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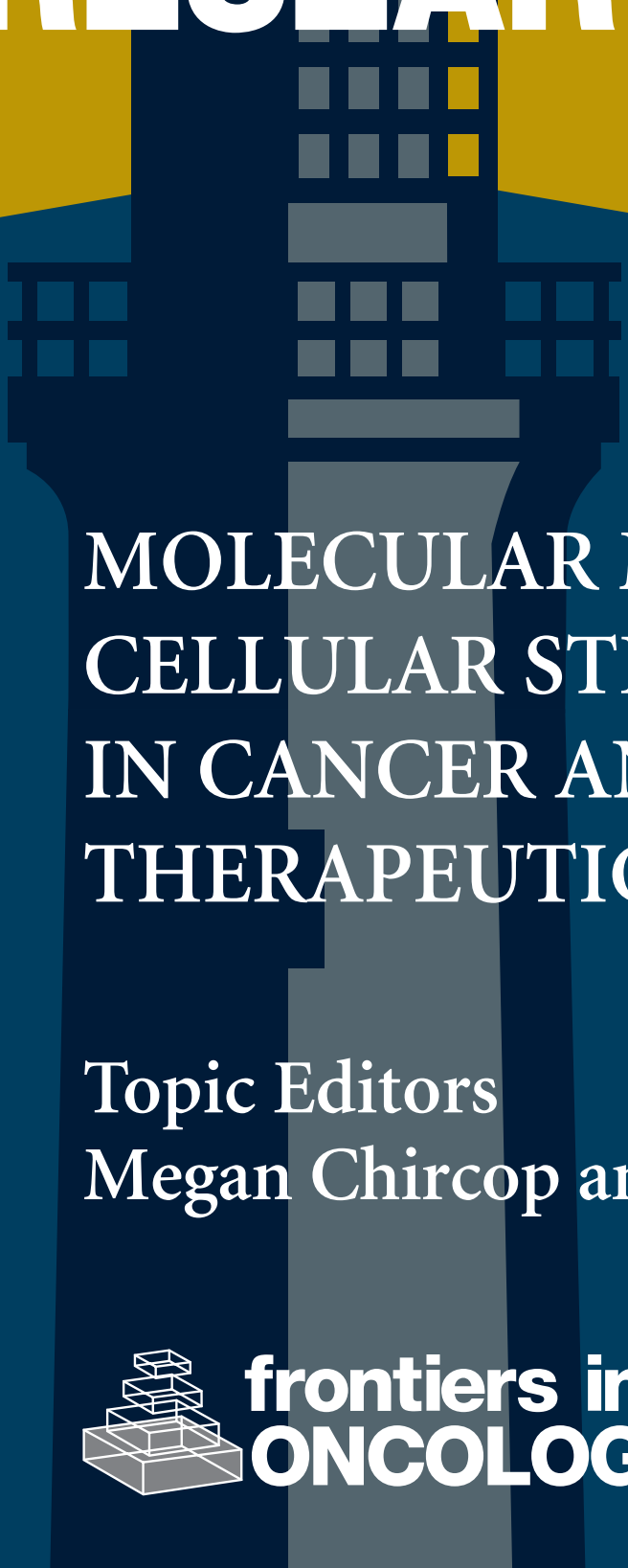

MOLECULAR MECHANISMS OF CELLULAR STRESS RESPONSES IN CANCER AND THEIR THERAPEUTIC IMPLICATIONS

Topic Editors
Megan Chircop and Daniel Speidel

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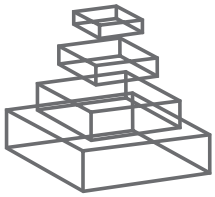

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MOLECULAR MECHANISMS OF CELLULAR STRESS RESPONSES IN CANCER AND THEIR THERAPEUTIC IMPLICATIONS

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In response to stress, cells can activate a myriad of signalling pathways to bring about a specific cellular outcome, including cell cycle arrest, DNA repair, senescence and apoptosis. This response is pivotal for tumour suppression as all of these outcomes result in restriction of the growth and/or elimination of damaged and pre-malignant cells. Thus, a large number of anti-cancer agents target specific components of stress response signalling pathways with the aim of causing tumour regression by stimulating cell death. However, the efficacy of these agents is often impaired due to mutations in genes that are involved in these stress-responsive signalling pathways and instead the oncogenic potential of a cell is increased leading to the initiation and/or progression of tumourigenesis. Moreover, these genetic defects can increase or contribute to resistance to chemotherapeutic agents and/or radiotherapy. Modulating the outcome of cellular stress responses towards cell death in tumour cells without affecting surrounding normal cells is thus one of the ultimate aims in the development of new cancer therapeutics. To achieve this aim, a detailed understanding of cellular stress response pathways and their aberrations in cancer is required.

This Research topic aims to reflect the broadness and complexity of this important area of cancer research.

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EDITORIAL
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Cellular stress responses in cancer and cancer therapy

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Keywords: apoptosis, DNA damage, DNA repair, chemotherapy, cellular stress, tumorigenesis, therapeutic targets, therapy resistance

In response to stress, cells activate so-called checkpoints – complex signaling pathways that induce a plethora of cellular outcomes. Checkpoints primarily initiate cell cycle arrest to provide the cell with time to repair the damage. However, if the damage is too severe then cells can permanently arrest the cell cycle (senescence) or trigger cell death, thereby preventing the transmission of genetic defects. These responses are pivotal for tumor suppression as all of these outcomes result in restriction of the growth and/or elimination of damaged and pre-malignant cells. Thus, a large number of anti-cancer agents target specific components of stress response signaling pathways with the aim of causing tumor regression by stimulating cell death or at least stopping cell growth. However, the efficacy of these agents is often impaired by mutations in genes that are involved in stress-responsive signaling pathways. Moreover, these cancer-specific genetic defects often contribute to resistance against chemotherapeutic agents and/or radiotherapy. Modulating the outcome of cellular stress responses toward cell death in tumor cells without affecting surrounding normal cells is thus one of the ultimate aims in the development of new cancer therapeutics. To achieve this aim, a detailed understanding of cellular stress response pathways and their aberrations in cancer is required.

The Research Topic titled "Molecular mechanisms of cellular stress responses in cancer and their therapeutic implications" features 11 articles that reflect the broadness and complexity of the processes induced by cellular stress. It begins with reviews on four different proteins/protein families that are critical for cellular stress responses and as such are important for both cancer development and the response to cytotoxic therapies.

Knippschild and colleagues discuss the complex functions of the casein kinase 1 (CK1) family and describe in depth how members of this family regulate signaling cascades that are relevant for the pathogenesis of inflammatory and proliferative diseases and, beyond this, for neurodegenerative disorders as well. They also summarize current knowledge on therapeutic modulation of CK1 activity and existing inhibitors (1).

In addition to phosphorylation, other post-translational modifications are an effective means to modify the activity of cellular proteins and hence respond to stress signals. Polonio-Valon et al. focus on the peptidyl-prolyl *cis/trans* isomerase Pin1, an enzyme that can induce conformational changes in its substrate proteins. In their article, they highlight the interactions of Pin1 with key proteins relevant to cancer and cancer therapy and discuss how Pin1 specifies cell fate decision in response to DNA damage (2).

One of the key proteins in cellular stress signaling is the tumor suppressor p53 and any collection of articles dealing with stress responses would be incomplete without an article on p53. Mueller and colleagues give a brief overview on the extensive literature on p53 and its family members, p63 and p73 with a specific focus on therapeutic implications (3).

Parker et al. discuss another well known protein family, the tubulins and their interacting partners. Tubulins are the building blocks of microtubules and therefore responsible for cell movement, intracellular trafficking, and cell division. They are also the target of a specific class of chemotherapeutics. As this article points out, microtubules and associated proteins play an important role in a range of cellular stress responses (4).

Understanding cellular processes that are differentially regulated in cancerous versus normal cells is a prerequisite for exploiting them therapeutically. Three articles focus on this aspect.

A hallmark of cancer as a proliferative disorder is the increased number of cell divisions and a high mitotic index. Mitotic cells respond differently to stress signals than interphase cells due to their condensed chromosomes. Burgess and colleagues review the pathways and outcomes activated by mitotic cells in response to stress and describe how this influences efficacy of chemotherapeutic drugs, especially those in the anti-mitotic class (5).

Abnormal DNA content is another common hallmark of cancer cells that has been recognized for a long time. In their hypothesis and theory article, Coward and Harding summarize evidence that links the acquisition of multiple chromosome copies (polyploidy) to tumor evolution and chemotherapy resistance. They argue that these polyploid cells themselves are critical drug targets (6).

Double-strand breaks are also prevalent in many cancer cells due to their increased proliferation and impaired DNA repair programs. Jekimovs et al. review the two DNA repair pathways activated by DNA double-strand-breaks and discuss the successes and failures of pre-clinical and clinical trials aiming to modulate these pathways (7).

Finally, four articles highlight some of the many factors that influence the success of cancer therapy with cytotoxic agents.

One of the most challenging problems is tumor heterogeneity, a topic discussed by Renovanz and Kim who argue that there is much to learn to be able to treat cancer patients more effectively (8).

Tumor hypoxia is another problematic aspect in many solid tumors as this has been linked to resistance against radiation and chemotherapy. In their original research article, Ontikatze and colleagues characterize a specific drug, dihydroartemisinin, that

may be instrumental in overcoming therapy resistance of hypoxic tumors (9).

Hormones can also affect the response to cytotoxic agents and this seems particularly obvious for estrogen. Caldon describes the complicated relationship of estrogen and DNA damage signaling in breast cancer and proposes that estrogen receptor signaling suppresses effective DNA repair and apoptosis in favor of proliferation (10).

Breast cancer is also the focus of the original article by Quante and colleagues who report new insights into the process leading to hyperplastic lesions in the mammary gland obtained from the analysis of a transgenic mouse model (11).

This collection of articles highlights some of the advances made in understanding the molecular mechanisms of cellular stress responses and the implications of this for cancer biology. Research in this field has already enabled improved clinical outcomes for cancer patients and we are hopeful that with continued investigation of this topic more discoveries will be translated into even better cancer treatments.

ACKNOWLEDGMENTS

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REVIEW ARTICLE
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The CK1 family: contribution to cellular stress response and its role in carcinogenesis

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Members of the highly conserved and ubiquitously expressed pleiotropic CK1 family play major regulatory roles in many cellular processes including DNA-processing and repair, proliferation, cytoskeleton dynamics, vesicular trafficking, apoptosis, and cell differentiation. As a consequence of cellular stress conditions, interaction of CK1 with the mitotic spindle is manifold increased pointing to regulatory functions at the mitotic checkpoint. Furthermore, CK1 is able to alter the activity of key proteins in signal transduction and signal integration molecules. In line with this notion, CK1 is tightly connected to the regulation and degradation of β-catenin, p53, and MDM2. Considering the importance of CK1 for accurate cell division and regulation of tumor suppressor functions, it is not surprising that mutations and alterations in the expression and/or activity of CK1 isoforms are often detected in various tumor entities including cancer of the kidney, choriocarcinomas, breast carcinomas, oral cancer, adenocarcinomas of the pancreas, and ovarian cancer. Therefore, scientific effort has enormously increased (i) to understand the regulation of CK1 and its involvement in tumorigenesis- and tumor progression-related signal transduction pathways and (ii) to develop CK1-specific inhibitors for the use in personalized therapy concepts. In this review, we summarize the current knowledge regarding CK1 regulation, function, and interaction with cellular proteins playing central roles in cellular stress-responses and carcinogenesis.

Keywords: casein kinase 1, cellular stress, centrosome, p53, signal transduction, tumorigenesis, inhibitor, disease

THE CK1 FAMILY

Members of the CK1 (formerly named casein kinase 1) family were among the first kinases described in literature (1). Although the milk protein component casein is not a physiological substrate for CK1, it reflects its preference for serine or threonine residues N-terminally flanked by already phosphorylated amino acid residues or acidic amino acids (2–7). Seven distinct genes encoding mammalian CK1 isoforms α, β, γ1, γ2, γ3, δ, and ε as well as various post-transcriptionally processed splice variants (transcription variants; TV) have been characterized (except for β all are expressed in humans). The closest relatives to the CK1 family are tau tubulin kinases 1 and 2 (TTBK1/2) and the vaccinia-related kinases 1–3 (VRK1-3) (**Figure 1A**). All CK1 isoforms are highly conserved within their kinase domains (51–98% identical) while the highly related isoforms CK1δ and ε display the highest homology. However, CK1 family members differ significantly in length and primary structure of their regulatory non-catalytic C-terminal domains, resulting in molecular weights ranging from 32 kDa (CK1α) to 52.2 kDa (CK1γ3) (**Figure 1B**) (5, 8–16). Meanwhile, CK1 homologous proteins have also been isolated from yeast, basidiomycetes, plants, algae, and protozoa (9, 15, 17–23). Since recognition motifs for CK1 are found on most cellular proteins, more than 140 *in vitro* and *in vivo* substrates have been reported thus far (see CK1 Substrate Specificity and **Table 1**). Therefore, in a cellular context a tight regulation of CK1 activity and expression is indispensable. Known general mechanisms for CK1 regulation include (i) phosphorylation by inhibitory autophosphorylation and/or (ii) phosphorylation by other cellular protein kinases, and (iii) interaction with cellular proteins or subcellular sequestration (see Regulation of CK1 Activity). Based on the broad spectrum of target proteins, CK1 family members are involved in modulating a variety of cellular functions: in immune response and inflammation (see CK1 in Immune Response and Inflammation), in spindle and centrosome-associated processes (see Interaction of CK1 with Centrosomes, Tubulin, and Microtubule-Associated Proteins), in DNA damage-related signal transduction (see CK1 in DNA Damage-Related Signal Transduction), in circadian rhythm (see CK1 in Circadian Rhythm and its Connections to Stress Response), and in apoptosis (see CK1-Signaling in Apoptotic Pathways). Consequently, a deregulation or dysfunction of CK1 in pathways responsible for regulation of growth, proliferation, and apoptosis may result in pathological conditions (see CK1 and the Wnt Pathway, CK1 in the Hedgehog Pathway to CK1 in the Hippo Pathway), such as tumorigenesis (see CK1-Related Tumorigenic Functions and CK1 in Metastatic Processes) or neurological diseases. Therefore, interest in CK1 isoforms as new drug targets has enormously increased within the last 15 years and led to development of several CK1-specific inhibitors (see CK1-Specific Inhibitors).

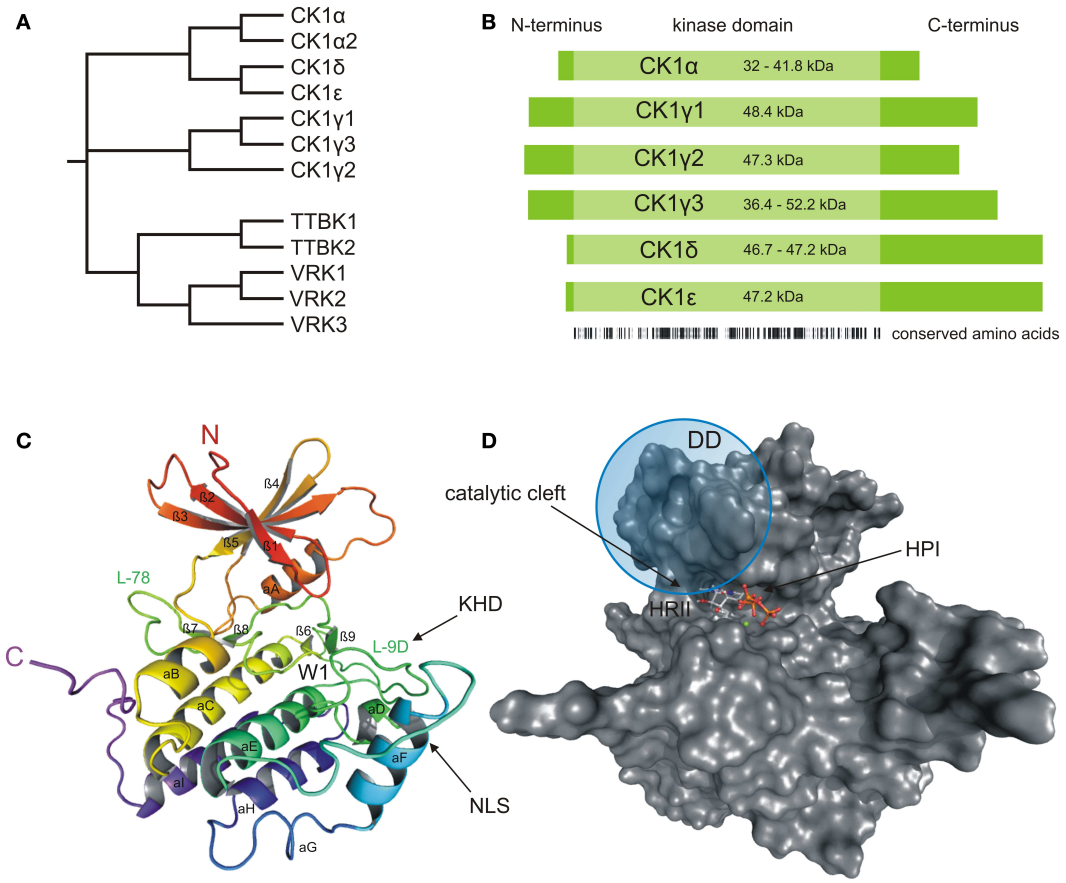


FIGURE 1 | Structural presentation of CK1δ. (A) Phylogenetic relation between CK1 isoforms of *Homo sapiens* (CK1α, γ1–3, δ, and ε) and other members of the human CK1 family (TTBK1–2, VRK1–3). **(B)** Schematic alignment of human CK1 isoforms α, γ1–3, δ, and ε. Their molecular weight varies between 32 (CK1α) and 52.2 kDa (CK1γ3). In case transcription variants have been reported for one isoform, the molecular weight is given as range from the smallest to the largest variant. All CK1 isoforms are highly conserved within their kinase domains (light green box, 286 aa), but differ within their variable N- (4–40 aa) and C-terminal (39–122 aa) non-catalytic domains (dark green boxes) [according to Knippschild et al. (333)]. Ribbon **(C)** and surface **(D)** diagram of the molecular structure of CK1δ (PDB code 4HGT) modeled in complex with Mg^{2+}-ATP at a resolution of 1.80 Å. The nomenclature is adapted from Xu et al. (24) and Longenecker et al. (25). Until today, crystal structures of human CK1 isoforms γ1 (PDB code 2CMW), γ2 (2C47), γ3 (2CHL, 2IZR, 2IZS, 2IZT, 2IZU, 4HGL, 4HGS, 4G16, 4G17), δ (4KB8, 4KBA, 4KBC, 4KBK, 4HNF, 3UYS, 3UYT, 3UZP), and ε (4HNI, 4HOK) are accessible as well. For reasons of clarity, we focused on CK1δ exemplarily, due to its superior relevance. The catalytic domain folds into two lobes primarily containing strands (N-terminal), respectively helices (C-terminal) forming a catalytic cleft between that represents the ATP binding pocket as well as a substrate binding site. KHD indicates the kinesin homology domain within L-9D. DD refers to a putative dimerization domain containing various amino acids of β1, β2, β5, L-5B, β7, and αB, whereas NLS displays a putative nuclear localization signal sequence at the junction between L-EF and αF. A tungstate molecule binding site identifies a specific phosphate moiety binding motif (W1). The active site contains a deep hydrophobic pocket (HPI) and a spacious hydrophobic region (HRII) (25–28). All modeling and docking studies were performed using Schrödinger software (Maestro, version 9.3, Schrödinger, LLC, New York, NY, 2012; Glide, version 5.8, Schrödinger, LLC, New York, NY, 2012). The illustration of modeling results was generated by the PyMOL Molecular Graphics System (Version 1.5.0.4, LLC) (29).

CK1 STRUCTURE AND DOMAINS

As a member of the superfamily of serine/threonine-specific kinases, CK1 represents the typical bi-lobal structure, which includes a smaller N-terminal lobe, primarily consisting of β-sheets, and a larger, mainly α-helical C-terminal lobe. The two lobes are connected by a hinge region forming a catalytic cleft for substrate and ATP binding (**Figures 1C,D**) (24, 25). In comparison to the general structural features of protein kinases, a prominent α-helix (αA-helix) within the N-terminal region is crucial for conformational regulation of kinase activity. A conserved glycine-rich loop (P-loop, bridging strands β1 and β2) forms the ceiling of the ATP active site and contributes to coordination of the γ-phosphate moiety of ATP (30). Contributing to structure-based inhibitor design, another loop (L-78) in close proximity to the hinge region has been demonstrated to trigger CK1 inhibitor selectivity (31). Within the C-terminal region, a specific phosphate moiety binding motif (W1) has been identified affording recognition of phosphorylated protein substrates and is further believed to be involved in CK1 regulatory interactions (9, 24, 25). In addition, a kinesin homology domain (KHD) within the T-loop (L-9D) and a putative dimerization domain (DD, containing various amino acids of strands β1, β2, β5, hinge region, β7, and αB) can be found inside the catalytic domain of CK1δ (**Figures 1C** and **2**) (26, 32–34). The KHD is thought to support the interaction of CK1 isoforms with

Table 1 | Reported substrates for CK1 family members and reported *in vitro* and *in vivo* substrates of CK1 family members of several species.

Functional groups	CK1 substrates
Cytoskeleton-associated proteins, adhesion factors, and scaffolding proteins	Myosin (56), troponin (56), ankyrin (57), spektrin 3 (58), filamin (59), vinculin (59), neurofilamentary proteins (60, 61), dynein (62), α-/β-tubulin (32), microtubule-associated protein (MAP) 1A (63), MAP 4 (32), stathmin (32), tau (32, 64), keratin 17 (65), desmolein (65), annexin II (65), centaurin-α (p42IP4) (66, 67), neural cell-adhesion molecule (NCAM) (68), E-cadherin (69), RhoB (70), myelin basic protein (MBP) (55), kinesin-like protein 10A (KLP10A) (71), lectin L-29 (72), galectin-3 (73), end binding 1 (EB1) (74), Sid4 (75), connexin-43 (76), metastasis suppressor 1 (MTSS1) (77), and Hsp79 and Hsp90 (78)
Receptors	β-Subunit of the insulin-receptor (79), TNFα-receptor (80), muscarin M3-receptor (81), Ste2p (α-factor-receptor) (82), Ste3p (α-factor-receptor) (83), platelet derived growth factor (PDGF) receptor (84), retinoid X receptor (RXR) (85), low density lipoprotein-related receptor protein (LRP) 6 (86, 87), type I interferon receptor (IFNAR1) (88), estrogen receptor α (ERα), amplified in breast cancer 1 (AIB1) (89), calmodulin (CaM) (90), and Ror2 (91)
Membrane transporters	Erythrocytes anion transporter (92), uracil permease (*Saccharomyces cerevisiae*) (93), translocase of the outer mitochondrial membrane 22 (Tom22) (94), and α-T663-hENaC (95)
DNA-/RNA-associated proteins	Non-histone chromatin proteins (96), RNA polymerase I and II (97), topoisomerase IIα (98), Star-poly(A) polymerase (Star-PAP) (99), Rec8 (100), DNA methyl-transferase (Dnmt1) (101), TAR DNA-binding protein of 43 kDa (TDP-43) (102), DEAD-box RNA helicase DDX3 (103), Ubiquitin-like, with PHD, and RING finger domains 1 (UHRF1) (104)
Ribosome-related proteins	15 kDa (105), 20 kDa (105), 35 kDa (105), L4 (65), L8 (65), L13 (65), ribosomal protein S6 (rpS6) (106), and ENP1/BYSL and LTV1 (107)
Transcription and splice factors	p53 (108), cyclic AMP responsive element modulator (CREM) (109), Swi6 (110), nuclear factor of activated T-cells (NFAT) (111), serine/arginine-rich (SR) proteins (112), T-cell factor (Tcf) 3 (113), brain and muscle Arnt-like protein (BMAL) 1 (114), cryptochrome 1 (CRY) (114), β-catenin (115, 116), armadillo (117), SMAD 1–3 and 5 (118), osmotic response element-binding protein (OREBP) (119), cubitus interruptus (Ci) (120), forkhead box G1 (FoxG1) (121), SNAIL (122), tafazzin (TAZ) (123), yes-associated protein (YAP) (124), proliferator-activated receptor γ co-activator 1α (PGC-1α) (125), *Drosophila* Myc (d-Myc) (126), cyclic AMP response element-binding protein (CREB) (127), Sre1N (yeast sterol regulatory element-binding protein homolog) (128), and NFκB (nuclear factor "kappa-light-chain-enhancer" of activated B-cells) subunit p65 (129)
Translation factors	Initiation factors (IF) 4B (130), 4E(5, 6, 130, 131)
Viral proteins	Simian virus 40 large T-antigen (SV40 T-Ag) (132), hepatitis C virus non-structural 5A (NS5A) (133), human cytomegalovirus ppUL44 (134), Poa semilatent hordeivirus triple gene block 1 (TGB1) (135), Kaposi sarcoma-associated herpesvirus latency associated nuclear antigen (LANA) (136), and yellow fever virus methyl-transferase (137)
Kinases and phosphatases	Cyclin-dependent kinase 5 (Cdk5) (138), protein kinase C (PKC) (139), protein kinase D2 (PKD2) (140), cell division cycle 25 (Cdc25) (141–143), and PH domain and leucine rich repeat protein phosphatase 1 (PHLPP1) (144)
Inhibitors and modulators	Inhibitor 2 of PPA 1 (145, 146), dopamine and cAMP regulated phosphoprotein of 32 kDa (DARPP-32) (147), disheveled (148), mammalian period circadian protein (mPER) (149), adenomatous polyposis coli (APC) (150), Bid (151), protein kinase C potentiated myosin phosphatase inhibitor of 17 kDa (CPI-17) (152), nm23-H1 (153), 14-3-3 proteins (154), MDM2 (155), MDMX (156), FREQUENCY (FRQ) (157), WHITE COLLAR-1 (WC-1) (158), CARD containing MAGUK protein (CARMA1)/caspase recruitment domain (CARD11) (159), SLR1 (160), endogenous meiotic inhibitor 2 (Emi2) (161), Chk1-activating domain (CKAD) of claspin (162), PER2 (163), protein S (164), Rap guanine nucleotide exchange factor 2 (RAPGEF2) (165), and Sprouty2 (SPRY2) (166)
Enzymes (miscellaneous)	Acetyl-CoA carboxylase (167), glycogen synthase (168, 169), yeast endoprotease Ssy5 (170), and neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4) (171)
Vesicle- and trafficking-associated proteins	SV2 (172), β3A- and β3B-subunit of the AP-3 complex (173), snapin (174), and ceramide transfer protein (CERT) (175)
Receptor-associated proteins	Fas-associated death domain (FADD) (176), receptor interacting protein 1 (RIP1) (177)
Factors of neuro-degenerative diseases	Presenilin-2 (178), tau (64), β-secretase (179), parkin (180), and α-synuclein (181)
Metastatic tumor antigens	Metastatic tumor antigen 1, short form (MTA1s) (182)

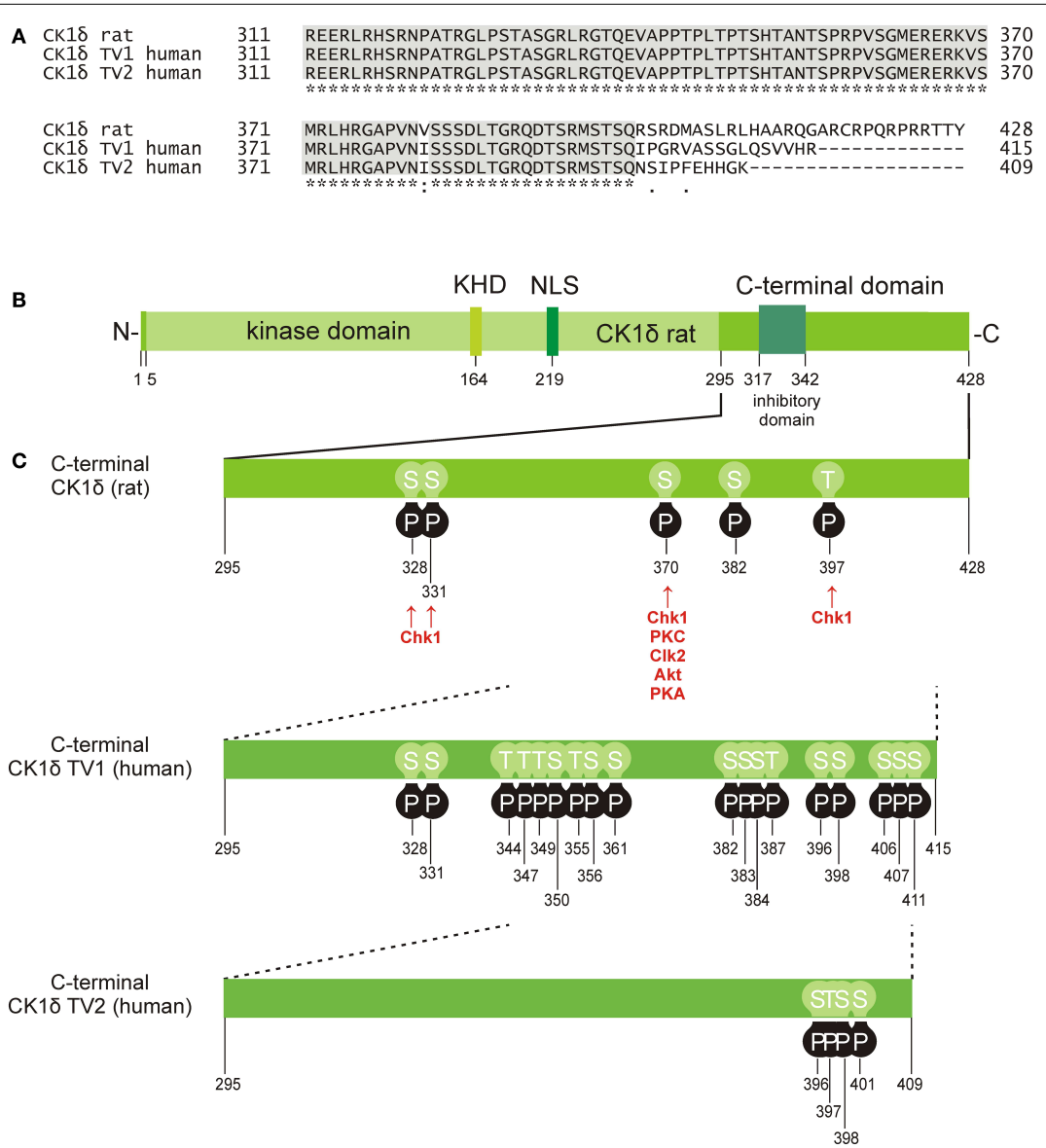


FIGURE 2 | Phosphorylation sites located in the C-terminal domain of CK1δ. **(A)** Alignment of the rat CK1δ C-terminal sequence with the human CK1δ transcription variants 1 and 2 C-terminal sequences (accession numbers L07578, NM001893, and NM139062, respectively) generated by using the program ClustalW (36, 37), showing conserved amino acids (gray) and obvious differences in the C-terminal domain beyond amino acid 399. **(B)** Domain structure of rat CK1δ (NLS: nuclear localization signal, KHD: kinesin homology domain). **(C)** Phosphorylation sites in the C-terminal regulatory domains of CK1δ rat and human transcription variants 1 and 2, that have so far been confirmed experimentally (38–53). Kinases identified for phosphorylation of the C-terminal domain are shown for rat CK1δ (38, 39).

components of the cytoskeleton as this domain has been shown to be necessary for the interaction of kinesins with microtubules (MT) (26, 32–34). Furthermore, a putative nuclear localization signal sequence (NLS) at the junction between L-EF and αF has been reported to affect substrate binding (**Figure 1C**). The present NLS however seems to be not sufficient for nuclear localization of CK1δ because only CK1αL variants, carrying an additional NLS in the L-exon, are able to localize to the nucleus (35).The L-9D loop represents the homolog of the so-called activation-loop identified in other protein kinases and may therefore play a role in CK1 regulation. Moreover, loops L-9D and L-EF may be of importance in substrate recognition (**Figure 1C**) (24–27). The ATP active site itself mainly consists of a deep hydrophobic pocket (HPI, selectivity pocket) lined by the gatekeeper (Met-82 in CK1δ) and a second spacious hydrophobic region (HRII) adjacent to the hinge region as well as sugar and phosphate binding domains (**Figure 1D**) (31).

