28) (*left graph*) and pLATS2(Ser 83) (*right graph*) labeling for the indicated conditions. Blue lines indicate the mean; black error bars are the SD. *p*-values are from unpaired *t*-tests. **(D)** Schematic of procedure used to process cells for labeling and quantification of pH3(Ser 28) and pLATS2(Ser 83). **(E)** Example images of control DMSO-treated (*top row*) and inhibitor-treated (*bottom two rows*) cells. Arrows in the control DMSO-treated panels highlight pLATS2(Ser 83) labeling of spindle poles (*green arrow*) and pH3(Ser 28) labeling of mitotic chromatin (*red arrow*). The concentrations shown for AZD1152-HQPA and MK-5108 exemplify selective inhibition of Aurora B and Aurora A activity, respectively.

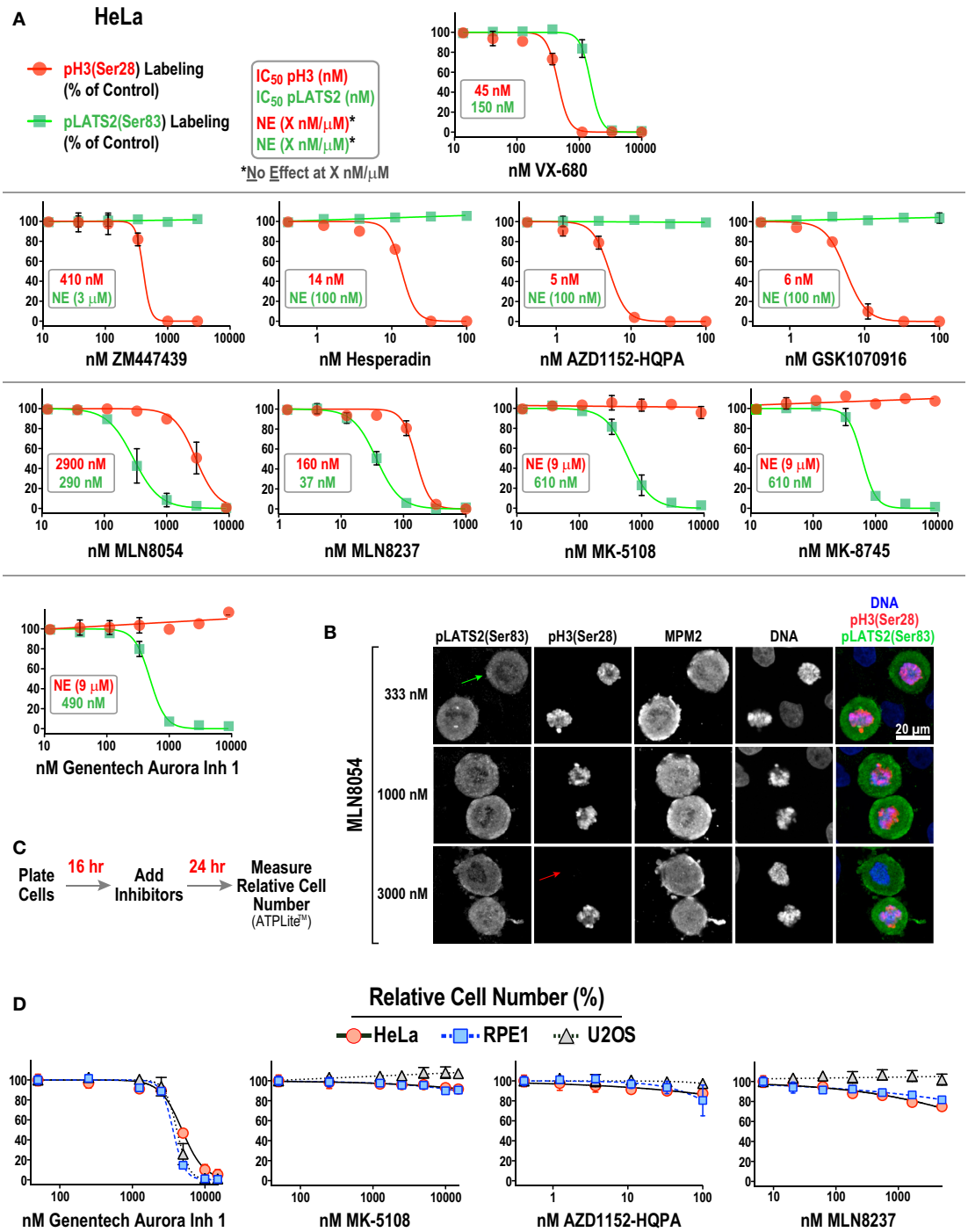

FIGURE 4 | Substrate phosphorylation-based analysis of all 10 Aurora inhibitors in HeLa cells. **(A)** Dose–response curves measuring pH3(Ser 28) (*red circles and line*) and pLATS2(Ser 83) (*green squares and line*) labeling intensity for all 10 inhibitors in HeLa cells. Each point on the graphs represents the mean of measurements performed on four separate plates (average of 350 cells per point), normalized relative to control; the SD is plotted when larger than the symbol size. IC_{50} values are listed on graphs, when applicable, and were derived by computing dose–response curves using a 4-parameter, variable slope fit in GraphPad Prism. When there was no effect on labeling intensity at the highest tested concentration (X μM/nM), NE(X μM/nM) is indicated on the graphs, e.g., in AZD1152-HQPA graph (NE, 100 nM, *green text*) indicates no effect on pLATS2(Ser 83) labeling of 100 nM AZD1152-HQPA. **(B)** Example images of HeLa cells treated with MLN8054 at different doses highlighting partial (333 nM), selective (1000 nM) and non-selective (3000 nM) inhibition of pLATS2(Ser 83) labeling. Green and red arrows highlight cells lacking pLATS2(Ser 83) and pH3(Ser 28) labeling, respectively. **(C)** Protocol used to measure cellular proliferation after short-term (24 h) inhibitor exposure. Relative cell number was quantified by measuring ATP levels, using a luminescence-based assay (ATPLite™ from PerkinElmer). **(D)** Dose–response curves measuring cellular proliferation for the indicated four inhibitors in three cell lines: HeLa, RPE1, and U2OS. Each point represents the mean of six measurements from two independent experiments. Error bars are the SD. See Figure S2B in Supplementary Material for the graphs for the other six inhibitors.

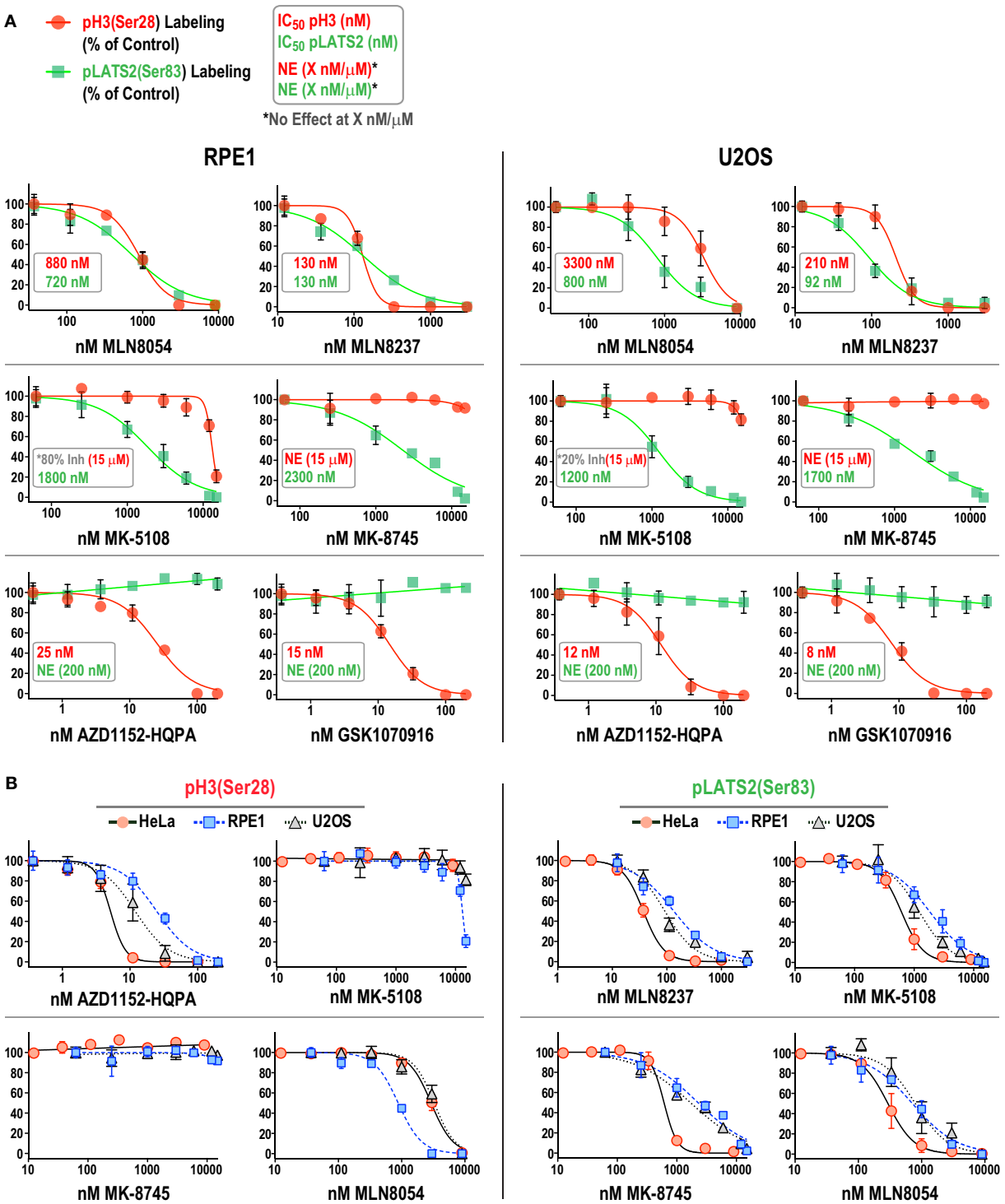

FIGURE 5 | Comparison of inhibitor specificity and potency between HeLa, RPE1, and U2OS cells. **(A)** Dose–response curves measuring pH3(Ser 28) and pLATS2(Ser 83) labeling intensity for the six indicated inhibitors in RPE1 and U2OS cells, plotted and labeled as in **Figure 4A**. Note that for MK-5108 and pH3(Ser 28) labeling, where full inhibition was not achieved in the concentration range tested, the inhibition observed at the highest concentration tested is indicated on the graphs. Each point on the graphs represents the mean of measurements performed on four separate plates (average of 200 cells per point), normalized relative to control. **(B)** Dose–response data for pH3(Ser 28) (*left set of graphs*) and pLATS2(Ser 83) (*right set of graphs*) labeling intensity plotted for all three cell lines for the indicated inhibitors. Note that AZD1152-HQPA, which potently inhibits pH3(Ser 28) and is plotted on the left, has no effect on pLATS2(Ser 83) labeling in any cell line over the tested concentration range (0–200 nM), and is thus not plotted on the right; instead MLN8237 is plotted. See also Figure S2C in Supplementary Material.

28) labeling assay in all experimental cell lines in order to identify the minimum concentration required for selective and complete Aurora kinase inhibition. The results of this analysis confirm MK-5108 and MK-8745 as the current best Aurora A-specific inhibitors, with the latter exhibiting the least effect on pH3(Ser 28) at doses that eliminate pLATS2(Ser 83) labeling. We additionally note that H3(Ser 28) may be targeted by Aurora C in tissues/cell types where this kinase is expressed. As Aurora C mRNA is present at modest levels in U2OS cells and all pH3(Ser 28) signal is abolished by AZD1152-HQPA and GSK1070916 in this cell line, we believe any minor Aurora C activity that may be present is inhibited by these compounds, a conclusion that is consistent with published biochemical studies (46, 47, 61).

Measurement of G2 Duration in a Live Imaging Assay Enables Assessment of Inhibitor Potency and Specificity for Aurora A

We next characterized the effect of selected inhibitors in single-cell live imaging assays, which provide high resolution, dynamic assessment of kinase function in a cellular context. For this purpose, the key challenge was to identify a specific readout for each kinase. For Aurora B, cytokinesis failure is a robust and well-established cellular phenotype of inhibition, which we confirmed with the four Aurora B-specific inhibitors (Figure S3 in Supplementary Material). However, for Aurora A, a specific quantifiable live imaging readout has been lacking. Prior work in *Xenopus* egg extracts (87), *Caenorhabditis elegans* embryos (88), and mammalian cells (89, 90) has suggested a role for Aurora A in controlling the kinetics of mitotic entry. Entry into mitosis, as defined by nuclear envelope breakdown (NEBD), is delayed in the absence of Aurora A. To quantitatively monitor this function of Aurora A in living cells, we employed an assay in which eGFP-tagged PCNA (GFP-PCNA) and mRFP-tagged histone H2B (H2B-RFP) are co-expressed and imaged in a cell population (91). PCNA concentrates in foci known as replication factories in S-phase (**Figure 6A**; Movie S1 in Supplementary Material) and the time interval from dissolution of PCNA foci to NEBD serves as a measure of G2 duration in living cells (**Figure 6A**; Movie S1 in Supplementary Material) (91–93). Using this assay in HeLa cells, we found that depletion of Aurora A, but not Aurora B, by RNAi significantly increased G2 duration (**Figure 6B**). We next measured G2 duration in HeLa cells following treatment with MK-5108 and AZD1152-HQPA, at concentrations that selectively eliminate labeling of pLATS2(Ser 83) or pH3(Ser 83), respectively (6 μM for MK-5108 and 100 nM for AZD1152-HQPA; **Figure 4A**). In agreement with the RNAi analysis, MK-5108, but not AZD1152-HQPA, significantly increased G2 duration (**Figure 6C**). Thus, measurement of G2 duration using the GFP-PCNA; H2B-RFP imaging assay provides a specific functional readout for Aurora A activity in living cells.

We next performed a dose–response analysis of the inhibitors developed to target Aurora A in the G2 duration assay in HeLa, RPE1, and U2OS. The results are shown in **Figure 7** (and Figure S4 in Supplementary Material) and highlight that measurement of G2 duration with this assay provides a sensitive and dose-responsive measure for Aurora A activity in cells. The concentrations where G2 duration was maximally extended by Aurora A inhibitors tracked well with the concentrations at which pLATS2(Ser 83) labeling was eliminated (see **Table 3** and text below). This concordance between distinct cell-based assays confirms that each assay specifically monitors Aurora A activity and gives us confidence that the inhibitor characterization performed using them is providing an accurate picture of efficacy in a cellular context.

Immunoblotting-Based Assessment of Inhibitor Potency and Specificity

Next, we sought to compare the inhibitor potency and specificity measurements obtained using the cellular assays to more proximal markers of cellular activity – namely phosphorylation of Aurora A and Aurora B. We developed methods to monitor kinase phosphorylation by immunoblotting because we found it to have higher signal-to-noise and greater consistency than immunofluorescence. For this analysis, we focused on the four inhibitors with the best overall cellular profiles as Aurora A-selective (MK-5108, MK-8745) or Aurora B-selective (AZD1152-HQPA and GSK1070916). After treating cells with different concentrations of these inhibitors, we performed Western blotting for eight targets for which commercial antibodies are available – pAuroraA(Thr 288), pAuroraA(Thr 288)/pAuroraB(Thr 232)/pAuroraC(Thr 198), total Aurora A, total Aurora B, pH3(Ser 28), pH3(Ser 10), total H3, and Cyclin B. We did not assess pLATS2(Ser 83), because the antibody used for immunofluorescence did not work well for immunoblots. The specific antibodies used for immunoblotting were selected based on extensive testing, employing both siRNA depletion (to assess specificity; **Figure 3A**; Figure S5 in Supplementary Material) and inhibitor treatments (to confirm detection of phospho-epitopes; **Figures 8A,B**); see Table S3 in Supplementary Material for descriptions and supplier information.

For analysis of the Aurora A-selective compounds, MK-5108 and MK-8745, we employed the protocol outlined in **Figure 8A**, based on taxol-induced mitotic checkpoint arrest. For analysis of Aurora B-selective inhibitors, AZD1152-HQPA and GSK1070916, we modified a previously described protocol [outlined in **Figure 8B**; (94)] whose design reflects the fact that Aurora B inhibition overrides taxol-induced arrest (44, 45). To ensure a fair comparison between different conditions, we immunoblotted Cyclin B to confirm that a similar number of mitotic cells were present in the analyzed lysates, in addition to blotting for total H3 as a general loading control. While optimizing the immuoblotting assays, we found that pAuroraA(Thr 288) exhibited low solubility compared to total Aurora A, pAuroraB(Thr 232), or total Aurora B in a typical cell lysis buffer containing non-ionic detergent; only with extensive sonication were we able to solubilize the pAuroraA(Thr 288) signal. This observation suggests that autophosphorylated Aurora A is associated with insoluble cytoskeletal elements, possibly microtubules or centrosomes. From a technical perspective, this observation highlights the importance of employing lysate preparation conditions that properly solubilize pAuroraA(Thr 288) in order

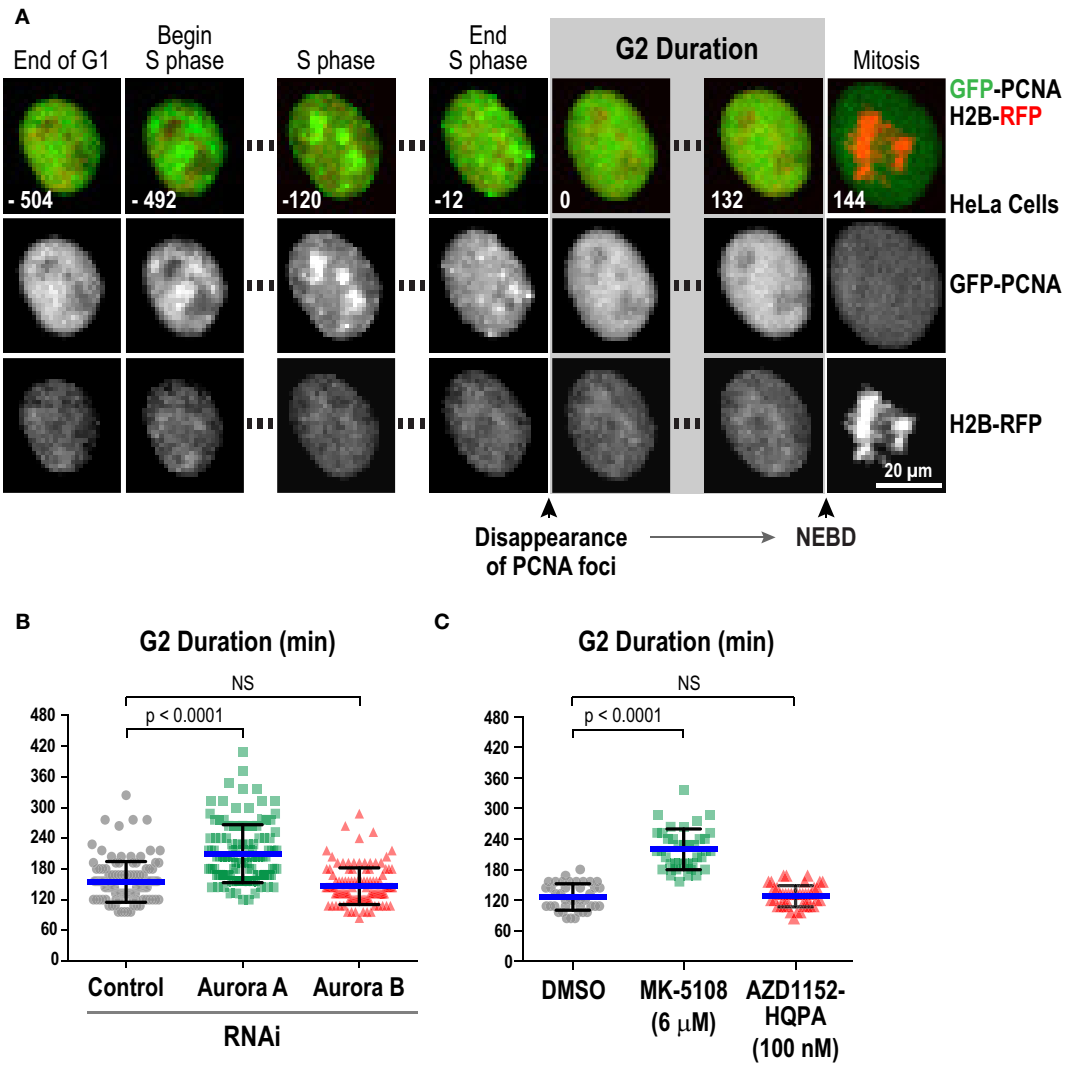

FIGURE 6 | Measurement of G2 duration provides a specific readout for Aurora A kinase activity. **(A)** Images from a timelapse sequence of HeLa cells stably expressing GFP-PCNA; H2B-RFP. Gray scale images for the two separate channels are located below each colored merge. G2 duration is measured as the time from dissolution of PCNA foci to nuclear envelope breakdown (NEBD). See also Movie S1 in Supplementary Material. **(B)** Analysis of G2 duration in HeLa cells following knockdown of Aurora A or Aurora B by RNAi. Blue lines indicate the mean; black error bars are the SD. *p*-values are from unpaired *t*-tests. **(C)** Analysis of G2 duration in HeLa cells using selective Aurora A versus Aurora B inhibition, with MK-5108 (6 µM) and AZD1152-HQPA (100 nM), respectively. Blue lines indicate the mean; black error bars are the SD. *p*-values are from unpaired *t*-tests.

to avoid false negative results and/or overestimates of inhibitor potencies. In situations where changes in cell number/viability are not expected (obviating the need to normalize loading by measuring lysate protein concentrations), samples could be prepared by lysing cells directly with SDS gel sample buffer.

The immunoblotting analysis of autophosphorylated Aurora A, pH3(Ser 28), and pH3(Ser 10), confirmed the specificity of MK-5108 and MK-8745 for Aurora A and AZD1152-HQPA and GSK1070916 for Aurora B. At concentrations of MK-5108 and MK-8745 that completely eliminate Thr 288 phosphorylation (and pLATS2(Ser 83) signal in the fixed immunofluorescence assay), there is no effect on pH3(Ser 10), pH3(Ser 28), or pAuroraB(Thr 232) (**Figure 8A**). Reciprocally, AZD1152-HQPA and GSK1070916 eliminated pH3(Ser 10), pH3(Ser 28), and pAuroraB(Thr 232) at concentrations that did not affect pAuroraA(Thr 288) (**Figure 8B**).