CK1 SUBSTRATE SPECIFICITY

Belonging to the group of acidotropic protein kinases, CK1 family members mainly recognize substrates containing acidic or

phosphorylated amino acid residues. The canonical consensus sequence for CK1 protein kinases is represented by the motif pSer/Thr-X-X-(X)-Ser/Thr whereas pSer/Thr indicates a phosphorylated serine or threonine residue. However, CK1 not only relies on phospho-primed motifs since the phospho-serine or phospho-threonine can also be replaced by an agglomeration of negatively charged acidic amino acids (2–7). In addition, non-canonical consensus sequences for CK1 family members have been described such as the SLS motif, found in β-catenin and nuclear factor of activated T-cells (NFAT), or the motif Lys/Arg-X-Lys/Arg-X-X-Ser/Thr occurring in sulfatide and cholesterol-3-sulfate (SCS) binding proteins (54, 55). Generally, substrate recognition motifs for CK1 protein kinases are massively distributed on cellular proteins. At present, more than 140 *in vitro* and *in vivo* substrates for CK1 isoforms have been reported, underlining its pleiotropic character (**Table 1**).

REGULATION OF CK1 ACTIVITY

Although members of the CK1 family are ubiquitously expressed, their expression levels differ depending on tissue and cell type (34, 183, 184). Certain factors seem to change the expression and activity of CK1, such as stimulation with insulin (185) or gastrin (140), viral transformation (186), treatment with topoisomerase inhibitors or other small molecules like calotropin (187), γ-irradiation (188), or altered membrane concentrations of phosphatidylinositol-4,5-bisphosphate (PIP2) (172). At the protein level, certain mechanisms regulating CK1 activity have been identified: structure-related regulation, subcellular localization, interaction with other proteins, and post-translational modifications.

In X-ray crystallography, CK1δ was found to form dimers. In the dimeric form, the adenine binding domain is occupied by the specific intramolecular contacts of the dimerization domain. As a consequence, ATP is excluded from the active center of the kinase. Therefore, formation of homodimers could possibly have a negative regulatory effect on CK1δ kinase activity *in vivo* (26). This hypothesis is supported by further observations: the expression of a mutant CK1δ with impaired kinase activity lead to down-regulation of endogenous CK1δ activity in a dominant-negative way in simian virus 40 (SV40)-transformed cell lines as well as to changes in mammary tumorigenesis in WAP-mutCK1δ/WAP-T bi-transgenic mice (189, 190).

Appropriate sequestration of CK1 proteins to particular cellular compartments is crucial for access to their pool of substrates (21, 191, 192). As an example, in *Saccharomyces cerevisiae* kinase activity of C-terminal deletion mutants of membrane-bound YCK1 and YCK2 could only be rescued by replacing the nuclear localization signal of the CK1 homolog Hrr25 with a prenylation motif, which is required for plasma membrane localization. Conversely, loss of Hrr25 function after deletion of its NLS could only be rescued by replacing the prenylation motif in YCK1 and YCK2 with a NLS. These observations led to the conclusion that merely partial cellular overlap of these three isoforms is not enough to rescue the deletion phenotype (192). In experiments using a CK1δ kinase-dead mutant, it has been shown that not only the existence of the kinase domain, but also the catalytic activity of the protein is essential for its appropriate subcellular localization (193). Additionally, a study designed to identify binding partners, which recruit CK1 to Alzheimer's disease (AD) ubiquitinated lesions identified a dysbindin structural homolog that interacts with CK1γ, δ, and ε, and in the case of CK1δ it has been shown to be a concentration-dependent inhibitor (194).

It is very common to find certain motifs in proteins that act as scaffolds, which direct the proper positioning of protein complexes. It has also been suggested that such scaffolds additionally exert complex allosteric control of their partners thereby regulating their activity [reviewed in Cheong and Virshup (195) and Good et al. (196)]. In general, proteins that function as scaffolds tether members of signaling pathways into complexes thereby increasing the interaction efficiency between partner molecules (196, 197). In the case of CK1, these scaffolds have an important regulatory role because they might change the affinity of CK1 isoforms for their substrates as well as the rate of phosphorylation and activation of CK1 kinase activity over the basal level (196, 198). In fact, protein scaffolds have been already found to exert substantial control over different kinase-mediated signaling pathways [reviewed in Brown et al. (199)], though they are not limited to the coordination of kinase cascades (196). Examples for such protein scaffolds include the centrosomal and Golgi N-kinase anchoring protein (CG-NAP), also known as A-kinase anchoring protein 450 (AKAP450) (191) and the DEAD-box RNA helicase DDX3, which has been previously identified as scaffolding adaptor that directly activates the kinase IκB (200). AKAP450 specifically interacts with CK1δ and ε and recruits them to the centrosome, where they can exert centrosome-specific functions coupled to the cell cycle. This interaction is confirmed by the ability of AKAP450 to re-localize CK1δ at the plasma membrane, when it itself is attached to the membrane (191). Recently, it has been suggested that the interaction of CK1δ with AKAP450 is necessary to mediate primary ciliogenesis (201). In addition, evidence is increasing that in Wnt-signaling CK1 activity depends on DDX3 as a co-factor. DDX3 directly interacts with CK1ε in a Wnt-dependent manner, and promotes phosphorylation of Disheveled (DVL) (103). DDX3 can therefore be seen as regulatory subunit of CK1 isoforms with the potential to increase the activity of CK1α, γ2, δ, and ε by up to five orders of magnitude (103). Since CK1 isoforms have been shown to phosphorylate DDX3, it could be speculated that CK1 isoforms might also play a role in regulating the functions of DDX3 (103).

Finally, CK1 activity can furthermore be regulated by post-translational modifications, mainly represented by reversible phosphorylation either through autophosphorylation or site-specific phosphorylation mediated by cellular kinases. Within the regulatory C-terminal domains of CK1δ and ε, sequences with the motif pSer/Thr-X-X-Y (Y: any amino acid except serine or threonine) can be generated by autophosphorylation events and can consecutively act as pseudo-substrates blocking the catalytic center of the kinase (202–205). By using CK1δ truncation mutants, Ser-318, Thr-323, Ser-328, Thr-329, Ser-331, and Thr-337 were detected as candidate sites for intramolecular autophosphorylation. Although not all of them influenced kinase activity, truncation of the C-terminal part up to amino acid (aa) 317 significantly enhanced activity of CK1δ (204). For CK1ε amino acid residues Ser-323, Thr-325, Thr-334, Thr-337, Ser-368, Ser-405, Thr-407, and Ser-408 within the C-terminal domain are

considered to be potential autophosphorylation sites (203). C-terminal inhibitory autophosphorylation could also be demonstrated for CK1γ1-3 as well as for CK1α and its splice variants CK1αL and CK1αS (16, 206).

Apart from intramolecular autophosphorylation, CK1 isoforms are also phosphorylated by other kinases. In the case of CK1δ, phosphorylation by PKA (cAMP-dependent protein kinase), Akt (protein kinase B), CLK2 (CDC-like kinase 2), protein kinase C isoform α (PKCα), and Chk1 (checkpoint kinase 1) has been demonstrated (38, 39) (**Figure 2**). PKA could be further characterized as a major CK1δ C-terminal targeting kinase predominantly phosphorylating Ser-370 both *in vitro* and *in vivo*. Mutation of Ser-370 to alanine increased kinase activity *in vitro* and enhanced formation of an ectopic dorsal axis during embryonic development of *Xenopus laevis* (39). More recently, Chk1 has been demonstrated to phosphorylate CK1δ at serine residues 328, 331, and 370, as well as threonine residue 397. Mutations at these sites proved to significantly increase kinase activity (38). Moreover, several residues in the C-terminal domain of CK1δ were found in a phosphorylated state in large-scale mass spectrometry analyses. However, the kinases responsible for the detected phosphorylation events were not specified (**Figure 2** and references therein).

Generally, dephosphorylation of CK1 by serine/threonine-specific protein phosphatases or low levels of H_2O_2 result in an increase of kinase activity (202, 203, 207). Proteolytic cleavage of the C-terminal domain also results in multiple increase of CK1 kinase activity *in vitro* (28, 202, 204). In addition, neddylation of CK1α seems to be involved in CK1 regulation (208).

CK1 IN STRESS-RELATED CELLULAR FUNCTIONS

In response to stress situations like mechanical damage, toxin exposure, or environmental stress exposure, cells experience a variety of molecular changes, which are generally referred to as cellular stress response. The purpose of these changes is to protect the cell against conditions, which may cause acute damage, but also to build some kind of resistance toward long term unfavorable conditions. In response to extreme temperature or toxic substances, expression of heat shock proteins (Hsp) is transcriptionally increased. Most of these proteins belong to a group of proteins, which are involved in the (un-)folding of other proteins (209). A quite recent report links phosphorylation events mediated by CK1, CK2, and GSK3β to the regulation of Hsp70 and Hsp90. In more detail, phosphorylation of Hsp70 and Hsp90, mediated by these kinases, plays an important role in regulating their binding to co-chaperones like HOP (protein folding activity) and CHIP (ubiquitin ligase activity). In highly proliferative cells, phosphorylated Hsp70 and 90 form complexes with HOP whereas CHIP-binding is prevented by phosphorylation of Hsp70 and 90. Therefore, CK1, CK2, and GSK3β together with the action of phosphatases might be involved in complex regulation of the C-terminal phosphorylation of Hsp70 and Hsp90 and their binding to co-chaperones (78). Moreover, apart from environmental or external stress conditions, cells may also be challenged by stress originating from pathological conditions as in the case of inflammatory or proliferative diseases. A detailed presentation of CK1 isoforms in regulating cellular stress response can be found in the following chapters.

CK1 IN IMMUNE RESPONSE AND INFLAMMATION

By analyzing lymphatic tissues of BALB/c mice, remarkable immunoreactivity of CK1δ and ε in granulocytic and megakaryotic cells as well as in a subpopulation of lymphocytes has been detected (183, 184, 210). Mitogenic activation of T-lymphocytes was accompanied by a significant increase in both CK1δ protein levels and kinase activity (210).

So far, several mechanisms have been reported by which CK1 isoforms might be involved in regulating lymphocyte activation and granulocyte physiology. Transcriptional activators of the NFAT family of proteins play a major role in T-cell activation. Their translocation to the nucleus can be blocked by phosphorylation of numerous sites present in the NFAT regulatory domain (211). Some of these are phosphorylated by various CK1 isoforms (rat liver CK1 and *Danio rerio* CK1α) with high efficiency. In a two-phase phosphorylation mechanism, first phosphorylation of the non-canonical site Ser-177 is initiated by CK1 binding to a cluster of acidic residues within the sequence of aa 173–218. This event enhances the subsequent phosphorylation of downstream residues in a hierarchical manner (212). In contrast, Okamura and colleagues reported NFAT1 to be phosphorylated by CK1 within the serine-rich region SRR-1 (aa 149–183) after binding of CK1 to a N-terminal motif between aa 1–98 (213).

Upon T-cell receptor engagement dynamic association of CK1α to the CBM (CARMA/BCL10/MALT1) complex has been shown. This complex acts as an NFκB (nuclear factor "kappa-light-chain-enhancer" of activated B cells) activating platform containing the scaffold protein CARMA1, the adaptor protein BCL10, and the paracaspase MALT1. Here, CK1α complex association is linked to NFκB activation, increased cytokine production, and lymphocyte proliferation. However, CK1α was found to be a bi-functional regulator of NFκB signaling since phosphorylation and subsequent inactivation of CARMA1 leads to termination of receptor-induced NFκB activation (159). Just recently, CK1γ1 has been demonstrated to be a negative regulator in innate immunity by directly phosphorylating the NFκB subunit p65 following RIG-I pathway stimulation after RNA virus infection. This phosphorylation event is sufficient to target p65 for its degradation (129). Following immune receptor engagement a signal transduction platform is assembled around the T-cell receptor. This specialized cell–cell junction is known as the immunological synapse whose formation also leads to remodeling of the actin cytoskeleton and to repositioning of the centrosome to the immunological synapse (214). Herein, the polarization process is supported by CK1δ phosphorylating the microtubule plus-end-binding protein 1 (EB1). Formation of CK1δ–EB1 complexes is associated with increased speed of microtubule growth and most likely also with subsequent centrosome translocation in activated T-cells (74).

In granulocytes as well as in solid tumors cell survival is significantly promoted by the transcriptional activator HIF-1 (hypoxia-inducible factor-1), which is able to respond to changes in cellular oxygen levels. HIF-1 is continuously produced and marked for degradation by a hydroxylation step involving oxygen-dependent hydroxylases. Under hypoxic conditions, the continuous destruction of HIF-1 is blocked (215). Additionally, HIF-1 expression and activity can be regulated by oxygen-independent mechanisms resulting in phosphorylation of critical residues in HIF-1

regulatory domains. CK1δ has been identified as one of these kinases able to phosphorylate Ser-247 in the PAS-B (Per-ARNT-Sim-B) domain of HIF-1α. This modification has no effect on HIF-1α stability but affects the formation of the transcriptionally active HIF-1α-ARNT heterodimer, which is seen as an obligatory step prior to DNA binding (216). Therefore, active CK1δ can be seen as negative regulator of HIF-1-mediated cell survival.

Additionally, for the highly CK1δ homologous isoform CK1ε, a major role for transcriptional regulation in granulocytes has been suggested. Along with human granulocytic differentiation, a down-regulation of CK1ε has been observed. Here, active CK1ε was shown to interact with and to stabilize SOCS3 (suppressor of cytokine signaling 3) leading to attenuation of STAT3. Consequently, overexpression of CK1ε inhibited granulocyte-colony-stimulating factor (G-CSF) induced differentiation of myeloid progenitor cells (217).

INTERACTION OF CK1 WITH CENTROSOMES, TUBULIN, AND MICROTUBULE-ASSOCIATED PROTEINS

Members of the CK1 family represent central components in the regulation of several cellular functions linked to cell cycle progression, spindle-dynamics, and chromosome segregation. CK1α has been shown to be located at the centrosome, microtubule asters, and the kinetochore (218–220). In addition, CK1δ especially associates with the spindle apparatus during mitosis and directly modulates MT by phosphorylation of α-, β-, and γ-tubulin, thereby exerting stress-induced functions at the spindle apparatus and the centrosome (221, 222). Recently, knockdown of CK1δ by siRNA was reported to inhibit microtubule nucleation at the Golgi apparatus (201). Furthermore, homologs of CK1, such as casein kinase 1-like 6 (CKL6), associate with cortical MT *in vivo* and phosphorylate tubulin *in vitro* (223).

In addition to the direct interaction of CK1 with MT, their polymerization and stability can also be regulated by CK1-mediated phosphorylation of microtubule-associated proteins (MAPs) (224). CK1δ regulates microtubule- and spindle-dynamics in response to genotoxic stress in order to maintain genomic stability by site-specific phosphorylation of tubulin, stathmin, and the MAPs MAP4, MAP1A, and tau (32, 63, 219, 225–227) as well as the phosphorylation of Sid4 that delays cytokinesis (75). An abnormal hyperphosphorylation of tau by CK1δ can lead to microtubule destabilization and is associated with the pathogenesis of AD (220, 225, 227).

Recent studies provide evidence that CK1 influences dynein-dependent transport along MT. For instance, CK1ε phosphorylates dynein intermediate chain (DIC) of the motor protein dynein thereby activating minus-end directed transport of membrane organelles along MT and regulating dynein activity by phosphorylation of the DIC component IC138 (**Figure 3**) (62, 228).

A particular interesting role of centrosome-associated CK1 has been proposed in regulating cell cycle progression by interaction with the Wnt pathway and p53 (**Figure 3**). CK1δ is associated to the centrosome and related to Wnt3-dependent neurite outgrowth. In this context, phosphorylation of DVL by centrosome-associated CK1δ facilitates neurite formation (32, 193, 229). CK1δ co-localizes with DVL2 at basal bodies and gradually accumulates at centrosomes when cells proceed through the cell cycle (230). The hypothesis of CK1 fulfilling regulatory roles at the centrosome is further underlined by the already discussed findings that CK1δ and ε are anchored at the centrosome through interaction with AKAP450 (see Regulation of CK1 Activity) (191) and that CK1δ phosphorylates EB1, which is relevant for centrosome positioning during T-cell activation (see CK1 in Immune Response and Inflammation) (74). Remarkably, further studies revealed that a subpopulation of p53 is located at the centrosome in order to prevent genomic instability. Therefore, the coordinated function of both CK1 and p53 could ensure the integrity of the centrosome and thereby maintain genomic stability (231–233).

CK1 IN DNA DAMAGE-RELATED SIGNAL TRANSDUCTION

CK1 family members can be considered as central components within the regulation of several cellular functions linked to DNA-processing or DNA damage [reviewed in Knippschild et al. (219)]. In context of DNA damage-associated signal transduction, p53 is activated initiating the activation of pathways ensuring centrosome integrity and genomic stability. This signaling network essentially involves coordinated action of CK1 and p53 (187, 231–233).

CK1α, δ, and ε are able to phosphorylate certain N-terminal target sites of p53 (Ser-6, Ser-9, Ser-15, Thr-18, and Ser-20) (187, 234–237). By phosphorylation of p53 (mostly at Ser-15 and Thr-18) CK1δ and ε decrease p53 binding affinity to its cellular counterpart Mouse double-minute 2 homolog (MDM2) resulting in increased levels of MDM2-released, active p53 (234, 238, 239). Conversely, phosphorylation of MDM2 at several serine residues within its central acidic domain (Ser-240, Ser-242, Ser-246, and Ser-383) results in increased MDM2-p53-binding and subsequent degradation of p53 under non-stress conditions. Phosphorylation of Ser-118 and Ser-121 by CK1δ, however, can mark MDM2 for SCF/β-TrCP (Skp1, Cullins, F-box/β-transducin repeat containing E3 ubiquitin protein ligase) binding and ubiquitination, finally leading to proteasomal degradation of MDM2 (**Figure 4**) (155, 239–241). Under normal conditions, CK1α has furthermore been suggested to be a key player promoting p53 inhibition and degradation by MDM2. Therefore, CK1α is physically interacting with MDM2 resulting in p53 degradation. Inhibition or depletion of CK1α as well as inhibition of CK1α-MDM2 association leads to p53 stabilization (208, 242). For the MDM2 homolog MDMX, phosphorylation of Ser-289 by CK1α has been confirmed resulting in increased binding to p53 and subsequent inhibition of p53 transcriptional function (156).

Among the target genes activated by p53 following genotoxic stress also transcription of CK1δ can be induced (187). Given the previously discussed fact that p53 can be activated by CK1δ-mediated phosphorylation in this network, an autoregulatory feedback loop between CK1δ and p53 has been suggested (**Figure 4**).

Apart from DNA damage, p53 activation can also be induced by hypoxia. Herein, p53 levels are stabilized via HIF-1α and its positive regulatory effect on ATM/ATR (ataxia telangiectasia-mutated/ataxia telangiectasia and Rad3-related) (243, 244). As discussed previously, HIF-1α represents a substrate for CK1 and its transcriptional activity can be negatively regulated by

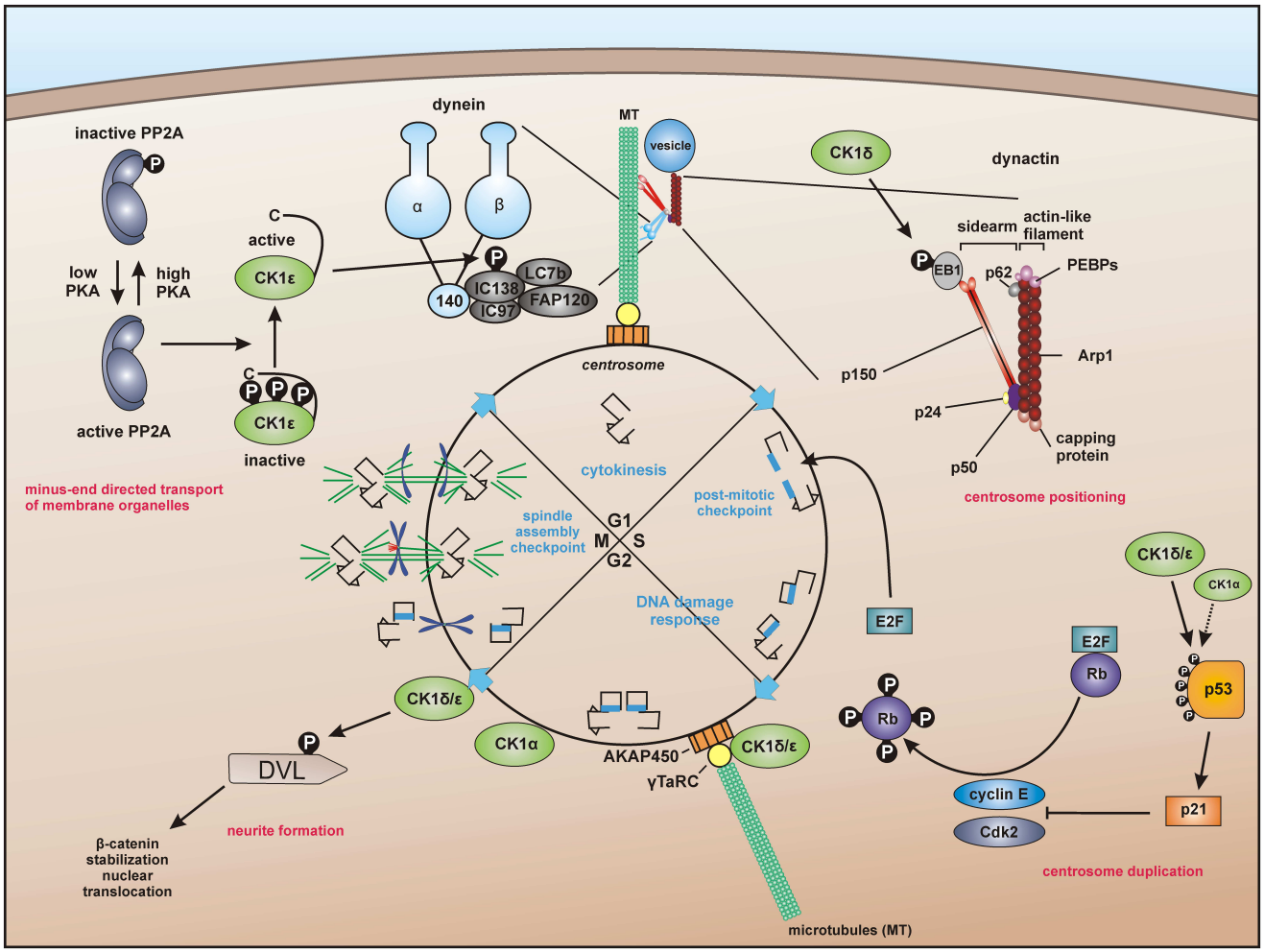


FIGURE 3 | Centrosome-associated functions of CK1. For dynein-dependent transport along microtubules (MT), CK1ε phosphorylates the dynein intermediate chain (DIC) of dynein, likely IC138, thereby activating minus-end directed transport of membrane organelles along MT (62, 228). CK1δ and CK1ε are associated with the centrosome mediated through interaction with the scaffold protein AKAP450 (A-kinase anchor protein 450) (191, 193, 203). Both isoforms are related to Wnt-signaling and neurite outgrowth by phosphorylation of DVL (229, 230). In addition, CK1δ phosphorylates the end binding protein 1 (EB1), which is relevant for centrosome positioning during T-cell activation (74). Furthermore, a subpopulation of p53 in coordinated function with CK1 at the centrosome could ensure the integrity of the centrosome and thereby maintain genomic stability (231–233).

CK1δ-mediated phosphorylation (216). However, since this modification has no effect on HIF-1α protein stability, the precise role of CK1δ-mediated HIF-1α phosphorylation in regulating ATM/ATR- and p53-specific functions under hypoxic conditions remains to be characterized.

More recent work suggested that interferon (IFN)-related signaling is able to activate p53 as a response to loss of epigenetic gene silencing (246). Among other critically involved epigenetic regulators, UHRF1 (ubiquitin-like, with PHD and RING finger domains 1) regulates the maintenance of DNA methylation during DNA replication (247). The stability of UHRF1 is regulated by proteasomal degradation including a priming step by CK1δ-mediated phosphorylation of Ser-108 thereby creating a recognition site for the SCF/β-TrCP ubiquitin ligase (104). Consequences of this negative regulatory connection between CK1δ and UHRF1 may also include the loss of stable DNA methylation and IFN-dependent activation of p53.

DNA/RNA virus infection has been described as a further mechanism resulting in p53 activation. This effect might be mediated via IFN-related p53 accumulation (245) but also via CK1-dependent signaling. In this context, CK1α-mediated phosphorylation of p53 at Ser-20 is induced after infection of T-cells with human Herpes virus 6B (HHV-6B). This phosphorylation event stabilizes the binding of p53 to the transcriptional co-activator p300. Therefore, CK1α takes part in gene regulation following virus infection induced p53 activation (236). Also infection with SV40 interferes with the p53 signaling network. SV40 large T-antigen (T-Ag) inactivates p53-dependent transcriptional activation whereas the oncogenic properties of T-Ag are enhanced by CK1-mediated phosphorylation (189, 190, 248). Moreover,

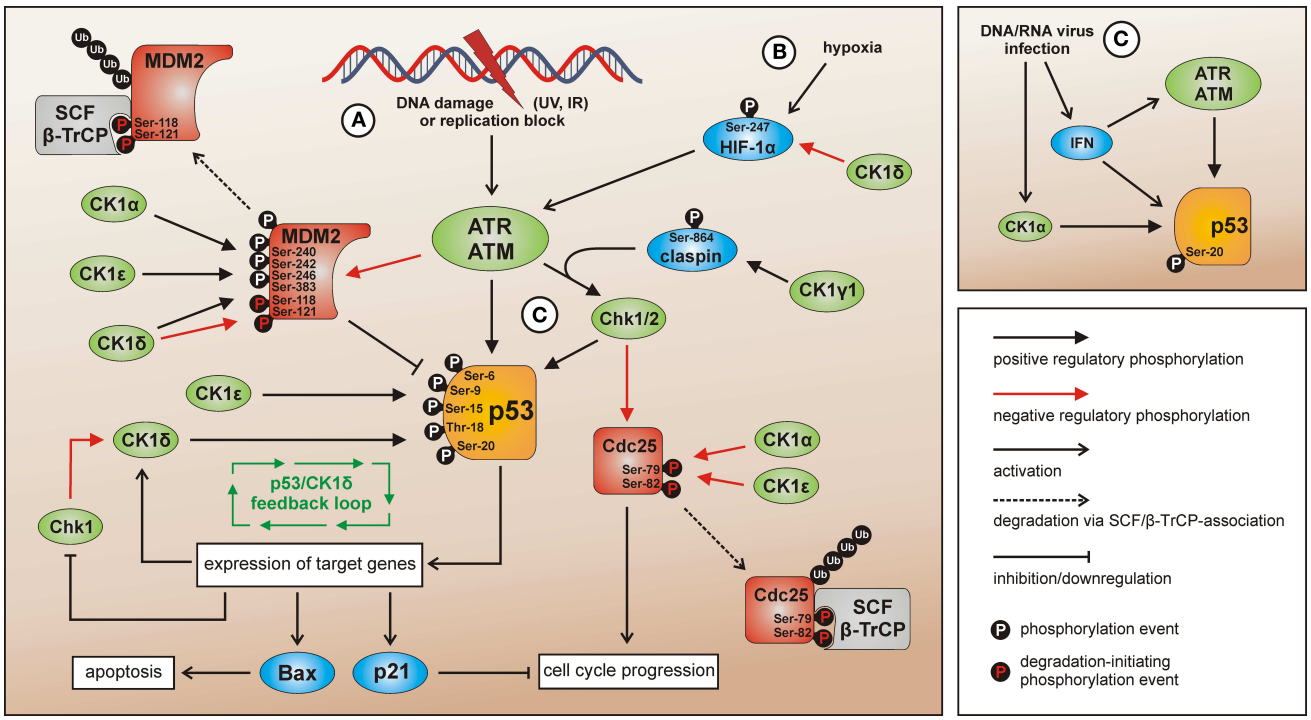


FIGURE 4 | CK1 isoforms in DNA damage-induced signal transduction. After induction of DNA damage (situation A) p53 and Chk1/2 are activated by ATR/ATM-mediated phosphorylation while the p53-regulatory component MDM2 is inhibited. The activation of Chk1 is supported by claspin whereas Chk1/claspin-binding is promoted by CK1γ1-mediated phosphorylation of claspin (162). The CK1 isoforms α, δ, and ε are able to activate p53 by site-specific phosphorylation (187, 234, 235, 237). Activated p53 in turn induces the expression of target genes like Bax (leading to apoptosis), p21 (leading to cell cycle arrest), and also CK1δ (autoregulatory feedback loop) (187). MDM2-mediated degradation of p53 can be activated via interaction with and phosphorylation by CK1α, but also through phosphorylation by CK1δ or ε leading to enhanced binding of MDM2 to p53. CK1δ-mediated phosphorylation of Ser-118 and Ser-121 however marks MDM2 for proteasomal degradation (155, 239–241). In case Chk1/2 gets activated after DNA damage the phosphatase Cdc25, normally initiating cell cycle progression, is blocked by inhibitory phosphorylation and subsequent degradation. In the regulation of Cdc25 inhibition and degradation also CK1 isoforms α and ε are involved (141, 143). Signaling mediated by p53 can also be initiated by hypoxia (via CK1δ-regulated HIF-1α; situation B) (216, 243, 244) or DNA/RNA virus infection (via IFN and/or CK1α-related signal transduction; situation C) (236, 245). Depicted phosphorylation events refer to reported CK1-specific target sites.

as a consequence of SV40 infection/transformation, MDM2 is metabolically stabilized, post-translationally altered, and able to build trimeric complexes with T-Ag and p53 as well as complexes with free p53 thereby inhibiting proteasomal degradation of p53 (249).

Abnormalities in p53 are also related to phenotypes of premature aging. Recently, a mechanistic connection between the proteasome activator REGγ, CK1δ, and p53 has been demonstrated using a mouse model for premature aging. In this pathway, CK1δ is degraded after direct binding to REGγ. Subsequently, degradation of MDM2 is disturbed due to the lack of CK1δ and p53 levels decrease. These findings provide new insights to the conversely discussed anti- and pro-aging effects of p53 (250).

Obviously, CK1 family members are involved in p53-related signal transduction in response to cellular stress conditions in numerous ways (**Figure 4**). However, in most cases upstream regulators and the mechanism of CK1 activity regulation in these pathways still remain unknown. Another component in DNA damage-initiated signal transduction, being targeted by CK1 isoforms, is the protein phosphatase Cdc25A (cell division cycle 25A). Activation of cyclin-dependent kinases (Cdks) by dephosphorylation mediated by Cdc25 is required for cell cycle progression from G1 to S phase (251). Among phosphorylation by other cellular kinases, site-specific phosphorylation of Cdc25A by CK1α and ε at residues Ser-79 and Ser-82 targets Cdc25A for degradation via the ubiquitin-proteasome pathway (141, 143). This CK1-regulated degradation of Cdc25A supports DNA damage-induced cell cycle arrest, which is mediated via inhibition of Cdks by p53 and p21 (252). Since CK1 isoforms are involved in both, the degradation of Cdc25A as well as of p53, CK1 family members might act in a synergistic way to initiate cell cycle arrest.

In addition, CK1γ1 is related to DNA damage signaling by catalyzing the phosphorylation of claspin, an adaptor protein critically involved in ATR-mediated activation of Chk1. In this context, CK1γ1-mediated phosphorylation of claspin enhances its binding to Chk1 (162). Chk1 in turn has been identified as a cellular kinase phosphorylating CK1δ leading to decreased CK1δ-specific activity (38). The significance of this observation for the p53/MDM2/CK1-signaling network remains to be determined. However, given the information that Chk1 is down-regulated by p53 activation the Chk1/CK1δ/p53-interconnection might be involved in fine-tuning the negative regulatory effect of p53 on Chk1 (253).

In hematopoietic cells, the physical interaction of CK1ε with PTEN (phosphatase and tensin homolog) has been proposed to modulate cell survival. Normally, constitutively active Akt kinase or Akt activated by the upstream phosphatidylinositol 3-kinase (PI3K) leads to the inhibition of p53 and p53-induced apoptosis, thereby providing a resistance mechanism for genotoxic stress (254, 255). However, in case PTEN is stimulated as shown for the interaction of PTEN with CK1ε, PI3K-mediated Akt activation is inhibited. Subsequent inhibition of p53 via active Akt is circumvented and the cells' sensitivity toward genotoxic stress can be restored (256).

In the context of Akt signaling, CK1α was reported to affect DEPTOR, an inhibitor of the mTOR (mammalian Target of Rapamycin) kinase, which regulates cell growth, proliferation, and survival [reviewed in Sarbassov et al. (257)]. Phosphorylation of DEPTOR by CK1α leads to βTrCP-mediated proteasomal degradation of DEPTOR resulting in activation of mTOR signaling, which is consistent with DEPTOR down-regulation and mTOR activation found in many cancers (258). Therefore, CK1α might provide a therapeutic target for the treatment of cancers characterized by low DEPTOR levels and activation of mTOR signaling, leading to increasing DEPTOR levels, and inhibition of mTOR signaling. Paradoxically, DEPTOR is overexpressed in multiple myeloma, which is necessary for PI3K-mediated activation of Akt and thereby inhibition of p53 and p53-induced apoptosis (259, 260).

Under conditions of genotoxic stress rapid changes in connexin-43 (Cx43) leading to alterations in gap junction-dependent intercellular communication have been observed in corneal endothelial cells associated with stabilization of gap junction communication (261). Earlier reports already showed phosphorylation of Cx43 by CK1δ, which stimulates the incorporation of Cx43 into gap junction plaques and which therefore most likely also takes part in long term cellular adaptations in response to genotoxic stress (76).

Further DNA-associated proteins being modulated by CK1 isoforms are topoisomerases. For topoisomerase IIα, phosphorylation of Ser-1106 by CK1δ and ε has been demonstrated (98). This phosphorylation event is linked to enhanced DNA-cleavage activity of topoisomerase IIα via the stabilization of topoisomerase-DNA cleavable complexes after etoposide treatment (98).

CK1 IN CIRCADIAN RHYTHM AND ITS CONNECTIONS TO STRESS RESPONSE

In almost every higher organism, an autonomous timer is known and referred to as the circadian clock. This timer consists of a signal transduction pathway to integrate external signals for time adjustment, a molecular oscillator generating the circadian signal, and a signal transduction pathway controlling the circadian periodicity of certain biological processes. Therefore, circadian proteins are closely connected to key regulators of the cell cycle, oxidative stress, and carcinogenesis. Basically, in the mammalian circadian clock the positive regulators CLOCK and brain and muscle ARNT-like protein (BMAL1) as well as the negative regulators PERIOD (PER) and CRYPTOCHROME (CRY) form an oscillating system controlling their own expression levels (**Figure 5**) [reviewed in more detail in Kelleher et al. (262)].

Linking circadian rhythm to cell cycle control, the heterodimer CLOCK/BMAL1 transcriptionally controls the expression of cell cycle regulators. PER1 interacts with ATM and Chk2 (264), whereas TIM, the mammalian homolog of *Drosophila* timeless protein, interacts with Chk1, ATR, and the ATR-related protein ATRIP (265). Furthermore, BMAL1 was identified to be necessary for p53-dependent growth arrest in response to DNA damage (266). Within the metabolism of reactive oxygen species (ROS) circadian proteins also seem to be involved, since the circadian clock could offer reliable control of daily variation in antioxidant response necessary to counteract increased oxidative stress. This connection is reasonable and important as oxidative stress is linked to the pathogenesis of cardiovascular diseases, atherosclerosis, and cancer (267). As an example, BMAL1 deficiency leads to chronic oxidative stress and an accelerated aging phenotype in mice (268). Conversely, activity of the circadian clock itself can be regulated by components of ROS metabolism (269). Finally, the circadian clock is also linked to the development of cancer. For PER2 mutant mice increased formation of radiation-induced lymphomas was reported and the frequency (FRQ) of intestinal and colonic polyps was increased in $APC^{min/+}PER2^{m/m}$ mice compared to $APC^{min/+}$ littermates (270).

In order to control the circadian rhythm involved, regulating components are subject to post-translational modifications like reversible phosphorylation (271). In general, CK1 isoforms δ and ε are able to phosphorylate and regulate the clock proteins BMAL1 and CRY and can modulate the expression of the period length modulator prohibitin 2 (PHB2) (114, 263). CK1δ is seen as an important regulator in circadian rhythm but also the involvement of other CK1 isoforms has been detected. CK1δ and ε are able to influence the length of the circadian period by regulating the stability and subcellular localization of PER (**Figure 5**) (149, 163, 272, 273). Phosphorylation of PER1 by CK1ε masks the nuclear localization signal of PER1 by conformational changes and marks PER for proteasomal degradation (149). CK1δ and ε interact with PER/CRY complexes thereby promoting nuclear localization of PER/CRY complexes (149, 274). In a high-throughput compound screening also CK1α was found to stimulate the degradation of PER1. In this screen, the protein kinases CK1α, CK1δ, and ERK2 were identified as targets for the compound *longdaysin*. However, CK1α binding affinity to PER1 is much weaker than for CK1δ or ε (275). The same is true for CK1γ (276). Thus, CK1δ and ε can be regarded as redundant for PER phosphorylation and essential for nuclear accumulation of PER (277). Inhibition of CK1δ and ε by the pan-CK1δ/ε inhibitor PF-670462 led to remarkably lengthened circadian rhythms (*in vivo* locomotor activity) and molecular oscillations (*in vitro* in the suprachiasmatic nucleus and peripheral tissue slices). These observations could not be made using the CK1ε-specific inhibitor PF-4800567 (278). PF-4800567 effectively blocked CK1ε-mediated nuclear localization of PER3 and degradation of PER2 but only showed minimal effect on the circadian clock in cycling Rat1 fibroblasts (273). The CK1ε *tau* mutation, however, which was discovered in the Syrian hamster as the first mammalian circadian mutation, was characterized as a gain of function mutation resulting in clock acceleration. In mice expressing the CK1ε *tau* mutation increased phosphorylation of PER1 and 2 can be detected leading to increased degradation of nuclear and

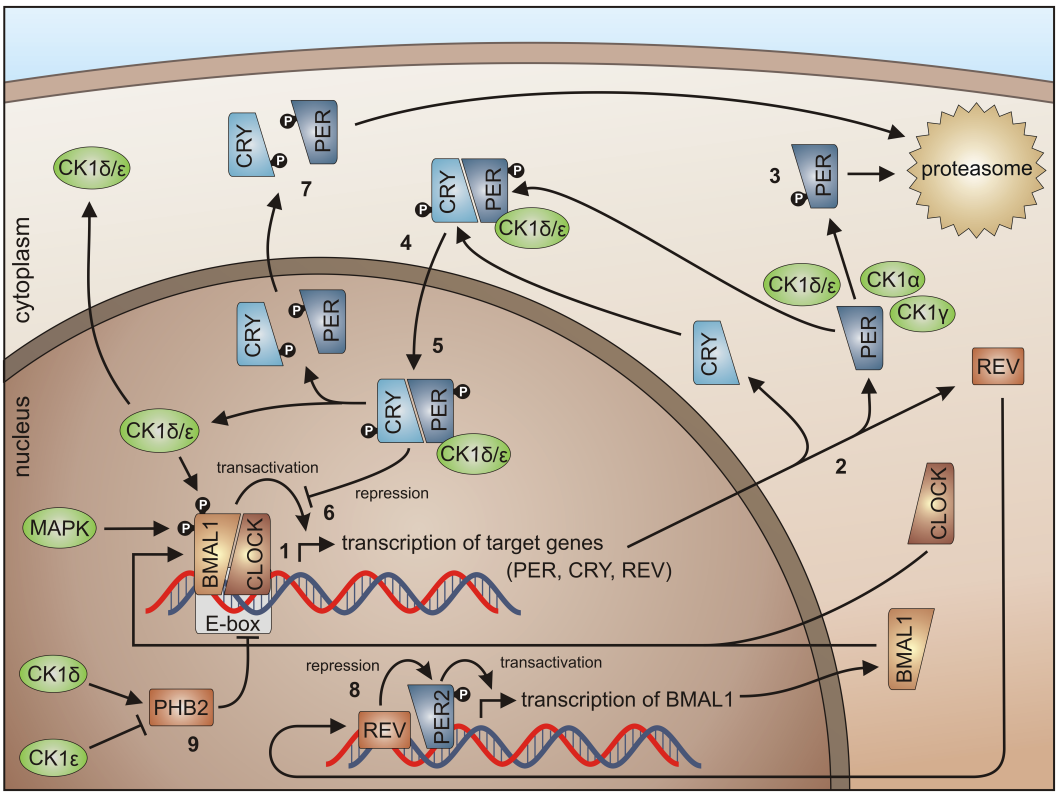


FIGURE 5 | CK1 in circadian rhythm regulation. By binding of the BMAL1/CLOCK heterodimer to the E-box, transcription of E-box-containing genes is initiated (1) (here shown for PER1-3, CRY1 and 2, and REV-ERBα), the transcripts are translated in the cytoplasm (2). Degradation of cytoplasmic PERs is triggered mainly by CK1δ and ε, but also by isoforms α and γ (3), while PER degradation is inhibited by binding of CRYs to PERs (4). Subsequently, complexes of CRY/PER and CK1δ/ε translocate to the nucleus (5). In the nucleus the CRY/PER complex represses the transcriptional activation of BMAL1/CLOCK target genes (6). CRYs and PERs finally shuttle back to the cytoplasm for proteasomal degradation (7). Repression of BMAL1 expression by REV-ERBα represents a second negative feedback loop (8). Together, these feedback loops are able to generate cyclic expression of BMAL1 and E-box-containing genes [for review see Knippschild et al. (219) and Cheong and Virshup (195)]. CK1δ and ε differentially effect expression of the period length modulator PHB2. Whereas CK1δ is able to promote PHB2 transcription, its expression is reduced by CK1ε (9) (8, 263).

cytoplasmic PER and acceleration of the mammalian clock (279, 280). Um and colleagues discovered, that the circadian period of Rat1 fibroblasts treated with the diabetes drug metformin was shortened by 1 h. By metformin treatment, AMP-activated kinase (AMPK) is activated, which phosphorylates CK1ε at Ser-389 leading to increased activity of CK1ε and subsequent degradation of Per2 (281). A higher level regulator of CK1ε activity in circadian rhythm is protein phosphatase 5 (PP5), which can raise the activity of CK1ε by dephosphorylation. As a consequence, phosphorylation by CK1ε and subsequent degradation of PER is also increased (282). Recently, CK1δ (but not CK1ε) has been shown to be crucial for the circadian timing mechanism in zebrafish (283).

Presented observations point to PER proteins as multikinase targets, which can be multiply phosphorylated and thereby regulated. Herein, the balance between phosphorylation and dephosphorylation by phosphatases is of certain importance. In cells deficient for CK1δ and ε, phosphorylation of PER is disturbed and PER proteins remain cytoplasmic. In case protein phosphatase 1 (PP1) is disrupted, phosphorylation of PER is accelerated. This effect is specific to PP1 and in contrast to previous *Drosophila* studies cannot be observed for PP2A (276).

CK1-SIGNALING IN APOPTOTIC PATHWAYS

For several CK1 isoforms, an involvement in the regulation of apoptotic signal transduction has been described. CK1α, δ, and ε are components of Fas-mediated apoptosis and induce an activation of initiator caspase 8. Here the pro-apoptotic protein Bid, which belongs to the Bcl-2 family, is of major interest. Amino acids Ser-64 and Ser-66 of Bid are supposed to be major targets for CK1-mediated phosphorylation while Thr-58 is targeted by CK2. Only unphosphorylated Bid can be processed by caspase 8-mediated proteolysis and can participate in cytochrome c-mediated apoptosis. Accordingly, inhibition of CK1 and CK2 induces accelerated Fas-triggered apoptosis by blocking inhibitory phosphorylation of Bid. Vice versa an overexpression of CK1ε and CK2 leads to a decreased number of apoptotic cells due to increased phosphorylation of Bid, blocking its caspase 8-mediated processing. Therefore, phosphorylation of Bid by CK1δ and ε and CK2 can inhibit Fas-mediated apoptosis (151).

Moreover, CK1 (isolated from pig spleen) can phosphorylate the p75 neurotrophin receptor, thereby negatively regulating p75-mediated apoptosis (284). CK1α is involved in apoptosis by interaction and phosphorylation of retinoid X receptor (RXR), a

class of retinoic acid receptors regulating cell survival by building heterodimers with NGF1B (nerve growth factor 1B), IGFBP-3 (insulin-like growth factor binding protein 3), and β-catenin. In this case, CK1 activity inhibits the induction of apoptosis by RXR agonists (85, 285–287).

Furthermore, CK1α is able to phosphorylate FADD (Fas-associated protein with death domain) at Ser-194 *in vitro* as well as *in vivo* and is supposed to be involved in regulating non-apoptotic functions of FADD like cell cycle interaction, sensitivity toward chemotherapeutics, and nuclear localization (176, 288). In erythrocytes, CK1α modulates cytosolic calcium activity and thereby regulates programed cell death (289).

PARTICIPATION OF CK1 IN THE DEVELOPMENT OF CANCER

During animal development, a precise coordination of cell patterning events is required to ensure appropriate organ architecture and size. Several developmental pathways control growth, proliferation, and apoptosis by strict regulation, which can result in pathological conditions when dysregulated. The Wnt (Wingless/Int-1), Hh (Hedgehog), and Hippo signaling pathways are important in tissue development, growth, and homeostasis (290–293). Aberrant activation of these pathways as well as mutations of components of these pathways has been linked to various cancer entities (294–298). Due to the contribution of CK1 family members in pathways associated with growth and development, the following sections concentrate on the current knowledge of CK1 participation and regulation in the Wnt, Hh, and Hippo signaling pathways.

CK1 AND THE WNT PATHWAY

Components of the Wnt-signaling pathway are involved in many developmental processes including dorsal axis formation, tissue patterning, and establishment of cell polarity (299–302). In addition, Wnt/β-catenin-mediated signaling plays an important regulatory role in cell proliferation processes in both, embryonic and mature organisms. Mutations in Wnt pathway components have been found in various human cancers, including cancers of the skin, liver, brain, and colon (291, 303–312).

In the canonical Wnt/β-catenin signaling pathway, all CK1 family members are involved. However, this involvement is quite complex. So far, positive as well as negative regulatory functions have been described. In absence of the Wnt ligand CK1α interacts with and phosphorylates Axin, adenomatous polyposis coli (APC), and β-catenin (at Ser-45), thereby priming β-catenin for further phosphorylation by GSK3β and subsequent degradation (195, 313) (**Figure 6A**). After binding of Wnt ligand to Frizzled (Fzd) the Wnt co-receptor LRP5/6 is phosphorylated either by membrane-bound CK1γ (positive regulation) (86) or by CK1ε (negative regulation) (314). Phosphorylated LRP5/6 then recruits Axin and the β-catenin destruction complex to the membrane and inhibits GSK3β. Wnt-activated CK1δ and CK1ε phosphorylate Axin as well as the scaffold protein DVL at multiple sites and can introduce a conformational change to the β-catenin destruction complex followed by dissociation of several components, thereby preventing β-catenin from being phosphorylated and degraded (148, 195). Recently, RNA helicase DDX3 was identified as a regulatory subunit of CK1ε in Wnt-signaling. Wnt-activation promotes recruitment of DDX3 to CK1ε and binding directly stimulates kinase activity, promoting phosphorylation of DVL, finally leading to stabilization of β-catenin (103). Accumulated β-catenin then translocates to the nucleus to activate the expression of TCF/LEF (T cell factor/lymphoid enhancing factor)-triggered target genes (**Figure 6B**) (291). CK1ε is also involved in the formation of an

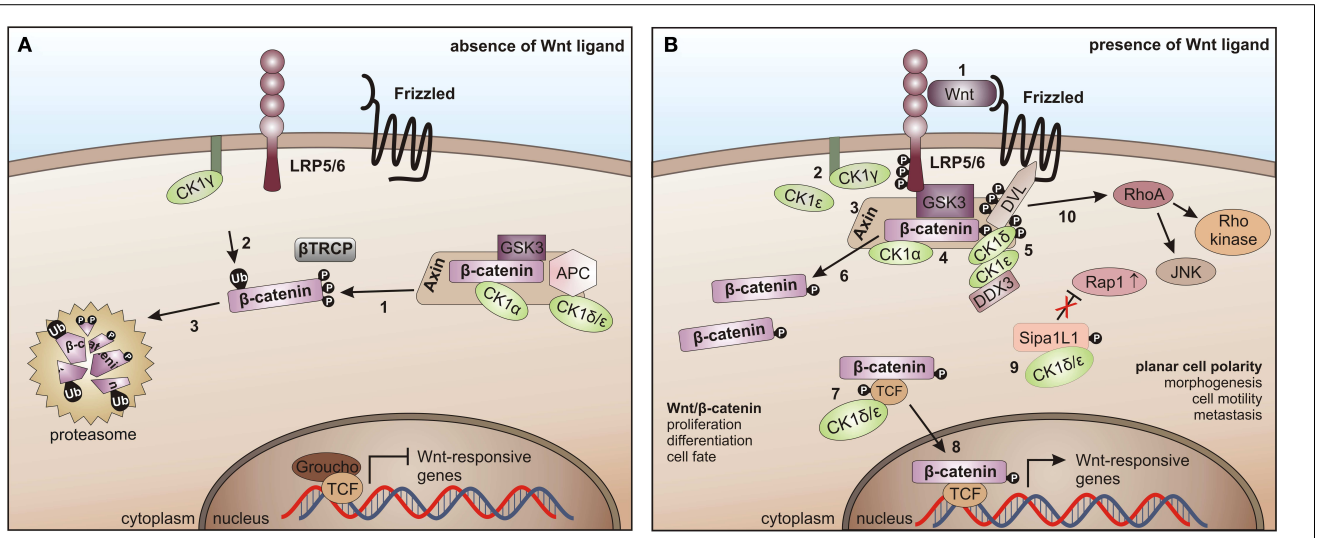


FIGURE 6 | CK1 in Wnt-signaling. **(A)** In the absence of the Wnt ligand, β-catenin is progressively phosphorylated by CK1α and GSK3 (1), recruited to β-TrCP for ubiquitination (2), and thereby primed for proteasome-dependent degradation (3). **(B)** After binding of Wnt to Frizzled and LRP5/6 (1), LRP5/6 is phosphorylated by CK1γ (positive regulation) and CK1ε (negative regulation) (2). It then recruits Axin and the β-catenin destruction complex to the membrane and inhibits GSK3 (3, 4). Wnt-activated CK1δ and ε phosphorylate Disheveled (DVL) and Axin (5), induce a conformational change in the β-catenin destruction complex and initiate the dissociation of various components (6). CK1ε cooperates with DDX3 in phosphorylating DVL (7). Also, TCF3 can be phosphorylated by CK1δ and ε thereby increasing its binding to β-catenin followed by the nuclear translocation of TCF3/β-catenin (8). The non-canonical Wnt pathway is positively regulated by CK1δ- and ε-dependent release of Rap1 from Sipa1L1 inhibition (9). The Rho/JNK signaling cascade is activated after phosphorylation of DVL (10) [adapted from Cheong and Virshup (195)].

active transcription complex by phosphorylating TCF3 thereby mediating its activation and binding to β-catenin (113).

CK1ε is associated with a positive regulatory function by joining the Wnt multi-protein complex to phosphorylate DVL, which in turn gets activated and inhibits GSK3β, finally resulting in stabilization of β-catenin (148).

Signaling in the non-canonical Wnt pathway is positively regulated by CK1δ and ε, which release Rap1 from Sipa1L1 inhibition. Subsequent to phosphorylation of DVL the Rho/JNK signaling cascade can be activated (**Figure 6B**) [reviewed in Cheong and Virshup (195)].

CK1 IN THE HEDGEHOG PATHWAY

The Hh signaling pathway regulates a variety of processes during embryonic development such as differentiation, proliferation, and organogenesis (290). In the adult organism, Hh signaling is significantly reduced but plays a critical role in regulating epithelial maintenance and regeneration of organs, which undergo constant renewal; among them, epithelia of internal organs and brain (315). Therefore, mutations or dysregulation of components of this pathway are associated with tumorigenesis and cancer development, including basal cell carcinomas, medulloblastomas, gliomas, gastrointestinal tumors, prostate cancer, and hematological malignancies (315–318).

In mammals, major components of the Hh pathway are represented by the three Hh homologous ligands Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh), the negative regulatory 12-pass membrane receptor Patched (PTCH), the positive regulatory 7-pass membrane protein smoothened (SMO), the glioma-associated oncogene (GLI) transcription factors GLI1, GLI2, GLI3, a multi-protein complex consisting of intraflagellar transport proteins, protein kinase A (PKA), GSK3, CK1, and suppressor of fused (SUFU) (319). In absence of Hh ligands, PTCH inhibits the localization of SMO to the cilia cell surface and represses SMO activity, thereby suppressing signal transmission via the GLI transcription factors into the nucleus. PKA, GSK3β, and CK1 phosphorylate the GLI transcription factors leading to their proteolytic processing into the repressor forms, which cannot activate target gene transcription (**Figure 7A**) (320, 321). Hh signal transduction is initiated upon binding of a Hh ligand to PTCH, thereby releasing SMO from PTCH-mediated inhibition, leading to its accumulation on cilia cell surface and consequent activation and release of the GLI transcription factors from the multi-protein complex. Activated GLIs then translocate to the nucleus, where they induce transcription of Hh target genes (**Figure 7B**) (319).

In 2002, Price and Kalderon postulated a negative regulatory role of CK1 in Hh signaling in *Drosophila melanogaster* (322). They demonstrated that CK1δ- and GSK3-mediated phosphorylation of Ci-155 (full-length Cubitus interruptus, the *Drosophila* homolog of GLI2 and GLI3) at PKA primed sites is required for the partial proteolysis of the transcription factors, thereby preventing Hh target gene transcription [reviewed in Price (323)]. The PKA, GSK3, and CK1 sites are conserved in Ci, GLI2, and GLI3, which are all similarly processed and may play similar roles in *Drosophila* and vertebrates (324–327). Furthermore, Wang and Li demonstrated, that CK1 and GSK3 phosphorylation sites are needed to process GLI3 (327). CK1 has also been implicated in positive regulation of SMO. Chen and co-workers demonstrated that mammalian SMO is activated via multiple phosphorylation events mediated by CK1α and G protein coupled receptor kinase 2 (GRK2), thereby inducing its cilia accumulation and active conformation (328).

CK1 IN THE HIPPO PATHWAY

During development, the evolutionary conserved Hippo pathway contributes to several processes, which restrict organ size by controlling cell proliferation and apoptosis [reviewed in Zhao et al. (124)]. Consequently, pathway deregulation can trigger tumorigenesis and occurs in a broad range of human cancers. Abnormal Hippo activity is associated with cancer cell proliferation, enhanced cell survival, and maintenance of a stem cell phenotype [reviewed in Harvey et al. (329)].