For AZD1152-HQPA and GSK1070916, there was strong correspondence between the concentration-dependent effects on H3 and Aurora B phosphorylation with those observed in the immunofluorescence and cytokinesis assays (**Figures 4A** and **8B**; Figure S3 in Supplementary Material; **Table 3**). However, for MK-5108 and MK-8745, complete loss of Aurora A Thr 288 phosphorylation was observed at significantly lower inhibitor concentrations than those necessary for full efficacy in the LATS2 phosphorylation and G2 duration assays [(IC_{50} pAuroraA(Thr 288) <<100 nM versus IC_{50} pLATS2 (Ser 83) and G2 duration: ~ 600-800 nM)]

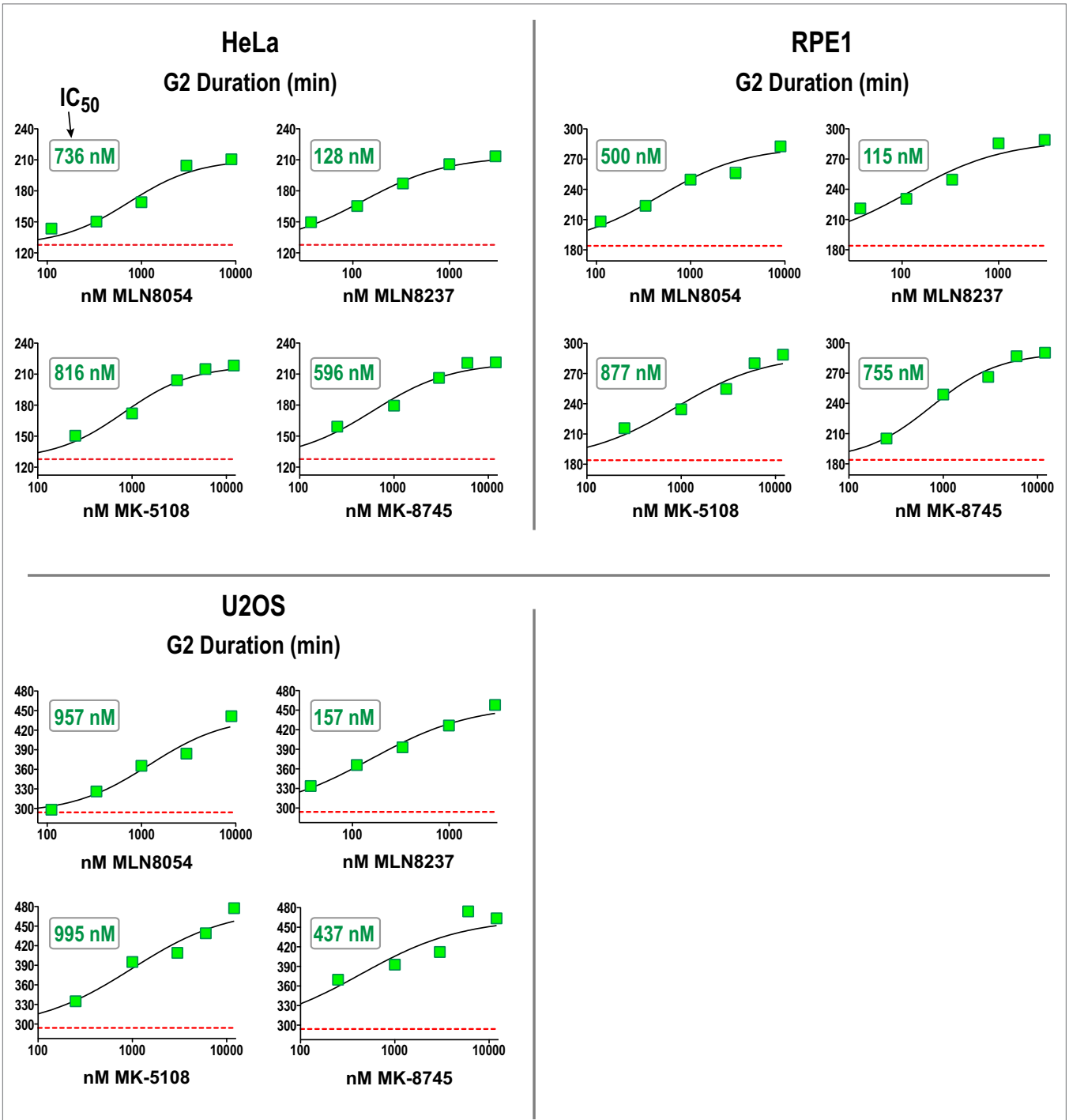

FIGURE 7 | Dose–response analysis of G2 duration with inhibitors specifically targeting Aurora A in 3 cell lines. G2 duration (in minutes), measured as in **Figure 6**, for the indicated inhibitors and cell lines. Each point represents the mean of 40–100 cells from two sets of measurements. See Figure S4 in Supplementary Material for scatter plots showing all measurements. IC_{50} values shown on the graphs were determined by computing dose–response curves using a four-parameter, variable slope fit in GraphPad Prism.

(**Figures 4A**, **7** and **8A**; **Table 3**). There are three potential explanations for this difference. First, the immunoblotting of activation loop phosphorylation, at least for Aurora A under the conditions employed here, may have a significantly lower dynamic range than the two cell-based assays. Second, the phosphatases that remove pAuroraA(Thr288) (27, 95, 96) may be more efficient than those that reverse pLATS2(Ser 83) and the Aurora A phosphorylation target(s) that contribute to G2 duration control.

TABLE 3 | Cellular inhibitory potencies of the Aurora inhibitor panel.

Aurora kinase inhibitor	Cell line	Assay			
		Substrate-phosphorylation		G2/M	Cytokinesis
		pLATS2 (Ser83) IC_{50} (nM)	pHistone H3 (Ser28) IC_{50} (nM)	IC_{50} (nM)	IC_{50} (nM)
VX-680	HeLa	150	45	nd	nd
	RPE1	nd	nd	nd	nd
	U2OS	nd	nd	nd	nd
MK-5108	HeLa	610	NE@9 μM	816	nd
	RPE1	1800	80% Inh @15 μM	877	nd
	U2OS	1200	20% Inh @15 μM	995	nd
MK-8745	HeLa	610	NE@9 μM	596	nd
	RPE1	2300	NE@15 μM	755	nd
	U2OS	1700	NE@15 μM	437	nd
MLN8054	HeLa	290	2900	736	nd
	RPE1	720	880	500	nd
	U2OS	800	3300	957	nd
MLN8237	HeLa	37	160	128	nd
	RPE1	130	130	115	nd
	U2OS	92	210	157	nd
Genentech Aurora Inhibitor 1	HeLa	490	NE@9 μM	nd	nd
	RPE1	nd	nd	nd	nd
	U2OS	nd	nd	nd	nd
ZM447439	HeLa	NE@3 μM	419	nd	615
	RPE1	nd	nd	nd	1315
	U2OS	nd	nd	nd	613
AZD1152-HQPA	HeLa	NE@100 nM	5	nd	4
	RPE1	NE@200 nM	25	nd	23
	U2OS	NE@200 nM	12	nd	20
Hesperadin	HeLa	NE@100 nM	14	nd	14
	RPE1	nd	nd	nd	47
	U2OS	nd	nd	nd	17
GSK1070916	HeLa	NE@100 nM	6	nd	6
	RPE1	NE@200 nM	15	nd	21
	U2OS	NE@200 nM	8	nd	6

NE, no effect; nd, not determined.

Third, this difference may arise from cellular Aurora A existing in multiple active but biochemically distinct forms, as proposed previously (28, 32). Recent biochemical data indicate that Thr 288 phosphorylation is not a prerequisite for Aurora A kinase activity if Aurora A is bound to TPX2 (28, 32). Further, our *in vitro* studies indicate that Aurora A/TPX2 is more difficult to inhibit than the free enzyme. Therefore, if LATS2 phosphorylation and mitotic entry kinetics are dependent upon Thr 288 unphosphorylated but active pools of Aurora A (bound to activators), sole assessment of Thr 288 phosphorylation may provide a misleading view of inhibitor potencies. Additional studies will be required to explore these possibilities.

Regardless of the underlying reasons, our data highlight that if a pAuroraA(Thr 288) immunoblot was employed with pH3 immunofluorescence/immunoblots to characterize inhibitor effects, one would conclude that MK-5108 and MK-8745 completely block cellular Aurora A activity at much lower concentrations than we measure for the pLATS2(Ser 83) immunofluorescence and live cell G2 duration assays, and that MK-5108 and MK-8745 have significantly greater selectivity in a cellular context than is actually the case. Thus, we caution on relying exclusively on immunoblotting, especially with activation loop phosphorylation antibodies for Aurora A, to measure inhibitor potency and specificity. Instead, we recommend performing quantitative fixed or live imaging-based analysis of kinase activity, and complementing with immunoblotting.

Synthesis of Biochemical and Cellular Profiling Data to Identify the Best Aurora A- and B-Selective Inhibitors

Differences in ATP concentrations (biochemical – micromolar; cellular milieu – millimolar) as well as compound solubility/stability, binding to serum proteins in media, and cellular penetration make direct correlation of biochemical and cellular inhibitor potencies difficult (97). However, the Aurora A/B selectivity ratios derived from our *in vitro* and *in vivo* data can be compared to prioritize inhibitors. In the biochemical assays, AZD1152-HQPA, ZM447439, Hesperadin, and GSK1070916 all exhibit >30-fold selectivity for Aurora B/INCENP$^{783–918}$ over Aurora A and Aurora

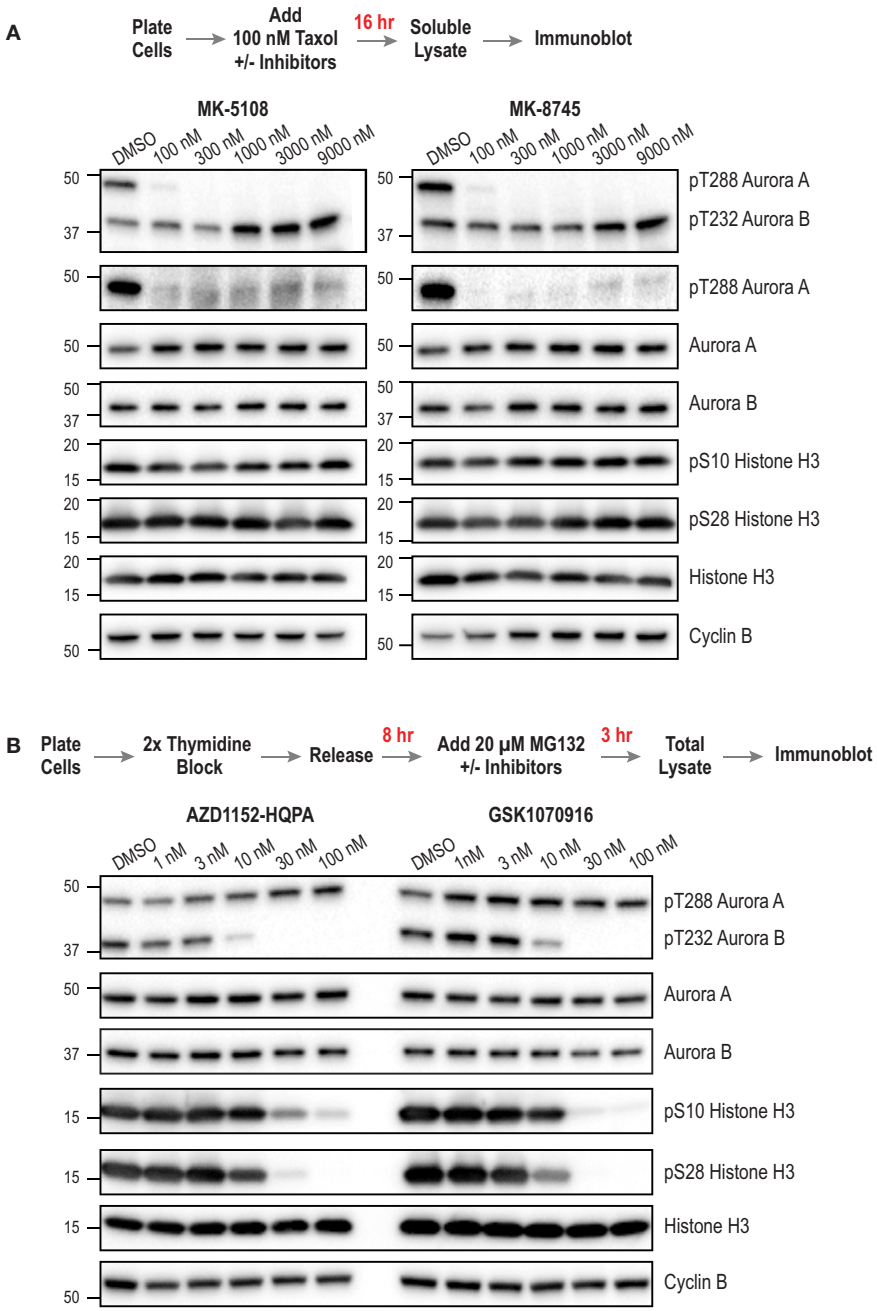

FIGURE 8 | Immunoblotting of activation loop phosphorylation of Aurora A/B and of pH3(Ser 28) and pH3(Ser 10) in HeLa cells following inhibitor treatments. **(A)** Analysis of MK-5108 and MK-8745. The targets blotted are indicated on the right; MW markers (in kD) are on the left. The topmost blot is with an antibody that recognizes the phosphorylated activation loops of both Aurora A and Aurora B. The protocol used to prepare mitotic taxol-arrested lysates is summarized above the blots. **(B)** Analysis of AZD1152-HQPA and GSK1070916 as in **(A)**. The only difference is the protocol used to prepare the mitotic lysate; as Aurora B inhibition overrides taxol-based mitotic arrest, a synchronization procedure followed by proteasome inhibition, schematized above the blots, was used. Blots were repeated in at least two independent experiments, which were highly consistent; blot sets from one representative experiment are shown. For **(B)**, see Figure S6 in Supplementary Material for an independent experiment performed at a higher top dose (300 nM).

A/TPX2[1–43] (**Table 2**). This selectivity is recapitulated in the cellular analysis. Complete inhibition of Aurora B(Thr 232) and H3(Ser 28) phosphorylation (as well as blockade of cytokinesis) can be achieved with each of these compounds in the absence of any effect on Aurora A(Thr 288) or LATS2(Ser 83) phosphorylation (**Table 3**). So, which of these compounds is the best for cell biology experiments? Hesperadin is potent and specific but appears to be unstable under long-term live imaging conditions (see legend of

Figure S3 in Supplementary Material). Within the quinazoline class of compounds (**Figure 1**), AZD1152-HQPA is preferable as it is approximately two orders of magnitude more potent *in vitro* and *in vivo* than ZM447439, from which it was derived. How then do AZD1152-HQPA and GSK1070916 compare? Although both are selective, AZD1152-HQPA exhibits significantly greater preference for Aurora B/INCENP$^{783–918}$ when compared to either Aurora A or Aurora A/TPX2$^{1–43}$ *in vitro* (**Table 2**). Consistent with this, immunoblotting revealed that, while both compounds eliminated Aurora B activity at <100 nM without affecting pAuroraA (Thr 288) (**Figure 8**), at 300 nM GSK1070916 inhibited Aurora A activity whereas AZD1152-HQPA did not (Figure S6 in Supplementary Material). In addition, when profiled against 363 human kinases (including Aurora A/B/C) at 100 nM concentration (Table S4 in Supplementary Material), both compounds exhibited high Aurora kinase-specificity but AZD1152-HQPA was slightly superior. Aurora B was the only enzyme inhibited ≥65% of control by AZD1152-HQPA whereas Aurora A and B as well as DDR1 are inhibited by GSK1070916 at or above this threshold (Table S4 in Supplementary Material). Therefore, we believe that AZD1152-HQPA is the current best choice for an Aurora B-selective inhibitor, and recommend use of GSK1070916 for confirmatory follow-up studies (*see below*).

In terms of the Aurora A inhibitors, MLN8054 and MLN8237 exhibit only modest selectivity *in vitro* and *in vivo* (**Tables 2** and **3**). Genentech Aurora Inhibitor I is cytotoxic within the range of concentrations required for full inhibition of Aurora A activity (**Figure 4D**). In the fixed and live imaging cell-based assays, the two Merck compounds were similar with MK-5108 being slightly more potent (**Table 3**) and MK-8745 being more selective in both RPE1 and U2OS cells (**Figure 5**; **Table 3**). By kinome profiling analysis, MK-8745 was significantly more Aurora kinase-specific. At 100 and 500 nM, MK-5108 inhibited 32 and 75 kinases (including the Aurora kinases), respectively, at >65% of control; Polo-like kinase 4 was the only cell cycle-related kinase affected (Table S4 in Supplementary Material). In contrast, MK-8745 at 500 nM only inhibited 16 kinases at >65% of control (Aurora A, AXL, BRK, DDR1, EphA6, GSK3α/β, IRAK1, JNK1, LKB1, ROS1, Trk A/B/C, TYRO3, YES). Thus, based on its Aurora and off-target selectivity, we believe that MK-8745 is the current best commercially available Aurora A-selective inhibitor for cellular studies.

The Structure of the Aurora A Kinase Domain Bound to MK-5108 Reveals Features Underlying Potency and Specificity

To gain insight into the remarkable biochemical potency and Aurora A-selective nature of the MK-8745/MK-5108 class of compounds, we determined the 2.2 Å X-ray crystal structure of the human Aurora A kinase domain bound to MK-5108 (**Figure 9A**; Table S5 and Figure S7 in Supplementary Material). In the inhibitor complex, the Aurora A kinase domain adopts an inactive conformation in which both the αC helix (*orange*; **Figure 9A**) and particularly the activation loop (*yellow*; **Figure 9A**) are improperly positioned for catalysis (**Figure 9B** – compare MK-5108-bound versus ADP-bound structures). Electron density maps indicate that both Thr 287 (which adopts two alternate conformations; only one is illustrated) and Thr 288 are phosphorylated, showing that the inhibitor is able to interact with the activated form of the enzyme (**Figure 9A**). Consistent with its action as an ATP-competitive inhibitor (56), MK-5108 inserts itself into the nucleotide-binding pocket between the two lobes of the kinase in the same orientation as its parent compound VX-680 (**Figures 9A,B**).

The picomolar affinity of MK-5108 for Aurora A is explained by the extensive polar and van der Waals interactions it forms with 22 residues throughout the active site (**Figure 9C**, Table S6 in Supplementary Material). The aminothiazole moiety (**Figures 9C,D**) is located adjacent to the gatekeeper residue Leu 210 enabling it to form two hydrogen bonds with the main chain amide nitrogen and carbonyl of Ala 213 within the hinge region (**Figure 9C**), thereby making it functionally analogous to the aminopyrazole moiety of VX-680 (**Figure 9D**). The 2-fluoro, 3-chlorophenol on the opposite end of the inhibitor (**Figure 9D**) packs against the side chains of the catalytic lysine (Lys 162), precluding its active conformation, as well as against Phe 275 of the DFG motif (**Figure 9C**). This interaction stabilizes a flipped, inactive conformation of the DFG motif that is intermediate between the active "DFG-in" state (**Figure 9B** – ADP-bound) (98) and the canonical "DFG-out" conformation (99). This conformation is distinct from the distorted conformation in the VX-680 Aurora A complex (**Figure 9B** – VX-680-bound) (74), and the "DFG-up" conformation linked to MLN8054 binding (59, 71, 75), and resembles that of Aurora A bound to adenosine (PDB: 1MUO) (100). Importantly, the 2-fluoro, 3-chlorophenol moiety of MK-5108 forms a likely highly energetically favorable edge-face aromatic pi stacking interaction with the indole of Trp 277 (**Figures 9C,D**). Because of the major differences in its chemical structure in this region (**Figure 9D**), VX-680 only forms hydrophobic contacts with Phe 275 (via its cyclopropylamide) and not Trp 277 (**Figure 9B**). The interaction between MK-5108 and the side chain of Trp 277 has the effect of "pinning down" the activation loop at its N-terminal end and disfavors its adoption of an active conformation (**Figure 9B**). We note that the phosphates on Thr 287 and Thr 288 form hydrogen bonds with the side chains of His 187 and Lys 250, respectively, from a symmetry-related molecule. However, since residues 281–285 are disordered (**Figure 9B**), we believe that residues 277–280 should not be constrained by these contacts, and that their positioning is a consequence of inhibitor binding. The inactive conformation of the activation loop that we describe here is, to our knowledge, unique among all known human Aurora A kinase domain – inhibitor complex structures. The conformations of the active site and activation loop residues stabilized by MK-5108 binding are distinct from those favored by TPX2 binding, which likely explains the reduced affinity of MK-5108 for the Aurora A/TPX2$^{1–43}$ complex.

The crystal structure of the MK-5108/Aurora A complex also suggests a potential explanation for the selectivity of this inhibitor. MK-5108 interacts with the side chains of two (Thr 217 and Arg 220) of the four residues in the vicinity of the active site that differ between Aurora A and B (Aurora A: Ala 141, Leu 215, Thr 217 and Arg 220; Aurora B: Lys 85, Arg 159, Glu 161, Lys

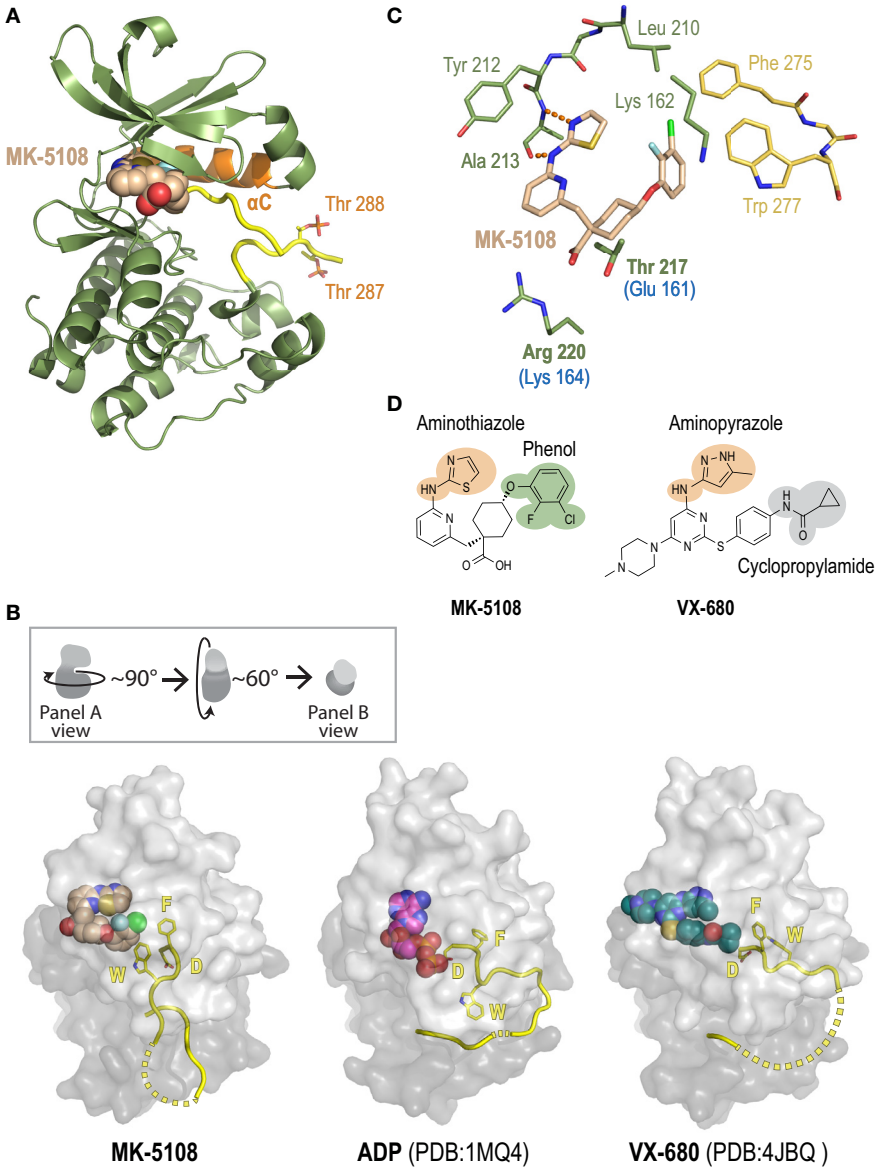

FIGURE 9 | A crystal structure of the Aurora A/MK-5108 complex reveals unique features compared to previous Aurora A-inhibitor complex structures. **(A)** The complex structure with the Aurora A kinase domain depicted as a ribbon diagram and MK-5108 as a spacefilling model. The majority of the kinase domain is colored green except for the activation loop, which is colored yellow, and the αC helix, which is colored orange. The phosphate groups on Thr 288 and Thr 287 are highlighted. **(B)** Comparison of Aurora A/MK-5108 structure to previously determined ADP-bound (PDB:1MQ4) and VX-680-bound (PDB: 4JBQ) structures. The orientation of the kinase domains relative to that in **(A)** is described in the inset. For all three structures, beyond the activation loop (yellow cartoon – dashed lines indicate disordered regions) only the protein surface is shown (light gray – N-terminal lobe, dark gray – C-terminal lobe). Asp 274 (D) and Phe 275 (F) of the DFG motif, as well as Trp 277 (W) are depicted. **(C)** Detailed view of the key contacts between MK-5108 and Aurora A residues. Hydrogen bonds between the aminothiazole of MK-5108 and Ala 213 are shown as orange dashes. MK-5108 interacts with the side chains of two residues which are different between Aurora A and B (Aurora A: Thr 217 and Arg 220; Aurora B: Glu 161 and Lys 164; the Aurora B residues are indicated in blue text below the corresponding Aurora A ones in the figure). See also Table S6 in Supplementary Material. For clarity, the main chain atoms of Lys 162, Thr 217 and Arg 220 as well as the side chain atoms of Glu 211 are not shown. **(D)** The chemical structures of MK-5108 and VX-680 with specific moieties highlighted.

164) (**Figure 9C**; Table S6 in Supplementary Material). Although the electron density for Aurora A Arg 220 is weak (indicative of mobility), the positively charged guanidinium of this residue is located close enough to the negatively charged MK-5108 carboxylate to form favorable electrostatic interactions (**Figures 9C,D**). However, the equivalent Aurora B residue is a lysine (Lys 164), which should also be able to form the same types of interactions. In contrast, the side chain of Thr 217 is wedged in between the carboxylate and the cyclohexyl ring of MK-5108 (**Figures 9C,D**). This tight fit would not be possible with the equivalent Glu 161 in Aurora B, likely significantly reducing binding affinity. Integrated mutagenesis, biochemical and structural studies of MLN8054

and Genentech Aurora A Inhibitor I (which both pack against Thr 217) have indicated that Thr 217 contributes heavily to the selectivity of these compounds (59, 71, 75). Equivalent efforts with MK-5108 and MK-8745 (which, by modeling, is predicted to bind in a highly similar manner as MK-5108 to the Aurora A active site) will be necessary to test if Thr 217 is the central determinant of Aurora A/B selectivity for these compounds, as suggested by our structural analysis.