The mammalian Hippo pathway is initiated by various growth suppressive signals like cell contact inhibition. The upstream kinases mammalian STE20-like protein kinase 1/2 (MST1/2), together with the scaffold proteins vertebrate homolog of *Drosophila* Salvador (WW45) and MOB kinase activator 1A/B (MOB1A/B) phosphorylate the large tumor suppressor 1 and 2 (LATS1/2). LATS1/2-dependent phosphorylation of the transcriptional co-activator Yes-associated protein (YAP) and its paralog Tafazzin (TAZ) then leads to YAP/TAZ inhibition by spatial separation from its nuclear target transcription factors TEAD (TEA domain) and SMAD (SMA/mothers against decapentaplegic) and additionally by phosphodegron-mediated degradation, preventing Hippo target gene transcription (**Figure 8A**) (330, 331).

Zhao and co-workers as well as Liu and co-workers identified CK1δ and ε as new temporal regulators of the Hippo pathway. YAP is phosphorylated by LATS on Ser-381 and this phosphorylation provides the priming signal for CK1δ or ε to phosphorylate a phosphodegron in YAP, which in turn recruits β-TrCP leading to YAP ubiquitination and degradation (124). Furthermore, TAZ phosphorylation at Ser-311 by LATS also leads to subsequent CK1ε-mediated phosphorylation of a phosphodegron in TAZ and consequently to its degradation (123). Xu and co-workers recently postulated the interaction of the Hippo and Wnt pathway via CK1ε. Herein, the Hippo upstream kinase MST1 is able to suppress the Wnt/β-catenin pathway by directly binding CK1ε, thereby preventing phosphorylation of DVL (**Figure 8B**) (332).

CK1-RELATED TUMORIGENIC FUNCTIONS

The important role of CK1 family members within various signaling pathways is furthermore supported by reports linking CK1 isoforms to modulation of key regulatory proteins such as p53, MDM2, and β-catenin, which act as signal integration molecules in stress situations and generally can be seen as a key regulatory connection to tumorigenesis [for more detailed review see Knippschild et al. (219, 333), and Cheong and Virshup (195)]. Considering the importance of signals mediated by CK1δ and ε to finally ensure genome stability, it is obvious that mutations leading to changes in the activity or expression levels of CK1 isoforms or mutations of CK1-specific target sites in its substrates can contribute to the development of cancer (**Table 2**). Foldynová-Trantírková and co-workers provided evidence that mutations in CK1ε, which are frequently found in breast cancer, lead to loss of function in the Wnt/β-catenin pathway but result in activation of

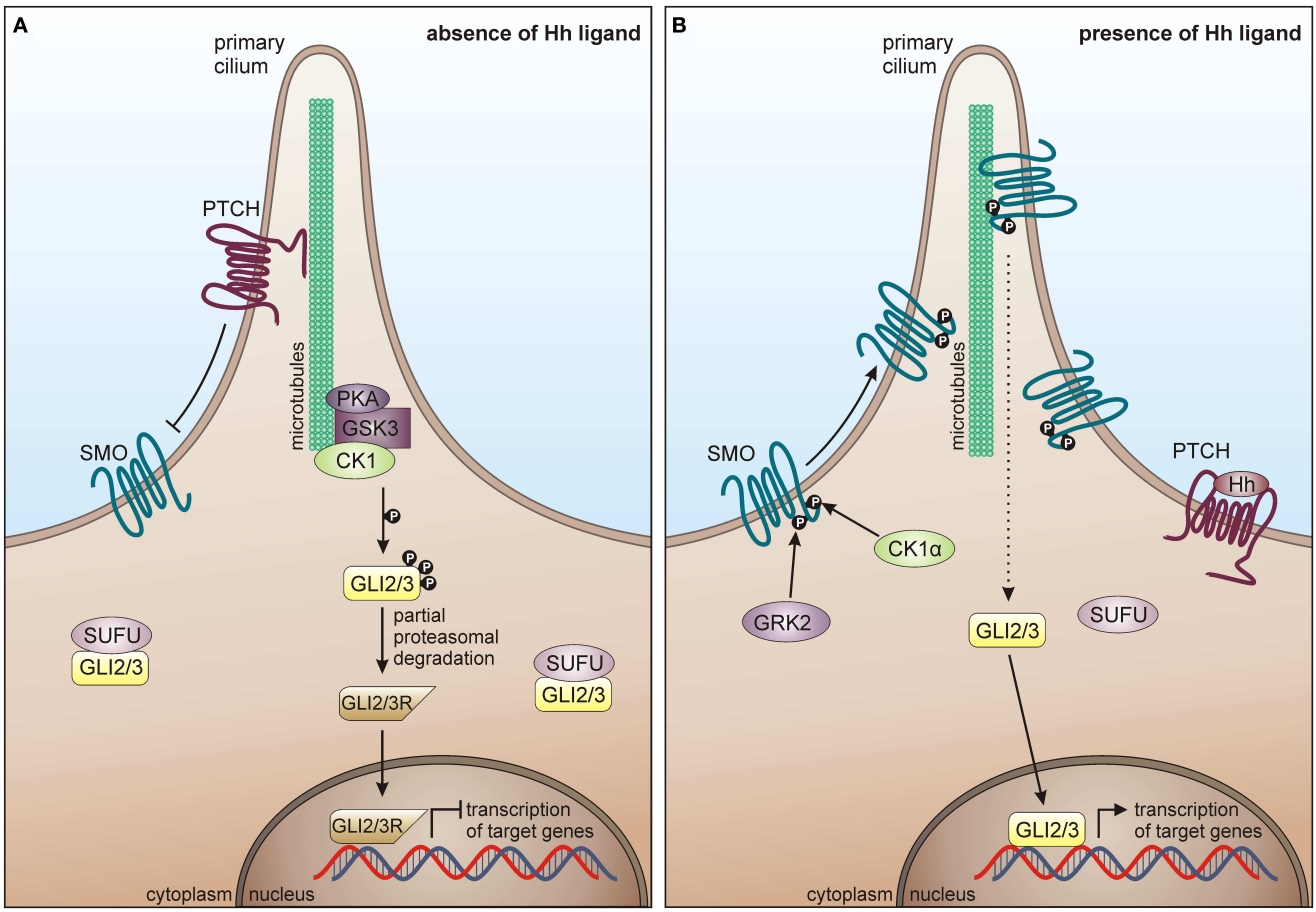


FIGURE 7 | CK1 in Hh signaling. **(A)** In absence of Hh ligand, PTCH localizes in the cilium and inhibits surface trafficking and cilia localization of SMO. GLI proteins are phosphorylated by PKA, GSK3β, and CK1, which lead to proteasome-dependent cleavage of GLI into a N-terminally truncated form, generating the repressor forms GLI2R and GLI3R. GLI2/3R translocate to the nucleus and inhibit translation of Hh target genes. Furthermore, SUFU prevents GLI from activating Hh target genes, by binding it in the cytoplasm and the nucleus. **(B)** In response to Hh, SMO is activated by GRK2 and CK1α-dependent phosphorylation and enters the primary cilium. Activated SMO orchestrates a signaling cascade, eventually resulting in the dissociation of the SUFU-GLI complex and the translocation of full-length GLI2/3 to the nucleus, where Hh target gene expression is induced.

the Wnt/Rac1/JNK and Wnt/Ca^{2+} pathway, consequently leading to increased migratory capacity and decreased cell-adhesion (334). A mutation within the C-terminal region of CK1δ detected in an adenomatous colorectal polyp leads to a higher oncogenic potential and promotes the development of adenomas in the intestinal mucosa (335). Furthermore, conditional knock-out of CK1α in the intestinal epithelium leads to activation of p53 and Wnt-signaling, while in p53 deficient gut, loss of heterozygosity of the CK1α gene causes a highly invasive carcinoma, indicating that CK1α acts as a tumor suppressor when p53 is inactivated (336).

In 1981, Elias and co-workers already reported an increased nuclear CK1 kinase activity in AML patients (186). Until now, several reports link altered CK1 expression and/or activity to cancer. Reduced CK1α protein and mRNA expression levels in primary melanomas and melanoma metastases compared to benign melanocytic lesions or early-stage melanomas have been detected. In the same study, reduced CK1α expression was also observed in lymphomas, ovarian, breast, and colon carcinomas, compared with the respective benign tissue (337). In renal cell carcinoma elevated CK1γ3 expression and activity levels have been described (338), whereas in choriocarcinomas strong expression levels of CK1δ were detected (222). Changes in the immunoreactivity of CK1δ have been observed in breast carcinomas, depending on the grade of tumor differentiation. High-grade ductal carcinomas *in situ* (DCIS) as well as invasive poorly differentiated carcinomas show reduced CK1δ immunostaining, whereas well differentiated carcinomas and low grade DCIS show strong staining of tumor cells (219). Regarding CK1ε, Fuja and co-workers observed similar correlations between tumor differentiation and immunohistological staining (341). Expression of CK1ε is also down-regulated in mammary cancers in SV40-transgenic mice expressing SV40 T-Ag (184). A recent study suggests that CK1ε is overexpressed in breast tumors and acts as a pivotal regulator of mRNA translation and cell proliferation. CK1ε phosphorylates the negative-acting factor 4E-BP1 (eukaryotic translation initiation factor 4E binding protein 1), thereby preventing its inhibitory function on the translation

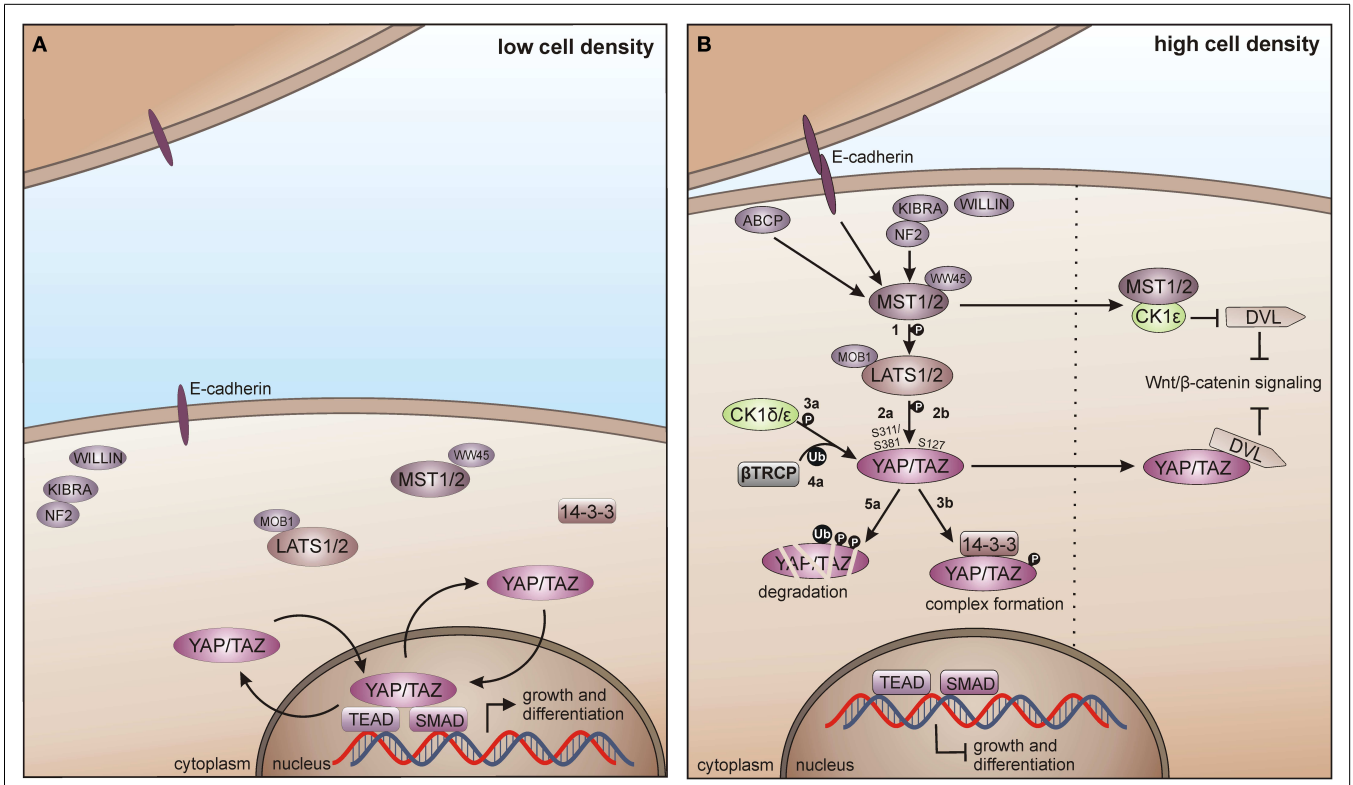


FIGURE 8 | CK1 in Hippo signaling in vertebrates. **(A)** In absence of growth suppressive signals YAP/TAZ promotes tissue growth and differentiation by regulating the activity of different transcription factors in the nucleus, including SMADs and TEADs. **(B)** Cell-density activated pathway regulation is controlled by multiple upstream branches by activating the core kinase cassette that represses YAP/TAZ driven gene transcription, either by degradation of TAP/TAZ or by forming physical complexes, preventing its nuclear access. Initially, MST1/2 is activated by various components and phosphorylates LATS1/2 (1), which in turn phosphorylates TAP/TAZ on Ser-311 or Ser-381 (2a). This phosphorylation primes YAP/TAZ for further phosphorylation by CK1δ/ε (3a) and consequent recruitment of and ubiquitination by β-TrCP (4a), priming YAP/TAZ for degradation (5a). However, LATS1/2 driven phosphorylation of TAP/TAZ on Ser-127 (2b) leads to the formation of 14-3-3-YAP/TAZ complexes, which accumulate in the cytoplasm preventing YAP/TAZ access to the nucleus (3b). Hippo pathway regulates Wnt/β-catenin signaling by inhibition of DVL, either by MST1/2-mediated prevention of CK1ε-dependent phosphorylation of DVL, or by direct inhibition of DVL by YAP/TAZ. ABCP: apicobasal cell polarity protein.

initiation complex elF4E (eukaryotic initiation factor 4E) and consequently leading to dysregulated mRNA translation and breast cancer cell growth (342). Elevated protein levels of CK1δ and ε were also observed in single tumor cells of grade 3 tumors of ductal pancreatic carcinomas and inhibition of CK1δ and ε by the CK1-specific inhibitor IC261 reduced pancreatic tumor cell growth in xenografts (339). In contrast, Relles and co-workers detected reduced expression levels in pancreatic ductal adenocarcinomas (340). CK1ε expression is increased in adenoid cystic carcinomas of the salivary gland (343), in epithelial ovarian cancer (344), in tumors of brain, head and neck, renal, bladder, lung, prostate, and salivary gland, in leukemia, melanoma, and seminoma (345). Toyoshima and co-workers found that CK1ε expression is significantly correlated with MYCN amplification in neuroblastoma and poor prognosis. In addition, CK1ε expression has been associated with c-MYC in several other tumors such as colon, lung, and breast cancer (346). In a recent study, Lin and co-workers demonstrated that loss of cytoplasmatic CK1ε expression correlates with poor survival rates in oral squamous cell carcinoma (347). Järas and co-workers recently found that CK1α is essential for AML cell survival and treatment with the CK1-specific inhibitor D4476 results in highly selective killing of leukemia stem cells by reducing Rsp6 (radial spoke protein 6) phosphorylation and activation of p53 (**Table 4**) (348).

In summary, the data reported so far provide evidence that CK1 isoforms exhibit oncogenic features by promoting proliferation, genome instability, and inhibition of apoptotic processes. This assumption is also supported by the fact that CK1 isoforms are often overexpressed in tumors and that overexpression of CK1ε correlates with poor survival as shown for patients with ovarian cancer (344). However, this finding cannot be generalized and might depend on additional factors, as in the case of oral squamous cell carcinoma loss of CK1ε expression correlates with poor survival rates (347). In addition, the functions of CK1α in tumorigenesis are manifold making it difficult to classify it as oncogene or tumor suppressor. In AML CK1α seems to exhibit oncogenic features (348), whereas in intestinal epithelium loss of heterozygosity of the CK1α gene causes a highly invasive carcinoma, indicating that CK1α acts as a tumor suppressor when p53 is inactivated (336).

Table 2 | CK1 isoforms in different tumor entities.

Isoform	Characteristic feature	Tumor entity	Reference
CK1α	Low/absent expression	Primary/metastatic melanoma, lymphomas, ovarian, breast, and colon carcinomas	(337)
CK1γ3	Altered activity/expression	Renal cell carcinoma	(338)
CK1δ	Increased expression levels	Choriocarcinomas	(222)
CK1δ	Reduced immunostaining	Poorly differentiated breast carcinomas and DCIS	(219)
CK1δ/ε	Elevated protein levels	High-grade ductal pancreatic carcinomas	(339)
CK1ε	Reduced expression levels	Pancreatic ductal adenocarcinoma	(340)
CK1ε	Increased immunoreactivity	Mammary DCIS	(184, 341)
	Decreased immunoreactivity	Invasive mammary carcinoma	
CK1ε	Overexpression	Breast cancer	(342)
CK1ε	High gene expression	Adenoid cystic carcinoma of the salivary gland	(343)
CK1ε	Overexpression	Epithelial ovarian cancer	(344)
CK1ε	Overexpression	Tumors of brain, head and neck, renal, bladder, lung, prostate, salivary gland, leukemia, melanoma, and seminoma	(345)
CK1ε	Overexpression	MYC-driven cancers (neuroblastoma, colon, lung, and breast cancer)	(346)
CK1ε	Loss of cytoplasmic expression	Poor prognosis in oral cancer patients	(347)

CK1 IN METASTATIC PROCESSES

In many cases, CK1 family members can also be involved in the regulation of metastatic processes. However, their potential to promote or suppress metastasis seems to depend not on the specific isoform but on their position in cellular signal transduction and the cellular context. Phosphorylation of nm23-H1 by CK1δ and ε has been shown to induce complex formation of nm23-H1 with a cellular partner called h-prune. Both proteins are linked to proliferative disorders and the nm23-H1-h-prune complex formation has even been proposed to positively influence cell motility (349). With this link of CK1 kinase activity to nm23-H1-h-prune complex formation an obvious role for CK1 in the mediation of metastasis has been established (153).

Quite recently, the stability of metastasis-related proteins has been shown to be regulated by CK1δ-mediated phosphorylation. First, the epigenetic sensor UHRF1 is critically involved in the maintenance of DNA methylation patterns during DNA replication and can be linked to carcinogenesis and metastasis if dysregulated (350, 351). Second, as mentioned before, proteasomal degradation of UHRF1 is regulated by CK1δ-mediated phosphorylation (104). Similar findings have been reported for metastasis suppressor 1 (MTSS1, also known as MIM, missing in metastasis), an anti-metastatic protein whose degradation also is triggered by CK1δ-mediated phosphorylation at Ser-322, thereby inducing its interaction with SCF/β-TrCP (77, 352).

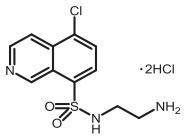
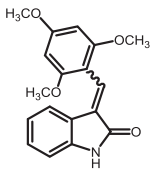
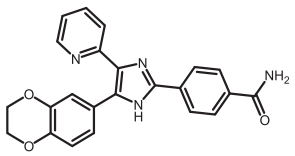
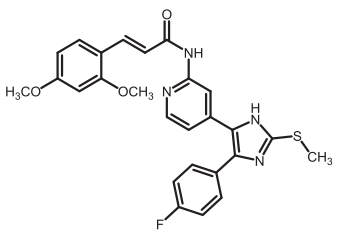
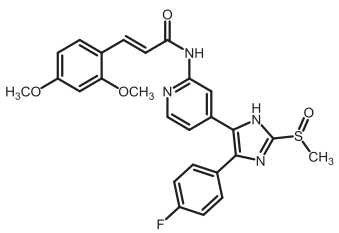
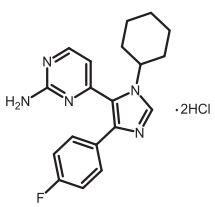
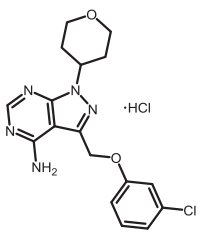
Furthermore, current reports demonstrate the involvement of CK1α in regulating the stability of metastasis-associated factors. When cell motility is induced the Rap guanine exchange factor (RAPGEF2) is phosphorylated by IKKβ and CK1α, initiating SCF/β-TrCP-mediated degradation. RAPGEF2 degradation-failure leads to inhibition of hepatocyte growth factor (HGF)-induced cell migration and expression of non-degradable RAPGEF2 suppressed metastasis of human breast cancer cells (165).

In canonical Wnt-signaling, CK1α has been positioned to be a tumor suppressor and cancer cells may activate proliferative processes via the Wnt/β-catenin pathway by suppressing CK1α expression. In the absence of CK1α, p53 is critically involved in controlling invasiveness as shown in a model for colon cancer (336). Re-expression of CK1α in metastatic melanoma cells reduced growth *in vitro* and metastasis formation *in vivo* (337). Consistent with these findings phosphorylation of β-catenin at Ser-45 by CK1α via activation by Wnt-5a has been shown to increase complex formation of β-catenin with E-cadherin thereby maintaining intercellular adhesion. Loss of Wnt-5a is thought to be associated with initial metastatic de-adhesion events (353, 354). Conversely, E-cadherin-mediated cell–cell contacts can be negatively regulated by CK1ε-mediated phosphorylation of E-cadherin at Ser-846 (69). In this context also, the Zn-finger transcription factor Snail is important as it can promote epithelial to mesenchymal transition (EMT) by down-regulating E-cadherin expression (355). Herein, CK1ε primes Snail for GSK3β-mediated phosphorylation, which marks Snail for degradation. Therefore, loss of CK1ε kinase activity prevents GSK3β-mediated phosphorylation and degradation of Snail supporting EMT and metastatic processes (122).

CK1-SPECIFIC INHIBITORS

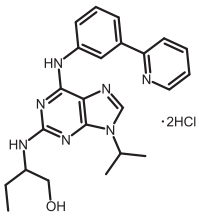
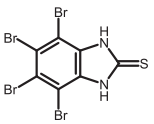
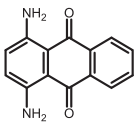
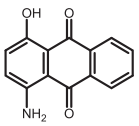
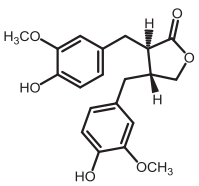
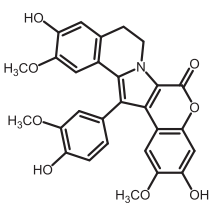
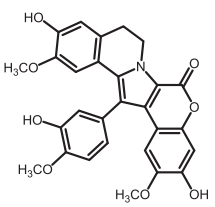
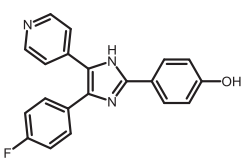
Due to the obvious involvement of CK1 isoforms in the pathogenesis of inflammatory and proliferative diseases and its contribution to the development of neuro-degenerative disorders, CK1 family members are attracting more and more attention as drug targets in regard to therapeutic applications. So far, several highly potent CK1-specific small molecule inhibitors have been identified (**Table 3**) and some have already been characterized for their therapeutic potential in animal models (**Table 4**). Most of these compounds are ATP-competitive type I inhibitors raising the problem of comparability of their effectiveness since their IC_{50}

Table 3 | CK1-specific small molecule inhibitors.

Inhibitor	Structure	IC_{50} (μM)	ATP (μM)	Reference
CKI-7		CK1: 6	100	(356, 357)
IC261		CK1δ/ε: 2.5	100	(357, 358)
D4476		CK1δ: 0.3	100	(357)
Peifer-17		CK1δ: 0.005; CK1ε: 0.073	100	(31)
Peifer-18		CK1δ: 0.011; CK1ε: 0.447	100	(31)
PF-670462		CK1δ: 0.013; CK1ε: 0.080	10	(273, 359)
PF-4800567		CK1δ: 0.711; CK1ε: 0.032	10	(273)

(Continued)

Table 3 | Continued

Inhibitor	Structure	IC_{50} (μM)	ATP (μM)	Reference
(R)-DRF053		CK1δ/ε: 0.014	15	(360)
4,5,6,7-Tetrabromo-2-mercaptobenzimidazole		CK1: 2.2	20	(361)
1,4-Diaminoanthra-quinone		CK1δ: 0.3	Not reported	(362)
1-Hydroxy-4-aminoanthra-quinone		CK1δ: 0.6	Not reported	(362)
(−)-Matairenisol		CK1: 10	10	(363)
Lamellarin 3		CK1δ/ε: 0.41	15	(364)
Lamellarin 6		CK1δ/ε: 0.8	15	(364)
SB-202190		CK1δ: 0.6	50	(365)

(Continued)

Table 3 | Continued

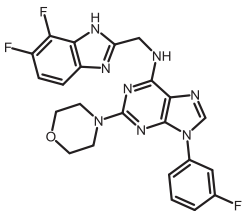
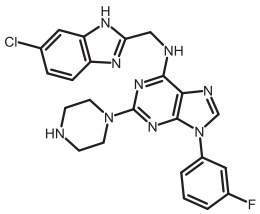
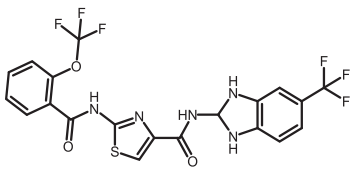
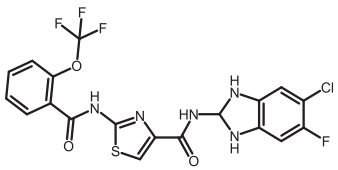
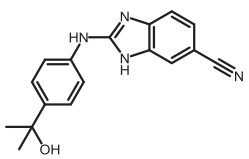
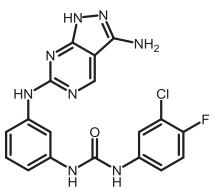
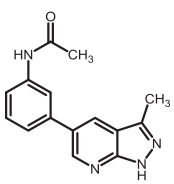
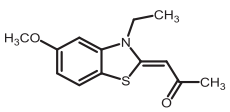
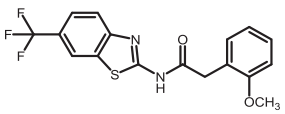
Inhibitor	Structure	IC_{50} (μM)	ATP (μM)	Reference
SR-3029		CK1δ: 0.044	10	(366)
SR-2890		CK1δ: 0.004	10	(366)
Bischof-5		CK1δ: 0.04; CK1ε: 0.199	10	(367)
Bischof-6		CK1δ: 0.042; CK1ε: 0.033	10	(367)
Hua-1h		CK1γ: 0.018	Not reported	(368)
Yang-2		CK1: 0.078	Not reported	(369)
CK01	similar to PF-670462	Not reported	Not reported	(370)
MRT00033659		CK1δ: 0.8935	20	(371)
TG0003		CK1δ: 0.4; CK1ε: 0.55	Not reported	(277, 372)
Salado-34		CK1δ: 0.01	10	(373)

Table 4 | Effects of CK1-specific inhibitors in selected animal models.

Process	Inhibitor	Model	Effects	Reference
Circadian rhythm	PF-670462	Rat	Inhibition of CK1δ/ε yields perturbation of oscillator function leading to phase delays in circadian rhythms	(359)
	PF-670462	Rat	Chronic treatment with the CK1δ/ε specific inhibitor PF-670462 yields cumulative phase delays in circadian rhythms	(374)
	PF-670462	Monkey	Inhibition of CK1δ/ε produces phase shifts in circadian rhythms of Cynomolgus monkeys	(375)
	PF-670462; PF-4800567	Mouse	Whereas PF-670462 causes a significant phase delay in animal models of circadian rhythm, CK1ε-specific PF-4800567 only shows a minimal effect on the circadian clock	(273)
	CK01	Mouse	Chronic administration of CK01 leads to a reversal of the anxiety-related behavior, and partial reversal of the depression-related phenotypes of the Clock mutant mouse	(370)
	PF-670462; PF-4800567	Mouse	Selective inhibition of CK1δ acts as a potent *in vivo* regulator of the circadian clock and may represent a mechanism for entrainment of disrupted or desynchronized circadian rhythms	(278)
	PF-670462; PF-4800567	Zebrafish	The use of a pan-CK1δ/ε inhibitor and a CK1ε-selective inhibitor revealed that activity of CK1δ is crucial for the functioning of the circadian timing mechanism in zebrafish at multiple levels	(283)
Drug use disorder	PF-670462	Rat	Inhibition of CK1δ/ε in the nucleus accumbens with the selective inhibitor PF-670462 blocks amphetamine-induced locomotion by regulating of the AMPA receptor phosphorylation	(376)
Sensitivity to opioids	PF-4800567	Mouse	Co-administration of the CK1ε specific inhibitor of PF-4800567 increased the locomotor stimulant response to methamphetamine and fentanyl	(377)
Alcoholism	PF-670462	Rat	The inhibition of CK1δ/ε with systemic PF-670462 injections dose-dependently prevented the alcohol deprivation effect	(378)
Cancer	IC261	Mouse	Inhibition of CK1 isoforms by IC261 influences the growth of induced pancreatic tumors in SCID mice	(339)
	IC261	Mouse	IC261 treatment blocks MYCN amplified neuroblastoma tumor growth *in vivo*	(346)
	D4476	Mouse	Inhibition of CK1α activity leads to reduced Rps6 phosphorylation and activation of p53, resulting in selective elimination of leukemia cells	(348)
Spinal inflammatory pain transmission	IC261; TG003	Mouse	Both compounds decreased the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) in inflammatory pain models	(379)

values have been determined at different ATP concentrations (see **Table 3**).

CKI-7 (*N*-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide), was the first ATP-competitive inhibitor being described to show selectivity toward CK1 (356). Later, IC261 (3-[(2,4,6-trimethoxyphenyl)-methylidenyl]-indolin-2-one) and D4476 (4-[4-(2,3-dihydro-benzo)[1,4]dioxin-6-yl)-5-pyridin-2-yl-1*H*-imidazol-2-yl]-benzamide) have been described as more potent and selective inhibitors, which also bind to the ATP binding pocket of CK1 (357, 358). Several effects reported for IC261-treated cells may however not be related to the selective inhibition of CK1 (380, 381). IC261 is also able to bind MT thereby inhibiting their polymerization similar to the spindle poison colchicine (380). Nevertheless, IC261 inhibits site-specific phosphorylation of p53 and Bid thereby inducing apoptosis in so-called type II cells (151, 187). Furthermore, its therapeutic potential has been demonstrated in xenotransplantation models for pancreatic cancer and neuroblastoma tumors (339, 346) (**Table 4**). However, it is still questionable whether the described anti-tumorigenic effects of IC261 are all mediated through selective inhibition of CK1δ and ε.

Two very potent and selective inhibitors for CK1δ and ε have been developed by Pfizer Global Research and Development: while PF-670462 possesses only poor isoform selectivity compound PF-4800567 shows a 22-fold stronger inhibition of CK1ε than CK1δ (273, 359). Furthermore, PF-4800567 demonstrated *in vivo*

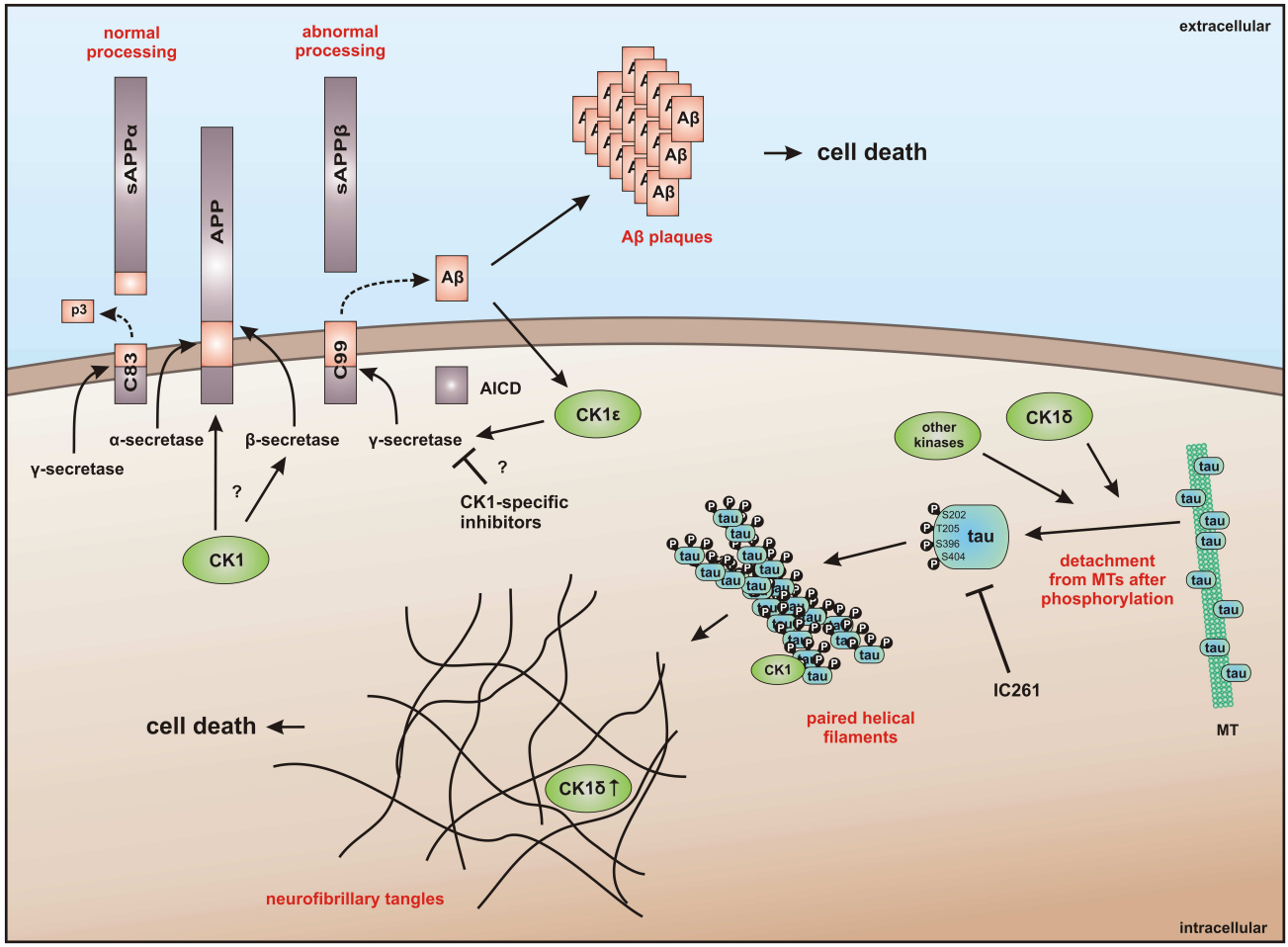


FIGURE 9 | CK1 in neuro-degenerative diseases. It has generally been accepted that overexpression of CK1 plays an important role in neuro-degenerative diseases, especially in tauopathies, such as Alzheimer's disease (AD). CK1δ is known to be up-regulated up to 30-fold on mRNA level in hippocampal regions of Alzheimer's disease (AD) brains (383). CK1δ plays a critical role in formation of neurofibrillary tangles through phosphorylation of tau at amino acids Ser-202/Thr-205 and Ser-396/Ser-404 (responsible for binding to tubulin) in human embryonic kidney 293 cells, thereby leading to a release of tau from MT and to destablilization of MT. Phosphorylation of these sites could be inhibited by the CK1-specific inhibitor IC261 (227). It is further known, that CK1 is associated to paired helical filaments in AD (384) and to tau-containing neurofibrillary tangles, in AD, Down syndrome, progressive supranuclear palsy, Parkinsonism–dementia complex and pallido-ponto-nigral degeneration (383, 385). The overexpression of constitutively active CK1ε, proposed to be involved in processing of amyloid precursor protein (APP) on γ-secretase level, results in an increase of amyloid-beta (Aβ) production, which is attenuated by use of CK1-specific inhibitors (386). In addition, Höttecke et al. (381) could show that the inhibition of γ-secretase by one of these inhibitors does not depend on CK1δ. An *in silico* analysis further revealed multiple CK1 consensus phosphorylation sites in the intracellular regions of APP, β-secretase, and γ-secretase subunits. Conversely, Aβ seems to influence CK1 activity (387). sAPPα/β: secreted amyloid precursor protein α/β; AICD: amyloid precursor protein intracellular domain.

potency by altering the circadian clock in cycling Rat1 fibroblasts and in a mouse model for circadian rhythm (273). Recently, the use of PF-670462 (and the similar compound CK01) proofed to be beneficial in the treatments of bipolar disorder (370), addictive behavior (378), and in perturbed circadian behavior (278), respectively.

By using structure-based virtual screening Cozza and co-workers identified two amino-anthraquinone analogs as CK1δ-specific inhibitors (362). Furthermore, several roscovitine-derivatives, among them (R)-DRF053, have been shown to inhibit both CK1 and CDK family members (360). In 2009, imidazole- (compounds 17 and 18) and isoxazole-derivatives have been found to be highly potent inhibitors for CK1δ and ε (31). Furthermore, a 2-phenylamino-6-cyano-1*H*-benzimidazole derivate (compound 1h) was identified as CK1γ-specific inhibitor with excellent selectivity, cellular potency, and acceptable pharmacokinetic properties (368). A new lead compound (a *N*6-phenyl-1*H*-pyrazolo[3,4-d]pyrimidine-3,6-diamine derivative), which inhibits CK1 with an IC_{50} value of 0.078 μM was discovered by Yang and colleagues (369). By using a pyrazolo-pyridine analog as CK1/Chk1 dual-specific inhibitor the p53 pathway could be stabilized and reactivated (MRT00033659) (371). Benzimidazole-based CK1-specific inhibitors were reported by several recent studies (**Table 3**) [SR-3029 and SR-2890 (366), Bischof-5 and Bischof-6 (367),

and Hua-1h (368)]. Furthermore, *N*-(benzothiazolyl)-2-phenylacetamides have been characterized as inhibitors for CK1δ-mediated phosphorylation of TDP-43 and may offer new therapeutic possibilities for the treatment of amyotrophic lateral sclerosis (ALS) (373). Quite recently, also the Clk-specific inhibitor TG003 was used to inhibit CK1 isoforms in a mouse model for mechanical allodynia and thermal hyperalgesia (**Table 4**) (379).

The potential of CK1-specific inhibitors for the treatment of neuro-degenerative diseases, like AD and Parkinson's disease, have been recently reviewed in detail by Perez and colleagues (382). The involvement of CK1 isoforms in the pathogenesis of AD is illustrated in **Figure 9**.

As an alternative to small molecule inhibitors lacking appropriate ADME (absorption, distribution, metabolism, and excretion) properties or showing unfavorable side effects synthetic peptides can also be used, which copy naturally occurring motifs that specifically influence the activity of the kinase or its interaction with cellular binding partners (388). Lately, small CK1α-derived peptides were used as *Biologic* tools to block CK1α binding to MDM2. At least, one peptide was identified to block the CK1α-MDM2 interaction (but not CK1α kinase activity) thus leading to decreased CK1α-MDM2-mediated degradation of p53 (208).

FINAL REMARKS

Summarizing the findings cumulated within many years regarding CK1 and its cellular functions, CK1 isoforms can be seen as central players in the regulation of numerous physiological cellular processes. Respecting this involvement in important cellular signal transduction pathways, it is reasonable that dysregulation of CK1 isoforms has been linked to the incidence of inflammatory and proliferative diseases but also to neuro-degenerative disorders. A summary of CK1-associated functions in neuro-degenerative diseases can be found in **Figure 9** and its associated figure legend. If potent CK1 (isoform)-specific inhibitors were available new therapeutic possibilities for personalized medicine could be provided. However, the development of isoform-selective compounds available for *in vivo* application still remains challenging and inhibitor development should include not only conventional small molecule design, but also novel peptide inhibitor approaches.

AUTHORS CONTRIBUTION

All Authors (Uwe Knippschild, Marc Krüger, Julia Richter, Pengfei Xu, Balbina García-Reyes, Christian Peifer, Jakob Halekotte, Vasiliy Bakulev, and Joachim Bischof) were involved in writing passages of the present review article and participated in final approval and revision. Figures and tables were created by Uwe Knippschild, Marc Krüger, Julia Richter, Pengfei Xu, Balbina García-Reyes, Christian Peifer, Jakob Halekotte, Vasiliy Bakulev, and Joachim Bischof.

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ShaPINg cell fate upon DNA damage: role of Pin1 isomerase in DNA damage-induced cell death and repair

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The peptidyl–prolyl *cis/trans* isomerase Pin1 acts as a molecular timer in proline-directed Ser/Thr kinase signaling and shapes cellular responses based on recognition of phosphorylation marks and implementing conformational changes in its substrates. Accordingly, Pin1 has been linked to numerous phosphorylation-controlled signaling pathways and cellular processes such as cell cycle progression, proliferation, and differentiation. In addition, Pin1 plays a pivotal role in DNA damage-triggered cell fate decisions. Whereas moderate DNA damage is balanced by DNA repair, cells confronted with massive genotoxic stress are eliminated by the induction of programed cell death or cellular senescence. In this review, we summarize and discuss the current knowledge on how Pin1 specifies cell fate through regulating key players of the apoptotic and the repair branch of the DNA-damage response.

Keywords: pin1, apoptosis, DNA repair, p53, p73, HIPK2, CtIP

INTRODUCTION

Proline residues bear the unique intrinsic feature of being able to convert between two distinct conformational states in a protein. Since the dihedral angle (ω) of the proline bond is large, a switch can introduce significant changes in total protein structure (1, 2). The interconversion between the two isomeric *cis* and *trans* states of peptide bonds preceding the amino acid proline can be catalyzed by peptidyl–prolyl *cis/trans* isomerases (PPIases) (3).

Three distinct families of PPIases that can facilitate prolyl isomerization have been identified so far. Among those are the family of Cyclophilins (Cyp), FK506-binding proteins (FKBPs), and the parvulins (4–7). Both Cyp and FKBPs have strong implications in immune responses due to their function as receptors for the immunosuppressive drugs cyclosporin A and FK506, respectively (4, 8, 9). The family of parvulins consists of the PPIase NIMA-interacting 1 (Pin1) and the more distantly related subgroup of proteins Par14 (Pin4) and Par17, which are both encoded within a single locus in the human genome (10, 11). In contrast to Pin1, the biological functions of Par14 and Par17 remain currently largely obscure. In the following section, we will introduce Pin1, which shows a unique feature among the PPIase protein family members, as it recognizes its client proteins in a phosphorylation-specific manner.

Abbreviations: ASPP, apoptosis stimulating protein of p53; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia related; CDK, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; Chk2, checkpoint kinase 2; DSB, double-strand breaks; DYRK2, dual-specificity regulated kinase 2; HIPK2, homeodomain-interacting protein kinase 2; HR, homologous recombination; iASSP, inhibitory member of the ASPP family; JNK, c-Jun N-terminal kinase; MEF, mouse embryonic fibroblast; NHEJ, Non-homologous end-joining; PPIase, peptidyl–prolyl isomerase; Siah-1, seven *in absentia* homolog 1; TRF1, telomeric repeat binding factor 1.

PHOSPHO-SPECIFIC ISOMERASE Pin1

Pin1 is a small enzyme consisting of 163 amino acids. It contains a WW protein interaction domain, which recognizes short proline-rich motifs at its N-terminus, and a C-terminal PPIase domain. The enzymatic conversion of peptide bonds between *cis* and *trans* conformation is dependent on the phosphorylation state of the Ser/Thr–Pro motif, which is the target sequence of Pin1 (12–14). In contrast to other known PPIases, Pin1 has the unique property of recognizing phosphorylation-specific motifs for isomerization. This feature links occurrence of specific phosphorylation-marks sites to conformational changes of its client proteins by *cis/trans* isomerization of the phospho-Ser/Thr–Pro bond (15) (**Figure 1**). Ser/Thr phosphorylation is a key mechanism of signal transduction and the most frequent post-translational modification in the cell. Phosphorylation at serine and threonine residues accounts for around 96% of all protein phosphorylation in the cell as revealed by global mass spectrometry analysis (16). Although phosphorylation has been shown to be sufficient for inducing conformational changes *per se* (17, 18), Pin1-catalyzed isomerization of phospho-Serine/Threonine residues represents a central mechanism in signaling and acts as a trigger to alter protein conformation (19–23).

Many Ser/Thr–Pro-directed kinases are predominantly localized in the nucleus (24) and play a major role in cell cycle regulation and cellular stress responses. This is evident from the well-studied functions of some representatives of this subgroup of kinases, such as cyclin-dependent kinases (CDKs), Jun-N-terminal protein kinases (JNKs), polo-like kinases (PLKs), and glycogen synthase kinase 3 (GSK-3). Accordingly, Pin1 also predominantly localizes to the nucleus, where it exerts its versatile signaling functions in regulating mitosis and mediating stress responses (14, 15, 25).

In this review, we are going to focus on the role of Pin1 in DNA-damage signaling. Excellent comprehensive reviews covering the

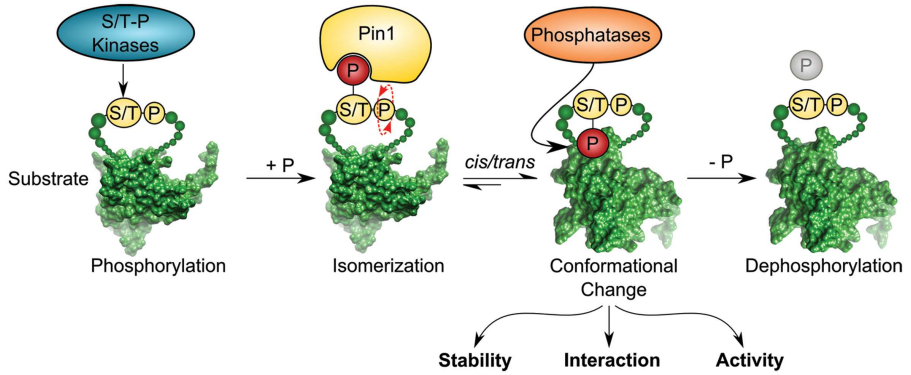

FIGURE 1 | Pin1 isomerase induces *cis/trans* conformational change of substrates containing pSer/Thr–Pro motifs. Ser/Thr–Pro-directed kinases phosphorylate diverse substrates thereby creating a putative binding site for the foldase Pin1, which catalyzes *cis/trans* isomerization of the previously phosphorylated protein in a subsequent step. Isomerization can regulate different functions of the substrate, such as stability, interaction, and activity. Pro-directed phosphatases, such as PP2A can dephosphorylate the pSer/Thr–Pro isomer [depicted substrate has been downloaded from PDB database; Protein-ID: 2FEJ (111)].

function of Pin1 in mitosis, Alzheimer disease, immune response, proliferation control, and cancer biology have been published recently (19, 22, 26–31) and are recommended to those readers interested in obtaining a global view on Pin1 function. In the following sections, we attempt to summarize the current knowledge about the function of Pin1 in DNA damage-induced cell fate with a focus on its role in the cell death response and DNA repair.

Pin1 AND p53

The tumor suppressor *p53* is mutated in more than 50% of human cancer (32) and cancer cells expressing wild-type p53 commonly functionally inactivate p53 by other means including overexpression of the p53-degrading E3 ubiquitin ligases MDM2 (33). In response to genotoxic stress such as UV, ionizing irradiation (IR), or chemotherapeutic drug treatment, p53 is stabilized, activated, and drives transcription of target genes leading to cell cycle arrest, senescence, or apoptosis, mostly depending on the strength of the insult and the cellular background (34). Activation of p53 upon genotoxic stress is largely determined by post-translational modifications, including site-specific phosphorylation and acetylation. Notably, p53 is phosphorylated by a set of stress-activated, proline-directed protein kinases such as p38, homeodomain-interacting protein kinase-2 (HIPK2), and DYRK2 (35–39). Pin1 binds to phosphorylated Ser/Thr–Pro sites on p53 upon genotoxic stress. In particular, phosphorylation at Ser33, Ser46, Thr81, and Ser315 has been shown to mediate the interaction of p53 and Pin1 (40–42). Subsequent conformational changes driven by Pin1 are crucial for the functional activation and stabilization of p53 upon DNA damage, which is achieved, at least in part, due to impaired interaction with the E3 ubiquitin ligase MDM2. Since MDM2 is a direct target gene of p53 (43), the Pin1-mediated accumulation of p53 is additionally regulated by a transcription-dependent increase of MDM2 (41). In line with these observations, cell cycle checkpoint function is impaired in Pin1-deficient MEFs as shown by higher re-entry into S-phase upon DNA damage. Overall, these studies demonstrate that Pin1 is important for the timely accumulation and the functional activation of p53 resulting in cell cycle arrest or apoptosis (**Figure 2**).

An important activation mark of p53 is the phosphorylation of p53 at Ser20, which is under control of checkpoint kinase 2 (Chk2) (44, 45). Phosphorylation of p53 at Ser20 impairs the interaction of p53 with its E3 ubiquitin ligase MDM2 (46, 47). Mutation of p53 Pro82 that precedes the phosphorylation site at Thr81 results in impairment of DNA damage-induced phosphorylation of p53 at Ser20 (48). Notably, genotoxic stress has also been shown to result in JNK-mediated phosphorylation of p53 at Thr81, which is important for JNK-dependent p53 transcriptional activation and apoptosis (49). Mechanistically, Pro82 mutation results in reduction of the DNA damage-induced interaction of Chk2 and p53. Exogenous expression of Pin1 enhances the interaction of Chk2 and p53 upon DNA damage and Ser20 phosphorylation is strongly impaired in Pin1-deficient MEFs. These findings indicate that Pin1-mediated isomerization of the Thr81–Pro82 bond is important for the binding of Chk2 to p53 and for DNA damage-induced phosphorylation of p53 at Ser20. In conclusion, this mechanism provides a model of how Pin1 facilitates Chk2-mediated p53 phosphorylation at Ser20, and as a functional consequence, leads to the disruption of the p53–MDM2 complex (48, 50).

Since Pin1 has a potent role in the activation of p53, one might wonder whether Pin1 is also able to trigger the activation of mutant p53. Somatic mutations in the *TP53* gene are frequent in many cancer types and have a huge impact on the clinical outcome of those cancers (51, 52). Pin1 is frequently overexpressed in cancer and mediates proliferative signals through client proteins such as Cyclin D1 (53, 54). *TP53* R172H mutation corresponds to the hot-spot mutation 175 in human cancers and has been linked to gain of function mechanisms associated with tumor progression, resembling Li–Fraumeni syndrome. In fact, *TP53* missense mutations exhibit enhanced oncogenic potential beyond the loss of physiological p53 functions (52, 55).

Comparison between mice harboring a mono-allelic mutant $p53^{R172H}$ or *p53* KO mice in a *Pin1* wild-type or *Pin1*-deficient background revealed that absence of Pin1 results in a reduced

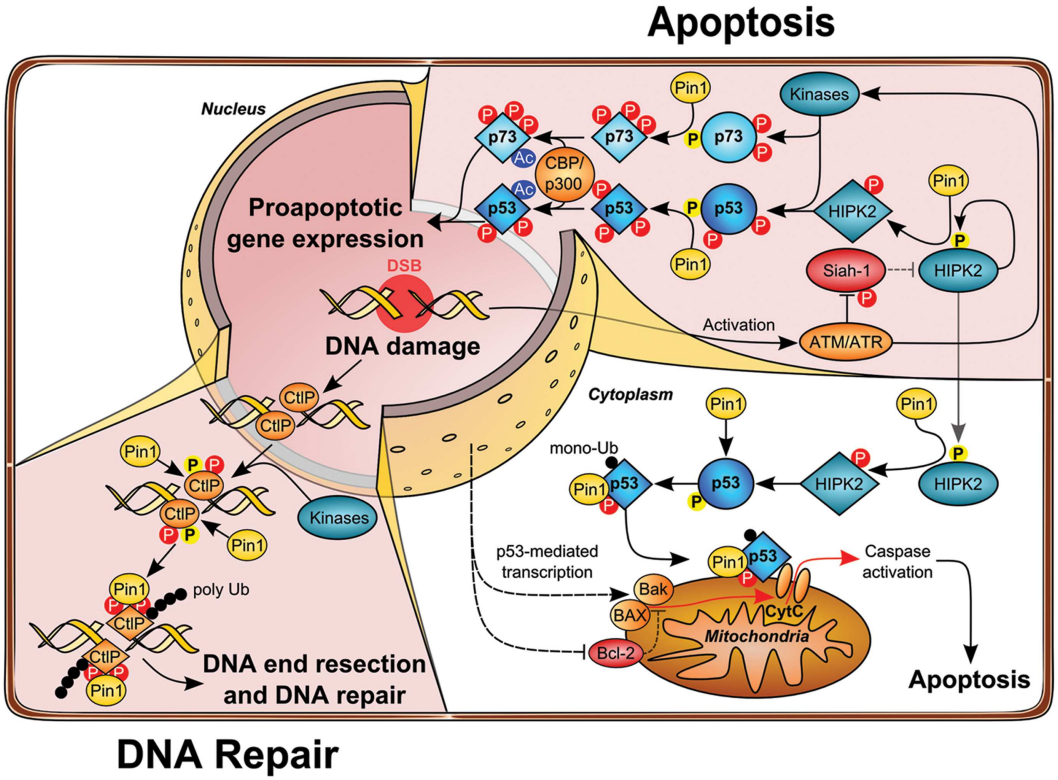

FIGURE 2 | Role of Pin1 in DNA damage-induced apoptosis and DNA repair pathways. Upon DNA damage ATM loosens the HIPK2–Siah1 complex by phosphorylation of Siah1, thereby allowing HIPK2 autophosphorylation. Phosphorylated HIPK2 recruits Pin1, which in turn changes HIPK2 conformation and potentiates disruption of the HIPK2–Siah1 complex. HIPK2 gets stabilized and phosphorylates p53 Ser46 to induce apoptosis. Moreover, p53 and Pin1 form a complex which further unleashes p53 apoptotic functions by changing p53 conformation and facilitating acetylation of p53 by the acetyltransferase CBP and loading of p53 on pro-apoptotic target gene promotors. At the same time, prolyl-isomerization of p53 Ser46–Pro47 by Pin1 leads to increased monoubiquitination of p53, which in turn triggers the translocation of cytosolic p53 to mitochondria, thereby initiating mitochondrial outer membrane permeabilization (MOMP) and intrinsic apoptosis release of cytochrome *c* into the cytoplasm. In a parallel signaling branch, ATM activates other kinases, such as c-Abl and p38, which leads to phosphorylation of p73. Recruitment and Pin1 binding leads to association with the acetyltransferase p300 and stimulates acetylation, which enhances transcriptional activity of p73 toward apoptotic genes. Beyond its function in apoptosis, Pin1 plays also a crucial role in double-strand break (DSB) repair. During S/G2 phase CtIP promotes end resection of DNA lesions. Phosphorylation of CtIP by CDK2 and most likely other kinases leads to Pin1 binding and isomerization of CtIP, which promotes its ubiquitylation and proteasomal degradation, thereby counteracting DSB end resection in favor of NHEJ.

tumor frequency and a decreased incidence of hematopoietic cancers. Most importantly, cancers of epithelial origin were completely absent in $p53^{R172H}$; *Pin1* KO mice (56). Interestingly, mutant p53 appears to be constitutively phosphorylated on Ser46 and Ser33 in breast cancer cell lines, which is potentiated upon oncogenic Ras^{G12V} induced signaling, thus creating permanent target sites for Pin1. Furthermore, mutant p53 exhibits a highly metastatic phenotype that is dependent on Pin1, since Pin1-depletion results in strongly reduced migration and invasion capacity *in vitro* and *in vivo*. Inversely, Pin1 overexpression potentiated migration in a mutant p53-dependent manner. Moreover, Ser46 phosphorylation appears to be critical for the migration phenotype observed in breast cancer cell lines bearing mutant p53. Most importantly, both Pin1 and mutant p53 synergize in the positive regulation of a set of genes that are relevant for migration and invasion. Furthermore, the oncogenic functions of mutant p53 are further enhanced by augmenting p63 transcription in a Pin1-dependent fashion, thereby reprograming gene expression in breast cancer cells. Overall survival appears to be drastically diminished in breast cancer cases bearing p53 missense mutation and Pin1 overexpression, which suggests that p53 status in combination with Pin1 expression level can be used as an independent prognostic marker for poor clinical outcome.

Pin1 AND p73

The p73 protein is a member of the p53 protein family. Similar to its homolog p53, p73 harbors tumor suppressive functions such as growth suppression, apoptosis, DNA repair, senescence, and differentiation. DNA-damage induced by chemotherapeutic drugs such as Doxorubicin or Cisplatin also activate p73 (in the absence of p53), which exerts transcription-dependent and independent functions (57–60). Apart from being implicated in cytotoxic stress-mediated cell cycle arrest or apoptosis, p73 also plays a pivotal role in development, especially in that of the neuronal system (61).

Interestingly, it has been demonstrated that DNA damage-induced p53-dependent apoptosis requires functional p63 and p73

(62). Along these lines, Pin1 is not only essential for proper activation of p53, but it is also required for p73's pro-apoptotic function (63). Pin1-depletion leads to defective induction of apoptosis and p73 accumulation upon Cisplatin administration. Accordingly, Pin1 controls p73 turnover in unstressed cells and upon cytotoxic stress and is required for proper activation of p73 target genes such as Bax and PIG3, as revealed in cell lines lacking p53 expression (63). In contrast to p53, Pin1 interacts with p73 even without any indication of cellular stress, implying that p73 is constitutively phosphorylated at Pin1 binding sites. Nevertheless, treatment with Doxorubicin and Cisplatin enhances binding of Pin1 to p73 via Ser412, Thr442, and Thr482 target sites, which is partly due to activation of p73 by c-Abl and p38 kinase (63). In fact, Pin1 is required for the c-Abl induced p73 activation and accumulation upon cytotoxic stress and p300-mediated p73 acetylation is strongly impaired in Pin1-deficient cells, since Pin1-mediated isomerization regulates p300–p73 binding (63). In conclusion, these data support the indispensable role of Pin1 activity to synergistically drive p53 and p73-mediated apoptosis under given stress conditions (as shown in **Figure 2**).