DISCUSSION

The current tool chest of Aurora inhibitors is the product of extensive chemical optimization in the pursuit of suitable clinical candidates, rather than optimal inhibitors for cell biology studies. As a consequence, careful comparison of these inhibitors in parallel biochemical and cell-based assays has been lacking. The systematic profiling data presented here should provide a resource for future studies employing these compounds. Based on our results, we provide the following 'good practice' guidelines with respect to their use:

(1) pH3(Ser 28) and pLATS2(Ser 83) labeling, which we validate as specific cellular readouts for Aurora B and A, respectively, provides a convenient and robust means to characterize existing and newly developed Aurora kinase inhibitors, and should be used prior to any detailed functional analysis conducted with this class of compounds. Inhibitor sensitivity can be modulated by biological factors (such as kinase expression levels) or technical factors (such as changes in serum/media and growth conditions) (97). Hence, dose–response analysis employing the pH3(Ser 28)/pLATS2(Ser 83) labeling assay is particularly important when extending inhibitor use to new cell lines not analyzed here. While we present a large dataset for HeLa, RPE1, and U2OS cells that should serve as a benchmark for future studies, we still recommend performing a dose–response even when using these three lines given inter-lab variability. The goal of such preliminary analysis should be to identify the *minimum* concentration that achieves complete inhibition of the relevant marker without affecting the other. We strongly advise against the "more is better" urge as unnecessarily high doses will likely lead to loss of specificity and potential unanticipated off-target effects.

(2) AZD1152-HQPA is a highly potent, selective, and efficacious Aurora B inhibitor and the best current choice for targeting this kinase. While AZD1152-HQPA does not exhibit any obvious effects against any of the other kinases that we tested, this profiling exercise was not exhaustive. Further, there is relatively little published about binding of this compound to non-kinase proteins, and even very well-characterized molecules can have unexpected off-target effects. For example, recent studies have revealed that the commonly used Plk1 inhibitor, BI-2536, and several other known kinase inhibitors, are potent inhibitors of BET bromodomain proteins (101–103). Therefore, we highly recommend that any results from studies using AZD1152-HQPA be corroborated with GSK10701916, which likely has a different off-target profile based on its unrelated chemical structure. Indeed, this strategy of using structurally distinct compounds with common mechanisms should be applied when using any of the inhibitors analyzed here and when performing chemical cell biology studies in general.

(3) MK-8745 represents the best current commercially available option for selective and potent Aurora A inhibition. We note, however, that the lowest concentration of MK-8745 that is necessary to maximally inhibit Aurora A in cells is >100-fold higher than the lowest concentration of AZD1152-HQPA that is necessary to fully inhibit Aurora B. Hence, based on current chemical biology standards [on-target cellular activity <1 μM; (104, 105)], AZD1152-HQPA is an ideal chemical tool whereas MK-8745 is not. In addition, validation of any findings with MK-8745 with an orthogonal chemical scaffold is not currently straightforward. If only partial inhibition of Aurora A is required, MLN8054 (and possibly MLN8237) could be used but only under carefully controlled circumstances. Alternatively, MK-5108 could be used but this is also not optimal given its chemical similarity to MK-8745. In the recent literature, at least five classes of compounds with Aurora A-selective behavior (which are not commercially available or only became available near the end of this study) have been reported (106–110). Assessment of these inhibitors should reveal if one or more of them can be paired with MK-8745 for analysis of Aurora A function in cellular experiments.

(4) Immunoblotting of activation loop phosphorylation should not be used in isolation to estimate inhibitor potency and specificity. As we show here, immunoblotting with pAuroraA(Thr 288) antibodies suggests significantly higher Aurora A inhibitor potency than is observed in validated fixed and live imaging-based cellular assays. Consequently, if only immunoblotting were performed, one could overestimate not just potency but also selectivity for Aurora A versus Aurora B. We recommend that the fixed or live imaging-based cellular assays described here be employed first, with immunoblotting serving as confirmation. The imaging-based cellular assays also have the advantage of revealing potential off-target effects, such as the toxicity of the Genentech Aurora Inhibitor 1 reported here.

Our analysis highlights that, while highly selective and potent tools for Aurora B inhibition are readily available, there is significant room for improved small molecule inhibitors of Aurora A. Part of the challenge in targeting Aurora A likely arises from its multiple activation mechanisms, which makes uniformly inhibiting the different active states of the kinase difficult. A second limitation is the prior lack of a consistent and rigorous assay paradigm for Aurora A activity in a cellular context – as we show here, immunoblotting of activation loop phosphorylation can be misleading when compared to other kinase activity readouts – a fact that can be rationalized by recent findings that activation loop phosphorylation is not essential for high levels of kinase activity in the presence of an activator such as TPX2. Our findings suggest new avenues to help address the challenge of developing a more potent and highly selective Aurora A inhibitor. First, the pLATS2(Ser 83) and the G2 duration assays provide

independent, robust, and dose-responsive cellular readouts that specifically report on Aurora A but not Aurora B activity. These assays could be used for optimization of novel classes of Aurora A inhibitors in a cell-based context, analogous to the strategy we employed recently to develop a Plk4 inhibitor, centrinone, that prevents centriole duplication (92). The target specificity of centrinone was confirmed through the extensive use of an engineered inhibitor-resistant mutant. Analogous approaches could also be applied using previously described inhibitor-resistant Aurora kinase mutants (75, 111, 112). Further, the crystal structure of the MK-5108/Aurora A kinase domain complex we determined, which revealed a previously unobserved protein conformation and active site interactions, could be used to generate more potent versions of MK-5108/MK-8745 and potentially design new molecules as well. Given the renewed interest in Aurora A as a drug target based on the recently discovered role of Aurora A in controlling c-Myc protein levels in cancers such as neuroblastoma (113, 114), new inhibitor discovery efforts leveraging the approaches described here could aid not only in developing better tools for cell biology experiments but also in fully realizing the therapeutic potential of inhibiting Aurora A.

MATERIALS AND METHODS

Inhibitors and Antibodies

Inhibitors and antibodies used in this study are described in Table S1 and S3 in Supplementary Material, respectively.

Kinase Assays

For the Aurora A assays, purified full-length human Aurora A (Millipore) was diluted to ~0.8 nM (based on enzyme activity) in 7.5 μL of a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 270 mM sucrose, 0.03% Brij 35, and 1 mM DTT in Corning #4512 white 384-well plates. Inhibitors arrayed in dose–response were added from DMSO stocks using a V&P 384-pintool head mounted on a Beckman Multimek chassis. Reactions were then initiated via the addition of 7.5 μL of a buffer containing 50 mM Tris (pH 7.5), 20 mM $MgCl_2$, 1 mM DTT, 0.2 mg/ml BSA, 70 μM ATP, and 800 μM Kemptide (amino acid sequence: LRRASLG (InnoPep)) using a NSX-384 384-channel liquid handler (Nanoscreen), and allowed to proceed for 2 h at 25°C. The final reaction buffer contained 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 75 mM NaCl, 135 mM sucrose, 0.015% Brij 35, 1 mM DTT, 0.1 mg/mL BSA, 35 μM ATP, and 400 μM Kemptide. The final [ATP] in the reaction mix (35 μM) is at the K_m(ATP) for Aurora A. Detection using a 5 μL aliquot of each reaction was performed with ADP-Glo™ reagents (Promega), following the manufacturer's instructions, in PerkinElmer #6008281 plates. Luminescence was measured on an Infinite M1000 plate reader (Tecan). Data were fit using a 4-parameter, variable slope fit in Prism (GraphPad), and K_is were calculated from IC_{50} data using the equation in **Figure 2B**.

For the Aurora A/TPX2$^{1–43}$ assays, purified full-length human Aurora A (Millipore) was diluted to ~0.8 nM (based on enzyme activity) in 7.5 μL of a buffer containing 80 nM TPX2$^{1–43}$ (InnoPep), 50 mM Tris (pH 7.5), 150 mM NaCl, 270 mM sucrose, 0.03% Brij 35, and 1 mM DTT in Corning #4512 white 384-well plates. The TPX2$^{1–43}$ concentration was determined using a calculated molar extinction coefficient (280 nm) of 8480 M^{-1} cm^{-1}. Inhibitors arrayed in dose–response were added from DMSO stocks using a V&P 384-pintool head mounted on a Beckman Multimek chassis. Reactions were then initiated via the addition of 7.5 μL of a buffer containing 50 mM Tris (pH 7.5), 20 mM $MgCl_2$, 1 mM DTT, 0.2 mg/ml BSA, 6 μM ATP, and 1,200 μM Kemptide using a NSX-384 384-channel liquid handler (Nanoscreen), and allowed to proceed for 1 h at 25°C. The final reaction buffer contained 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 75 mM NaCl, 135 mM sucrose, 0.015% Brij 35, 1 mM DTT, 0.1 mg/mL BSA, 3 μM ATP, and 600 μM Kemptide. The final [ATP] in the reaction mix (3 μM) is at the K_m(ATP) for Aurora A/TPX2$^{1–43}$. At the final concentration of 40 nM, TPX2$^{1–43}$ is >10 times the concentration required to achieve half-maximal activation of Aurora A under these reaction conditions (3 nM) and the previously reported K_d of TPX2$^{1–43}$ [2.3 nM (70)]. Detection, measurement, and data analysis were performed as described above.

For the Aurora B/INCENP$^{783–918}$ assays, purified full-length human Aurora B/INCENP$^{783–918}$ (SignalChem) was diluted to ~0.5 nM (based on enzymatic activity) in 12 μL of a buffer containing 31.25 mM Tris (pH 7.5), 12.5 mM $MgCl_2$, 93.75 mM NaCl, 168.75 mM sucrose, 0.0125% Tween 20, 0.625 mM DTT, 0.1875 mg/mL BSA, and 500 μM Kemptide in Corning #3657 clear 384-well plates. Inhibitors arrayed in dose–response were added from DMSO stocks using a V&P 384-pintool head mounted on a Beckman Multimek chassis. After 15 min at 25°C, reactions were initiated via the addition of 3 μL of 50 μM ATP using a NSX-384 384-channel liquid handler (Nanoscreen), and allowed to proceed for 1 h at 25°C. The final reaction buffer contained 25 mM Tris (pH 7.5), 10 mM $MgCl_2$, 75 mM NaCl, 135 mM sucrose, 0.01% Tween 20, 0.5 mM DTT, 0.15 mg/mL BSA, 10 μM ATP, 400 μM Kemptide. The final [ATP] in the reaction mix (10 μM) is at the K_m(ATP) for Aurora B/INCENP$^{783–918}$. Detection, measurement and data analysis were performed as described above.

Radiometric assay-based kinome profiling of AZD1152-HQPA, GSK1070916, MK-5108 and MK-8745 was performed by Reaction Biology Corporation (Malvern, PA, USA) using [ATP] ~ K_m(ATP) for all enzymes.

Cell Lines

RPE1 (hTERT-immortalized RPE cells) and U2OS osteosarcoma cells were obtained from ATCC. HeLa cervical carcinoma cells were from a laboratory stock. RPE1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 plus glutamine medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. U2OS and HeLa cells were maintained in DMEM + Glutamax supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin.

For generation of HeLa, U2OS, and RPE1 lines co-expressing H2B-RFP and either GFP-PCNA or YFP-tubulin, cells were infected first with an H2B-RFP expressing retrovirus. A pBABE-puro vector, encoding human histone H2B with mRFP1.3 fused at its C-terminus (H2B-RFP) obtained from the laboratory of Don Cleveland, and pBSK-VSV-G were

co-transfected into the packaging cell line GP2-293 (Clontech) using FuGENE HD (Promega). Virus-containing culture supernatant was collected 48 h after transfection and added to the growth medium of cells, followed by addition of Polybrene (Millipore) to 8 μg/ml.

An MGC collection human PCNA cDNA with eGFP fused at its N-terminus (GFP-PCNA) was cloned into pBABE-hygro. A pBABE-bla (blasticidin) vector encoding human alpha 1B tubulin with eYFP fused to its N-terminus (YFP-tubulin) was obtained from the laboratory of Don Cleveland. Virus production and infection of cells previously transduced with H2B-RFP was performed similarly. FACS was used to select cell populations expressing transgenes at moderate levels.

RNAi

HeLa cells co-expressing GFP-PCNA and H2B-RFP were used for all imaging-based RNAi experiments. ON-TARGETplus SMARTpool siRNAs (GE Healthcare) targeting Aurora A and Aurora B, as well as a non-targeting control pool, were transfected into cells using Lipofectamine RNAiMAX (Thermo Fisher Scientific) at a final concentration of 50 μM in 6-well plates. Five hours after transfection, cells were trypsinized and seeded into a 96-well cycloolefin plate (Greiner) at 10,000 cells/well in fresh medium supplemented with 2.5 mM thymidine. Cells were incubated in the presence of thymidine for 18–20 h, and then 300 ng/mL nocodazole for 6 h. Knockdown was confirmed by immunoblotting with the Aurora kinase antibodies specified in Table S3 in Supplementary Material and an anti-tubulin antibody (DM1A; 1:1000; Sigma).

For fixed analysis to quantify pLATS2(Ser 83), pH3(Ser 10), and pH3(Ser 28) intensities, plates were washed twice with fresh medium and returned to the incubator for 8 h. Cells were then fixed with either 4% PFA = paraformaldehyde (in phosphate-buffered saline, PBS) (for pH3 analysis) or 100% ice-cold methanol (for pLATS2 analysis). The following primary antibodies were used: pLATS2(Ser 83) (see Table S3 in Supplementary Material); pH3(Ser 10) (1:100; Cell Signaling); pH3(Ser 28) (see Table S3 in Supplementary Material). Cells were imaged on the CV7000 spinning disk confocal system (Yokogawa Electric Corporation) using a 40 × 0.95 NA U-PlanApo objective and 2560 × 2160 sCMOS camera with 2 × 2 binning. 5 μm × 2 μm z-sections of 50 fields/well were imaged, with replicate wells per RNAi condition.

For quantification, maximum intensity projections were generated by the CV7000 acquisition software and transferred to ImageJ for analysis. For pLATS2(Ser 83) measurements, the integrated signal from a 10 × 10 pixel box centered on each mitotic spindle pole was measured. For background subtraction, a 10 × 10 pixel box in the cytoplasm was used. Mean values of measurements were normalized to the control RNAi condition. A total of 186–230 measurements from two independent experiments were made. For pH3(Ser 10) and pH3(Ser 28), the DNA signal was used to threshold and define a binary mask, which was transferred to the pH3 channel. The mean intensity of this region was then measured in the pH3 channel. For background subtraction, the masked region was expanded by 20 pixels, and the mean intensity of the peripheral region was used. Mean values of measurements were normalized to the DMSO-treated condition. A total of 88–147 measurements from three independent experiments were made.

For live imaging experiments to measure G2 duration, plates were washed twice with fresh medium, and immediately mounted onto the CV1000 spinning disk confocal system (Yokogawa Electric Corporation). The imaging chamber was maintained at 37°C and 5% CO_2. Cells were imaged using a 20 × 0.75 NA U-PlanApo objective and 512 × 512 EM-CCD camera with 2 × 2 binning. Twelve fields/well were imaged, with 4 replicate wells per RNAi condition. 3 μm × 2 μm z-sections in the GFP (25% power, 200 ms, 35% gain) and RFP (20% power, 200 ms, 35% gain) channels were captured in each field, at 12-min intervals for 24 h. Quantification was performed as described in the G2 duration assay section (see below).

Cellular Proliferation Analysis

Eight thousand HeLa cells, 8,000 U2OS cells or 4,000 RPE1 cells were seeded into white 96-well assay plates (Corning #3610) 16 h before inhibitor addition. All inhibitors were diluted in DMSO and added to cells in complete growth media (2× desired concentrations were prepared in complete growth medium and added to wells). After 24 h, relative cell number was measured using ATPLite™ reagent (PerkinElmer) following the manufacturer's instructions. DMSO-treated cells were used as controls. Two independent experiments with triplicate measurements per condition were performed. Luminescence was measured on an Infinite M1000 plate reader (Tecan).

Live Cell-Activated Caspase 3/7 Assay

HeLa cells (6,000/well) were seeded in 96-well μCLEAR plates (Greiner) in 100 μL DMEM plus serum, and incubated for 16 h at 37°C and 5% CO_2. MK-5108 and Genentech Aurora Inhibitor 1 were diluted 1:100 from DMSO stocks into serum-free DMEM and 11 μL of the diluted compound was added to cells. After 24 h 2 μM CellEvent Caspase-3/7 Green Reagent (Life Technologies), and NucBlue Live ReadyProbes Reagent (Hoechst 33342; Life Technologies) were added. Cells were imaged after 60 min on a CV7000 spinning disk confocal system (Yokogawa Electric Corporation) with a 20 × 0.75 NA U-PlanApo objective and 2560 × 2160 sCMOS camera with 2 × 2 binning. The imaging chamber was maintained at 37°C and 5% CO_2. Six to eight fields/well were imaged, with duplicate wells for each condition. 3 μm × 2 μm z-sections in the blue (40% power, 300 ms, 35% gain) and green (40% power, 300 ms, 35% gain) channels were captured in each field. The apoptotic fraction was calculated by dividing the number of cells fluorescing at 530 nm (corresponding to the cleaved caspase reporter reagent) by the number of nuclei (Hoechst staining). Image analysis was done using the CV7000 image analysis software (Yokogawa Electric Corporation).

Substrate Phosphorylation Assay

Twelve thousand HeLa cells, 10,000 U2OS cells, or 8,000 RPE1 cells were seeded into 96-well glass-bottom Sensoplates (Greiner) 16 h before inhibitor addition. Prior to seeding, the glass-bottom plates were coated with poly-L-lysine (Sigma). All inhibitors were diluted in DMSO and added to cells in complete growth media (2× desired concentrations were prepared in complete growth

medium and added to wells). After 8 h cells were fixed with 4% PFA for 20 min at room temperature. The fixed cells were washed with PBS. For immunostaining, cells were permeabilized and blocked with PBS containing 10% normal donkey serum (Jackson ImmunoResearch) and 0.1% Triton-X100 for 1 h at room temperature. Primary antibodies against phospho-LATS2 (Ser 83), phospho-histone H3(Ser 28), and anti-phospho-MPM2 (see Table S3 in Supplementary Material) were incubated for 1 h at room temperature. Cells were stained with Cy3-conjugated goat anti-rat, Alexa Fluor 488-conjugated goat anti-mouse IgG2b, and Alexa Fluor 647-conjugated goat anti-mouse IgG1 secondary antibodies (see Table S3 in Supplementary Material) and Hoechst 33342 for 1 h at room temperature. Cells were then washed twice with PBS containing 0.1% Triton-X100. Image acquisition in four channels was performed using a CV7000 spinning disk confocal system (Yokogawa Electric Corporation) with a 40 × 0.95 NA U-PlanApo objective and 2560 × 2160 pixel sCMOS camera. Fluorophores (Hoechst 33342, Alexa Fluor 488, Cy3 and Alexa Fluor 647) were excited with 50% laser power for 300 ms and maximum projections of 8–14 μm × 1 μm z-sections were recorded. Fifty fields per well were imaged with quadruplicate wells for each condition. Image analysis was done using the CV7000 image analysis software (Yokogawa Electric Corporation). Between 100 and 1,000 mitotic cells per condition were segmented applying object identification parameters to select for bright MPM2 labeling. Using a nuclear identifier protocol, minimum intensity thresholds were set for pLATS2(Ser 83) and the pH3(Ser 28) signals, and the resulting identified objects were eroded, dilated, and filtered for size by user-defined thresholds. For only the MPM2-positive mitotic cells, the mean fluorescence intensity of the identified pLATS2(Ser 83) and pH3(Ser 28) objects was measured, and the average intensity per cell per well was calculated. The same thresholds were applied for all of the inhibitor-treated samples, which were processed, imaged, and analyzed in parallel with control DMSO-treated cells. Data were fit using a four-parameter, variable slope fit in Prism (GraphPad). Primary and secondary antibody dilutions can be found in Table S3 in Supplementary Material.

G2 Duration Assay

HeLa, U2OS, and RPE1 cells co-expressing GFP-PCNA and H2B-RFP were seeded into 96-well glass bottom Sensoplates (Greiner) at 10,000 cells/well 16 h before inhibitor addition. Prior to seeding, glass-bottom plates were coated with poly-L-lysine (Sigma). All inhibitors were diluted in DMSO and added to cells in complete growth media (2× desired concentrations were prepared in complete growth medium and added to wells). Movies were acquired on a CV1000 spinning disk confocal system (Yokogawa Electric Corporation) with a 20× U-PlanApo 0.75 NA objective and 512 × 512 EM-CCD camera with 2 × 2 binning. The humidity controlled imaging chamber was maintained at 37°C and 5% CO_2. Three fields per well were imaged, with duplicate wells for each condition. 3 μm × 2 μm z-sections in the GFP (25% power, 100 ms, 20% gain) and RFP (20% power, 100 ms, 20% gain) channels were captured in each field at 12-min intervals for 24 h. Cells were manually tracked from appearance of GFP-PCNA foci to the beginning of the next mitosis (NEBD). GFP-PCNA foci appear in the nucleus during mid to late S-phase, and the first frame in which these foci are no longer visible was defined as the beginning of G2 phase. Results represent combined measurements of 40-100 cells per condition from two independent experiments. Data were fit using a 4-parameter, variable slope fit in Prism (GraphPad).

Cytokinesis Assay

HeLa, U2OS, and RPE1 cells co-expressing YFP-α-tubulin and H2B-RFP were seeded into 96-well glass-bottom Sensoplates (Greiner) at 8,000 cells/well 16 h before inhibitor addition. Prior to seeding, glass-bottom plates were coated with poly-L-lysine (Sigma). All inhibitors were diluted in DMSO and added to cells in complete growth media (2× desired concentrations were prepared in complete growth medium and added to wells). Movies were acquired on a CV1000 spinning disk confocal system (Yokogawa Electric Corporation) with a 40× U-PlanApo 0.95 NA objective and 512 × 512 EM-CCD camera. The humidity controlled imaging chamber was maintained at 37°C and 5% CO_2. Eight fields per well were imaged, with duplicate wells for each condition. 5 μm × 2 μm z-sections in the YFP (25% power, 100 ms, 20% gain) and RFP (20% power, 100 ms, 20% gain) channels were captured in each field at 5-min intervals for 24 h. Cells were manually tracked from mitosis to G1, and the appearance of microtubule midbodies and mono/binucleated daughter cells were analyzed to assess cytokinesis success. Results represent combined measurements of 50-100 cells per condition from two independent experiments. Data were fit using a 4-parameter, variable slope fit in Prism (GraphPad).

Western Blot Analysis

For Aurora A inhibitors, HeLa cells were seeded into 10 cm dishes and treated with 100 nM taxol and DMSO or compounds in dose–response for 16 h. Cells were harvested at 50–80% confluence and lysed in RIPA buffer supplemented with a protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific) using a Qsonica Q800R sonicator (10 min, 50% amplitude, 15 s on/15 s off). Before loading, concentrations of cleared extracts were normalized using a Bio-Rad Protein Assay (Bio-Rad). For every sample, 25–50 μg protein per lane was run on Mini-PROTEAN gels (Bio-Rad) and transferred to PVDF membranes using a TransBlot Turbo system (Bio-Rad). For primary anti-phospho-Histone H3(Ser 10), anti-phospho-Histone H3(Ser 28), anti-Aurora A, anti-Aurora B, anti-phospho-Aurora A(Thr 288)/Aurora B(Thr 232)/Aurora C(Thr 198), anti-Histone H3, anti-Cyclin B1, and anti-phospho-Aurora A (Thr 288) antibodies (see Table S3 in Supplementary Material), blocking and incubations were performed in TBS-Tween with 5% BSA or non-fat dry milk. Detection was performed using HRP-conjugated secondary antibodies (see Table S3 in Supplementary Material), with SuperSignal West Femto (Thermo Fisher Scientific) substrates. Membranes were imaged on a ChemiDoc MP system (Bio-Rad).

Aurora B inhibitor analysis was done as described above with the following adaptations: seeded HeLa cells were synchronized using a 2.5 mM double-thymidine block. Eight hours after release, cells were treated with 10 μM MG132 and DMSO or compound in dose–response for 3 h. After PBS washing, treated cells were harvested with sample buffer, and the total cell lysate was heated

for 5 min at 95°C before sonication. Primary anti-phospho Histone H3(Ser 10), anti-phospho-Histone H3 (Ser 28), anti-Histone H3, anti-phospho-Aurora A(Thr 288)/Aurora B(Thr 232)/Aurora C(Thr 198), anti-Aurora B, and anti-Cyclin B1 antibodies were incubated and detected as described above. Primary and secondary antibody dilutions can be found in Table S3 in Supplementary Material.

Aurora B Transcript Variant Analysis

HeLa total cellular RNA was prepared using RNeasy (Qiagen) according to the manufacturer's instructions. Random primer-based cDNA synthesis was performed with MultiScribe reverse transcriptase (Applied Biosystems) from 500 ng RNA (20 µL reaction volume, 10 min at 25°C, 120 min at 37°C, 5 min at 85°C). The cDNA was diluted 1:5, and 10 µl was used in a 50 µl PCR reaction with Q5 DNA polymerase (New England Biolabs) and the following primers: GGTCATTTGTAGCCACATCCTGTC (specific to human Aurora B transcript 5; nucleotides 108–131 of RefSeq NM_001313951) and GCATCTGCCAACTCCTCCATGATC (universal primer for human Aurora B transcripts; nucleotides 687–664 of RefSeq NM_001313951). The PCR amplification conditions were (10 s at 98°C, 30 s at 69°C, 30 s at 72°C, 35 cycles). Reaction products were visualized by fluorescence on a 3% NuSieve GTG agarose gel. Identical PCR conditions were used for amplification with T7 and SP6 promoter sequences appended to the primers for direct sequencing after gel purification.

Crystal Structure of Aurora A Bound to MK-5108

The kinase domain of human Aurora A (amino acids 123-390) was cloned into pET28a with an N-terminal 6XHis tag and an intervening rhinovirus 3C protease cleavage site. The protein was expressed in *E. coli* BL21 Rosetta 2(DE3) cells (Novagen) at 16°C overnight. Cells were harvested by centrifugation, resuspended in a buffer containing 50 mM Tris (pH 8.0), 300 mM NaCl, 40 mM imidazole, 20 mM $MgCl_2$, 10% glycerol, 0.5 mM TCEP, and an EDTA-free protease inhibitor cocktail, and lysed using a microfluidizer. After clarification via centrifugation, the lysate was loaded onto a HisTrap HP column (GE Healthcare), and the bound protein was eluted in a buffer containing 50 mM Tris (pH 8.0), 300 mM NaCl, 200 mM imidazole, 20 mM $MgCl_2$, 10% glycerol, and 0.5 mM TCEP. The tag was cleaved with Turbo3C protease (ETON) overnight at 4°C while being dialyzed against a buffer containing 20 mM Tris (pH 7.0), 200 mM NaCl, 20 mM $MgCl_2$, 10% glycerol, and 0.5 mM TCEP. Since both the 6× His-tagged and untagged species bind metal affinity resins in this buffer, the cleavage reaction was loaded onto a HisTrap HP column and the untagged protein was selectively eluted in a buffer containing 20 mM Tris (pH 7.0), 200 mM NaCl, 40 mM imidazole, 20 mM $MgCl_2$, 10% glycerol, and 0.5 mM TCEP. Trace amounts of the Turbo3C protease were removed using a GSTrap HP column (GE Healthcare). The untagged protein was further purified using size exclusion chromatography on a Superdex 75 16/600 column (GE Healthcare). The final eluate [in 20 mM Tris (pH 7.0), 200 mM NaCl, 20 mM $MgCl_2$, 10% glycerol, and 0.5 mM TCEP] was concentrated to 6.2 mg/mL using Amicon Ultra 10K MWCO concentrators (Millipore), and MK-5108 was added from a 50 mM DMSO stock to a final concentration of 500 µM.

The inhibitor bound protein was crystallized by hanging drop vapor diffusion using a reservoir buffer consisting of 100 mM BisTris (pH 6.5), 30% PEG3350 at 21°C. A total of 1.5 µL protein solution was mixed with 1.5 µL reservoir buffer and sealed in a chamber containing 400 µL of reservoir solution. After 1 week, a rod-shaped crystal (~100 µm × 5 µm × 5 µm) was transferred to a cryoprotectant containing 100 mM BisTris (pH 6.5), 200 mM NaCl, 20 mM $MgCl_2$, 25% PEG3350, 10% glycerol, 30 µM MK-5108, and flash-frozen in liquid nitrogen.

X-ray diffraction data were measured using Beamline 7-1 at the Stanford Synchrotron Radiation Lightsource and processed with HKL2000 (115). The structure was determined by molecular replacement using PHASER (116) and sequential searches with the large and then the small lobes of an ensemble model (PDB: 1MQ4, 2J4Z, 3FDN, 3LAU, 4UYN). Refinement was performed using PHENIX (117) interspersed with iterative cycles of rebuilding using Moloc (118). Figures were made using PyMol (Schrödinger).

AUTHOR CONTRIBUTIONS

JH, MY, and HL performed biochemical experiments to determine inhibitor potencies and selectivities. CdG, JA, YW, and RD performed cell-based experiments to determine inhibitor potencies and selectivities. CdG and DJ assessed inhibitor effects on cellular proliferation and apoptosis. DJ performed Aurora B and C expression analysis. MM and RD performed PCR analysis of Aurora B transcript variants. AM and AS determined the structure of Aurora A bound to MK-5108. TG supplied the inhibitors and arranged for kinome profiling. CdG, YW, AD, TG, and AS conceived and designed experiments. AD and AS wrote the manuscript with input from CdG and all other authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fonc.2015.00285

MOVIE S1 | Timelapse imaging of HeLa cells stably expressing GFP-PCNA; H2B-RFP. Images were acquired at 12-min intervals; playback rate is 10 frames per second (7200 × real time).

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METHODS
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Phenotypic Screening Approaches to Develop Aurora Kinase Inhibitors: Drug Discovery Perspectives

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Targeting mitotic regulators as a strategy to fight cancer implies the development of drugs against key proteins, such as Aurora-A and -B. Current drugs, which target mitosis through a general mechanism of action (stabilization/destabilization of microtubules), have several side effects (neutropenia, alopecia, and emesis). Pharmaceutical companies aim at avoiding these unwanted effects by generating improved and selective drugs that increase the quality of life of the patients. However, the development of these drugs is an ambitious task that involves testing thousands of compounds through biochemical and cell-based assays. In addition, molecules usually target complex biological processes, involving several proteins and different molecular pathways, further emphasizing the need for high-throughput screening techniques and multiplexing technologies in order to identify drugs with the desired phenotype. We will briefly describe two multiplexing technologies [high-content imaging (HCI) and flow cytometry] and two key processes for drug discovery research (assay development and validation) following our own published industry quality standards. We will further focus on HCI as a useful tool for phenotypic screening and will provide a concrete example of HCI assay to detect Aurora-A or -B selective inhibitors discriminating the off-target effects related to the inhibition of other cell cycle or non-cell cycle key regulators. Finally, we will describe other assays that can help to characterize the *in vitro* pharmacology of the inhibitors.

Keywords: high-content imaging, assay development, cell cycle, pH3S10, Aurora kinases

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INTRODUCTION

Aurora-A and Aurora-B are two serine threonine kinases that regulate cell cycle progression from G2 through to cytokinesis in a coordinated manner even though their localization and activation timing during the cell cycle varies. Aurora-A is required for mitotic entry, centrosome maturation and separation, and chromosome alignment (1), whereas Aurora-B is involved in chromosome condensation, segregation, and cytokinesis by regulating microtubule kinetochore associations (2). Inhibition of any of these two kinases will produce a different phenotype, while Aurora-A inhibition delays mitotic entry and progression and accumulates cells in G2/M phase (3, 4), Aurora-B inhibition prevents proper alignment of chromosomes to the spindle plate, inhibits cytokinesis, and results in the formation of multinucleated cells (5). However, the fact that human Aurora-A and -B share 71% identity in its carboxy-terminal catalytic domain is critical for evaluating the specificity of inhibitors (6).

Classic antimitotic drugs such as spindle poisons (e.g., taxanes and vinka alkaloids) prevent microtubule dynamics. Since microtubules, besides its mitotic role, are also necessary for multiple cellular functions during interphase, the use of these drugs is associated with side-effects such as neurotoxicity that can lead to irreversible neuropathologies (7). As opposed to previous molecules, inhibitors against specific therapeutical targets and focused on patients with specific characteristics (patient tailoring) need to be discovered. The pharmaceutical industry is evolving to fulfill this need, a tendency that can be observed in the development of CDK inhibitors. With approximately 14 molecules in clinical trials, the first generation molecules are often pan-CDK inhibitors (e.g., flavopiridol), whereas the more recent molecules tend to focus in specific CDKs (e.g., palbociclib and abemaciclib against CDK4/6) (8). The same concept applies for molecules against Aurora kinases, where most clinical trials have focused on Aurora-A selective agents (53%) as opposed to Pan-Aurora (32%) and Aurora-B-specific compounds (15%) (9).

In cancer treatment, there are mainly two approaches to inhibit a target: small molecules (e.g., gemcitabine against lung and pancreatic cancer) and large molecules (e.g., trastuzumab against ERBB2-overexpressing/amplified tumors). Large molecules include recombinant proteins and monoclonal antibodies and are often referred to as "biotech" drugs. (10). Whereas large molecules tend to be administered intravenously, small molecules usually allow easier administration (oral) but tend to be less selective. To discover a small molecule against a new target, pharmaceutical companies usually test thousands of compounds through biochemical assays, followed by a reduced number of compounds through cell-based assays and an even minor quantity through *in vivo* assays. Testing such an amount of compounds rapidly required the development of automation platforms and other technologies that allow the use of high-throughput screening (HTS) techniques. Usually, the molecular targets for cancer therapy are involved in complex biological processes and they interact with others from the same or even different molecular pathways. This adds a degree of difficulty to drug discovery in general and to assay development in particular. All of the above highlights the need for multiplexing technologies that allow for the evaluation of several readouts in the same experiment. Both, on-target and off-target effects will indicate the selectivity of the compounds, which ultimately, together with oral administration and safety profile, are the main desirable properties of a final drug candidate.

MULTIPLEXING TECHNOLOGIES

Singleplex technologies such as cell viability assays fall short in guaranteeing that the observed cellular effect upon compound treatment is due to inhibiting the target of interest. Off-target effects could create false positives and considering the challenge of selective compound properties, new technologies to monitor phenotypic changes associated with target inhibition are required. High-content imaging (HCI) and flow cytometry are two of the most commonly used techniques.

High-Content Imaging

Also called high-content screening, HCI is a technique where a few hundred or a few thousand perturbagens (compounds, drugs, siRNAs, and cDNAs) are tested and scores of parameters are recorded from each individual cell using multiple imaging channels. The readouts can be kinetic and single endpoint using live and fixed cells, respectively (11).

The technology is based on obtaining one or several images of every sample, usually placed in wells of 96-well, 384-well, or even 1536-well microplates to achieve high throughput. For that purpose, two major types of detectors can be used: digital cameras and photomultiplier tubes (PMTs). The images can later be analyzed and managed by using specific software that usually comes with the instrument.

The assay type is an immunocytofluorescence assay and the selection of the proper antibody that recognizes the protein of interest is of importance. Usually, a secondary antibody is used to increase specificity and amplify the signal. These secondary antibodies are conjugated with fluorescent dyes that have a wide variety of absorption and emission wavelengths, allowing multiplexing while minimizing overlapping spectra (e.g., Alexa Fluor®).

There are basically three types of instruments according to the detection technology used: wide field imagers (often built around inverted research microscopes), confocal HCA imagers (confocal microscopes, preferred for live cell imaging and best used for imaging small intra-cellular structures, small cells, complex 3-D structures and samples with strong background fluorescence), and laser scanning cytometers (conceptually similar to a flatbed scanner with laser beams scanned across the entire surface of the plate and fluorescence detected with PMTs, good at detecting cells but not subcellular features or processes) (11).

Flow Cytometry

This technique goes back to the invention of the first devices based on the Coulter principle to sort cell populations (12). Nowadays, fluorescence-based methods are used for the detection of biomarkers, cell counting, and sorting.

One of the key principles of flow cytometry is a process called hydrodynamic focusing. Basically, the fluidics system of the machine allows it to order the sample in solution that has been injected (where particles are randomly distributed in three-dimensional space) into a stream of single particles that can be interrogated by the detection system. Subsequently, each particle passes through one or more beams of light. Light scattering or fluorescence emission provides information about the particle's properties. The laser and the arc lamp are the most commonly used light sources in modern flow cytometry (13).

With the possibility to analyze single cell events out of cellular aggregates or clusters, flow cytometry overcomes one of the main disadvantages of HCI. However, working with formats such as 384-well plates in flow cytometry is more complex than in HCI, requiring additional optimization to improve signal homogeneity and reading time.

A wide array of phenotypic changes can be chosen as readout: changes in morphology, protein translocation and expression,

alteration of the phosphorylation status of proteins, changes in DNA content (e.g., Aur B inhibition usually leads to endoreplication and an increase of cells with 8N and beyond), and epigenetic modifications (e.g., H3K27). Both, HCI and flow cytometry allow for the development of novel cell-based assays to define the *in vitro* efficacy of new molecules. Through a combination of the multiple readouts mentioned above, we can detect both on-target and off-target effects of the drug and can evaluate the most appropriate concentration of a drug and the accurate cellular exposure to achieve the desired phenotype. These parameters are of importance for the design of the *in vivo* dosing schedule and eventually for a successful drug discovery process.

IMPLEMENTATION, OPTIMIZATION, AND VALIDATION OF HIGH-THROUGHPUT ASSAYS

Assay Development

When setting up a cell-based assay for screening for the first time, there are several steps that should be followed [**Figure 1**; (11)]. One of the first steps is to select the cell lines for the study. Through the use of internal or external cell sensitivity panels and published scientific articles, cell lines with an anticipated response to the target of interest (e.g., Aurora kinases) could be identified. This will not only help to select the most appropriate cell lines for the assay but also open the possibility to study the genetic background of those cell lines to establish a connection with target inhibition leading to a proper patient-tailoring strategy. As a counterscreening for toxicity, a non-sensitive cell line can be selected or other readouts can be added to the sensitive cell lines (for example, apoptosis or senescence) to confirm the cause of cell proliferation inhibition.

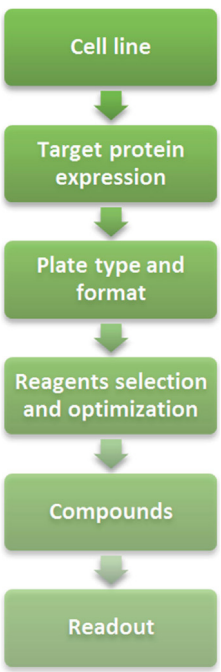

FIGURE 1 | Assay development flow chart.

Another parameter that would need to be considered is if and how the target protein is expressed in the cellular model of interest. This will provide an idea of the possible readouts for the assay: monitor directly changes in protein expression, phosphorylation, or location; surrogate readouts such as phenotypic changes; or even a combination of both.

Once the cell lines have been selected, according to the expected throughput of the assay generally either 96-well or 384-well plates will be chosen to seed the cells. Growth conditions and the appropriate cell seeding density will also need to be determined. Clear bottoms are required in these wells, so the lasers can excite the sample. If using poorly adherent cells, it is useful to plate the cells in wells coated with extracellular matrix components (e.g., poly-D-lysine, collagen, etc.).

Fixing and staining steps are quite similar to those of an immunofluorescence assay. The reagents used can be optimized according to the cell line and antibody that have been selected. Reagents to be optimized include salt-based solutions (Hank's balanced salt solution, phosphate-buffered saline, and Tris-buffered saline), fixatives (formaldehyde, methanol, or other non-toxic fixative reagents), permeabilization buffers (salt solutions or water containing detergents, such as Triton X-100, Tween-20, SDS, and NP-40), blocking buffers (BSA, milk, and FBS), and the antibodies (concentration).

It is critical which type of compounds will be used (agonists, antagonists or both) and whether there is an available reference compound. Moreover, the DMSO tolerance of the cells and the period of time that the cells will be incubated with the compounds are important factors for the assay. The treatment duration depends on the type of response and the doubling time of the cell line used, e.g., changes in phosphorylation usually can be monitored within hours whereas changes in DNA content will require more time.

The number of lasers and detection channels available in the HCI instrument is essential for the readouts for the assay. These instruments will allow the use of DAPI/Hoechst or propidium iodide to stain the nuclei and different secondary antibodies.

Setting Up a Flow Scheme

Biochemical assays are an easy way to rapidly evaluate thousands of molecules and select a reduced number of molecules for their further characterization in cell-based assays.

Basically, several biochemical assays are set up for the enzymes of interest and others closely related, either from the same family (Aurora-A and -B) or involved in the same pathway (CDKs, PLK1, etc.). Usually, the inhibition of the latter ones should be avoided to ensure that the phenotypic outcome of the cell-based assay is due to inhibition of the target of interest (Aurora). Several techniques can be used to monitor biochemically the effect produced by the compounds: radioactivity, fluorescence, luminescence, mass spectrometry, etc. These assays will allow for the selection of the best molecules according to potency and selectivity to be tested in the cell-based assays.

Both, biochemical and cell-based assays, along with novel biophysical techniques, are used to evaluate structure–activity

relationship (SAR) and design improved versions of the molecule. The more advanced molecules would be studied in-depth by testing several cell lines to confirm a link between genetic background and drug sensitivity. Drug combination with standards of care could also be addressed. Finally, the molecules will be tested *in vivo* to confirm the efficacy, and to evaluate whether they will proceed to clinical studies.

Thus, the flow scheme determines the different stages the compounds will go through before determining a candidate molecule for clinical trials. The assays included in the flow scheme need to be biologically significant for the targeted disease and the different stages need to show a desirable degree of connectivity.

Assay Validation

Due to the high number of compounds to be evaluated, the assays need to be reproducible overtime and independently of the operator performing the assay. For that reason, there is a clear need for strict assay validation criteria that assure high quality data. When validating a new assay, we will require two different types of validation assays: a 3-day plate uniformity study and a replicate-experiment study.

Plate Uniformity

Uniformity assays are performed at the maximum and minimum signal or response levels to ensure that the signal window is adequate to detect active compounds during the screen. Therefore, the variability tests are conducted on three types of signals: "Max" signal (the maximum signal as determined by the assay design), "Min" signal (the background signal as determined by the assays design), and also "Mid" signal (this parameter estimates the signal variability at some point between the maximum and minimum signals).

Two different plate formats exist for the plate uniformity studies: interleaved-signal format – where all signals are on all plates but varied systematically, so that on all plates, on a given day, each signal is measured in each plate; and concentration–response curve plate format – where a reference compound is tested at multiple concentrations with production control wells (Max and Min, **Figure 2A**). The last one also includes uniform signal plates for "Max" (**Figure 2B**) and "Min" (**Figure 2C**) where each signal is run uniformly instead of the concentration–response curve for the reference compound. In both cases, the recommended acceptance criterion is Z' factor ≥ 0.4 (which is comparable to a Signal Window ≥ 2), coefficient of variation <20%, absence of edge, drift or other spatial effects, and minimum significant ratio <3 or the normalized average Mid-signal should not translate into a twofold shift (within days or across any 2 days).

Replicate-Experiment Study

Replicate-experiment studies are used to formally evaluate the within-run assay variability and are a diagnostic and decision tool used to establish that the assay is ready to go into production by showing that the endpoints of the assay are reproducible over a range of potencies.

The analysis approach used in the replicate-experiment study is to estimate and factor out between-run variability, and then

A

Min	CRC1	CRC1	CRC1	CRC1	CRC1	CRC1	CRC1	CRC1	CRC1	CRC1	Max
Min	CRC2	CRC2	CRC2	CRC2	CRC2	CRC2	CRC2	CRC2	CRC2	CRC2	Max
Min	CRC3	CRC3	CRC3	CRC3	CRC3	CRC3	CRC3	CRC3	CRC3	CRC3	Max
Min	CRC4	CRC4	CRC4	CRC4	CRC4	CRC4	CRC4	CRC4	CRC4	CRC4	Max
Min	CRC5	CRC5	CRC5	CRC5	CRC5	CRC5	CRC5	CRC5	CRC5	CRC5	Max
Min	CRC6	CRC6	CRC6	CRC6	CRC6	CRC6	CRC6	CRC6	CRC6	CRC6	Max
Min	CRC7	CRC7	CRC7	CRC7	CRC7	CRC7	CRC7	CRC7	CRC7	CRC7	Max
Min	CRC8	CRC8	CRC8	CRC8	CRC8	CRC8	CRC8	CRC8	CRC8	CRC8	Max

B

Min	Max	Max	Max	Max	Max	Max	Max	Max	Max	Max	Max
Min	Max	Max	Max	Max	Max	Max	Max	Max	Max	Max	Max
Min	Max	Max	Max	Max	Max	Max	Max	Max	Max	Max	Max
Min	Max	Max	Max	Max	Max	Max	Max	Max	Max	Max	Max
Min	Max	Max	Max	Max	Max	Max	Max	Max	Max	Max	Max
Min	Max	Max	Max	Max	Max	Max	Max	Max	Max	Max	Max
Min	Max	Max	Max	Max	Max	Max	Max	Max	Max	Max	Max
Min	Max	Max	Max	Max	Max	Max	Max	Max	Max	Max	Max

C

Min	Min	Min	Min	Min	Min	Min	Min	Min	Min	Min	Max
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FIGURE 2 | Example of concentration–response curve plate format for plate uniformity assessment (96-well plates). **(A)** CRC plate. **(B)** "Max" plate. **(C)** "Min" plate.

estimate the magnitude of within-run variability. The procedure has mainly three steps:

1. Select 20–30 compounds that have potencies covering the concentration range being tested and, if applicable, efficacy measures that cover the range of interest. The compounds should be well spaced over these ranges.
2. Run all compounds in each of two runs of the assay.
3. Compare the two runs. A series of statistical parameters will be calculated (mean-ratio, ratio limits, minimum significant ratio, and limits of agreement). MSR should be <3 and both limits of agreement should be between 0.33 and 3.0.

After successfully passing both validation studies (plate uniformity and replicate experiment), the assay is ready and the different libraries of compounds can be tested.

For a more comprehensive explanation on HTS assay validation, please refer to Iversen et al. (14).

PRACTICAL EXAMPLES APPLIED TO AURORA INHIBITORS

Development of pH3S10 and PI Multiplexing Assay

To evaluate Aurora-A or -B phenotype for different libraries of compounds, a multiplexing assay monitoring pH3S10 and DNA content was developed.