Pin1 AND iASPP

p53 activity needs to be tightly regulated in unstressed cells to prevent an unscheduled activation of the apoptotic response. To this end, p53 is sustained at low levels under physiological conditions, which is achieved by constant proteasomal degradation of p53 mediated by the E3 ubiquitin ligase MDM2. On the other hand, also association with iASPP inhibits p53 by preventing its binding to promoters of pro-apoptotic target genes (64). ChIP experiments revealed that under genotoxic stress conditions, Pin1-depletion results in decreased binding of p53 to p21 and Bax promoters. Upon genotoxic stress, Pin1 is directly associated with p53 on chromatin mainly via its binding sites Ser33, Ser46, Thr81, and Ser315. In fact, Pin1 activates p53-mediated transcription in a direct manner by potentiating the binding of the acetyltransferase p300 and subsequent acetylation of p53 at Lys373 and Lys382. Moreover, Pin1 also enhances the binding of p300 on p53-occupied promoters, which results in transcriptional activation. In addition, Thr81–Pro82 also appears to be a critical binding site for Pin1 and is required for proper acetylation of p53. The Thr81 residue lies within the Proline-rich domain of p53, which mediates binding of the p53 inhibitor iASPP. iASPP has been shown to regulate the transcriptional activity of p53 and is also pivotal for the stabilization of p53 in response to genotoxic stress (64). Intriguingly, Pin1 regulates the dissociation of the iASPP–p53 complex upon cytotoxic stress and thereby unmasks p53 transcriptional activity. Notably, dissociation of iASPP from p53 is dependent on stress-induced Ser46 phosphorylation but not on Pin1 stimulated acetylation of p53. Taken together, these findings indicate that Pin1 regulates p53 activity at different levels: (1) Pin1 controls p53 stabilization, (2) Pin1 is required for p53 binding to its target promoters, (3) Pin1 associates with chromatin and promotes p300-mediated acetylation of p53, and (4) Pin1 initiates the dissociation of p53 from its inhibitor iASPP. As described in the next chapter, Pin1 is also essential for stabilization of the p53 Ser46 kinase HIPK2. By facilitating efficient p53 Ser46 phosphorylation, Pin1 may regulate its own complex formation with p53 and drive the apoptotic response.

Pin1 AND THE p53 Ser46 KINASE HIPK2

Homeodomain-interacting protein kinase-2 is a central activator of the apoptotic cell death in development and in response to cellular stress and also acts as a tumor suppressor targeted by cellular and viral oncogenes (37, 38, 65–70). HIPK2 triggers apoptosis induction upon various types of DNA damage including UV radiation, ionizing radiation, and chemotherapeutic drug treatment through catalyzing phosphorylation of p53 at Serine 46, a phosphorylation mark, which drives expression of pro-apoptotic target genes (37, 68, 71–73). In addition, HIPK2 also activates the apoptotic response independent of p53 by regulating the JNK pathway and by targeting the anti-apoptotic transcriptional repressor CtBP for degradation (74, 75). In healthy cells and cells recovering from sublethal DNA damage, HIPK2 is kept inactive through proteasome-dependent degradation mediated by the ubiquitin ligases MDM2, WSB1, and Siah-1 (76–79). In the wake of DNA damage, HIPK2 is stabilized by a mechanism involving the checkpoint kinases ATM and ATR, which facilitate dissociation of the HIPK2–Siah-1 complex by phosphorylation of Siah-1 at Ser19 (77).

Interestingly, HIPK2 stabilization upon DNA damage also requires Pin1 activity (80). It has been recognized that HIPK2 autophosphorylates at multiple sites, which influences its kinase activity (81, 82). In particular, autophosphorylation *in trans* at Thr880/Ser882 through an intermolecular mechanism activates its kinase activity and apoptotic function upon genotoxic stress. Thr880/Ser882 autophosphorylation takes place at an early phase of HIPK2 activation and decreases when HIPK2 is fully stabilized. Furthermore, pThr880/pSer882 is followed by Pro residues and thus represent *bona fide* Pin1 binding sites. In fact, Pin1 binds HIPK2 through this phospho-motif and alters the conformation of the autophosphorylated HIPK2 isoform and mediates stabilization by inhibiting its proteasomal degradation. Loss of Pin1 by genetic deletion or RNA interference results in a lack of DNA damage-induced HIPK2 stabilization and apoptosis induction (80) (as shown in **Figure 2**). Interestingly, HIPK2 autophosphorylation appears to be evolutionary conserved and is also detected on the zebrafish HIPK2 protein, and induction of apoptosis in zebrafish embryos by ionizing radiation (IR) is regulated by the autophosphorylation of HIPK2. Finally, Pin1 is essential for IR-induced cell death in zebrafish embryos and in human cancer cells, highlighting a fundamental role in the DNA damage-triggered apoptotic response (80). Taken together, these findings support a major role of Pin1 in DNA damage-activated apoptosis signaling.

Pin1 AND HUNTINGTIN

It has recently been shown, that mutant huntingtin (mHtt) is able to induce p53-dependent apoptosis via a Pin1-mediated mechanism (83). Activation and accumulation of p53 has been observed in Huntington disease and recapitulated in transgenic mouse models (84). mHtt, which bears an elongated segment of polyglutamine, is able to trigger the DNA-damage response (DDR) and induce the accumulation and phosphorylation of p53 at Ser46 and Ser15 (83, 85). In fact, Ser46 is critical and sufficient for the induction of apoptosis upon mHtt generated cellular stress. Interestingly, the other Pin1 binding sites identified in previous studies (40–42) are dispensable in mHtt-triggered neuronal death. p53 Ser46 is a Ser/Thr–Pro target site for Pin1 and expression of

mHtt can stimulate the interaction of p53 and Pin1. As previously mentioned, isomerization of Ser46–Pro47 bond has been demonstrated to be a prerequisite for the dissociation of the inhibitor iASPP from p53 in order to fully activate p53 transcriptional activity (86). In accordance with these previous findings, disruption of the iASPP–p53 complex is also critical in mHtt-mediated p53 activation. Moreover, Pin1 is necessary for the transcriptional activity of p53 and PUMA-mediated apoptosis induction in mHtt-stimulated cells and neurons derived from a mHtt-knock-in mouse model (83). Furthermore, it has been shown that Ser46 phosphorylation upon mHtt expression is synergistically mediated by HIPK2 and PKC δ (**Figure 2**). These findings provide an unexpected link between neurodegenerative disease and the apoptotic DDR mediated by HIPK2 and Pin1.

Pin1 AND MITOCHONDRIAL APOPTOSIS SIGNALING

p53 can regulate apoptosis by transcription-dependent and -independent mechanisms. Recent studies have demonstrated that the interplay of the nuclear and cytoplasmic functions of p53 is crucial for shaping its full apoptotic activity (87). The mitochondrial pathway is predominantly activated in response to IR or other types of cytotoxic stress and is therefore exploited in cancer therapy by ionizing-IR or chemotherapeutic drugs such as doxorubicin. Remarkably, p53 has been demonstrated to modulate Bcl-2 family members that regulate apoptosis by controlling mitochondrial permeability (88, 89). Upon cytotoxic stresses, p53 is partially localized at the mitochondrion and has been demonstrated to induce mitochondrial outer membrane permeabilization (MOMP) (90). In fact, the cytosolic apoptotic function of p53 is rapidly induced upon cytotoxic stress and precedes its transcription-dependent functions. Pin1 has also been linked to the regulation of cytosolic functions of p53. In this context, Pin1 potentiates mitochondrial damage induced by p53 and triggers apoptosis by releasing cytochrome *c* from the mitochondria (91). These effects require Pin1–p53 binding, since mutation of target sites on p53 diminished mitochondrial damage and induction of apoptosis when compared to wild-type p53. In addition, Pin1 is important for the efficient translocation of cytosolic p53 to mitochondria upon treatment with the chemotherapeutic drugs Doxorubicin and Etoposide. Mechanistically, Pin1 binds cytosolic fractions of p53 mainly via its target site at Ser46–Pro47. Ser46 is a target site for the protein kinase HIPK2 that has been shown to increase p53-dependent apoptosis (37, 38). Indeed, HIPK2 is able to cooperate with Pin1 to induce mitochondrial apoptosis and increases the fraction of p53 bound to mitochondria, which is dependent on the catalytic activity of the kinase and presence of Pin1 (91). These results show that HIPK2 is not only critical for the nuclear, transcription-dependent function of p53 but – in cooperation with Pin1 – also regulates activation of p53 at mitochondria.

Pin1 AND TELOMERES

Telomeres are chromatin structures capping the ends of chromosomes in order to shield the free DNA ends from degradation and damage. Erosion of telomeres below a critical length has been linked to chromosome end fusion and premature aging (92–95). Dysfunctional telomeres are considered as a permanent source of DNA damage leading to the activation of p53 (96). To avoid detection of chromosome ends as aberrant DNA structures, telomeres are organized in a higher order duplex lariat structure, the T-loop, and sequestered by a specialized macromolecular shelterin protein complex. The telomeric repeat binding factor 1 (TRF1) is part of the shelterin complex and is essential for telomere function. Pin1 has been shown to regulate TRF1 function on telomeres by directly binding to TRF1 through the phospho-Thr149–Pro150 motif (97), which is phosphorylated by CDKs during mitosis. Furthermore, Pin1 negatively regulates TRF1 stability, which requires its PPIase activity and leads to increased binding of TRF1 to telomeres (97). Strikingly, TRF1 stability is increased not only upon Pin1-depletion in human cells, but also *in vivo* as determined in the Pin1 KO mouse model. TRF1 has previously been shown to regulate telomere length (98, 99). Remarkably, Pin1 inhibition leads to progressive telomere shortening through a TRF1-dependent mechanism in human cells and in splenocytes derived from Pin1 KO mice (97). Accordingly, Pin1 nullizygous animals show premature aging phenotypes such as reduced bone radio-density and thinner dermal and epidermal layers along with further hallmarks of accelerated aging (54, 100, 101). Of note, the widely used telomerase-deficient mouse models, which are frequently used to study aging by telomere-erosion require five to six generations to develop efficient telomere shortening and premature aging phenotypes. The fact that Pin1 KO mice show massive telomere erosion and a severe aging phenotype even in the first generation suggests that active resection takes place at the telomere in the absence of Pin1. The molecular basis of this interesting phenotype still remains to be elucidated.

ROLE OF Pin1 IN DNA DOUBLE-STRAND REPAIR: THE Pin1–CtIP LINK

DNA damage is one of the major factors driving genomic instability and carcinogenesis in multicellular organisms. Numerous factors lead to the activation of the DDR, including ionizing radiation, chemotherapeutic drug treatment, and strong hyperproliferative signals as induced by oncogene expression (102). Proper cellular response to DNA damage is needed to suppress carcinogenesis, and this involves a set of gene products that elicit cell cycle arrest, DNA repair, premature senescence, or apoptosis, in accordance with the damage inflicted. The maintenance of genome integrity is accomplished by an elaborate signaling network termed DDR. A major mechanism underlying the cellular response to DNA damage is protein-phosphorylation (103). Accordingly, the master checkpoint kinases ATM, ATR, and DNA-PK play a central role in coordination of the DDR. Double-strand breaks (DSBs) are considered to be a particularly deleterious form of DNA lesion that needs to be repaired by the cell to facilitate cell survival and proliferation.

Two main pathways orchestrate the DNA repair process of DSBs, namely homologous recombination (HR) and non-homologous end-joining (NHEJ). HR represents a highly accurate, error-free repair mechanism, whereas NHEJ is more error-prone. In mammalian cells, DSBs produced for instance by IR are mostly repaired by NHEJ, however, when DNA replication is impeded this leads to stalled replication fork collapses and resulting DSBs are resolved by the HR pathway (104). The NHEJ

pathway operates mainly in the G_0/G_1 phase of the cell cycle by joining DSB ends, whereas HR employs the sister homolog as the template for repair and thereby is restricted to late S and G_2 phase (105).

A recent study demonstrated that Pin1 plays an important role in the DNA repair pathway (106, 107). Using a proteomics approach, the authors elegantly isolated a set of novel Pin1 binding proteins, among them a couple of factors known to play a role in DNA repair including MDC1, 53BP1, BRCA1, and CtIP (106). They focused on the Pin1–CtIP link and demonstrated that depletion of Pin1 by RNAi leads to an increased DNA-end resection and decreased NHEJ frequency. In good accordance with these findings, Pin1 overexpression strongly diminished HR frequency. The authors also provided insight in the mechanism by showing that Pin1-depletion leads to aberrant hyper-phosphorylation of the single-strand DNA binding protein RPA2, which serves as a surrogate marker for DNA-end resection (106). The DNA repair protein CtIP is a key regulator of DNA-end resection at DSBs and is essential for the recruitment of additional key factors to the DSBs in S/G_2 phase (108–110). Interestingly, the authors found that the Ser/Thr–Pro-directed kinase CDK2 phosphorylates CtIP on Thr315 and Ser276 and thereby facilitates complex formation with Pin1, which subsequently mediated isomerization of CtIP. The resulting altered protein conformation impacts on the stability of CtIP and promotes its polyubiquitylation and proteasomal degradation (**Figure 2**). This regulatory principle facilitates timing of DNA-end resection at break sites during late S/G_2 phase. Of note, since Pin1 overexpression is frequently observed in cancer, this mechanism is presumably involved in the increased genomic instability observed in human cancer cells. Taken together, Pin1 obviously plays a critical role in coordinating DNA repair pathway choice by suppressing HR and promoting the NHEJ pathway.

CONCLUSION AND POTENTIAL FUTURE ASPECTS

The identification of Pin1 and the clarification of its underlying enzymology have greatly put forward our knowledge on mechanisms of signal transduction. The intense research activities on Pin1 during the last years generated fascinating novel insight in the function and regulation of this remarkable enzyme and its role in cell signaling and disease. Pin1 provides a highly sophisticated, elegant means to translate pSer/pThr–Pro phosphorylation marks into conformational changes and thus in altered protein function. Since Ser/Thr–Pro phosphorylation is the most abundant post-translational modification in mammalian cells, it appears not very surprising that Pin1 emerged as a central specifier in signal transduction. Despite these facts, however, there is still much to be learned about the biochemistry and biology of Pin1.

For example, numerous Pin1 substrates including p53, HIPK2, and CtIP harbor several pSer/pThr–Pro sites, which critically contribute to Pin1 binding. This raises a number of questions: how is the exact stoichiometry of the Pin1–substrate complexes? Does Pin1 isomerize all pSer/pThr–Pro bonds in its substrates or only a subset? In addition, it is currently unclear whether Pin1 also cross-talks to phosphatases. The timed removal of the pSer/pThr–Pro marks by a given phosphatase would contribute to lock a substrate in a particular conformational state by preventing a backward isomerization reaction.

Even though there is evidence that Pin1 is subject to regulation by post-translational modifications including phosphorylation and SUMOylation, it currently remains unclear whether Pin1 function is also regulated in response to DNA damage. Such regulation might facilitate functional dissection of the DNA damage-associated functions and the cell growth regulatory and mitotic activities.

Beyond its impact on CtIP function, Pin1 has been found to interact with numerous additional factors implicated in DNA repair (106, 107) suggesting a currently unexplored broader function of Pin1 in coordinating DNA repair.

Finally, Pin1 appears to play important roles both in oncogenic and tumor suppressive signaling pathways. To exploit Pin1 function in diseases such as cancer it will be a major effort in the future to dissect the molecular determinants in order to design small molecules for specific interference with its oncogenic and mitotic functions, but to conserve its pro-apoptotic activities, which are necessary for the efficacy of genotoxic stress-inducing cancer therapies.

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p53 family and cellular stress responses in cancer

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p53 is an important tumor suppressor gene, which is stimulated by cellular stress like ionizing radiation, hypoxia, carcinogens, and oxidative stress. Upon activation, p53 leads to cell-cycle arrest and promotes DNA repair or induces apoptosis via several pathways. p63 and p73 are structural homologs of p53 that can act similarly to the protein and also hold functions distinct from p53. Today more than 40 different isoforms of the p53 family members are known. They result from transcription via different promoters and alternative splicing. Some isoforms have carcinogenic properties and mediate resistance to chemotherapy. Therefore, expression patterns of the p53 family genes can offer prognostic information in several malignant tumors. Furthermore, the p53 family constitutes a potential target for cancer therapy. Small molecules (e.g., Nutlins, RITA, PRIMA-1, and MIRA-1 among others) have been objects of intense research interest in recent years. They restore pro-apoptotic wild-type p53 function and were shown to break chemotherapeutic resistance. Due to p53 family interactions small molecules also influence p63 and p73 activity. Thus, the members of the p53 family are key players in the cellular stress response in cancer and are expected to grow in importance as therapeutic targets.

Keywords: p53, p63, p73, cellular stress, cancer, chemosensitivity, apoptosis

INTRODUCTION

Human cells are constantly exposed to external and internal stressors, which cause damage to the integrity of the cell and to its genome. In order to guarantee the survival of the organism, cells have developed numerous strategies to adapt to stressors. In this review, we would like to discuss the influence of cellular stress on tumor development as well as strategies in cancer therapy targeting pathways involved in cell-cycle control and apoptosis. Special emphasis is put on the members of the p53 family.

CELLULAR STRESS RESPONSE IN CANCER DEVELOPMENT

The development of cancer is a multistep process that involves a series of mutations in the progenitor cell (1). It enables clonal proliferation, uncontrolled growth, and finally invasion (2, 3). Cellular stress can be caused by a multitude of external or internal influences such as ultraviolet radiation (4–6), ionizing radiation (7), hypoxia (8), carcinogens (e.g., aflatoxin) (9, 10), cigarette smoke (11), oxidative stress (12–14), and oncogene activation (15). This can lead to DNA damage and, in consequence, to malignant transformation of the cell. In order to restore its integrity, the cell disposes of a number of damage control mechanisms. These mechanisms are older than the human species and can already be found 1 billion years ago in descendants of choanoflagellates and the early metazoan sea anemone (16). Human tumor protein p53, often described as the "guardian of the genome," and its target genes play key roles in cell-cycle control and induction of apoptosis. In its capacity as tumor suppressor protein, p53 is not only able to act as transcription factor for genes of pro-apoptotic effector proteins but it is also involved in transcription-independent cellular signaling leading directly to cell death via pathways originating from the mitochondria or the cytosol (17–19). Furthermore, p53 induces transcription of DNA repair enzymes, thereby promoting cell survival (20–22). This shows the functional dichotomy of p53. To date, the exact mechanisms deciding about death or survival of the damaged cell still remain to be elucidated. Under physiological conditions, cellular p53 levels are low and the protein has a relatively short-half-life of 20 min. Upon DNA damage, p53 levels rise primarily through stabilization of the protein (23).

While p53 has been known for more than three decades, two further members of the p53 family, p63 and p73, have been discovered more recently. The three genes exhibit a high degree of homology and there is increasing evidence that they have risen from the triplication of a common ancestral gene (24, 25). All three genes consist of important structural elements including a DNA-binding domain (DBD), an oligomerization domain (OD), and a transactivation domain (TAD) (26). p63 (27, 28) and p73 (29) have been shown to induce apoptosis similarly to p53 via activation of several of its downstream target genes (30–32). Yet, both family members also exhibit functions distinct from p53 (**Figure 1**).

While p63 is crucially involved in craniofacial, limb, and skin development (33), p73 plays an important role during neurogenesis (34). Multiple isoforms of the p53 family members are generated using different promoters and alternative splicing. They can carry out contrary functions. Whereas some isoforms have oncogenic potential, others can act as tumor suppressors (35). However, many isoforms seem to have both capacities depending on the entity of the cell they are expressed in and the tissue context. To date, regulation and interactions of the three members of the p53 family are still under investigation.

APOPTOSIS

Malignant tumors often exhibit defects in apoptosis signaling pathways, resulting in tumor cell survival. Therefore, understanding the exact mechanisms of apoptosis can provide

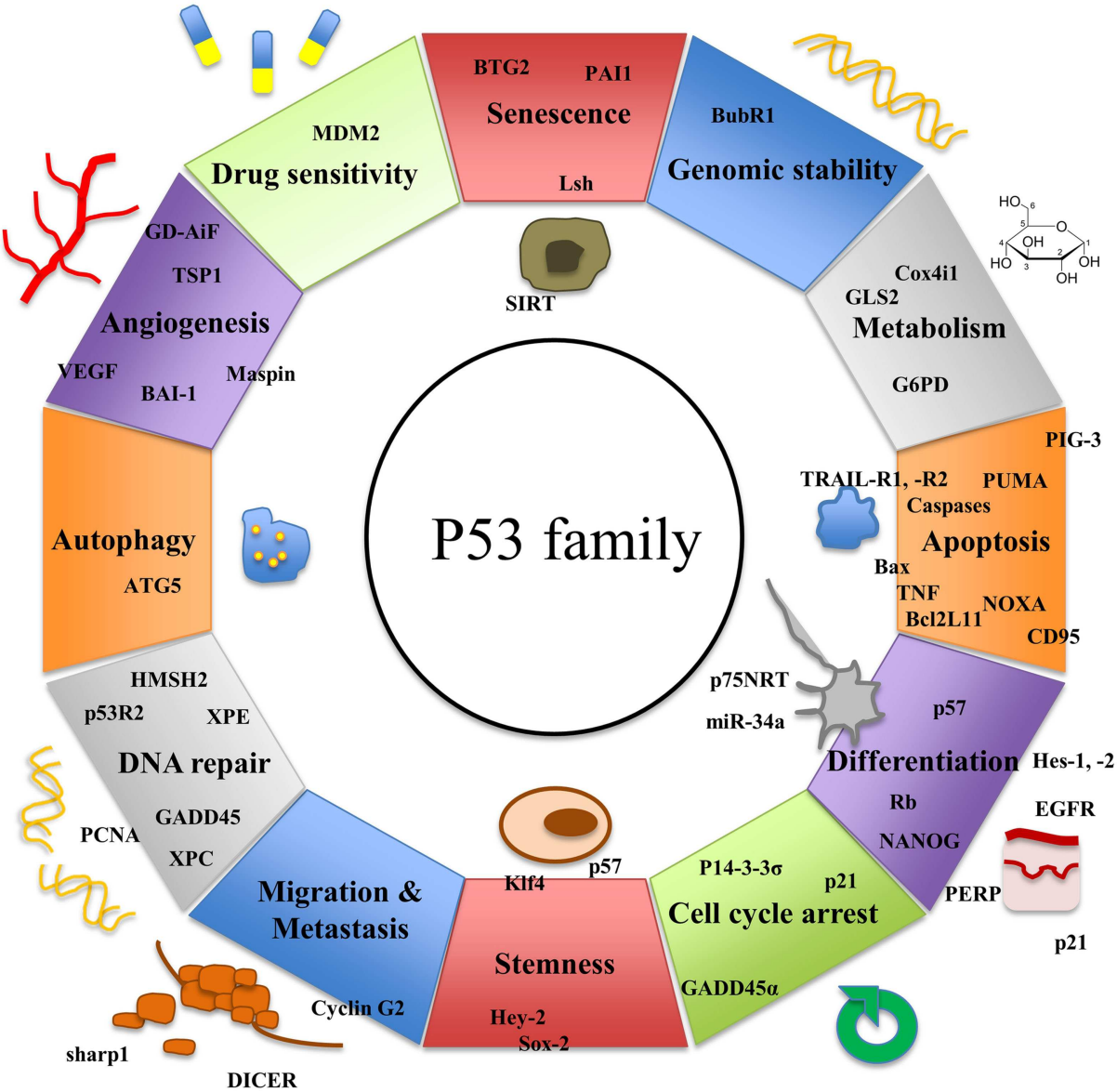

FIGURE 1 | Functions of p53 and its homologs p63 and p73 and their target genes.

new strategies for the development of anti-cancer treatments. The extrinsic apoptosis signaling pathway is initiated by ligands such as TNFα, CD95L, and TRAIL binding to death receptors (36–38). The best characterized members of the death receptor family are TNFR1, CD95, DR3, TRAIL-R1 (CD4), TRAIL-R2 (CD5), and DR6 (39, 40).

Death receptor signaling leads to activation of caspases. Caspases are cysteinyl aspartate proteinases, which are synthesized as inactive zymogens and, upon stimulation, are initialized by autolytic cleavage (41). Initiator caspases, such as caspase 8 und 9, form signaling complexes, which activate downstream effector caspases, including caspase 3 and 7, through proteolytic cleavage (41, 42). Effector caspases cannot self-activate but process a multitude of cellular substrates during cell death (43). The intrinsic apoptosis signaling pathway originates in the mitochondria and is part of the cellular stress response. It is regulated by proteins of the Bcl-2 family. Pro-apoptotic members of the protein family include Bax, Bak, and their subclass of BH-3 only proteins such as BAD, BID, BIM, Hrk, PUMA, BMF, and Noxa, whereas A1, Bcl-2, Bcl-w, Bcl-XL, and Mcl-1 are among the anti-apoptotic members (44). The anti-apoptotic Bcl-2 proteins exert their function by stabilizing the outer mitochondrial membrane (45). Upon cellular stress, Bid and Bim mediate homo-oligomerization of Bax and Bak, which leads to the release of cytochrome *c* from the mitochondrial intermembrane space (46). By binding Bcl-2 proteins Bad, Noxa, and PUMA lead to inhibition of the proteins (44).

Being released into the cytosol, cytochrome *c* forms a complex with APAF-1 and pro-caspase 9. After cleavage, caspase 9 activates effector caspase 3 (44).

p53 AND ITS ISOFORMS

p53 is encoded by the TP53 gene on the short arm of chromosome 17 and has a molecular mass of 43.7 kDa (25). It spans 19,200 bp including 11 exons (**Figure 2**). There are three known promoters within the p53 gene: two sites upstream of exon 1 producing full-length p53 and one internal site within intron 4 leading to transcription of amino-terminally truncated Δ133p53 (47). Δ40p53 isoforms, which have lost a part of the N-terminal TAD, can be obtained by alternative splicing of exon 2 and alternative initiation of translation at ATG40 (24), while Δ160p53 isoforms, which lack the first 159 residues, arise from translational initiation at ATG160 (48). Alternative splicing of intron 9 generates additional three isoforms, full-length p53, p53β, and p53γ (24). Both 53β and p53γ lack the OD (24). To date, a total of 12 p53 isoforms have been described: p53, p53β, p53γ, Δ40p53α, Δ40p53β, Δ40p53γ, Δ133p53α, Δ133p53β, Δ133p53γ, Δ160p53α, Δ160p53β, and Δ160p53γ (49, 50). While some p53 isoforms exert functions similar to full-length p53, others have antagonizing properties. Δ133p53, for example, inhibits p53-mediated apoptosis and causes cell-cycle arrest at the G2/M checkpoint (47, 50). Δ40p53 isoforms control the development of pluripotent embryonic stem cells into differentiated somatic cells by modulating IGF-1-R levels (51). Very little is known about the clinical role of p53 isoforms and further investigation is needed to determine if they could prove valuable as targets for anti-cancer therapy.

Human p53 protein consists of several domains. The central DNA-binding domain (DBD) (core domain) is shared by most p53 isoforms and binds to response elements of target genes. A large number of p53 mutations occur within this region of the gene (52). The N-terminal transcription–activation domain (TA) is the binding-site for positive (e.g., p300/CBP, TAFII40/60) or negative regulators (e.g., MDM2 and MDMX) of p53 gene transcription (53). The C-terminal oligomerization (CTD) domain is subject to alternative splicing and post-translational modification. The CTD has been shown to influence DNA binding and transcriptional activity of the p53 family members (54).

p53 REGULATES CELL-CYCLE, INDUCES APOPTOSIS, AND PROMOTES CELL DIFFERENTIATION

p53 controls a large number of genes mediating G2/M and G1 cell-cycle arrest, DNA damage recognition, DNA repair, apoptosis, and senescence (25) (**Figure 1**). Absence of one parental copy of p53 through germline mutation of TP53, a condition called Li–Fraumeni syndrome, leads to development of several tumors, particularly sarcomas and cancers of the breast, brain, and adrenal glands (55, 56). Even in young individuals suffering from this condition multiple malignant tumors may develop. p53 knock-out mice have been shown to be prone to development of various types of malignancies demonstrating the important role of p53 in cancer biology (57). When initiated during the cellular stress response, p53 activates transcription of p21, a cyclin-dependent kinase inhibitor. p21 blocks CDK-1 and -2 leading to cell-cycle arrest at G1 and S phase (58). Since p53 counteracts cell growth and development, it is crucial that p53 function is strictly regulated. The E3 ubiquitin ligase MDM2 blocks p53's transcriptional activity by binding to the N-terminal TA domain of the protein (59, 60). MDM2 is also capable of inducing the ubiquitin-mediated proteasomal degradation of the tumor suppressor protein (61, 62). In return, p53 positively regulates expression of MDM2. Thereby, it creates an auto-regulatory loop that controls the level of active p53 in the cell (63–65). During the cellular stress response, MDM2 is inhibited by different regulator proteins leading to accumulation of p53 in the cell (66).

Another important upstream regulator of p53 activity is p14ARF, a protein transcribed from an alternate reading frame of the CDKN2A gene locus that also encodes for the tumor suppressor p16INK4a (67, 68). p14ARF is part of the cell's response to oncogenic activation (69–73). It acts as an inhibitor of MDM2-medited degradation of p53 (74). Therefore, ARF-deficient mice are prone to developing tumors of various entities (75). In a negative feedback loop, ARF promotes degradation of its activator E2F-1 and is suppressed by its downstream target p53 (76, 77).