Cell Model

HeLa cells (ATCC# CCL-2) are epithelial cells isolated from cervix adenocarcinoma. These cells were selected based on their

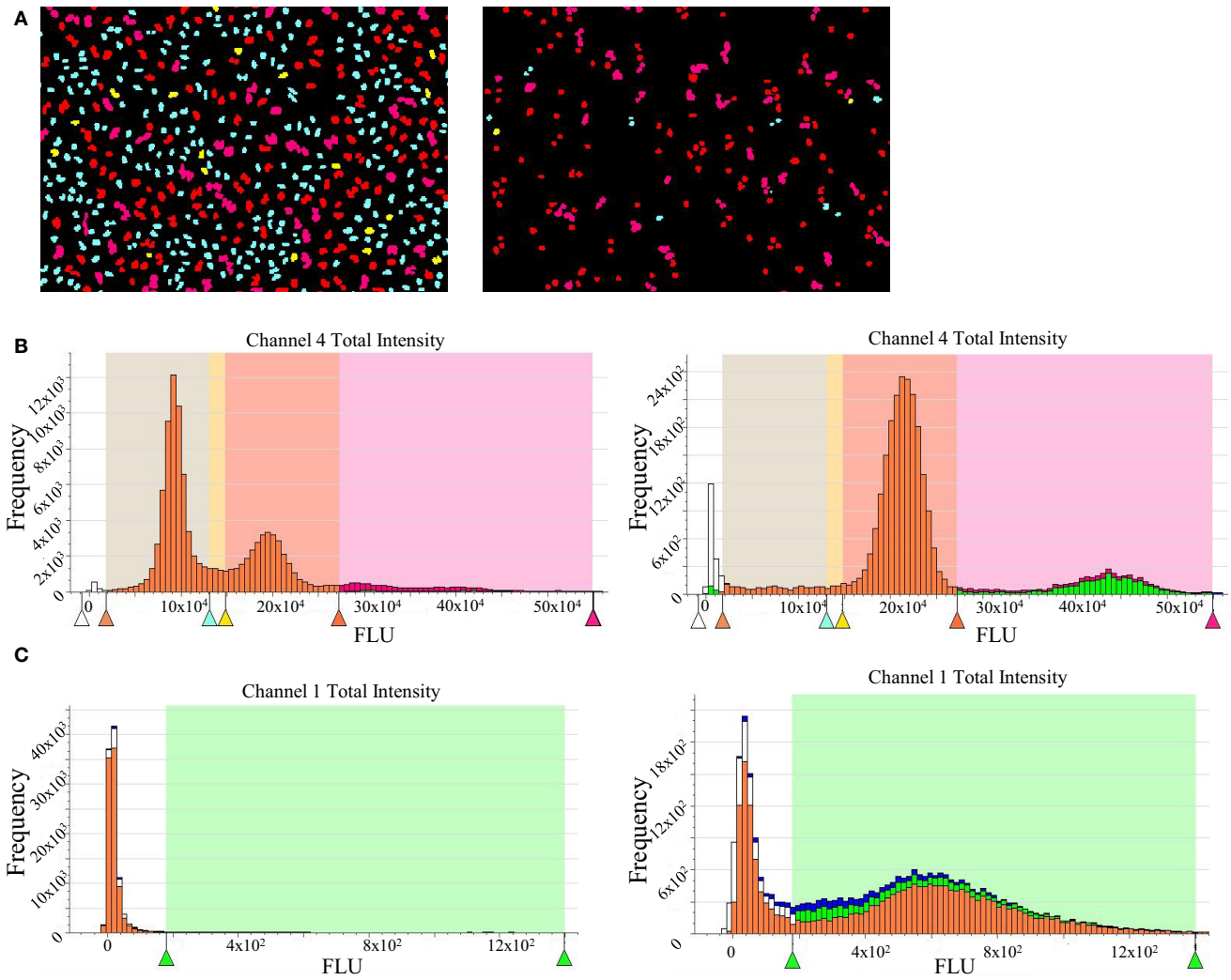

FIGURE 3 | High-content imaging data for propidium iodide staining and pH3 detection. **(A)** Acumen screenshots of untreated (left) and nocodazole-treated HeLa cells (right). **(B)** Histograms showing cell cycle distribution generated after raw data processing from untreated (left) and nocodazole-treated cells (right). The G1 subpopulation is in cyan blue; S is represented in yellow, G2M phase in red, and cells with >4N DNA content are in pink. **(C)** Histogram showing pH3 signal after raw data processing from untreated (left) and nocodazole-treated cells (right).

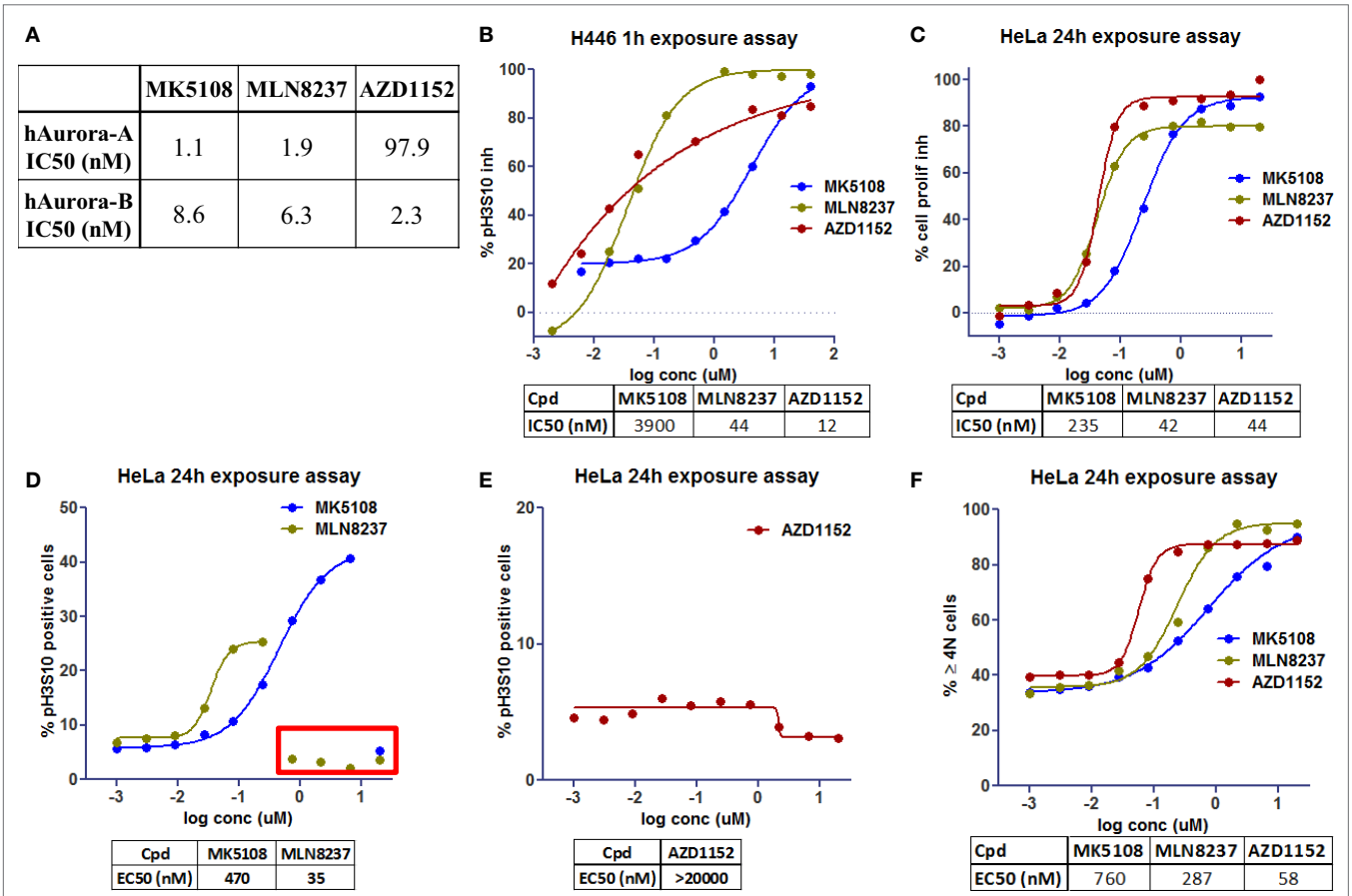

FIGURE 4 | Dose–response curves for Aurora kinases inhibitors in clinical trials [modified from Ref. (18)]. **(A)** Inhibition of recombinant human Aurora-A and -B in biochemical assays. **(B)** Inhibition profile for pH3S10 after 1h of exposure (NCI-H446 cells). **(C)** Inhibition profile for cell proliferation after 24 h of exposure (HeLa cells). **(D,E)** Accumulation profile for pH3S10 after 24 h of exposure (HeLa cells). **(F)** Accumulation profile for cells with 4N and >4N DNA content after 24 h of exposure (HeLa cells).

morphology for the imaging assay and because of the selected target of interest.

The optimal cell seeding density was evaluated and 5000 cells per well (96-well plates) was chosen as it produces a strong enough signal while cells remain well separated to allow single cell identification. To avoid loss of responsiveness, cells with as low passage number as possible were used and never exceeding a passage of 20. Cells were plated 18–24 h prior to compound dosing and were incubated at 37°C with 5% CO_2.

Compound Treatment

DMSO tolerance experiments determined that 0.25% DMSO should not be exceeded. As the cell doubling time is around 20 h, 24 h of incubation with compounds was chosen as an appropriate dosing time that would allow monitoring changes in mitotic index.

To perform compounds dose–response titration, threefold serial dilutions (in complete growth media containing 0.75% DMSO) were carried out in 96-well plates. This created a 10-point curve starting from 20 µM (final concentration in the assay). Then, 50 µL of compound solution was transferred from a dilution plate onto a cell plate containing 100 µL of culture media.

Assay Performance

HeLa cells were incubated with compounds for 24 h at 37°C/5% CO_2, fixed with Prefer (Anatech) for 30 min at room temperature, and permeated with 0.1% Triton X-100 in PBS for 15 min. After a couple of washing steps with PBS, cells were blocked with 1% BSA in PBS. Then, the blocking solution was removed and the primary antibody solution [rabbit anti-phospho-histone H3 (ser10), Millipore] was added to the cells (1:1000 in 1% BSA in PBS) and they were incubated overnight at 4°C with a gentle shake. Next day, the primary antibody was washed away with PBS and cells were treated with 1:1000 Alexa Fluor 488-conjugated secondary antibody for 1 h at room temperature in the dark. Finally, upon washing steps with PBS, cells were treated with 1.4 µg/mL PI solution containing 50 µg/mL ribonuclease for 2 h at room temperature (DNA staining). An Acumen Explorer (TTP Labtech) was used for reading and image analysis.

Nocodazole was used as a tool compound for assay validation. It is a molecule that affects microtubule dynamics by preventing polymerization. This will result in the activation of the spindle assembly checkpoint and, therefore, a cell cycle arrest at G_2/M phase. As a negative control, untreated cells were used. This assay allows the measurement of several readouts with single cell resolution and we focused our interest mainly in the following:

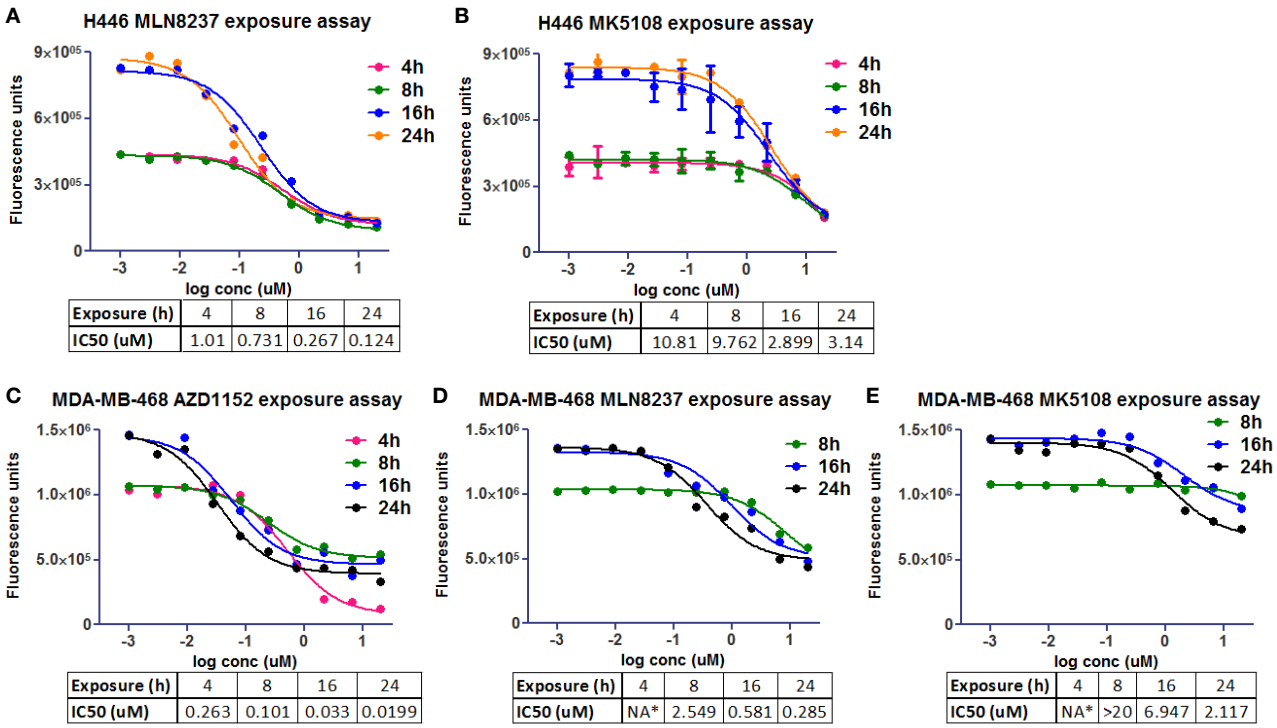

FIGURE 5 | Cell viability *in vitro* assays to determine the optimal pharmacological dose schedule for AZD1152, MLN8237, and MK5108 [modified from Ref. (18)]. **(A,B)** H446 cells. **(C–E)** MDA-MB-468 cells. NA*, not available.

cell number, 2N, 4N, >4N, and pH3S10. Aurora-B kinases are responsible for one of the classic modifications of chromatin in mitosis, phosphorylation of histone H3 on S10 (6), that is why pH3S10 was chosen as both mitotic marker and readout of Aurora-B inhibition. Data from treated and untreated cells are plotted to generate histograms that can be gated to separate diploid cells, tetraploid, and polyploid cells, and cells in S phase. As expected, nocodazole-treated cells show an accumulation in G_2M as well as an accumulation of pH3 positive cells (**Figure 3**).

Results Interpretation and Phenotype Deconvolution

To evaluate the phenotypic outcome of the assay and thus help with results' interpretation, three molecules that are or have been in clinical trials showing different Aurora-A and -B selectivity profiles were selected: AZD1152, a selective Aurora-B inhibitor (15), and selective Aurora-A inhibitors, MLN8237 (16) and MK5108 (17). These molecules were first tested in biochemical assays (**Figure 4A**) to confirm their potency against Aurora-A and -B.

By performing 10-point dose–response curves in the previously mentioned multiplexing assay, we evaluated the effect of the compounds in the different readouts. Those readouts allow distinguishing Aurora-A and -B phenotypes, whereas Aurora-A leads to mitotic arrest (increase of 4N subpopulation and pH3S10, and therefore cell proliferation inhibition), Aurora-B leads to endoreplication (increase of 4N but mainly >4N subpopulations, cell proliferation inhibition, and a decrease in pH3S10).

To confirm the Aurora-B phenotype of the compounds a singleplex assay was also developed, using NCI-H446 cells (human small cell lung carcinoma, ATCC# HTB-171) and evaluating inhibition of pH3S10 at a shorter time (1 h incubation, **Figure 4B**).

AZD1152 shows a clear Aurora-B phenotype with pH3S10 inhibition and accumulation of >4N subpopulation (**Figures 4B,E,F**), whereas MLN8237 and MK5108 show an Aurora-A phenotype with accumulation of pH3S10 positive cells and 4N subpopulation (**Figures 4D,F**). With regards to cell proliferation inhibition (**Figure 4C**), MK5108 seems to be less potent than the other two molecules.

In **Figure 4D**, we can see inside the red rectangle, a possible effect not related to Aurora-A inhibition (higher in MLN than in MK) when evaluating pH3S10. At high concentrations of these compounds, there is a decrease in this readout that might be a consequence of Aurora-B inhibition (as seen in **Figure 4B**). Although in **Figure 4F**, the % of 4N and >4N positive cells was represented as one readout, it could be separated into two to further differentiate Aurora-A and -B phenotype.

By looking at the cell cycle subpopulations, with this type of assay we can also identify effects caused by inhibition of other targets (e.g., G_1S arrest for CDK4/6 inhibitors).

In vitro Pharmacological Characterization Through Multiplexing Assays

To further extend the use of this technology, more in-depth assays can be designed for advanced molecules as a bridging step between

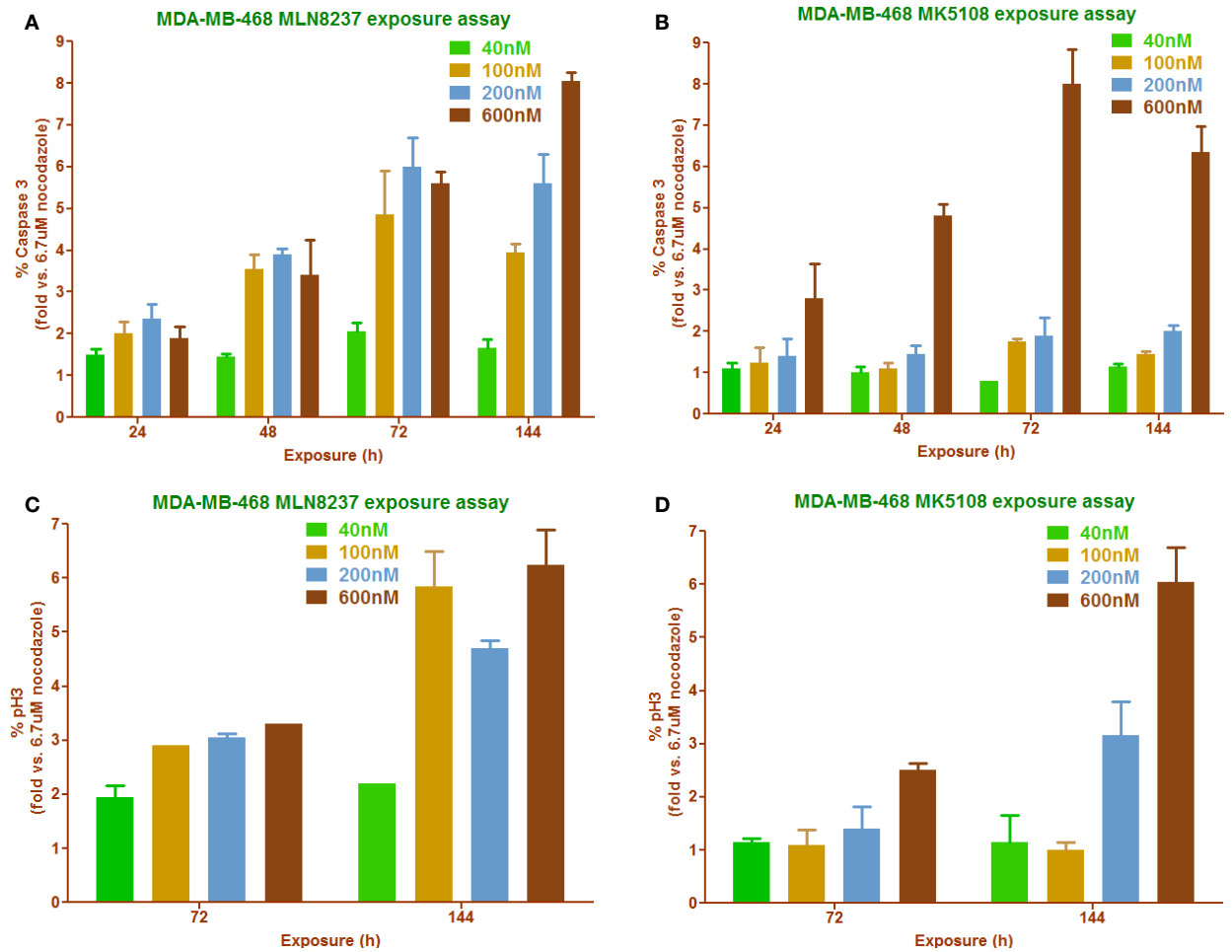

FIGURE 6 | Phenotypic readouts, permanent exposure to MLN8237 and MK5108 (18). (A,B) Percentage of caspase 3 positive cells determination in a time course manner carried out in a range of compounds concentrations. *n* = 2 per conc. **(C,D)** Percentage of mitotic index determination in a time course manner carried out in a range of compounds concentrations. *n* = 2 per conc.

biochemical assays and *in vivo* assays. By using different exposure times to the compounds and performing washout experiments, we can try to investigate the *in vitro* pharmacology (required time on target and sustainability of the response). Adding different readouts as apoptosis or senescence will also help to identify the cause of cell proliferation inhibition.

The same molecules were used in an experiment to estimate the exposure time needed in two different cell lines, NCI-H446 and MDA-MB-468 (human breast adenocarcinoma ATCC# HTB-132), to promote growth inhibition and achieve the desired phenotype (**Figure 5**). CellTiter-Glo® was used to evaluate cell viability inhibition as one of the cell lines (NCI-H446) is mixed, with both adherent and suspension cells. It seemed that at least 24 h on target were required to promote cell growth inhibition.

High-content imaging follow-up experiments were performed in MDA-MB-468 to correlate cell proliferation inhibition with phenotypic readouts (% pH3S10 accumulation and % caspase 3 induction) as well as to try to find the most appropriate dose of the compounds to promote these effects (**Figure 6**). As already shown in **Figure 4C**, MK5108 was found to be less potent than MLN8237 when used at the same dose. This observation correlates with the two different readouts used in this experiment: caspase 3 (**Figures 6A,B**) and pH3S10 (**Figures 6C,D**), where MK5108 seems to require a higher dose (600 nM) and longer exposure time (72–144 h) to produce a considerable response, whereas MLN8237 seems to work at 200 nM and 48–72 h.

To summarize, we have reviewed a couple of multiplexing technologies focusing on HCI as a powerful technique for HTS. This technique can be used not only for screening purposes but also to go in-depth and try to characterize the *in vitro* pharmacology of the molecules. This could build a bridge between *in vitro* and *in vivo*, saving resources and helping to design more appropriate *in vivo* experiments. All of this integrated in a flow scheme will generate key data to select the best candidate molecule (with desired properties such as oral administration, safety, and selectivity) improving its possibilities to move into clinical trials.

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REVIEW
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Scientific rationale supporting the clinical development strategy for the investigational Aurora A kinase inhibitor alisertib in cancer

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Alisertib (MLN8237) is a selective small molecule inhibitor of Aurora A kinase that is being developed in multiple cancer indications as a single agent and in combination with other therapies. A significant amount of research has elucidated a role for Aurora A in orchestrating numerous activities of cells transiting through mitosis and has begun to shed light on potential non-mitotic roles for Aurora A as well. These biological insights laid the foundation for multiple clinical trials evaluating the antitumor activity of alisertib in both solid cancers and heme-lymphatic malignancies. Several key facets of Aurora A biology as well as empirical data collected in experimental systems and early clinical trials have directed the development of alisertib toward certain cancer types, including neuroblastoma, small cell lung cancer, neuroendocrine prostate cancer, atypical teratoid/rhabdoid tumors, and breast cancer among others. In addition, these scientific insights provided the rationale for combining alisertib with other therapies, including microtubule perturbing agents, such as taxanes, EGFR inhibitors, hormonal therapies, platinums, and HDAC inhibitors among others. Here, we link the key aspects of the current clinical development of alisertib to the originating scientific rationale and provide an overview of the alisertib clinical experience to date.

Keywords: Aurora, combination therapy, biomarkers, alisertib, mitosis

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Alisertib: A Highly Selective Aurora A Kinase Inhibitor

Early interest in targeting Aurora A for cancer treatment stemmed in part from the fact that the gene, localized to chromosome 20q13.2, is commonly amplified and overexpressed in a diversity of cancer types (1–7). Aurora A amplification and overexpression is correlated to a worsened prognosis for patients. For example, a meta-analysis study of 5523 cancer patients from thirty-nine studies demonstrated that patients with higher Aurora A expression levels had a significantly worsened survival outcome irrespective of disease type or stage (8). Aurora A overexpression is also thought to drive oncogenesis by causing genomic instability; this proposal is supported by evidence demonstrating that Aurora A overexpression transforms normal cells into cancer cells in experimental studies (7, 9–13). As such, Aurora A has been considered an attractive target for treating cancer and multiple Aurora kinase inhibitors have been developed and tested in cancer patients, including alisertib (MLN8237).

Alisertib is a benzazepine containing small molecule inhibitor of Aurora A (14). In enzymatic, cell and *in vivo* assays, alisertib has proven to selectively inhibit Aurora A (14). For example, alisertib

demonstrated selectivity for Aurora A relative to other kinases in an *in vitro* screen of 205 kinases, and was >200-fold more potent against Aurora A than the structurally related kinase Aurora B in cellular assays. The selectivity for Aurora A was substantiated by mechanism of action studies in cultured cancer cells and tumors grown as xenografts in immunocompromised mice. Alisertib concentrations that lead to cell cycle arrest and death are preceded by phenotypic changes consistent with Aurora A inhibition; including increased incidence of tetraploid (4N) cells as well as mitotic cells with abnormal mitotic spindles and misaligned chromosomes (**Figure 1**). Furthermore, alisertib did not affect the viability of cancer cell lines expressing a drug-resistant Aurora A mutation, suggesting that its antitumor activity occurs predominantly through Aurora A inhibition (15).

Alisertib has demonstrated antitumor activity across a broad array of solid cancers and heme-lymphatic experimental tumor models when grown *in vitro* and *in vivo* (14, 16–24). In addition, single-agent alisertib has been evaluated in multiple clinical trials and has shown clinical activity across a diversity of cancer types, including solid and hematological cancers in adult and pediatric populations. Though alisertib displays differential antitumor activity across experimental tumor models and in cancer patients, the biological underpinnings for alisertib sensitivity remain unclear. Multiple hypotheses have been developed based on Aurora A biology and data collected in experimental models that predict which cancers will most likely respond to alisertib as a single agent or in combination with other therapeutic agents. In this review, the data supporting some of these concepts is shared.

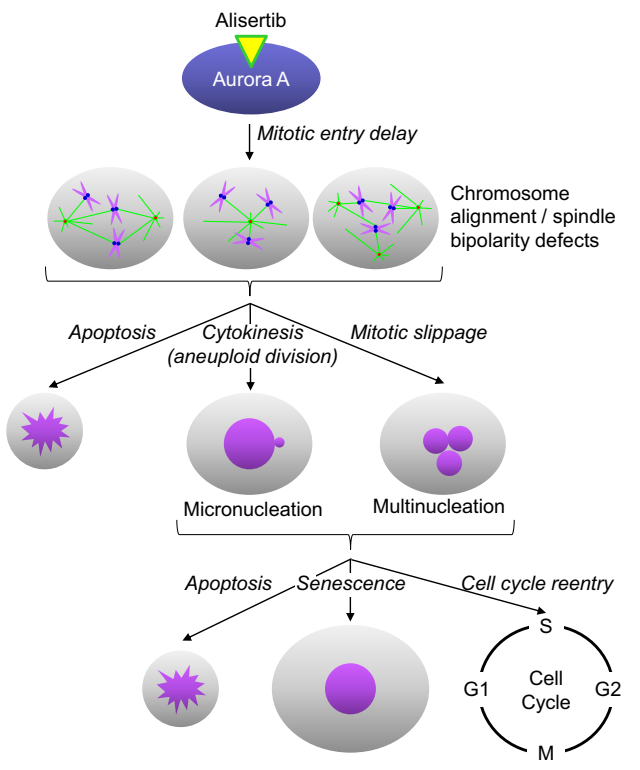

FIGURE 1 | Alisertib mechanism of action. Alisertib selectively binds to and inhibits Aurora A kinase in cells. Inhibition of Aurora A results in delayed mitotic entry and progression through mitosis leading to an accumulation of cells with a tetraploid DNA content. Mitotic cells treated with alisertib display a variety of defects, including monopolar, bipolar, and multipolar spindles, all with misaligned chromosomes. These cells can die directly out of mitosis via apoptosis, undergo aneuploid cytokinesis or exit mitosis without undergoing cytokenesis through a process known as mitotic slippage. The resulting interphase cells typically display gross nuclear defects including micronucleation and multinucleation. These cells will then undergo apoptosis, senescence or reenter the cell cycle; the specific fate is likely dictated by the extent of DNA damage/aneuploidy that occurred in any cells as a result of the abnormal mitotic division as well as other underlying genetic factors.

Early Clinical Studies for Dose/Schedule Selection and Proof of Mechanism

Alisertib has been formulated for oral administration in patients and is available as an enteric-coated tablet and as a liquid solution for pediatric cancers. In two phase 1 studies of alisertib in adults with advanced solid malignancies (25, 26), and in one phase 1 study of alisertib in adults with hematological cancers (27), the single agent maximum tolerated dose was determined to be 50 mg dosed orally twice daily for 7 days followed by 14 days of non-treatment. This dose was selected for further single-agent alisertib evaluation in additional clinical trials of adult cancer patients. Alisertib was also evaluated once daily for 21 days followed by 14 days of non-treatment; 50 mg was the maximum tolerated dose on this schedule (25, 26). The most common dose limiting toxicities (DLTs) observed with alisertib were fatigue, nausea, neutropenia, and stomatitis. These toxicities reflect the pharmacologic activity of alisertib as a cell cycle inhibitor in highly proliferative tissues. Other common alisertib-associated toxicities included alopecia, anorexia, leukopenia, anemia, thrombocytopenia, asthenia, vomiting, diarrhea, and somnolence. The occurrence of somnolence was likely due to the benzodiazepine-like structure of alisertib.

Alisertib has also been evaluated in pediatric cancer patients. This was in part based on the observation that alisertib was active against a range of pediatric tumors grown *in vitro* and *in vivo*, in particular, neuroblastoma and acute lymphocytic leukemia (28, 29). In a phase 1 study of children with solid tumors, the maximum tolerated dose of alisertib in children with solid tumors was 80 mg/m^2 administered orally once daily for 7 days followed by 14 days of non-treatment (30). The exposures achieved with this dose is approximately 1.5-fold greater than the adult maximum tolerated dose of 50 mg twice daily. Mucositis/stomatitis, mood alteration/depression, neutropenia, and elevated alkaline phosphatase were the DLTs in these patients; neutropenia being the most frequently occurring dose-limiting toxicity. In addition to depression, other mood alterations included impaired memory, agitation, euphoria, and somnolence, predominantly grade 1 and 2. Hand–foot–skin reactions were also observed in these patients.

The selectivity of alisertib for Aurora A relative to Aurora B observed in non-clinical experimental models also translated into the cancer patients. Pharmacodynamic studies performed on tumor biopsies obtained from patients prior to and after alisertib dosing demonstrated an exposure-related decreases in tumor mitotic cells with aligned chromosomes and bipolar spindles in the post-dose samples; phenotypes consistent with Aurora A inhibition (25, 31). Moreover, skin and tumor biopsies

taken prior to and after alisertib dosing had increased in mitotic cells in the post-treatment biopsies with serine 10 phosphorylated Histone H3. As serine 10 phosphorylation of histone H3 is catalyzed by Aurora B in cells, these data demonstrate that alisertib does not significantly inhibit Aurora B at the single agent maximum tolerated dose (25, 26, 31). Confirmation of alisertib's functional selectivity for Aurora A in cancer patients allows for its rational development for treating multiple types of cancers as single agent or in combination with other therapeutic agents.

Population-based pharmacokinetic–pharmacodynamic modeling demonstrate that alisertib steady-state exposures achieved with 50 mg twice daily for 7 days is associated with pharmacodynamic activity in tumors and a low probability for DLTs (31). Moreover, patients with intolerable treatment related toxicities at 50 mg twice daily can be dose reduced to 40 or 30 mg on the same schedule and still maintain tumor pharmacodynamic effects. Overall, multiple tolerated and pharmacodynamically active dose/schedules have been identified in adult and pediatric patients allowing for sufficient flexibility in alisertib dosing that can be taken advantage of for single-agent evaluation and for combining with multiple other therapeutic agents.

Alisertib Single-Agent Rationale

Neuroblastoma

Interest for developing alisertib in neuroblastoma initially derived from an evaluation of alisertib antitumor activity in a large set of pediatric cancer models executed by the Pediatric Preclinical Testing Program which maintains the mission for identifying novel therapies for treating childhood cancers. Alisertib potently inhibited the growth of neuroblastoma cells *in vitro* and resulted in maintained complete responses in three of seven neuroblastoma xenograft models grown in immunocompromised mice; responses which surpassed the activity of other agents tested in these models (29). Subsequent to these findings it was proposed that Aurora A is essential for the growth and survival of *MYCN*-amplified neuroblastoma cells. Aurora A binds to and stabilizes N-MYC by protecting it from FBXW7 E3 ubiquitin ligase-mediated proteasomal degradation in a manner that is independent from Aurora A catalytic activity (**Figure 2**) (32). Furthermore, alisertib and the structurally related molecule MLN8054 bind to Aurora A's catalytic domain in manner that causes an allosteric shift in the protein thereby disrupting its' interaction with N-Myc (33, 34). Interestingly, the allosteric shift at the Aurora A/N-Myc interaction site caused by alisertib does not occur with all catalytic inhibitors of Aurora A kinase. Several studies have also demonstrated antitumor activity of Aurora A inhibition in *MYCN*-amplified neuroblastoma models. For example, treatment of TH-*MYCN* neuroblastoma mice with MLN8054 resulted in decreased N-Myc protein expression, diminished expression of N-Myc target genes, tumor regressions and increased survival (33). Other Aurora A inhibitors also decreased N-Myc expression resulting in inhibited tumor growth of other *MYCN*-amplified tumors (34, 35).

As a result of these findings, the Children's Oncology Group led a phase 1 study of single-agent alisertib in children with relapsed/

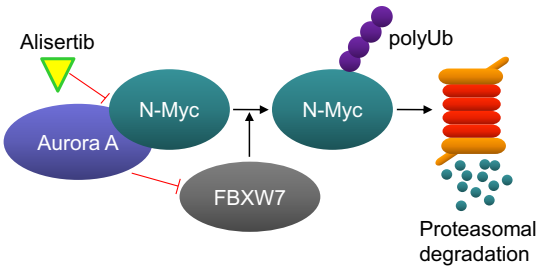

FIGURE 2 | Aurora A protects N-Myc from proteasome-mediated degradation. Aurora A binds to N-Myc, thereby preventing it from being ubiquitinated by the E3-ligase FBXW7. Alisertib binds to Aurora A and changes its confirmation in a manner that disrupts its interaction with N-Myc. N-Myc is than ubiquitinated by FBXW7 and subsequently degraded by the proteasome.

recurrent solid tumors including neuroblastoma to determine the maximum tolerated dose, safety profile and pharmacokinetics of alisertib. In this study, 4 out of 11 evaluable neuroblastoma patients treated with alisertib had stable disease (≥6 cycles) (30). As described above, the DLTs in these patients was mucositis, neutropenia, and mood alteration. A phase 2 study of alisertib in young patients with recurrent or refractory solid tumors or leukemias including neuroblastoma has also recently been completed (NCT01154816). Currently, there is an ongoing study being led by the New Approaches to Neuroblastoma Therapy (NANT) consortium in recurrent or resistant neuroblastoma patients combining alisertib with the FDA-approved drugs for neuroblastoma treatment, irinotecan and temozolomide (NCT01601535). In this study, there is a plan to compare *MYCN* status to patient outcome.

Small Cell Lung Cancer

Similar to neuroblastoma, SCLC has an etiological link to Myc-family of oncogenes including *MYC* (c-Myc), *MYCN* (N-Myc) and *MYCL1* (L-Myc). Amplification and overexpression of these genes is thought to constitute 18–31% of SCLCs (36–38). Multiple preclinical studies have suggested that SCLCs with Myc activation or amplification are notably sensitive to Aurora kinase inhibitors. For example, SCLC cell lines with *MYC*, *MYCN*, and *MYCL1* activation or amplification were the most sensitive in a viability screen of 87 cell lines using the dual Aurora A and Aurora B kinase inhibitor PF-03814735 (39). In a separate screen of 34 SCLC cell lines, four structurally diverse Aurora kinase inhibitors VX680, alisertib, PHA680632, and ZM447439 were most effective against the *MYC*-amplified cell lines (37). Studies with the dual Aurora A and Aurora B kinase inhibitor VX680 demonstrated that it selectively killed human retinal pigment epithelial cells that overexpress c-Myc (40).

In a phase 2 study of single-agent alisertib in five types of advanced refractory or relapsed solid cancers, encouraging activity was seen in SCLC (41). Objective partial responses were observed in 10 of the 48 (21%) SCLC enrolled in this study; these responses occurred in both chemotherapy-sensitive and chemotherapy-resistant disease, the latter which has a worse prognosis. The most common grade 3–4 adverse events in the SCLC patients

from this phase 2 study were neutropenia, anemia, leucopenia, and thrombocytopenia, which are consistent with those noted in earlier trials of alisertib. Currently, a phase 2 study of alisertib in combination with paclitaxel compared to placebo in combination with paclitaxel in patients with second line relapsed or refractory SCLC is ongoing (NCT02038647).

Neuroendocrine Prostate Cancer

Neuroendocrine prostate cancer is thought to evolve from late stage prostate adenocarcinoma concurrent to become resistant to hormonal therapy (42, 43). As part of that transition, neuroendocrine prostate cancers become more genomically unstable than prostate adenocarcinoma and include co-amplification of MYCN and Aurora A (44, 45). Given this observation, the relative sensitivity of several prostate adenocarcinoma and neuroendocrine cancer models to the pan-Aurora inhibitor danusertib was tested (44). In a viability screen of four cell lines grown in cell culture, the one neuroendocrine prostate cancer model was significantly more sensitive to danusertib than the three adenocarcinoma cell lines. Danusertib also displayed greater antitumor activity in LNCaP cells transfected with MYCN than vector–control LNCaP cells and was more effective in inhibiting the growth *in vivo* of a neuroendocrine prostate cancer model relative to an adenocarcinoma model. As a result of these observations, a phase 2 in NEPC is ongoing with single-agent alisertib (NCT01799278).

Atypical Teratoid/Rhabdoid Tumors

Aurora A is a promising target for therapy in ATRT and alisertib has demonstrated to be a potent radiosensitizer in ATRT experimental models (46). ATRT is a rare and highly malignant central nervous system (CNS) tumor usually diagnosed in childhood. ATRT represents around 3% of CNS pediatric cancers and has a high mortality rate with a very poor prognosis. Mutation or deletion of the tumor suppressor gene INI1/hSNF5 occurs in the majority of ATRTs. hSNF5/INI1 is a component of the chromatin remodeling SWI/SNF complex which regulates many proteins involved in chromatin structure. Aurora A is a direct downstream target of hSNF5/INI1. hSNF5/INI1 acts to repress Aurora A expression; as such, loss of INI1/hSNF5 in rhabdoid tumors leads to aberrant overexpression of Aurora A which is required for tumor survival in non-clinical cancer models (47). These preclinical findings supported the use of alisertib for ATRT patients. Wetmore et al. reported an encouraging result for clinical use of alisertib as single agent in recurrent ATRT in four children (48). Patients with recurrent or progressive ATRT received oral administration of alisertib 80 mg/m^2 once daily for 7 days of a 21-day treatment cycle. Disease burden was evaluated by brain and spine MRI and by evaluation of spinal fluid cytology (lumbar puncture) after two cycles of alisertib and every 2–3 cycles thereafter for as long as the patients remained free from tumor progression. All four patients had disease stabilization and/or regression after three cycles of alisertib therapy. Two patients on therapy showed stable disease regression for 1 and 2 years. Consistent with other pediatric studies, alisertib in these patients had moderate but manageable toxicities, including neutropenia, leukopenia, thrombocytopenia, anemia, somnolence, and alopecia. Alisertib appears a promising therapeutic agent in this pediatric population. A phase 2 study is ongoing to further evaluate alisertib in the treatment of children with ATRT.

Breast Cancer

Single-agent alisertib efficacy was evaluated in a phase 2 study that comprised five advanced solid tumor indications including breast cancer (41). Among response-evaluable breast cancer patients, objective response (all partial responses) was observed in 9 [18%, 95% confidence interval (CI) = 9–32%] of 49 women with breast cancer. The most common grade 3–4 adverse events in the breast cancer patients from this study included neutropenia, fatigue, leucopenia, and stomatitis. The antitumor activity of alisertib was particularly encouraging in the hormone receptor-positive and HER2-negative subgroups. Median progression-free survival in this subgroup was 7.9 months (95% CI 4.2–12.2). This clinical finding is supported by previously reported preclinical results. D'Assoro et al. demonstrated that Aurora A drives the transition of estrogen receptor α-positive (ERα$^+$) breast cancer cells from an epithelial to a highly invasive mesenchymal phenotype (49). The transition from an epithelial-like to a mesenchymal-like phenotype was characterized by reduced expression of ERα, HER-2/Neu overexpression and loss of CD24 surface receptor (CD24$^{-/low}$) and overexpression of Aurora A (**Figure 3**). Aurora A overexpression induces epithelial–mesenchymal transition (EMT) and a cancer stem cell-like phenotype. Inhibition of Aurora A by alisertib *in vitro* reverses EMT and suppresses the self-renewal ability of CD24$^{-/low}$ breast cancer. Moreover, molecular targeting of Aurora A by shRNA *in vivo* restores a CD24$^+$ epithelial phenotype and inhibits the development of distant metastases. Other studies demonstrated that increased Aurora A activity may result in anti-hormonal therapy resistance in breast cancer (50). Aurora A induces endocrine resistance through down-regulation of ERα expression in initially ERα$^+$ breast cancer cells (51). In breast cancer patients, high Aurora A expression is associated with poor survival particularly in node-negative ER-positive breast cancer patients (50). Taken together, alisertib could be a novel promising therapeutic agent to selectively eliminate

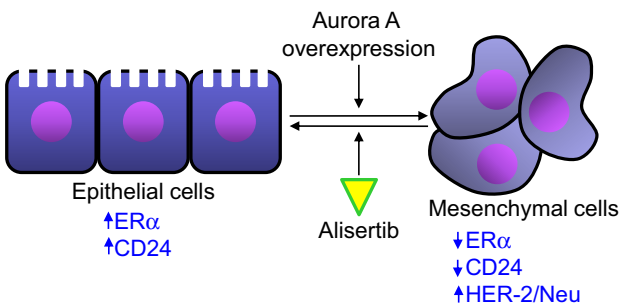

FIGURE 3 | Aurora A activity affects epithelial to mesenchymal transition in breast cancer cells. Aurora A overexpression leads to the transition of breast cancer cell from an epithelial phenotype to a mesenchymal phenotype, leading to decreased ERα and CD24 expression and HER-2/Neu overexpression. Alisertib counteracts the effects of Aurora A overexpression leading to an epithelial to mesenchymal transition reversion.

highly invasive cancer cells and improve the disease-free and overall survival of ER-positive breast cancer patients resistant to conventional endocrine therapy.

Alisertib Combination Development Rationale

Taxanes

A considerable amount of data has accumulated in preclinical studies suggesting the benefit of combining Aurora kinase inhibitors with antimicrotubule perturbing agents. This class of anticancer therapies which comprises the taxanes, vinka alkaloids, and the epothilones is among the most commonly used for treating both solid and hematological cancers. Multiple preclinical studies have demonstrated the beneficial combination of inhibiting Aurora kinase with this class of agents (52–60). For example, alisertib combined with the taxanes paclitaxel and docetaxel in triple-negative breast cancer tumors grown as xenografts in immunocompromised mice led to additive or synergistic antitumor activity with prolonged tumor growth delay and in some cases durable complete responses after discontinuing treatment (53). Though the underlying biological underpinnings explaining the beneficial combination between antimicrotubule agents remains uncertain, it has been shown that Aurora A inhibition using MLN8054 or RNA interference in the presence of paclitaxel caused cells to rapidly exit mitosis without completing cytokinesis, presumably due to a disruption of the spindle assembly checkpoint (61).

Alisertib administered as a single agent was evaluated in patients with platinum-resistant or -refractory epithelial ovarian, fallopian tube, or primary peritoneal carcinoma (62). Though active in these diseases as a single agent (overall response rate of 10%, durable for 6.9–11.1 months), the activity was not considered sufficient for further development in ovarian cancer as a single agent. Therefore, alisertib was tested in combination with paclitaxel in relapsed and refractory ovarian cancer (NCT01091428). During the phase 1b portion of this study weekly paclitaxel (QWx3) at 80 mg/m^2 and 60 mg/m^2 was administered with alisertib dosed twice daily on a 3 days on, 4 days off schedule for three consecutive weeks over 28-day cycles (63). Exposure efficacy modeling was used for selecting the phase 2 dose for this study (53). In addition, alisertib and paclitaxel are being tested in metastatic or locally recurrent breast cancer (NCT02187991) and SCLC (NCT02038647). Numerous other studies have been completed or are ongoing testing alisertib in combination with other microtubule perturbing agents, including Abraxane (nab-paclitaxel) in patients with advanced solid cancers (NCT01677559), docetaxel in patients with advanced solid tumors (NCT01094288), and vincristine and rituximab in patients with relapsed or refractory B-Cell lymphomas (NCT01397825).

EGFR Inhibitors

Epidermal growth factor receptor (EGFR)-targeting antibodies or small molecular EGFR inhibitors are widely used to treat patients with gastrointestinal (GI), breast, head and neck, and lung cancers. However, the clinical efficacy of these agents is limited by intrinsic and acquired resistance factors. Astsaturov and colleagues employed a synthetic lethal screening method and identified Aurora A as a promising hit necessary for cells to survive in the presence of an EGFR inhibitor (64). In addition, they observed synergistic activity of combined inhibition of the EGFR and Aurora A pathways in cancer cells. Combination of erlotinib and alisertib showed synergistic antitumor activity *in vitro* and *in vivo* in lung cancer models (65). Furthermore, Aurora A and EGFR protein expression were assessed by immunohistochemistry in patients with squamous cell cancer of the head and neck (SCCHN) ($n = 180$). Co-expression of elevated levels of Aurora A and EGFR was a poor prognostic factor in SCCHN (66). Recently, Crystal and colleagues established patient-derived resistant NSCLC models to identify effective drug combinations (67). Aurora kinase inhibitors were active in combination with EGFR inhibition in a number of EGFR-mutant cell lines. These data together suggest a potential benefit of such combination therapy in patients. Currently, there is an ongoing phase 1 study evaluating the safety and tolerability of combining alisertib with erlotinib in patients with non-SCLC (NCT01471964).

Hormonal Therapy in Breast Cancer

A number of evidences suggest alisertib may be a rationale combination partner for hormonal therapy. First, promising alisertib single-agent activity was observed in ER-positive and HER2-negative patients as described above (41); second, Aurora A plays a role in the development of endocrine resistance through activation of SMAD5 nuclear signaling and down-regulation of ERα expression in initially ERα$^+$ breast cancer cells (51); and third, aromatase inhibitors (AIs) are used for treatment of ER-positive breast cancer though resistance to AI is a major obstacle to optimal patient outcome. Aurora A is upregulated in AI-resistant cell lines and knockdown studies of Aurora A have shown that it is essential for AI-resistant cell growth. In AI-resistant cell lines, alisertib blocked cell cycle progression at the G2/M phase, interfered with chromosome alignment and spindle pole formation, and preferentially inhibited AI-resistant cell growth compared with parental control cells (68). Furthermore, combination of Aurora inhibitors (alisertib, JNJ-7706621, or danusertib) with fulvestrant is superior to treatment with either of the compounds alone, particularly in AI-resistant cell lines (68). Importantly, this combination may have minimal overlapping toxicities in breast cancer patients. A phase 1/2 trial of alisertib in combination with fulvestrant in patients with hormone receptor-positive metastatic or locally advanced breast cancer is ongoing (NCT02219789).

Platinums

Platinum-based drugs continue to be the mainstay of therapy for many cancers, such as ovarian and lung cancers; however, chemoresistance (intrinsic or acquired) is a major limitation for platinums as it is for other therapies. Increasing evidence suggests a role of Aurora A in platinum resistance. Elevated expression of Aurora A is associated with poor prognosis in epithelial ovarian cancer patients (69) and high Aurora A expression is correlated with cisplatin-based chemotherapeutic resistance and predicts poor patient overall survival (OS) and progression-free survival in NSCLC (70). Moreover, forced expression of Aurora A increased

the resistance of the lung cancer cells to cisplatin and knocked down of Aurora A expression in the cisplatin resistant cells by siRNA resulted in a significantly enhanced sensitivity to cisplatin (70). In addition, combination of alisertib and cisplatin resulted in enhanced antitumor activity *in vivo* in multiple preclinical models (21). In a recent phase 2 clinical trial, alisertib exhibits encouraging single-agent activity in SCLCs, particularly in refractory or chemotherapy-resistant/relapsed patients as described above. Three of twelve patients with refractory or chemotherapy-resistant disease had objective responses to alisertib (41). In earlier studies, alisertib also showed modest single-agent antitumor activity in patients with platinum-resistant ovarian cancers (62). Combination of alisertib with platinums may be a viable strategy for the treatment of patients with platinum-resistant recurrent SCLC and ovarian cancers.

HDAC Inhibitors

Alisertib has shown promising single-agent antitumor efficacy in a phase 2 trial for the treatment of various hematological malignances (71). The overall response rate was 27% (10% CRs) including 100% (1/1) in Burkitt lymphoma (BL), 29% (6/21) in diffuse large B cell lymphoma (DLBCL), and 50% (4/8) in peripheral T-cell lymphoma (PTCL). Recent data from a phase 2 study of alisertib in PTCL led by the South West Oncology Group (SWOG) showed two complete responses and seven partial responses and a response rate (ORR) of 24%. Among the most common subtypes (PTCL NOS, AITL, and ALCL), the ORR was 33% (72). Similar to previously described data with alisertib, myelosuppression was a common adverse effect and constituted the predominant toxicity requiring dose reduction. Mucositis, anorexia, and diarrhea occurred in less than one-quarter of patients and were largely grade 1 or 2 in severity. Grade 1 or 2 fatigue was also common, being observed in nearly half of patients. Nonetheless, two responding patients in this trial received alisertib for 1 year. On the basis of these results, a global phase 3, randomized registration-enabling trial (NCT01482962) was initiated comparing alisertib with investigator's choice (gemcitabine, pralatrexate, or romidepsin) in patients with relapsed/refractory PTCL. This study was discontinued as a pre-specified interim analysis indicated that the study was unlikely to meet the primary endpoint of superior progression-free survival (PFS) over the standard of care in this treatment setting, although single-agent activity of alisertib was confirmed. In this phase 3 study, alisertib showed a similar ORR compared to the control arm.