Primarily, p53 is a transcription factor. It is involved in the intrinsic and extrinsic apoptosis signaling pathways by initiating transcription of functional proteins such as PUMA, Bax, Bid, CD95, and TRAIL-R2 (78). Yet, transcription-independent functions have been described. In the cytosol, p53 induces cell death by forming inhibitory complexes with Bcl-XL and Bcl-2, which leads to the permeabilization of the mitochondrial membrane and cytochrome *c* release (79, 80). Furthermore, cytosolic p53 can activate pro-apoptotic proteins such as Bax and Bak through direct protein–protein interaction (18, 81, 82).

Recently, it was observed that p53 also plays an important role in stem cell biology. In embryonic stem cells, p53 guarantees genetic stability via induction of differentiation (83) while

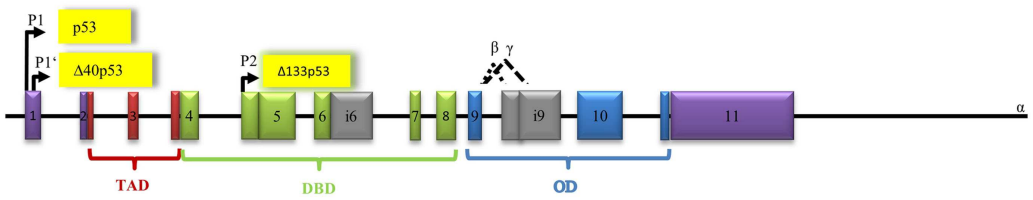

FIGURE 2 | Architecture of the human p53 gene structure: alternative splicing (α, β, γ), alternative promoters (P1, P1′, P2), transactivation domain (TAD), DNA-binding domain (DBD), and oligomerization domain (OD) are indicated. The P1 promoter generates full-length-proteins with a transactivation domain (TAD), whereas the P1′- and P2 promoters generate proteins lacking the TAD.

limiting generation of induced pluripotent stem cells and tightly controls reprogramming (84). The cancer stem cell (CSC) hypothesis suggests that every tumor holds a pool of CSCs capable of renewal. They are essential for sustenance and growth of the tumor and respond poorly to conventional chemotherapy (85). CSCs result from either dedifferentiation of somatic cells or mutations in existing stem or progenitor cells (84). Targeting CSCs via activation of p53-linked pathways could trigger cell differentiation. In consequence, malignant cells would be more susceptible to DNA damaging agents and their capacity of self-renewal would be reduced.

In 1997, the cloning of p73 as a new p53 family member was reported, this was followed by the discovery of p63 – the third member of the p53 family (54, 86–89). The protein architecture is highly conserved among the three members of the p53 family (30). The highest degree of sequence homology has been described for the DNA-binding core domain (30). In contrast, the C-terminal domains are diverse and subject to alternative splicing and post-translational modification. Sauer et al. demonstrated that the C-terminal domains influence DNA binding and transcriptional activity (54) and suggested that the diversity of the C-terminal domains of the p53 family influences cell fate decisions and cellular responses that are regulated by the p53 family members (90).

p63 AND ITS ISOFORMS

The p53 homolog p63 contains three promoters that are known to encode three types of isoforms (91). The first promoter has only recently been discovered by Beyer et al. In response to DNA damage, it leads to activation of human male germ-cell-encoded TAp63 protein, which is specifically expressed in testes and protects the genomic integrity of the male germline (91, 92). The second promoter mediates transcription of TA isoforms, which contain a N-terminal TAD (22% identical with the TAD of p53) followed by a DBD (60% identical with the DBD of p53), an OD (38% identical with the OD of p53), and the sterile alpha motif (SAM) (30). In contrast, there is no SAM in the p53 gene. The third promoter is located between exon 3 and 4. Loss of exons 2 and 3 and incorporation of exon 3′ through the third promoter results in different ΔN isoforms (93). Additionally, alternative splicing at the 3′-terminus leads to the generation of five isoforms (α, β, γ, δ, and ε) and contributes to the variety of proteins (93) Premature transcriptional termination in exon 10 generates isoform ε (94) (**Figure 3**).

TAp63 is predominantly expressed in oocytes, although it has also been identified in other tissues like epidermis. In TAp63 knock-out mice, a phenotype with ulcers, hair defects, and reduced wound healing can be observed (95).

When first discovered, ΔN isoforms were thought to exclusively repress transcription. But, ΔN isoforms gain their transcriptional activity from two additional TADs within the residue, one located between the OD and the SAM domain and another located in proximity to the proline-rich domain (96, 97). Therefore, they do not only repress functions of the TA isoforms by inhibiting transcription of TA dependent genes but also transactivate their own target genes (98). ΔN63 is found in epidermal cells, in particular (99). Knock-out mice with down-regulated ΔNp63 show severe skin wounds as well as delayed wound healing (100). ΔNp63 expression can be found in multiple tumors, particularly in those with unfavorable prognosis (101). Of importance for clinical use is the fact that ΔNp63α expression is a prognostic marker for poor response to cisplatin chemotherapy in HNSCC (102). However, categorizing ΔNp63 isoforms as proto-oncogenes and TAp63 isoforms as tumor suppressors would be far too simple (103). For instance, diffuse large human B-cell lymphomas do not show enhanced expression of ΔNp63 protein, but overexpression of TAp63 (104, 105).

p63 function is regulated by post-translational modifications that influence p63 protein stability. For example, E3 ligases like Pirh2 and ITCH lead to polyubiquitination and subsequent proteasomal degradation of the protein (106). RNA-binding proteins such as RNPC1, HuR, or PCB1 control stability of p63 by binding AU-, CU-, or U-rich elements in 5′ or 3′ UTRs of p63 mRNA (107–109).

p63 and p53 have common and distinct downstream target genes (110), thereby sharing functions in cell-cycle control and apoptosis (**Figure 1**). TAp63 causes G1 cell-cycle arrest through transcriptional up-regulation of p21 and p57/Kip2 (111). Furthermore, p63 induces apoptosis via the extrinsic and the intrinsic apoptosis signaling pathway by enhanced expression of Bax, RAD9, DAP3, APAF-1, CD95, TNF-R, or TRAIL-R death receptors (27).

In addition, p63 assumes defined functions within the cell distinct from those of p53. In oocytes, DNA damage directly induces phosphorylation of p63, which leads to oocyte death (112, 113). p63 knock-out mice show a phenotype that is lethal soon after birth. They suffer from significant epithelial abnormalities, concerning skin, glands, teeth, and hair follicles (114). Their limbs are

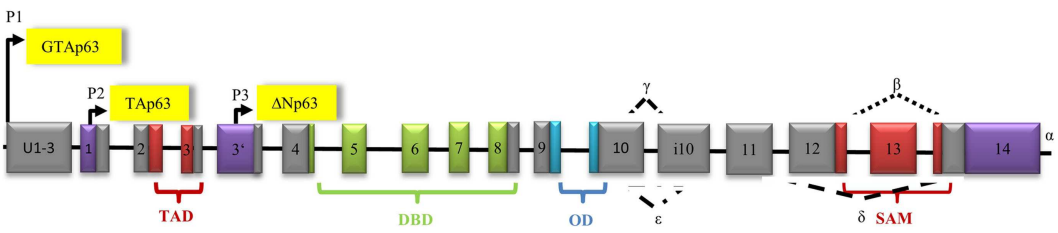

FIGURE 3 | Architecture of the human p63 gene structure: alternative splicing (α, β, γ, δ, ε), alternative promoters (P1, P2, P3), transactivation domain (TAD), DNA-binding domain (DBD), oligomerization domain (OD), and sterile alpha motif domain (SAM) are indicated.

truncated and craniofacial anomalies are characteristic (93, 115). Human heterozygous mutations of p63 result in dysplasia of hair, teeth, digits, sweat glands, and nails (93). Therefore, p63 is essential for epithelial development. Furthermore, in a recent study, D'Aguanno et al. suggested that p63 might be involved in cancer cell metabolism. Colon CSCs showed a higher glycolytic activity when expressing TAp63 instead of ΔNp63 (116). Consistent with these observations, Giacobbe et al. reported that TAp63 isoforms can enhance expression of the mitochondrial glutaminase 2 (GLS2) gene, both in primary cells and in tumor cell lines (117).

Loss of function mutations of p63 are extremely rare in malignancies in contrast to p53 mutations (30) and controversial phenotypes have been described. Development of spontaneous tumors could be found as well as no increase in tumor disposition (111, 118–120). However, alterations in p63 expression patterns play an important role in tumorigenesis (121). In addition, mice heterozygous for mutations in both p53 and p63 (p53+/−; p63+/−) show higher tumor burden in comparison to mice heterozygous for p53 only (118). Knock-down of p63 (p63−/−) can lead to loss of p53 and thereby to cancer development (118). In fact, mice lacking p53 and p63 show increased Ras-mediated sarcoma development (111) and are prone to malignant transformations of embryonic fibroblasts (122). Furthermore, TAp63 has been shown to play an important role in tumor dissemination. Interactions of TGFβ, Ras, and mutant p53 induce formation of a ternary complex of mutant p53, Smads, and the p63 protein, which opposes the anti-metastatic function of p53 (123, 124). TAp63 leads to overexpression of metastasis suppressor genes or microRNAs like DICER1, mir-130b, and integrin recycling genes (116). Mutant p53 can reduce Dicer expression via inhibition of TAp63, thus enabling tumor metastasis (125). The p63 gene controls transcription of the miR-200 family, which regulate CSCs and epithelial–mesenchymal transition (126). ΔNp63α induces miR-205 transcription and regulates epithelial–mesenchymal transition in human bladder cancer cells (127). Therefore, controlling p63 could be a promising approach to control or prevent metastasis in cancer.

p73 AND ITS ISOFORMS

The p73 gene consists of 15 exons and is located on chromosome 1p36. Like p63, p73 has several TA isoforms containing a specific TAD and ΔN isoforms lacking it (**Figure 4**). The first promoter, located on exon 1, can induce transcription of several truncated ΔNp73 isoforms. They are either lacking exon 2 or exon 2 and exon 3 (ΔEx2p73 and ΔEx2/3p73). In variant ΔN'p73, exon 3 is substituted by exon 3′. The TAD of p73 is 30% identical to p53. The consecutive p73 DBD shares 63% and the OD 38% identity with p53 (30). The OD is followed by the SAM domain, which is crucial for activating the molecule via tetramerization. At least seven different 3′ terminal splicing variants are known (α, β, γ, δ, ε, ζ, η) (128). Different cell types just express a selection of p73 isoforms (129). Splice variants α and β are rarely expressed in malignant cells (130). Expression of γ, δ, ∈, and θ isoforms has been described in acute myeloid leukemia (AML) and in chronic myeloid leukemia (CML) (131).

There are several molecular mechanisms that regulate p73 function on transcriptional, post-translational, and protein level (32). Enhancers of p73 transcription are p300 (132), E2F-1 (133), CREB-binding protein (CBP) (134), YAP (135), and MM1 (my modulator 1) (136), while MDM2 (137) and c-myc (136) inhibit p73 transcriptional activity. On the post-translational level, p73 activity is reduced by sumoylation by PIAS-1 (138), deacetylation by SIRT (139), threonine phosphorylation by CDK2/CDK-1 (140), neddylation by NEDD8 (141), and conjugation and ubiquitination by Itch (142). In contrast, acetylation by p300 and pCAF (143) or phosphorylation by c-Abl (144), p38MAPk or PKCδ (145) stimulate p73 activity. The RING finger E3 ubiquitin ligase PIR2 selectively ubiquitinates ΔNp73 variants (146). ASPP proteins are also able to regulate p73 function via their poly-C-binding domain (147).

Functions of p73 are diverse. Similarly to its family members p73 plays an important role at different regulatory checkpoints of the cell-cycle. TAp73 induces G1 cell-cycle arrest via enhanced expression of p21 and p57/Kip2 (148). Furthermore, TAp73 represses genes relevant in G2/M-phase like CDC25B and CDC25C (149), Cyclin B1 (150), and Cyclin B2 (149). p73 binds to FLASH and leads to cell-cycle arrest in S-phase (151). As known from p53, DNA damage stimulates p73 to induce apoptosis involving endoplasmic reticulum (ER) stress (152).

Neuronal differentiation is regarded as innate p73 function that is not shared with p53. Phenotype studies of genetically modified

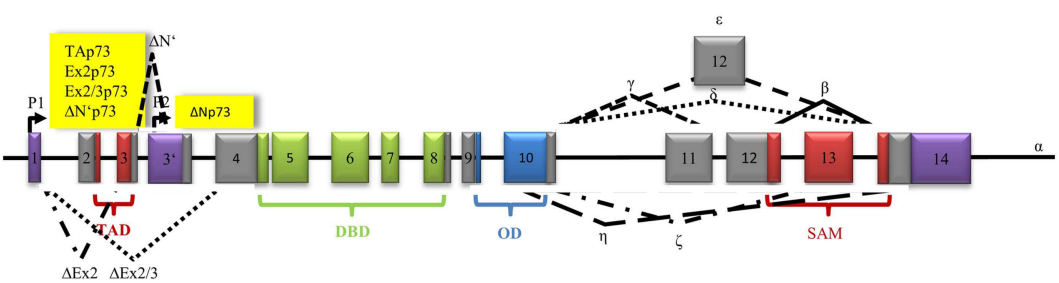

FIGURE 4 | Architecture of the human p73 gene structure: alternative splicing (α, β, γ, δ, ε, ζ, η), alternative promoters (P1, P2), transactivation domain (TAD), DNA-binding domain (DBD), oligomerization domain (OD), and sterile alpha motif domain (SAM) are indicated. The P1 promoter generates full-length-proteins with a transactivation domain (TAD), whereas the P2 promoter generates proteins lacking the TAD. Alternative splicing of exon 2 produces Ex2p73 proteins that contain part of the TAD, alternative splicing of exon 2 and 3 produces Ex2/3p73 proteins that have completely lost the TAD. Alternative splicing of exon 3′ generates ΔN′p73.

mice support this thesis. Most p73 knock-out mice die within the first 4 weeks after birth. They show hippocampal dysgenesis, hydrocephalus *ex vacuo*, atypical social and reproductive behavior, and often suffer from chronic infections (34). Heterozygous mice develop an Alzheimer's disease-like phenotype with impaired motor and cognitive functions (153, 154). Autopsy revealed accumulation of phosphor-tau positive filaments in the brain and in atrophic neurons (153). TAp73 knock-out mice develop a less severe phenotype characterized by malformations of the hippocampal dentate gyrus (155), whereas ΔNp73 knock-out mice present with reduced neuronal density in the motor cortex, loss of vomeronasal neurons, and Cajal–Retzius cells, as well as choroid plexus atrophy (156, 157). Latest research revealed that TAp73 is a transcriptional activator of the p75 neurotrophin receptor ($p75^{NTR}$), which plays an important role during neurogenesis. TAp73 knock-out mice show reduced levels of $p75^{NTR}$ and suffer from peripheral nerve defect, including myelin thickness and thermal sensitivity (158).

Similarly to p63, p73 executes a set of important functions in tumor metabolism. TAp73 induces the expression of glucose-6-phosphate dehydrogenase (G6PD), which is essential for the oxidative pentose phosphate pathway (159). Cox4il is another p73 target gene relevant in metabolism. Deletion of TAp73 leads to impairment of oxidative phosphorylation via Cox4il. As a result, levels of reactive oxygen species in cells accumulate (160).

p73 is rarely mutated in human cancer (<1%), but overexpression of p73 can be found in several malignancies, for example, in hepatocellular carcinoma (29, 161, 162), neuroblastoma (163), lung cancer (164), prostate cancer (165, 166), urothelial cancer (167), colorectal carcinoma (168), and breast cancer (>40%) (169). Seventy percent of TAp73 knock-out mice or mice heterozygous for p73 suffer from malignant tumors. Colorectal and breast cancer predominantly show an increase in ΔNp73 (170). Overexpression of both, TA and ΔN isoforms, has been detected in thyroid cancer and in chronic B-cell leukemia (171), whereas diminished p73 expression has been reported for pancreatic malignancies (172). p73 heterozygous mice (p73+/−) have an increased probability for the development of spontaneous tumors such as lung adenocarcinoma, lymphomas of the thyme, and hemangiosarcoma (118). Mice heterozygous for mutations in both p53 and p73 (p53+/−; p73+/−) develop a severe disease pattern due to a severe tumor burden and more aggressive tumor dissemination (118).

p53 FAMILY AS A TARGET OF SMALL MOLECULES

Large-scale genome sequencing has shown that over half of human malignancies exhibit point mutations in the p53 gene impairing p53 function. Most p53 mutations are missense point mutations located within the DBD. Many of them lead to destabilization of folding of the domain at physiological temperatures and interfere with its DNA-binding ability (173). Certain mutations lead to a gain-of-function of p53 and result in oncogenicity (52, 174, 175). In many other tumors p53, though intact, is inactive following enhanced degradation or reduced activation (176). Loss of wild-type p53 function or gain-of-function is often associated with aggressive tumor growth, poor prognosis, and resistance to chemotherapy. Restoration of p53 function in mice suffering from lymphomas or sarcomas has been shown to induce tumor regression (177, 178). Therefore, restoring wild-type function of p53 holds great promise as a future strategy for cancer treatment.

SMALL MOLECULES TARGETING WILD-TYPE p53

To date, a number of small molecules have been identified, which are able to restore wild-type p53 function to cancer cells (**Figure 5**). The first small molecule inhibitors, which target p53/MDM2-interaction, are Nutlins. Nutlins are a family of three (Nutlin-1, Nutlin-2, Nutlin-3) cis-imidazoline analogs. They occupy the deep hydrophobic pocket of MDM2 that mediates p53 interaction (179). Hence, Nutlins prevent p53 degradation and lead to p53 accumulation and stabilization. There is evidence that Nutlins do not only enhance p53 function but also upregulate p73 in different *in vitro* and *in vivo* settings (180). Nutlin-3a has even proven effective at inducing apoptosis in p53-deficient colorectal carcinoma cells and hepatocellular carcinoma cell lines via activation of p73 (181, 182). A number of preclinical studies, mostly using Nutlin-3 as a therapeutic agent, have been carried out focusing especially on hematological malignancies like AML (183, 184), ALL (185), and B-CLL (186, 187). However, Nutlins are also able to induce apoptosis in other cell lines including ovarian cancer (188), sarcoma (189, 190), as well as glioblastoma (191). Yet, effectiveness of Nutlin therapy ultimately presumes the presence of wild-type p53 and latest findings suggest that it strongly depends on the epigenetic profile of p53 target genes (190, 192). Moreover, Michaelis et al. and Aziz et al. reported on several different cancer cell lines that developed *de novo* p53 mutations and became resistant toward Nutlin-3 mediated apoptosis (193, 194).

Another small molecule that inhibits p53/MDM2 interaction is RITA (reactivation of p53 and induction of tumor cell apoptosis). RITA binds p53 and thereby induces conformational changes within the molecule that prevent MDM2 association (195, 196). In a human head and neck cancer cell line (HNC), RITA was able to restore p53 function contributing to cytotoxicity of cisplatin therapy and leading to apoptosis *in vitro* and *in vivo* (197). The anti-tumoral effect of RITA was also observed in neuroblastoma cell lines (198).

Rational design led to construction of the spiro-oxindole MI-219, which is a highly specific small molecule inhibitor of p53/HDM2-interaction (199). Later, it was discovered that MI-219 does not only induce dissociation of the two molecules but also leads to auto-ubiquitination and degradation of HDM2 (200). MI-219 has been shown to activate p53-dependent pathways, which initiated cell-cycle arrest and apoptosis in a number of cancer cell lines, whereas primary cells remained unaffected by these p53-mediated effects (199). In a preclinical trial, the pharmacological properties of MI-219 were tested and dosages were predicted for use in phase I clinical studies (201).

As an alternative to interfering with p53/MDM2-interaction, degradation of p53 can be prevented by inhibiting the E3 ligase activity of MDM2, and therefore, preventing ubiquitination of p53 (202). A series of 5-deazaflavin derivatives, named HDM2 ligase inhibitor 98 class (HLI98), which bind the C-terminal RING-domain of MDM2, were identified (203–205). Later, it was shown that the nitro group of the molecules is not needed to convey inhibitory function, which led to the synthesis of novel

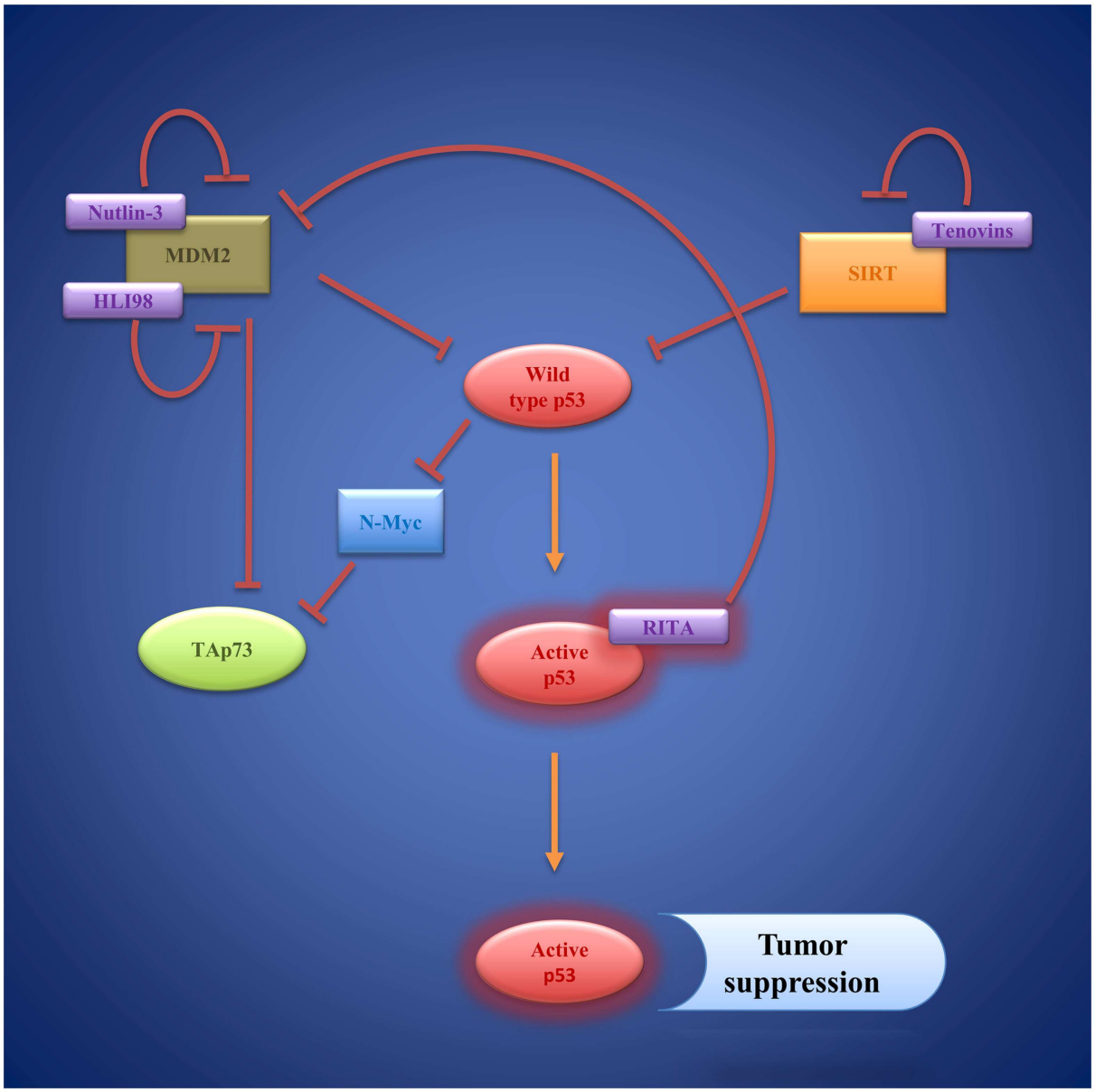

FIGURE 5 | Wild-type p53 as a target of small molecules: Nutlins, HLI98, and RITA compensate MDM2 inhibition of p53 via inhibition of MDM2. Tenovins have been identified as SIRT 1 and SIRT 2 inhibitors that indirectly activate p53. Activated p53 induces transcription of genes regulating cell-cycle arrest and apoptosis, resulting in tumor suppression.

5-deazaflavin derivatives named MDP compounds (206). While HLI98 and MDP compounds demonstrate an interesting proof of concept, there are still obstacles to overcome in terms of chemical properties such as solubility as well as selectivity for MDM2 (206). Another important question, which needs further attention, is whether inhibition of MDM2 function leads to induction of MDM2 formation via the p53 feedback loop.

The tryptamine JNJ-26854165 (Serdemetan) effectively prevents p53/HDM2 from binding to the proteasome, thereby inhibiting degradation of p53 (207). In acute myeloid and lymphoid leukemia cells, JNJ-26854165 induces apoptosis via p53 by transcription-dependent and -independent pathways (207). A phase I clinical trial assessing safety and dosage of Serdemetan in advance stage and refractory solid tumors showed good bioavailability of the substance and p53 levels in skin biopsies increased. Forty percent of patients showed stable disease, yet in some patients QTc prolongation was observed as an adverse effect (208). However, increased MDM2 levels could render substances like Nutlins, RITA, MDP compounds, and JNJ-26854165 less efficient (209).

SIRT1, a nicotinamide adenine dinucleotide-dependent class III histone deacetylase, deacetylates p53 at Lys382, thereby

reducing its activity (210). Hence, blocking SIRT function is a new strategy of restoring p53 function independent of MDM2 (211). Two small molecules, tenovin 1 and the more water-soluble tenovin 6, which block SIRT1 and SIRT2 function efficiently, were discovered by Lain et al. (212). Tenovin 1 was shown to induce apoptosis in cutaneous T-cell lymphoma cells (213). Interestingly, following tenovin 6 treatments cell death was observed in five different colon cancer cell lines independent of their p53 status (214). Also, tenovin 6 activated autophagy-lysosomal pathway genes in chronic lymphocytic leukemia cells without affecting p53 pathways (215). Both findings point toward additional cellular mechanisms mediating the anti-tumor effect of tenovins.

SMALL MOLECULES TARGETING MUTANT p53

In tumors that harbor p53 mutations, which often lead to loss of its DNA-binding function, targets for small molecules other than MDM2 are needed. An increasing number of p53 mutations have been described so far. Nevertheless, most mutations cause unfolding of the DBD rendering it unable to bind to target genes for transactivation (216, 217). Therefore, a number of small molecules aiming at restoring and stabilizing the original DBD conformation have been developed (**Figure 6**). Bykov et al. identified two small molecules by screening a library of low-molecular-weight compounds for substances, which are able to restore wild-type function of mutant p53: PRIMA-1 and MIRA-1 (218, 219). PRIMA-1 (p53 reactivation and induction of massive apoptosis) is a pro-drug (220). The molecule effectively induces apoptosis in bladder cancer cell lines (221). Later, PRIMA-1^{MET} (APR-246), a compound that bears great structural similarities to PRIMA-1, but has higher activity than its predecessor, was discovered (222). Interestingly, PRIMA-1^{MET} can not only restore the pro-apoptotic function of p53 but also of mutant TAp63γ and of TAp73β, while exerting little effect on TAp73α (223). Furthermore, PRIMA-1^{MET} is involved in activating downstream target genes of the p53 family (223–225).

PRIMA-1^{MET} alone and PRIMA-1^{MET} in combination with chemotherapeutic drugs are effective at inducing tumor cell apoptosis *in vivo* (221, 222, 225). Also, a phase one clinical trial using PRIMA-1^{MET} (APR-246) in advanced prostate cancer and hematological malignancies, as well as a phase Ib/II clinical trial using this compound in addition to carboplatin in recurrent high-grade serous ovarian cancer are under way and will offer more insight into the effectiveness and practicability of mutant p53 reactivation (National Cancer Institute: Safety Study of APR-246 in patients with refractory hematologic cancer or prostate cancer; p53 suppressor activation in recurrent high-grade serous ovarian cancer, a Phase Ib/II study of systemic carboplatin combination chemotherapy with or without APR-246).

MIRA-1 (mutant p53 reactivation and induction of rapid apoptosis) is a maleimide-derived molecule and has no structural similarity with PRIMA-1, but it is equally able to restore p53 function leading to cell death via apoptosis with even higher potency than PRIMA-1 (219). By reestablishing its DNA-binding capacity and transcriptional transactivation through p53, MIRA-1 leads to programed cell death in multiple myeloma *in vitro* and in a mouse model (226). To date, little is known about the molecular mechanisms and safety of MIRA-1 treatment and further research is needed before clinical evaluation.

Although PRIMA-1 and MIRA-1 seem to have stabilizing effect on a great variety of p53 mutants, they are not able to restore normal protein configuration to the Phe176 mutant (218). This shows the necessity to test p53 status and to identify the underlying p53 mutations before small molecule treatment (220). In fact, approaches have been made to target distinct mutations. Rational drug design led to the identification of the compound PhiKan083, which stabilizes the Cys 220 p53 mutant and prolongs its half-life, but does not rescue any other p53 mutant (227). PhiKan083 fits into a groove in the defective molecule and induces refolding of the protein (227). In consequence, the melting point of the mutant increases and denaturation is slowed down (227).