The histone deacetylase (HDAC) inhibitors (vorinostat and romidepsin) were approved in the United States for the treatment of cutaneous T-cell lymphoma and romidepsin for the treatment of PTCL. Preclinical data support combining Aurora A inhibitors with HDAC inhibitors. For example, several studies demonstrated that HDAC inhibitors reduce Aurora A expression leading to arrest in the G2/M portion of the cell cycle, abnormal mitotic spindles and followed by apoptosis (73–75). The pan-Aurora kinase inhibitor MK-0457 in combination with the vorinostat enhanced lymphoma cell death through repression of c-Myc and c-Myc responsive micro RNAs (76). Alisertib also demonstrated synergistic antitumor activity when combined with romidepsin in experimental models of T-cell lymphoma (77). Alisertib selectively synergizes with romidepsin by inducing cytokinesis failure in T-cell lymphoma. Cytokinesis failure was confirmed after a corresponding post-treatment increase in CENP-A protein levels. CENP-A is a chromatin-associated protein and plays a role in the final stages of cytokinesis. Overall, these collective data provide a rationale for evaluating alisertib in combination with romidepsin in patients with multiple lymphoma subtypes. A phase 1 trial of alisertib plus romidepsin for relapsed/refractory aggressive B- and T-cell lymphoma is ongoing (NCT01897012).

TABLE 1 | Most common treatment-emergent adverse events of alisertib dosed at 50 mg orally twice daily for 7 days followed by 14 days of non-treatment.

	All grades[a]	Grade ≥3[b]
Gastrointestinal disorders	Diarrhea, nausea, stomatitis, vomiting, abdominal pain, constipation	Stomatitis, diarrhea
Blood and lymphatic system disorders	Neutropenia, anemia, thrombocytopenia, leukopenia, febrile neutropenia	Neutropenia, anemia, thrombocytopenia, leukopenia, febrile neutropenia
General disorders and administration site conditions	Fatigue, pyrexia, asthenia, edema peripheral	Fatigue
Skin and subcutaneous tissue disorders	Alopecia	
Nervous system disorders	Somnolence, headache, dizziness	
Metabolism and nutrition disorders	Decreased appetite, dehydration	
Respiratory, thoracic and mediastinal disorders	Dyspnea, cough	

[a]*Treatment-emergent adverse events of alisertib in ≥10% patients.*
[b]*Treatment-emergent grade 3–4 drug-related adverse events in ≥5% patients.*

Summary

To date, many clinical studies have been conducted to evaluate antitumor efficacy of alisertib in patients with diverse solid tumors or hematologic malignancies. Treatment related adverse events (in ≥10% of patients) of single-agent alisertib are summarized in **Table 1** (25, 26, 41). Although alisertib has shown single-agent clinical activity in multiple tumor settings, identification of appropriate combination partners and sensitive patient populations is required to ensure that an acceptable risk/benefit profile can be achieved. Aurora A has been implicated in the development of resistance to multiple chemotherapies and targeted agents and preclinical data suggest that alisertib can be combined with multiple therapies to yield additive or synergistic antitumor activity. Furthermore, combinations with targeted therapies might yield more favorable clinical risk/benefit profile than combinations with chemotherapeutic partners due to decreased risk for overlapping toxicities. Lastly, identification of potential predictive biomarkers for alisertib will significantly increase the likelihood of expanding the clinical risk/benefit profile. As such, many correlative studies are ongoing to identify predictive biomarkers which could lead to a precision medicine strategy for alisertib.

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Opposing effects of inhibitors of Aurora-A and EGFR in autosomal-dominant polycystic kidney disease

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Aurora-A kinase (AURKA) overexpression in numerous tumors induces aneuploidy, in part because of cytokinetic defects. Alisertib and other small-molecule inhibitors targeting AURKA are effective in some patients as monotherapies or combination therapies. Epidermal growth factor receptor (EGFR) pro-proliferative signaling activity is commonly elevated in cancer, and the EGFR inhibitor erlotinib is commonly used as a standard of care agent for cancer. An erlotinib/alisertib combination therapy is currently under assessment in clinical trials, following pre-clinical studies that indicated synergy of these drugs in cancer. We were interested in further exploring the activity of this drug combination. Beyond well-established functions for AURKA in mitotic progression, additional non-mitotic AURKA functions include control of ciliary stability and calcium signaling. Interestingly, alisertib exacerbates the disease phenotype in mouse models for autosomal-dominant polycystic kidney disease (ADPKD), a common inherited syndrome induced by aberrant signaling from PKD1 and PKD2, cilia-localized proteins that have calcium channel activity. EGFR is also more active in ADPKD, making erlotinib also of potential interest in this disease setting. In this study, we have explored the interaction of alisertib and erlotinib in an ADPKD model. These experiments indicated erlotinib-restrained cystogenesis, opposing alisertib action. Erlotinib also interacted with alisertib to regulate proliferative signaling proteins, albeit in a complicated manner. Results suggest a nuanced role of AURKA signaling in different pathogenic conditions and inform the clinical use of AURKA inhibitors in cancer patients with comorbidities.

Keywords: PKD1, Aurora-A kinase, mouse models, renal cyst, EGFR, SRC

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INTRODUCTION

In its role as a mitotic regulator, Aurora-A kinase (AURKA) accumulates through G2 at the centrosome, becomes active at G2/M transition, and remains active through M phase as it translocates along the mitotic spindle to the midzone, with the bulk of AURKA degraded at the midbody at cytokinesis. A large number of proteins have been identified that directly associate with AURKA either in its N-terminal unstructured domain or C-terminal kinase domain, and regulate AURKA activation, including the highly studied TPX2 (1–7), but in addition, the scaffolding factors NEDD9,

nucleophosmin/B23, PAK kinases, CEP192, and others (8–12). Human AURKA is overexpressed in many tumors arising from breast, colon, ovary, and other tissues, and to function as an oncogene when exogenously expressed in numerous cell line models (13–18). AURKA overexpression is invariably associated with increased number of centrosomes and multipolar spindles, which arise as consequence of failed cytokinesis, and reflect failure to downregulate AURKA activity at the end of mitosis. Inhibitors designed to block AURKA mitotic activity are currently undergoing clinical assessment as cancer therapeutics, with MLN8237/alisertib in multiple late-stage trials (19, 20).

Although most work on AURKA focuses on the activity of this protein in mitotic cells, a number of studies have now identified additional functions of AURKA in non-mitotic cells. For example, AURKA activity is required for neurite extension, in a post-mitotic cell population (21, 22). AURKA is also transiently activated by elevated cytoplasmic calcium, which triggers calmodulin binding to the N-terminal unstructured domain of AURKA and enhances binding to some partners, such as NEDD9, and reciprocally, AURKA phosphorylation of the polycystin 2 (PC2) calcium channel can inhibit its activity in interphase cells (23, 24). In addition, growth factor stimulation of quiescent ciliated cells induces NEDD9 expression and AURKA activation, leading to resorption of the cilium (25). These latter activities were of particular interest, as they not only potentially informed some roles of AURKA relevant to cancer (26, 27) but also connected AURKA activity to another pathological condition, autosomal-dominant polycystic kidney disease (ADPKD).

Autosomal-dominant polycystic kidney disease arises from inactivating mutations in the genes *PKD1* or *PKD2*, and currently has few treatment options (28). Formation of cysts is marked by multiple phenotypic changes in the cells lining renal tubules [reviewed in Ref. (28)]. These pleiotropic changes reflect the complex cellular action of the polycystins PC1 and PC2, products of the *PKD1* and *PKD2* genes. ADPKD is classified as a ciliopathy (29), based on the obligate functional heterodimerization of PC1 and PC2 on cell cilia, where PC1 normally acts as a flow sensor to trigger the calcium channel activity of PC2: calcium influx and other signaling interactions of the PC1/PC2 heterodimer act to restrain cell growth and govern the polarity of cell division in normal cells (30). Loss of cilia or defects in ciliary function can independently induce cyst formation (31).

As ADPKD signaling defects have become better understood, an unexpected feature has been the recognition that they possess extensive similarity to signaling defects seen in cancer (32). Exploiting these convergences, current research into the effective clinical management of ADPKD has been exploring the inhibition of signaling proteins, such as mTOR and SRC, that typically have elevated expression or activity in response to mutation of PC1/PC2 signaling, and actively contribute to cystic growth [reviewed in Ref. (30)]. Given the connections described above among AURKA, PC2, and cilia, and the identification that AURKA itself is elevated in cystic epithelia (23, 33), we previously explored efficacy of AURKA inhibition in controlling cyst growth in a mouse model of ADPKD (33). The initially surprising result of this study was that alisertib strongly exacerbated cyst formation. However, this outcome was compatible with an independent study that in the specific context of driver lesions in PKD1 or PKD2, genetic ablation of cilia reduces symptoms, suggesting the hypothesis that it is abnormal signaling rather than loss of signaling from the cilium that induces cyst formation (34). If so, then inhibiting signaling processes downstream of polycystins would potentially oppose the activity of alisertib. Epidermal growth factor receptor (EGFR) is activated in ADPKD (35, 36), and interacts with polycystins (37). In cancer, the combination of erlotinib and alisertib was first suggested by an siRNA screen that identified genes that influenced cellular response to inhibition of EGFR (38). In this work, AURKA inhibitors were shown to combine effectively with both small molecule and antibody inhibitors of EGFR *in vitro* and *in vivo*, providing the conceptual basis for two ongoing clinical trials (NCT01471964 and NCT01540682, clinicaltrials.gov). In the current study, to probe these novel actions of AURKA in ADPKD, we have evaluated the interaction of the EGFR inhibitor erlotinib with alisertib in control of cyst formation.

MATERIALS AND METHODS

Mouse Strains and Drug Treatment

Conditional *Pkd1*$^{-/-}$ mice in which tamoxifen induction of the Cre-flox regulatory system permits targeted inactivation of the Pkd1 gene *in vivo* have been described (33, 39, 40). *Pkd1fl/fl;Cre/Esr1*$^{+}$ (referred to as *Pkd1*$^{-/-}$), and control mice lacking an intact Cre-flox system (*Pkd1fl/fl;Cre/Esr1*$^{-}$) mice were injected intraperitoneally with tamoxifen [250 mg/kg body weight (BW), formulated in corn oil] on post-natal days P2 and P3 for the early cyst induction, or post-natal days P35 and P36 for late cyst induction, to induce *Pkd1* deletion in the test group, as described (39). Alisertib (Millennium Pharmaceuticals, Inc., Cambridge, MA, USA) was formulated in 10% 2-hydroxypropyl-β-cyclodextrin (Sigma Aldrich, St. Louis, MO, USA) with 1% sodium bicarbonate and 20 mg/kg administered orally twice daily (BID), using a 5-day on/2-day off schedule. Erlotinib was formulated in 10% DMSO saline and 10 mg/kg administered orally once daily (QD), using a 5-day on/2-day off schedule. Treatment began at the age of 4 months and cyst growth monitored by magnetic resonance micro-imaging (MRI); mice were euthanized 10 weeks after the beginning of treatment to collect kidneys and liver for analysis. The Institutional Animal Care and Use Committee (IACUC) of Fox Chase Cancer Center approved all experiments involving mice.

MRI Protocol and Image Analysis

Magnetic resonance micro-imaging was performed exactly as described in Ref. (33, 41, 42). Briefly, mice were anesthetized with 1–2% isoflurane in O_2 and then imaged using a vertical bore 7-T magnet, Bruker DRX300 spectrometer, ParaVision 3.0.2 software (Bruker), and a single tuned ^{1}H cylindrical radiofrequency coil. Kidney and cyst volume were quantified using Image J (43). For estimation of kidney volume, the kidney parenchyma was manually surrounded while excluding the renal pelvis, and summing up the products of area measurements of contiguous images and slice thickness, as in Ref. (44). Subsequently isolated kidney areas

were prepared using defined settings for background subtraction and band passing, with a threshold set for each kidney based on the original images by targeting threshold values designating the transition between parenchyma and cyst at the border of the larger cysts in the kidneys. Cyst volume was estimated using a semi-automatic threshold approach (45, 46).

Tissue Preparation and Histology

All tissues were collected and fixed in 10% phosphate-buffered formaldehyde (formalin) for 24–48 h, dehydrated and embedded in paraffin. Hematoxylin and eosin (H&E) stained 5 μm sections were used for morphological evaluation.

Western Blotting

To analyze the expression levels of individual proteins, kidney tissues were lysed and resolved by SDS-PAGE. Western blotting was performed using standard procedures, and developed by chemiluminescence using Luminata Western HRP substrates (Classico, Crescendo and Forte) (EMD Millipore) and Immun-Star AP Substrate (Bio-Rad Laboratories). Quantification of signals on Western blots was done using the NIH ImageJ Imaging and Processing Analysis Software with signaling intensity normalized to loading control (β-actin or vinculin). Primary antibodies included anti-Src (Cell Signaling, #2110), anti-phospho-Src Tyr418 (Abcam, #ab4816), anti-S6 (Cell Signaling, #4858), anti-phospho-S6 S235/236 (Cell Signaling, #2317), anti-phospho-ERK Thr202/Tyr204 (Cell Signaling, #9101), anti-phospho-EGFR Y1068 (Cell Signaling, #3777), anti-phospho-EGFR Y1173 (Invitrogen, #44794G), anti-EGFR (Cell Signaling, #2646), anti-phospho-Akt S473 (Cell Signaling, #4060), anti-Akt (Cell Signaling, #2920), anti-Aurora-A (mouse, BD Transduction, #610939 and rabbit, Cell Signaling, #3092), anti-histone H3 (Cell Signaling, #3638S), anti-vinculin (Sigma, #V9131), and mouse anti-β-actin conjugated to HRP (Abcam, #ab49900). Secondary anti-mouse and anti-rabbit HRP-conjugated antibodies (GE Healthcare) were used at a dilution of 1:10,000 and secondary anti-mouse and anti-rabbit AP-conjugated antibodies (Jackson Immunoresearch Labs) were used at a dilution of 1:5,000.

Phosphorylation Assay

Histone H3 (Upstate, Charlottesville, VA, USA) was used as substrate for AURKA kinase activity, using standard methods. Parallel aliquots without [γ32P]ATP were processed for SDS-PAGE. To assess Aurora-A activation, we performed an *in vitro* kinase assay using AURKA immunoprecipitated from whole kidney lysates using beads conjugated with anti-Aurora A antibody (Bethyl Laboratories, S300-070-3). Immunoprecipitation samples were incubated overnight with antibody at 4°C, washed, and resolved by SDS-PAGE.

Statistical Analysis

Analyses were performed using STATA version 12. Data were analyzed using Wilcoxon rank-sum tests and generalized linear models with appropriate family and link functions (e.g., Gaussian or Gamma families with log or identity links). Where necessary, we estimated growth curves using generalized estimating equations (GEE) with exchangeable or Markov working correlation matrices to account for correlated data (46).

RESULTS

Alisertib and Erlotinib Treatment of a Conditional Knockout Model for ADPKD: Modest Effect on Kidney Volume and Weight

We used a previously described *Pkd1* conditional knockout mouse model in which tamoxifen induction of a Cre-flox regulatory system allows targeted inactivation of the *Pkd1* gene *in vivo* (39, 40). In this system, the loss of *Pkd1* at post-natal day 28 results in development of renal cysts at ~4.5–5 months of age, progressing to severe enlargement of the kidney and renal failure at 6–7 months of age. The experimental outline is shown in **Figure 1A**. We defined four cohorts of $Pkd1^{-/-}$ mice: Cohort 1 ($n = 11$), vehicle (10% cyclodextrin, 1% sodium hydrocarbonate, and 5% dextrose, with 10% DMSO mixed in 1:1 ratio) twice a day; Cohort 2 ($n = 16$), alisertib, 20 mg/kg, twice a day (40 mg/kg daily); Cohort 3 ($n = 13$), erlotinib, 10 mg/kg, once a day; and Cohort 4 ($n = 14$), alisertib 20 mg/kg, twice a day plus erlotinib, 10 mg/kg, once a day (2 h after the morning dose of alisertib). Parallel cohorts 5–8 were also run, with wild type mice that received the same dosing regimen: each of these cohorts contained 8–10 animals. Starting at the time of injection, mice were weighed weekly. Treatment with alisertib or alisertib plus erlotinib resulted in slower weight gain over 10 weeks in both wild type and $Pkd1^{-/-}$ groups, while erlotinib alone had no effect on weight gain (**Figure 1B**).

For analysis of kidney enlargement over time, $Pkd1^{-/-}$ and wt mice were assessed at 4, 5.5, and 6.5 months of age using a MRI approach (41) (**Figure 2A**). In general, drug effects on rate of kidney growth did not rise to statistical significance. Following normalization to BW, alisertib slightly increased the rate of kidney growth versus vehicle-treated $Pkd1^{-/-}$ mice at all time points (**Figure 2B**). Erlotinib did not significantly affect growth, at all time periods. The alisertib/erlotinib combination initially resulted in a rate of kidney growth similar to vehicle or erlotinib-treated mice, but at latter time points, the ratio of kidney volume to BW indicated a phenotype more similar to alisertib. However, it is important to note that mice treated with this drug combination had a significantly lower BW (**Figure 1B**), which likely contributes to the difference. As a control, we established that no drug affected kidney volume increase in wild type mice (**Figure 2C**). After 10 weeks, mice were euthanized and kidney weight to BW ratio directly determined. This confirmed findings from MRI, with a non-statistically significantly trend toward elevated BW in $Pkd1^{-/-}$ mice treated with alisertib or alisertib/erlotinib, and all mice with a $Pkd1^{-/-}$ genotype having a statistically significant greater kidney weight than all wt mice (**Figure 2D**). Hepatic cysts are a common feature of ADPKD in humans, occurring in a significant number of patients. In a previous study, we showed that inhibition of HSP90 significantly reduced liver cyst burden (42), while also reducing the rate of development of kidney cysts (41). In the present study, the alisertib effect was specific to kidney

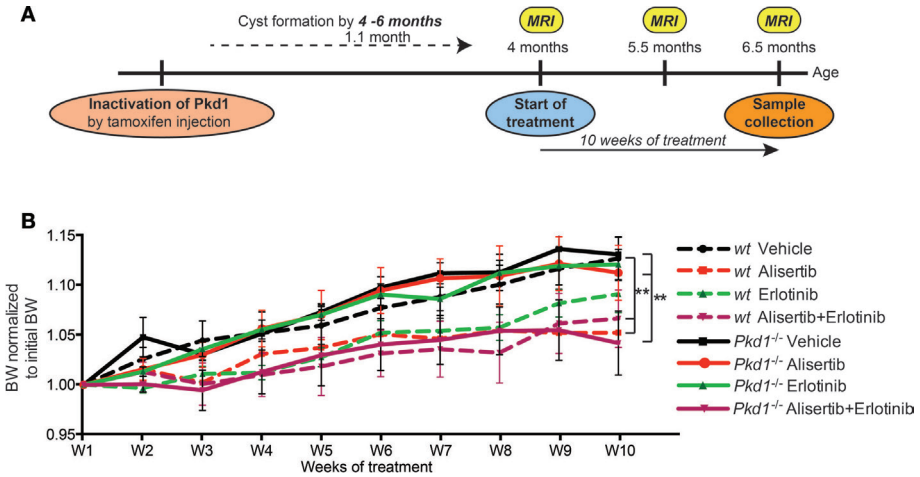


FIGURE 1 | Alisertib and erlotinib treatment of a conditional knockout model for ADPKD. **(A)** Experimental design. *In vivo* experiments were performed using a conditional knockout mouse model for tamoxifen-induced, Cre-dependent ablation of *Pkd1* (39). **(B)** Body weight (BW) dynamics for the course of the treatment (vehicle, alisertib, erlotinib, alisertib + erlotinib), measured weekly. Differences between genotypes and drug treatment groups at 10 weeks were statistically significant between alisertib and alisertib + erlotinib versus the vehicle-treated groups in both wt and $Pkd1^{-/-}$ groups. *$P < 0.01$ relative to vehicle.

tissue, and no effect was seen with any drug treatment in liver from wild type or $Pkd1^{-/-}$ mice (**Figure 2E**).

Alisertib and Erlotinib Treatment of a Conditional Knockout Model for ADPKD: Drug Interactions in Control of Cyst Development

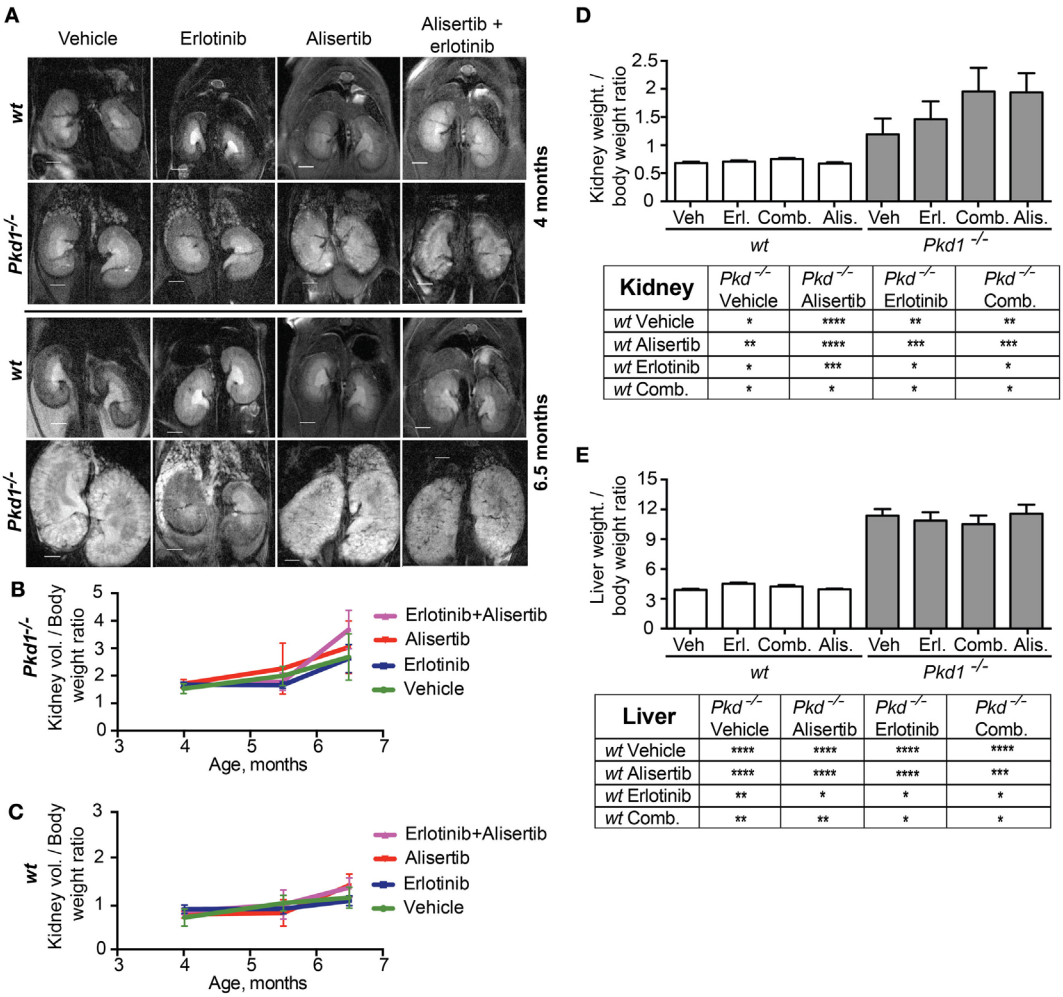
The development of cysts was analyzed by quantification of MRI imaging (**Figures 2A** and **3A**), and subsequently confirmed by visual assessment of hematoxylin and eosin (H&E) stained tissues collected after 10 weeks of treatment (**Figures 3B,C**). No wild type mice developed cysts. Among the $Pkd1^{-/-}$ mice, there was some heterogeneity in cyst development between individual animals, in concordance with the basic biology of the disease and previous reports using the model (39–41). Notably, the erlotinib treatment strikingly reduced cystogenesis in most animals, at all time points, in a statistically significant effect (**Figure 3A**). Alisertib treatment elevated cyst growth early, and cystogenesis was much greater than in vehicle-treated animals by the experimental endpoint, as previously noted (33). Interestingly, the alisertib/erlotinib combination treatment caused an initial delay in the formation of cysts, similar to erlotinib. However, at later time points, the beneficial effect was lost, and at experimental endpoint, the overall phenotype resembled alisertib-treated mice. Mice treated with alisertib or erlotinib plus alisertib had an extremely heterogeneous phenotype at the experimental endpoint. Although the majority had highly cystic kidneys, some had only limited cysts, suggesting a stochastic effect in response to drug between individual animals, and accounting for the large error bars.

Signaling Consequences of Alisertib and Erlotinib Treatment *Pkd1*$^{-/-}$ and wt Kidneys

To better understand the functional interaction of inhibition of AURKA and EGFR, we analyzed activation of the signaling of these drug targets and of signaling pathways relevant to ADPKD in kidney tissue collected from $Pkd1^{-/-}$ and wt mice after 10 weeks of drug treatment.

Under conditions of vehicle treatment, AURKA expression was elevated in $Pkd1^{-/-}$ versus wt kidneys, as previously reported (33) (**Figure 4A**). *In vitro* kinase analysis of phosphorylation of the substrate histone H3 (HH3) by AURKA immunoprecipitated from kidney lysates, or the autophosphorylation of immunoprecipitated AURKA, normalized to total levels of immunoprecipitated kinase (**Figure 4B**), surprisingly indicated that drug treatments did not produce statistically significant effects on AURKA activity. However, parallel Western analysis (**Figures 4C,D**) indicated that total levels of AURKA were significantly depleted in tissue treated with each of the drugs, particularly in those treated with alisertib or alisertib plus erlotinib. Generally, similar effects of drug treatment were observed in wt kidneys. Hence, the primary consequence of alisertib treatment was to reduce overall AURKA activity by reducing total AURKA expression.

Epidermal growth factor receptor activation is reflected by phosphorylation of EGFR at Y1068, which allows it to associate with GRB2 to activate downstream signaling cascades (47), and at Y1173, which is important for SHC binding and EGFR internalization (48). Total EGFR expression was not elevated in wt versus $Pkd1^{-/-}$ kidneys (**Figure 4A**). However, in $Pkd1^{-/-}$ kidneys, Y1068 phosphorylation was significantly increased by treatment with alisertib, or alisertib plus erlotinib, and Y1173 phosphorylation was increased, albeit to a lesser degree (**Figures 4E–G**). Furthermore, in $Pkd1^{-/-}$ kidneys, total EGFR expression was also elevated by treatment with alisertib or the alisertib/erlotinib combination (**Figures 4E,H**). Together, these results emphasized the role of AURKA inhibition in potentiating proliferative signaling relevant to a severe cystic phenotype. By contrast, no drug treatment significantly affected EGFR expression or phosphorylation on Y1173 in relation to vehicle in wt kidneys, although interestingly, the alisertib/erlotinib combination



Kidney	*Pkd* $^{-/-}$ Vehicle	*Pkd* $^{-/-}$ Alisertib	*Pkd* $^{-/-}$ Erlotinib	*Pkd* $^{-/-}$ Comb.
wt Vehicle	*	****	**	**
wt Alisertib	**	****	***	***
wt Erlotinib	*	***	*	*
wt Comb.	*	*	*	*

Liver	*Pkd* $^{-/-}$ Vehicle	*Pkd* $^{-/-}$ Alisertib	*Pkd* $^{-/-}$ Erlotinib	*Pkd* $^{-/-}$ Comb.
wt Vehicle	****	****	****	****
wt Alisertib	****	****	****	***
wt Erlotinib	**	*	*	*
wt Comb.	**	**	*	*

FIGURE 2 | Effects of alisertib and erlotinib on kidney growth. **(A)** Representative images of murine kidneys acquired by using magnetic resonance micro-imaging (MRI) approach, at 4 (top) and 6.5 (bottom) months of age following treatment with vehicle, alisertib, erlotinib, and a combination of these drugs. **(B,C)** Quantification of the MRI imaging results for kidney volume, normalized to body weight for *Pkd1*$^{-/-}$ **(B)** and wt **(C)** mice. Differences at experimental endpoint (10 weeks of treatment) are not statistically significant. **(D,E)** Direct measurement of kidney **(D)** and liver **(E)** weight normalized to body weight for *Pkd1*$^{-/-}$ and wt mice at experimental endpoint (10 weeks of treatment). Top – graphs representing results; bottom – summary of *P*-values for the presented data. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

significantly induced Y1068 phosphorylation in a subset of wt kidneys (**Figures 4E–H**).

We then analyzed the activity and expression of the ADPKD-related proteins S6, SRC, ERK1/2, and AKT (**Figures 4** and **5**). For S6, AKT, and SRC, total levels were elevated in *Pkd1*$^{-/-}$ versus wt kidneys (**Figure 4A**). The patterns of response to drug treatment were complicated for these downstream pro-proliferative proteins. Focusing first on alisertib in *Pkd1*$^{-/-}$ kidneys, this treatment significantly reduced levels of total S6 and SRC, and increased total levels of ERK1/2 (**Figure 5**). However, alisertib also resulted in a very significant increase in the ratio of active (phosphorylated) S6, leading to a net gain in S6 activity in kidney lysates, compatible with an increased cystic phenotype. By contrast, activity of SRC and ERK was reduced by alisertib in *Pkd1*$^{-/-}$ kidneys. These patterns of expression and activation were very different from those evoked by alisertib treatment of wt kidneys. In wt kidneys, alisertib very significantly reduced S6 and ERK1/2 activation, and reduced SRC expression. Alisertib treatment also resulted in a variable pattern of SRC activation, with three mice having very high levels of phosphorylated SRC, but most having SRC activity reduced. Whereas erlotinib or erlotinib plus alisertib effectively reduced ERK1/2 and S6 activity in wt kidneys, these treatments were less effective in *Pkd1*$^{-/-}$ kidneys. With the exception of effect on total ERK1/2 expression in *Pkd1*$^{-/-}$ kidneys, erlotinib, and erlotinib plus alisertib resulted in statistically non-distinct effects on the expression and activation of the signaling proteins analyzed. This was surprising, given the very different results of these treatments on cystic phenotype.

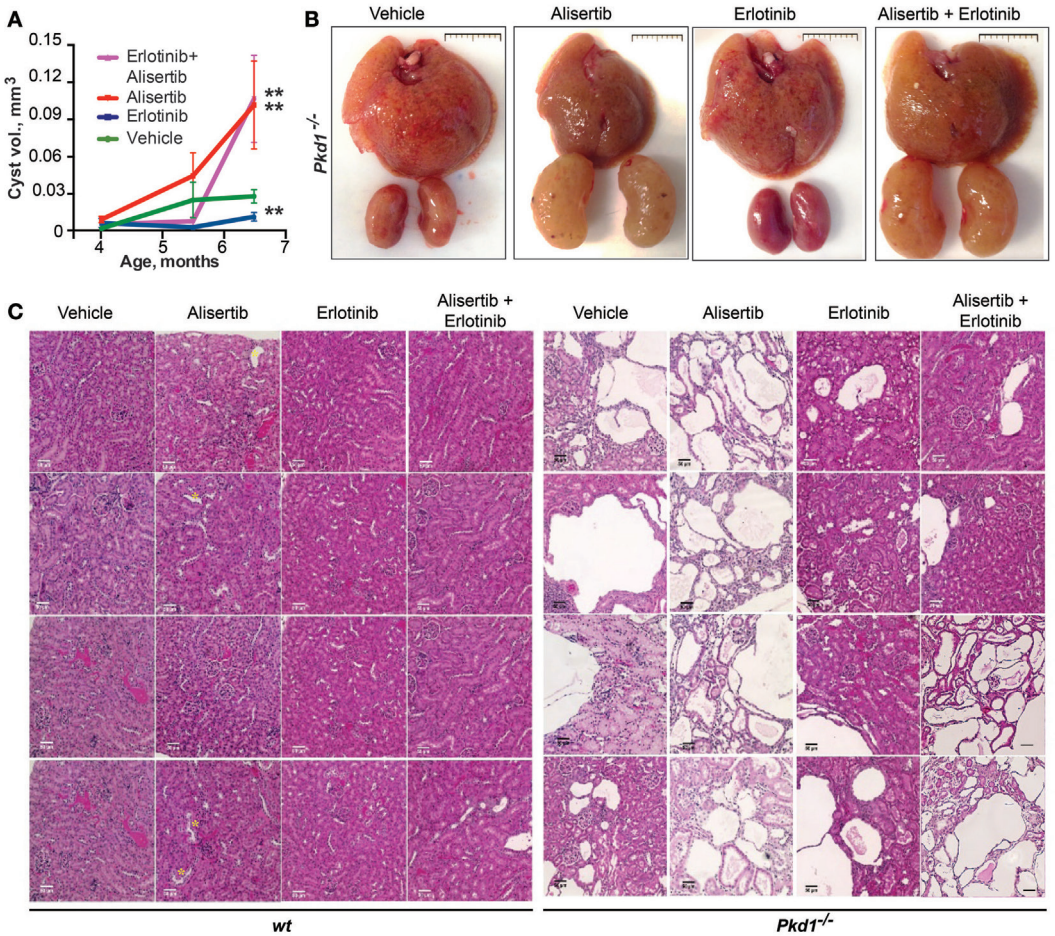


FIGURE 3 | Cystogenesis during drug treatment in *Pkd1*$^{-/-}$ and wt mice. **(A)** Ratio of renal cyst volume to body weight in *Pkd1*$^{-/-}$ mice in the four drug treatment groups. *$P < 0.05$ and **$P < 0.01$ relative to vehicle treatment. **(B)** Representative images of collected livers (top) and kidneys (bottom) from each treatment cohort; scale bar, 1 cm. **(C)** Representative light microscopy images from H&E slides, reflecting cystic burden of *Pkd1*$^{-/-}$ and wt mice in all treatment groups at experimental endpoint (10 weeks of treatment). Images taken at 20× magnification; scale bar, 50 μm.

DISCUSSION

As significant findings, this study has established that the cystogenic activity of the AURKA inhibitor in ADPKD can be partially reversed by treatment with erlotinib, and for the first time showed that erlotinib itself has a potent activity in limiting cystic growth. Second, it also demonstrated that erlotinib and alisertib elicited distinct response profiles in the kidneys of wt versus *Pkd1*$^{-/-}$ mice, potentially reflecting differences in signaling landscape associated with the replacement of the normal renal parenchyma with cysts. Third, it also demonstrated that in the context of drug treatment, changes in degree of cystogenesis in *Pkd1*$^{-/-}$ mice could not be aligned with specific changes in expression or activity of AKT, S6, ERK1/2, or SRC, in spite of the common association of elevated activity of these signaling proteins with disease etiology. Finally, as discussed below, this work emphasized some significant differences between the interactions of alisertib and erlotinib in the context of ADPKD versus cancer, such that this treatment is potentially beneficial in cancer but not ADPKD.

These findings confirmed earlier reports that AURKA activity was associated with cyst formation, and that inhibition of AURKA exacerbated cystogenesis in the context of genetic loss of *Pkd1* (23, 24, 33). A particularly interesting finding was that treatment with alisertib alone or in combination with erlotinib was more strongly associated with loss of AURKA protein, rather than inhibition of AURKA activity. There are two potential explanations for this observation. First, AURKA expression is highly regulated by protein degradation, and the protein is more susceptible to degradation when catalytically inactive (49, 50). Hence, alisertib treatment may be elevating the rate of AURKA destruction, and in this context, the elevated activity of the remaining AURKA may represent a sub-population effectively protected by interaction partners (49, 50). Second, AURKA expression has been reported as most abundant and active in the renal epithelia of early cysts (23, 24, 33). However, in the

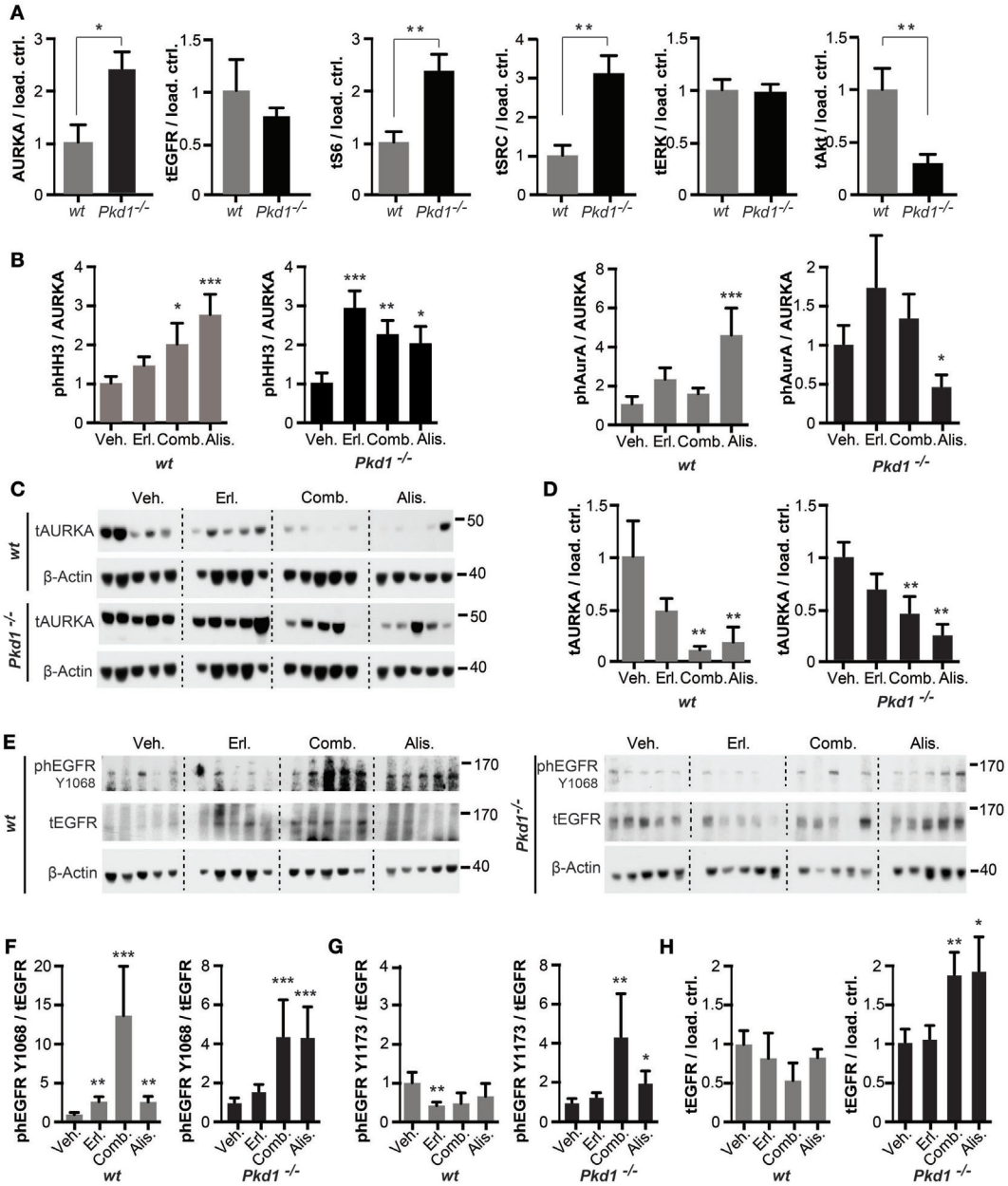


FIGURE 4 | Drug inhibition of targets in *Pkd1*$^{-/-}$ and wt mice. **(A)** Quantification of Western data for indicated proteins from kidney lysates prepared from vehicle-treated wt or *Pkd1*$^{-/-}$ mice, normalized to β-actin or vinculin-loading control. *$P < 0.05$ and **$P < 0.01$ relative to vehicle treated. **(B)** Aurora-A (AURKA) was immunoprecipitated from kidney lysates and used for *in vitro* kinase with γ-32P-ATP to indicate autophosphorylation and phosphorylation of histone H3 kinase (HH3) (top two rows), with parallel blots probed by Western to allow normalization to total AURKA and HH3 in reaction. Quantitation of data from complete cohort of mice in each treatment group, indicating ratio of phosphorylated HH3 or AURKA to total immunoprecipitated AURKA in *wt* and *Pkd1*$^{-/-}$ mice following indicated drug treatments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ relative to vehicle treated. **(C)** Western analysis showing representative expression of total Aurora-A (tAURKA) in kidney lysates after 10 weeks of treatment with indicated drugs, with β-actin loading control. **(D)** Quantitation of data from complete cohort of mice in each treatment group, indicating ratio of total AURKA to β-actin in *wt* and *Pkd1*$^{-/-}$ mice following indicated drug treatments. **$P < 0.01$ relative to vehicle treated. **(E)** Western blot with representative images of kidney lysates from each treatment cohort showing expression of EGFRphosphorylated (ph) at the indicated amino acids, total EGFR (tEGFR) or β-actin loading control. **(F–H)** Quantitation of data from complete cohort of mice in each treatment group, indicating ratio of phEGFR-Y1068 to tEGFR **(F)**, phEGFR-Y1173 to tEGFR **(G)**, or tEGFR to loading control **(H)**. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, relative to vehicle treated.

tissue isolated at the end of this experiment and used for signaling analysis, the majority of tissue in alisertib- and alisertib/erlotinib-treated cells reflects loss of normal renal structure and replacement with enlarged late-stage cysts that have in many cases lost epithelial lining, and fibrotic tissue. In this interpretation, a difference in tissue composition explains the loss of

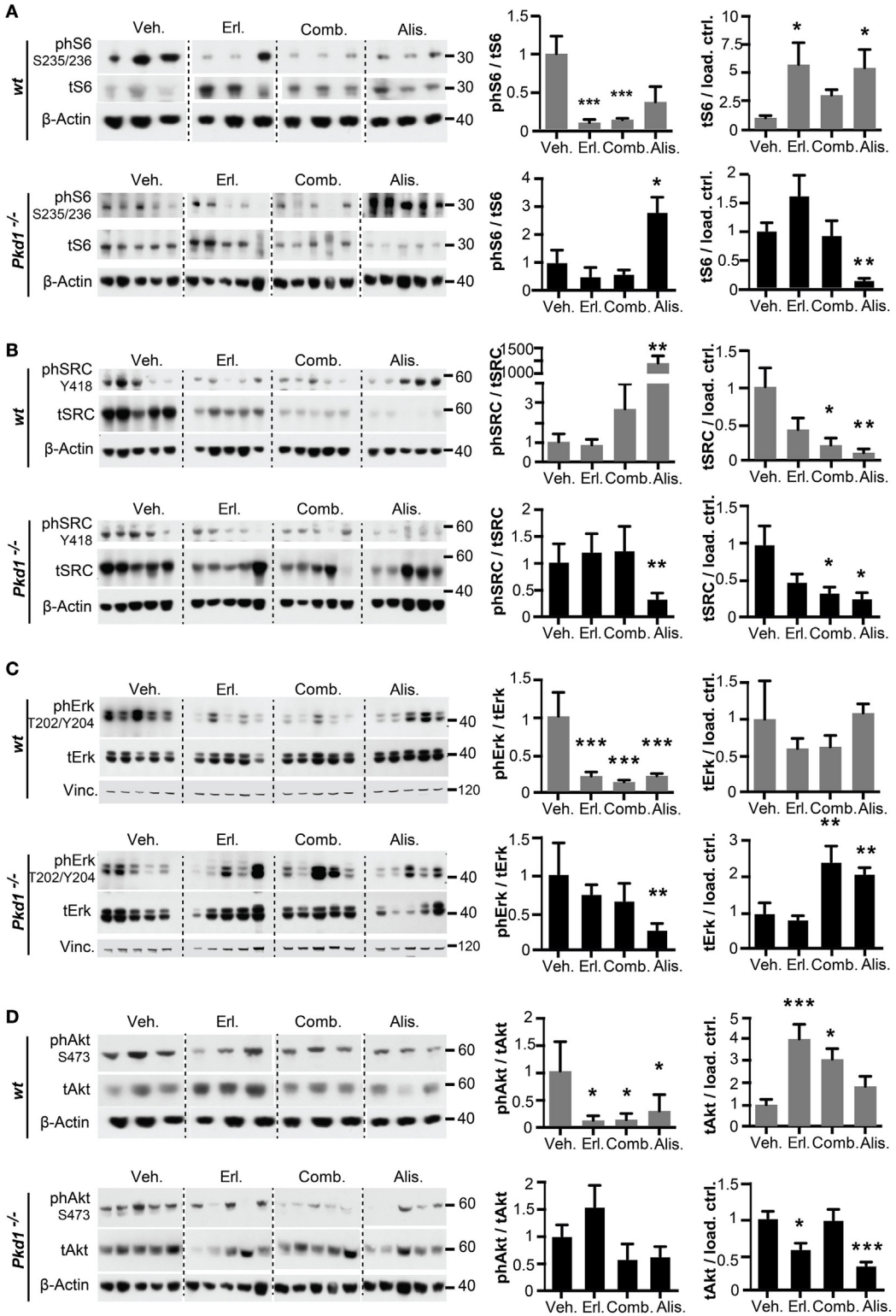


FIGURE 5 | Drug inhibition of ADPKD-related signaling proteins in *Pkd1*$^{-/-}$ and wt mice. S6 **(A)**, SRC **(B)**, ERK1/2 **(C)**, and AKT **(D)** in kidney lysates after 10 weeks of treatment with indicated drugs. Western analysis (left) showing representative expression of phosphorylated and total expression of proteins and quantitation of data (right) from complete cohort of mice in each treatment group with analysis, as described in **Figure 4**. **(A–D)** Ratios of phosphorylated to total protein for S6 **(A)**, SRC **(B)**, ERK1/2 **(C)**, and AKT **(D)** are shown in the left graph, and ratios of total protein to β-actin or vinculin (vinc) loading control are shown in the right graph. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ relative to vehicle treated.

AURKA activity. Potentially, both explanations contribute to the observed phenotypes.

In analysis of signaling consequences of AURKA inhibition, alisertib reduction of SRC expression and activity was notable. One previous study has shown direct AURKA phosphorylation of SRC enhances SRC activity (51); it is possible that this phosphorylation also contributes to SRC stability, so that alisertib destabilizes SRC. The significant effect of AURKA inhibition on S6 phosphorylation is compatible with the enhanced cystogenesis seen in alisertib-treated mice. However, comparison of alisertib versus alisertib/erlotinib-treated mice confounds simple interpretation of specific signaling changes as relevant to the experimental endpoint, given the cystogenic phenotype of the combination resembles alisertib, whereas the signaling phenotype resembles erlotinib. This discordance suggests that while proteins such as S6 and SRC may have elevated expression and activity in ADPKD, their contribution to disease pathogenesis is not an essential driver of disease progression. This interpretation would be compatible with the limited activity of inhibitors of the S6 activator mTOR (52–54), or inhibitors of SRC (55), in assessment for treatment of polycystic kidney disease.

Although erlotinib has not previously been assessed, some prior studies have used alternative inhibitors of EGFR (56–58) or related ERBB-family kinases (59) in various models for cyst formation. Typically although not invariably, these studies have found inhibition of EGFR/HER2 signaling reduced cystic burden. In this study, we show that erlotinib is well tolerated and effective in controlling cyst growth in the context of loss of Pkd1, and that erlotinib can delay the growth of cysts that alisertib promotes. These results suggest that erlotinib may be useful for clinical evaluation in ADPKD. It is also interesting and important to note that erlotinib treatment reduces the level of AURKA in wt but not *Pkd1*$^{-/-}$ kidneys (**Figure 4**). In this case, one possible explanation is that erlotinib treatment causes wt cells to accumulate in the G1 phase of cell cycle by inhibiting multiple mitogenic effector pathways (60), hence reducing the population of G2/M phase cells in which AURKA is most abundant, whereas this inhibition is partially overcome in ADPKD tissue. This interpretation would suggest that small pool of AURKA at the basal body of cilia (61) – the pool potentially most relevant to the control of cystic severity, based on the model developed by Ma and colleagues (34) – might not be affected by erlotinib, as it is active in G0/G1. Test of this idea requires development of antibodies or probes suitable for analysis of AURKA activity by immunofluorescence in mouse tissue, currently a technical limitation on performing this work (50). Additional analyses of future interest would be the profiling of renal function (rather than cystic burden) following treatment with alisertib, erlotinib, or the combination, as well as broader profiling of gene expression changes following such treatments.

Finally, understanding the basis of AURKA and EGFR activity in ADPKD is of considerable interest for the field of oncology. One in 500 individuals suffers from ADPKD, many of whom will ultimately develop cancer and could potentially be treated with alisertib or erlotinib, given the common use of these agents. This work emphasizes not only the importance of avoiding alisertib but also suggests these patients would safely receive erlotinib. This study also emphasizes the different functional and signaling interactions of targeted inhibitors in distinct cellular contexts. Typically, AURKA inhibitors are used in therapeutic combinations with cytotoxic agents or other targeted agents (62): there is great interest in identifying productive therapeutic combinations. The positive interaction of alisertib and erlotinib in the context of oncogenic drivers (38), versus the opposing activity in the context of lesions in *Pkd1*, emphasizes the dynamic nature of signaling networks. Given the large number of comorbidities that are commonly experienced by cancer patients, including diabetes, cardiovascular disease, and other conditions that can affect cell signaling, there is clearly a need for further study.

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