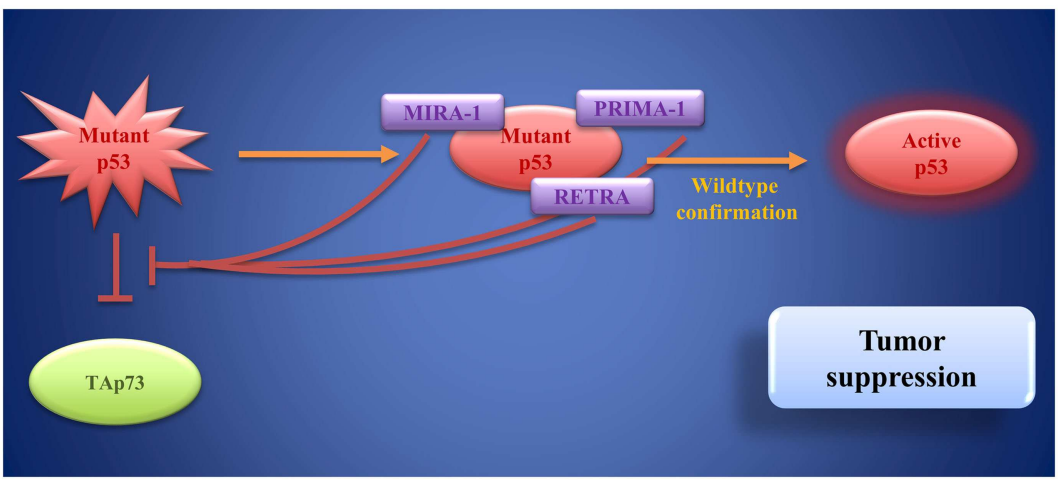

FIGURE 6 | Mutant p53 as a target of small molecules: PRIMA-1, MIRA-1, and RETRA bind to mutant p53 and restore wild-type p53 function. Moreover, they block mutant p53-induced inhibition of TAp73. These activities result in tumor suppression.

CP-31398 was discovered by screening a library of more than 100,000 synthetic compounds for substances that effectively stabilize p53 conformation (228). Initially, CP-31398 was thought to prevent unfolding of wild-type and mutant p53 and increase levels of wild-type p53 by blocking ubiquitination and degradation (229). Yet, further research revealed that it yields a number of p53-independent functions, which mediate its cytotoxic effects (230). In a mouse model of urothelial cancer of the bladder CP-31398 effectively reduced tumor growth and invasion (231).

However, increased p53 activity bares risks for non-cancerous cells that might also be subject to apoptosis and further research is needed to find the adequate dose-response relationship, specific to the compound used (209). In an attempt to identify molecules, which restore p53's transcriptional activity exclusively in cancer cells holding p53 mutations, reactivation of transcriptional reporter activity (RETRA) was identified by screening compounds from a chemical library (232). Further analysis revealed that RETRA, rather than restoring a functional p53 molecule, leads to an increase in TAp73 levels and to its release from a blocking complex with mutant p53 (232). As mentioned above, p73 can activate various target genes of p53 involved in cell-cycle arrest and apoptosis, thereby mediating tumor cell death (232). *In vivo*, in a xenograft mouse model, tumor growth could be decelerated by intraperitoneal injection of RETRA (232). Although still in the very early stages of development, RETRA opens up new perspectives for p63- and 73-based cancer treatment options.

Moreover, restoring p53 apoptotic function and modulation of p63 and p73 expression is often essential for sensitivity toward chemotherapeutic drugs or radiation, as lack of p53 and unfavorable expression patterns of p63 and p73 can lead to resistance toward treatment in different malignant tumors (233–235). Reconstitution of p53 function or activation of certain p63 and p73 isoforms might allow reducing the dose of cytotoxic drugs while still maintaining their anti-tumor effects. Simultaneously, this would permit to protect normal tissues from side effects of chemotherapy.

However, restoration of wild-type p53 might not be beneficial in all types of tumors. Jackson et al. showed that doxorubicin lead to cell-cycle arrest and senescence instead of cell death in breast cancer expressing wild-type p53, thereby promoting tumor cell survival and resistance to chemotherapy (236). This shows the necessity to elucidate which p53-dependent pathways are favored in certain malignancies before considering small molecule treatment. Novel treatment approaches could lead to the development of substances that selectively activate p53-mediated apoptosis signaling pathways.

CONCLUSION

The p53 family plays a central role in cancer development and treatment response. Whereas p53 is often mutated in tumors, p63 and p73 function is preserved, yet altered by different expression patterns of their TA and ΔN isoforms. Increasingly, these expression patterns are evaluated to estimate prognosis and adapt anti-cancer therapy. Nevertheless, the molecular mechanisms regulating the interplay between the different isoforms of the p53 family are only partly understood and are focus of current research. Identifying compounds that interfere with oncogenic signaling induced by certain p63 and p73 isoforms could be a novel approach in anti-cancer therapy.

An increasing number of compounds that re-establish pro-apoptotic p53 function in cancer cells have emerged over the past decade. A variety of small molecules, which aim at increasing p53 function in cancers expressing wild-type p53, have been discovered. Among them are Nutlins, which are already undergoing clinical evaluation, RITA, tenovins, and many others.

In tumors with underlying p53 mutation restoring wild-type activity of p53 has proven more difficult, but nevertheless feasible. PRIMA-1 and MIRA-1 are effective at inducing apoptosis via p53 in tumors that exhibit a great variety of p53 mutations. Yet, there are other small molecules, like PhiKan083, which are more specific and restore wild-type configuration of specific mutants only.

A number of *in vivo* studies and clinical trials have shown synergistic effects of small molecule treatment and chemotherapeutic drugs in a variety of malignancies. Especially cancer cells, which are resistant to chemotherapy due to impaired p53 function, become more susceptible to treatment.

Taking the approaches of p53 reactivation further, there might be new possibilities of targeting CSCs, which are often insusceptible to chemotherapy. Induction of p53 in these cells could lead to activation of pro-apoptotic pathways via differentiation.

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Microtubules and their role in cellular stress in cancer

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Microtubules are highly dynamic structures, which consist of α- and β-tubulin heterodimers, and are involved in cell movement, intracellular trafficking, and mitosis. In the context of cancer, the tubulin family of proteins is recognized as the target of the tubulin-binding chemotherapeutics, which suppress the dynamics of the mitotic spindle to cause mitotic arrest and cell death. Importantly, changes in microtubule stability and the expression of different tubulin isotypes as well as altered post-translational modifications have been reported for a range of cancers. These changes have been correlated with poor prognosis and chemotherapy resistance in solid and hematological cancers. However, the mechanisms underlying these observations have remained poorly understood. Emerging evidence suggests that tubulins and microtubule-associated proteins may play a role in a range of cellular stress responses, thus conferring survival advantage to cancer cells. This review will focus on the importance of the microtubule–protein network in regulating critical cellular processes in response to stress. Understanding the role of microtubules in this context may offer novel therapeutic approaches for the treatment of cancer.

Keywords: microtubules, tubulin, post-translational modifications, microtubule-associated proteins, stress response

INTRODUCTION

Microtubules, together with microfilaments and intermediate filaments, form the cell cytoskeleton. The microtubule network is recognized for its role in regulating cell growth and movement as well as key signaling events, which modulate fundamental cellular processes. Emerging evidence also suggests that it is critically involved in cell stress responses. This review will focus on the role of microtubules in this context in cancer.

Microtubules are composed of α- and β-tubulin heterodimers that associate to form hollow cylindrical structures (1) (**Figure 1**). They are highly dynamic, and are constantly lengthening and shortening throughout all phases of the cell cycle. During interphase, microtubules are nucleated at the centrosome (minus end) and radiate toward the cell periphery (plus end). Interphase microtubules are involved in the maintenance of cell shape and in the trafficking of proteins and organelles (1). Motor proteins translocate cell components on microtubule tracks, and protein–protein interactions with other adaptor proteins co-ordinate this process. Tubulin heterodimers also exist in soluble form in cells, and protein interactions with this tubulin population regulate microtubule behavior.

The addition and removal of soluble tubulin heterodimers to dynamic microtubule ends is a highly regulated process (**Figure 1**). Tubulin dimers are nucleotide binding proteins, with β-tubulin also possessing GTPase activity. The manner in which tubulin heterodimers are orientated in microtubules gives rise to a polar molecule that differs in both structure and kinetics at each end of the microtubule. The dynamics of tubulin addition and release are much slower at the minus end of the microtubule, which terminates with α-tubulin proteins, compared with the plus end of the microtubule, which terminates with β-tubulin proteins. The addition of a tubulin heterodimer to a microtubule activates the GTPase activity of β-tubulin, locking the β-tubulins in the microtubule in a GDP-bound state. The β-tubulins exposed to the solvent at the end of the microtubule form a GTP cap that is important in preventing microtubule depolymerization. Therefore, the binding of GTP at the microtubule plus end imparts structural and kinetic polarity to microtubules and is an important regulator of microtubule stability. It is believed that the polymerized and soluble tubulin pools interact with different signaling networks, however, the dynamic exchange of tubulin subunits between these pools makes it difficult to distinguish the functional roles of soluble and polymerized tubulin experimentally. The reader is referred to several excellent reviews for more detailed information on microtubule structure and dynamics (1, 2).

During mitosis, microtubules form the spindle to enable correct chromosomal segregation (3). Tubulin-binding agents (TBAs; e.g., taxanes, vinca alkaloids, epothilones, and eribulin) are important chemotherapeutic drugs that suppress spindle dynamics, causing subsequent mitotic arrest and cell death in rapidly dividing cells (3). Recent evidence suggests that the induction of cell stress in interphase cells also contributes significantly to TBA-mediated cell death (4–6), highlighting the importance of tubulin in cell stress responses in cancer.

In humans, microtubules are composed of combinations of eight α-tubulin isotypes and seven β-tubulin isotypes, with the different tubulin isotypes possessing specific tissue and developmental distributions (7) (**Table 1**). The members of the tubulin family share a high degree of structural homology and are distinguished from one another by highly divergent sequences at their carboxy-terminal (C-terminal) tail (8).

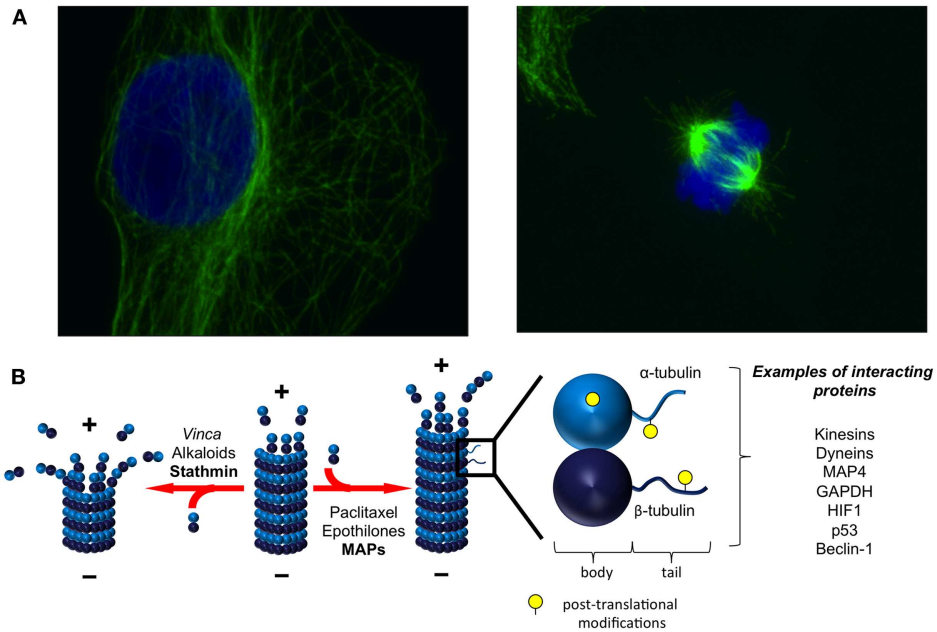

FIGURE 1 | Microtubules are dynamic structures that interact with diverse proteins. **(A)** Microtubules form a dynamic network and are constantly lengthening and shortening. In interphase [**(A)**, left], microtubules are anchored at the centrosome (minus end) and radiate toward the cell periphery (plus end). The microtubule network undergoes dramatic remodeling throughout the cell cycle, from interphase and through mitosis [**(A)**, right]. Green: α-tubulin, blue: DAPI. Images courtesy of Dr. Sela Po'uha. **(B)** Heterodimers of α- and β-tubulin associate to form microtubules. The dynamic addition and removal of tubulin heterodimers is faster at microtubule plus ends than at microtubule minus ends. Both endogenous factors and TBAs regulate and influence microtubule dynamics. A variety of proteins involved in cellular homeostatic mechanisms and stress responses also interact with tubulins either in their soluble or polymerized forms. Post-translational modifications on tubulins influence these interactions. Adapted with permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology [Ref. (9)], Copyright 2011 and Nature Reviews Cancer [Ref. (15)], Copyright 2010.

Table 1 | Tubulin isotypes present in humans [Adapted with permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Ref. (15)) Copyright 2010 and Elsevier (Ref. (233)) Copyright 2009].

Tubulin isotype	Gene name	Accession number
α-TUBULIN		
α1A-Tubulin	TUBA1A	NP_006000
α1B-Tubulin	TUBA1B	AAC31959
α1C-Tubulin	TUBA1C	Q9BQE3
α3C-Tubulin	TUBA3C	Q13748
α3E-Tubulin	TUBA3E	NP_997195
α4A-Tubulin	TUBA4A	NP_005991
α8-Tubulin	TUBA8	Q9NY65
α-Like 3-Tubulin	TUBAL3	NP_079079
β-TUBULIN		
βI-Tubulin	TUBB	NM_178014
βII-Tubulin	TUBB2A, TUBB2B	NM_001069; NM_178012
βIII-Tubulin	TUBB3	NM_006086
βIVa-Tubulin	TUBB4	NM_006087
βIVb-Tubulin	TUBB2C	NM_006088
βV-Tubulin	TUBB6	NM_032525
βVI-Tubulin	TUBB1	NM_030773

The authors direct readers to comprehensive reviews (233) for further information on tubulin isotype structure.

The C-terminal tails of tubulin are also thought to mediate protein–protein interactions and act as sites of post-translational modifications to confer unique functionality to each isotype (9).

TUBULIN ALTERATIONS IN CANCER

Diverse changes in the microtubule network have been identified and characterized in a wide variety of cancers, including altered expression of tubulin isotypes, alterations in tubulin post-translational modifications, and changes in the expression of microtubule-associated proteins (MAPs) (**Table 2**). Despite evidence from *in vitro* studies associating tubulin mutations with resistance to TBAs (10–13), tubulin mutations are not clinically prevalent and their importance in disease progression and chemotherapy resistance is controversial (14). Microtubule alterations are thought to influence cellular responses to chemotherapeutic and microenvironmental stressors, thereby contributing to broad spectrum chemotherapy resistance, tumor development, and cell survival.

CHANGES IN TUBULIN ISOTYPE COMPOSITION

Altered tubulin isotype expression is the most widely characterized microtubule alteration reported in cancer and has been observed in both solid and hematological tumors. These changes are often associated with chemotherapy resistance and poor

Table 2 | Clinical studies of tubulin alterations in cancer.

Microtubule alteration	Observation	Effect	Cancer	Reference
Altered isotype expression	High βI-tubulin	Poor response to docetaxel treatment	Breast cancer	(234)
	High βIII-tubulin expression	Poor survival, poor outcome for surgical resection or TBA response; correlates with subtype	Non-small cell lung cancer (NSCLC)	(21, 31, 108, 235–238)
		Correlates with poor survival, poor response to platinum and taxane treatment, advanced stage, or aggressive disease	Ovarian cancer	(13, 16, 35, 239–242)
		Favorable response to taxane treatment	Ovarian (clear cell adenocarcinoma)	(243)
		Poor response to taxane treatment	Breast cancer	(234, 244)
		Correlates with disease stage	Pancreatic ductal adenocarcinoma	(17)
		Correlates with disease stage	Glioblastoma	(101)
		Localized to invasive edge	Colorectal cancer	(245)
		Poor response to taxane/platinum treatment	Uterine serous carcinoma	(246)
		Poor response to taxane treatment	Gastric cancer	(247)
		Aggressive disease, patient outcome	Prostate cancer	(36, 248, 249)
	Low βII-tubulin expression	Correlates with poor response to taxane treatment or advanced stage disease	Breast and ovarian cancer	(239, 250)
	High βIVa-tubulin expression	Poor response to taxol treatment	Ovarian cancer	(240)
	High βV-tubulin expression	Favorable response to taxane treatment	NSCLC	(251)
	High α1b-tubulin expression	Histological grade	Hepatocellular carcinoma	(252)
	High γ-tubulin expression	Poorly differentiated	Medulloblastoma	(253)
Altered post-translational modification	High Δ2α-tubulin	Poor response to vinca alkaloid treatment	Advanced NSCLC	(238)
	High detyrosinated tubulin	Disease aggressiveness	Breast cancer	(48)
	Active tyrosination cycle	Favorable patient outcome	Neuroblastoma	(50)

prognosis (**Table 2**) [reviewed in Ref. (15)]. Compared with α-tubulin isotypes, β-tubulin isotypes have received more attention in this context, largely due to the availability of isotype-specific antibodies, and the fact that TBAs bind to the β-tubulin subunit to exert their toxic effect. Furthermore, βIII-tubulin is the most comprehensively examined isotype across a variety of cancers.

Elevated βIII-tubulin levels are associated with poor prognosis in a host of different epithelial cancers. In addition to TBA resistance, βIII-tubulin levels influence sensitivity to non-tubulin-targeted agents [reviewed in Ref. (15)]. The clinical observations are supported by numerous *in vitro* studies where altered βIII-tubulin levels confer resistance to a broad spectrum of drug classes in solid and hematological tumors [reviewed in Ref. (15)]. Coupled with evidence that βIII-tubulin is also involved in tumor development and disease aggressiveness (16–18), these results suggest that βIII-tubulin may be acting as a survival factor in cancer.

Altered levels of βII-, βIVa-, βIVb-, and βV-tubulins have also been associated with resistance to TBAs in a number of drug resistant cancer cell types (19–26). However, the clinical relevance of these specific tubulin isotypes is limited and requires further investigation. Moreover, the involvement of tubulin isotypes in disease progression is complex, and depends on both the treatment regime and disease stage (27). Additional complexity may be conferred by interactions between different isotypes, since the overexpression of specific β-tubulin isotypes, such as βI, βII, and βIVb, does not affect TBA resistance in Chinese Hamster Ovary cells (28, 29). For βIII-tubulin the results have been conflicting. Overexpression of βIII-tubulin failed to confer resistance to TBAs in prostate cancer (28, 29). In contrast, overexpressing this isotype in Chinese Hamster Ovary cells conferred resistance to paclitaxel (30).

In cancer, alterations in the tubulin isotype composition have been detected at both the gene and protein level and result from increased gene transcription and enhanced mRNA stability (24). However, tubulin mRNA levels do not always reflect protein expression due to the complexity of post-translational mechanisms that control tubulin expression (24, 31). For instance, the tumor suppressor miR-100 and the miR-200 family of microRNAs (24, 32, 33) as well as epigenetic mechanisms (34, 35) are implicated in coordinating β-tubulin isotype expression. Therefore, dysregulation of miRNA networks and epigenetic mechanisms in cancer may also contribute to aberrant tubulin isotype expression in cancer. Recent evidence showing an association between elevated βIII-tubulin expression and PTEN deletions in prostate cancer also suggest that changes in the levels of this isotype may result from PTEN-mediated genetic reprograming (36).

Cell stress influences the tubulin isotype composition. For example, βIII-tubulin expression can be induced (24, 37) or decreased (16) by chemotherapy treatment. The induction of βIII-tubulin has been observed in response to vinca alkaloid treatment in breast cancer cells through an activator protein-1 (AP-1) site on the βIII-tubulin promoter (38), while its induction in hypoxic and hypoglycemic conditions in ovarian cancer cells is mediated by hypoxia-inducible factor (HIF) 1α and Hu antigen (HuR), respectively, at the 3′ untranslated region (UTR) (39, 40). The latter mechanism is a regulatory feature commonly utilized by proteins involved in cell stress, and enables rapid changes in protein levels (41). However, it is to be noted that the regulation of βIII-tubulin levels in cell stress responses may depend upon the basal expression of the protein and may also be cell type specific.

Initially, differences in the drug binding affinity and structural characteristics of microtubules composed of different β-tubulin isotypes were thought to explain correlations between aberrant tubulin isotype compositions and resistance to TBAs. However, recent observations correlating changes in isotype expression with tumor development and resistance to non-TBA agents have challenged the simplicity of this model. With increased recognition of the importance of cell stress responses in chemotherapy efficacy, isotype-mediated modulation of these responses may contribute to chemotherapy resistance. In particular, cellular homeostasis relies on a dynamic microtubule network and may be perturbed by alterations in microtubule stability and dynamics. The microtubule isotype composition does affect microtubule stability, with consequences for TBA sensitivity (7, 23, 42). Stable microtubules play an important role in cellular trafficking and their role in multiple stress responses are discussed below. Chemotherapy agents that do not bind to tubulin can also affect microtubule stability by unknown mechanisms (43), and this may represent a mechanism common to chemotherapy agents of different classes.

The tubulin isotype composition can also influence microtubule dynamics. In non-small cell lung cancer (NSCLC) cells, suppression of βIII-tubulin using RNA interference technology decreases microtubule dynamics in the presence of TBAs, but has no effect under basal conditions (44). These observations suggest that changes in isotype composition may influence microtubule dynamics in the presence of chemotherapeutic stressors but not under basal conditions; however, a direct causal relationship between isotype expression, microtubule dynamics, and cell survival in response to these and other stressors has not been established. In general, the importance of microtubule dynamics in homeostatic cell signaling suggests that cell stress responses, and not just spindle dynamics, may be impacted by aberrant isotype expression in cancer, thus offering an additional determinant of chemosensitivity.

TUBULIN POST-TRANSLATIONAL MODIFICATIONS

Tubulins are subject to diverse post-translational modifications (PTMs) [reviewed in Ref. (9)]. The majority of tubulin PTMs are highly heterogeneous, and little is understood about the regulation and impact of these modifications. Post-translational modifications are thought to regulate protein–protein interactions with the microtubule cytoskeleton, thereby affecting signaling events within the cell. The majority of these modifications are localized to the tubulin C-terminus and potentially impart specific functions to the different tubulin isotypes.

Removal and addition of the α-tubulin C-terminal tyrosine occurs cyclically in cells. Tyrosine addition and removal are catalyzed by tubulin tyrosine ligase (TTL), and carboxypeptidases, respectively (9). Highly dynamic microtubules are more likely to be detyrosinated, due to the kinetic balance between higher TTL and carboxypeptidase activities on the soluble and polymerized tubulin pools, respectively (45). While traditionally viewed as an intrinsic hallmark of stable microtubules, the detyrosination motif alters motor protein recruitment to microtubules, thereby stabilizing microtubules and influencing trafficking functions within the cell (46).

Tyrosination modifications of α-tubulin are known to be critical for differentiation, cell cycle progression, organelle trafficking, and vesicular transport (9). Altered levels of tyrosination modifications and the enzymes responsible for them have been detected in a range of cancers and are associated with more aggressive disease (47–50). For instance, loss of TTL induces mesenchymal transition in breast cancer cells, which may contribute to increased metastatic potential and altered cell stress responses (51).

Increased acetylation of α-tubulin on Lys40 has also been observed in tumor cells (52). Elevated HDAC6 expression, one of several regulators of tubulin acetylation, is associated with better prognosis in breast cancer (53). Sirtuin-2 is also responsible for tubulin deacetylation (54) and has been linked with the regulation of autophagy in response to stress [reviewed in

Ref. (55)]. HDAC6 does influence microtubule stability (56), however, whether acetylation itself influences microtubule stability remains uncertain. Acetylated tubulin is implicated in intracellular trafficking (57), endoplasmic reticulum (ER) localization, and ER–mitochondria interactions (58), as well as the regulation of microtubule dynamics (59). The involvement of α-tubulin acetylation in a broad range of cell functions may reflect its importance in the maintenance of cellular homeostasis.

Other post-translational modifications have been detected in prostate and hepatic cancers. Removal of the final two residues of the βIVb-tubulin C-terminal tail was identified in higher stage liver cancer and in a mouse model of hepatic carcinoma (60). Polyglutamylated α-tubulins (47) and the polyglutamylation enzyme TTL-like 12 are elevated in prostate cancer and correlate with more aggressive disease (61).

Overall, despite a lack of clarity surrounding the mechanistic details of the function of tubulin PTMs, mounting evidence points to their role in fundamental cell processes. The diverse PTM alterations observed in a range of cancers are likely to perturb homeostatic processes, thereby contributing to stress response signaling. Detailed spatiotemporal mapping of tubulin PTMs and proteomic studies investigating their role in signaling networks are required to elucidate the influence of tubulin PTMs on cellular stress responses.

MICROTUBULE-ASSOCIATED PROTEINS

A wide variety of proteins are known to interact with tubulins. Interactions between tubulin and MAPs influence microtubule stability and dynamics, and are known to affect chemotherapy sensitivity and tumor growth in cancer [reviewed in Ref. (62)]. Aberrant expression of primarily neuronal MAPs (e.g., Tau, MAP2) has been detected in non-neuronal cancer tissue. For example, tau overexpression is correlated with poor outcome in breast cancer, and this protein may influence taxane sensitivity by decreasing the affinity of the drug for β-tubulin (63). Altered MAP2 expression is also associated with taxane resistance (22, 64), with differential effects in primary and metastatic melanoma (65).

Increased MAP4 expression and altered expression of multiple MAP4 isoforms have been detected in TBA-resistant leukemia and NSCLC cells *in vitro* (10, 11, 66). In addition, changes in stathmin, survivin, BRCA1, CLIP170, and VHL expression have all been associated with chemotherapy resistance and disease progression (62, 67). For instance, stathmin was recently shown to play an important role in regulating neuroblastoma cell migration and invasion (68). Moreover, silencing its expression using RNAi gene-silencing technology significantly reduced lung metastases in a clinically relevant orthotopic neuroblastoma mouse model (68). The overexpression of kinesins also influences chemotherapy sensitivity and disease progression through mitotic and non-mitotic mechanisms [reviewed in Ref. (69)]. A recent study has shown that kinesins interact differentially and specifically with tubulin isotypes and tubulin post-translational modifications (70). In this way, changes in tubulin isotype expression and post-translational modifications seen in cancer may also influence motor protein function and the numerous basic processes that depend upon these interactions.

The effect of MAPs on cell function in cancer is complex, with interactions between individual MAPs influencing survival and metastases. Progress toward understanding the functional consequences of these proteins and their signaling networks in cancer relies upon more comprehensive characterization of the interactions between tubulins and MAPs, and the influence of tubulin isotypes and PTMs on these interactions.

MICROTUBULE CYTOSKELETON IN STRESS RESPONSES

Microtubules influence homeostatic mechanisms and cell stress responses by regulating intracellular trafficking, acting as a scaffold for the co-localization and sequestration of stress response proteins, transmitting stress signals through cytoskeletal remodeling and modulating the induction of cell death pathways. Examples of their role in these processes are described below.

MICROTUBULES AND CELLULAR SIGNALING

While microtubules possess distinct functions in particular stress responses, the microtubule network also influences common signaling pathways engaged by a variety of cellular stresses. Stress response signaling requires trafficking of proteins and organelles throughout the cell and modulation of the microtubule network is expected to influence signal transduction events. For example, TBAs differentially suppress microtubule-mediated intracellular transport in neuronal cells (71).

In addition to general effects on signal transduction, microtubules regulate mitogen activated protein kinase (MAPK) signaling. The MAPK superfamily includes extracellular regulated kinases (ERK), c-Jun N-terminal protein kinase (JNK), and p38 families and is critically involved in mediating the initiation and execution of a range of cellular stress responses [reviewed in Ref. (72)]. MAPK proteins interact extensively with the microtubule network, with one-third of the total MAPKs associating with microtubules through kinesin motor proteins (73). Interactions between microtubules and these signaling proteins can regulate and co-ordinate widespread cellular stress signaling events.

The JNK signaling pathway is induced by a wide range of environmental stressors (72) and TBAs activate this pathway in the induction of apoptosis (74–76). In particular, JNK signaling is required for the execution of apoptosis in response to ER stress and autophagy (77). JNK co-ordinates cytoskeletal architecture in normal cells and JNK1 regulates microtubule dynamics (78, 79). JNK1 also phosphorylates MAP1 and MAP2 to alter their distribution and microtubule architecture (79). In this context, JNK, the heavy chain kinesin family-5B protein and βIII-tubulin form a complex, raising the possibility that alterations in β-tubulin isotype composition may affect JNK pathway activation and cell death responses.

While TBAs generally activate JNK signaling to initiate apoptosis [reviewed in Ref. (80)], microtubule stabilizing and destabilizing agents differentially influence downstream signaling events, suggesting that microtubule stability regulates JNK signaling (81). Compared with etoposide and doxorubicin, vinblastine uniquely causes c-Jun phosphorylation, AP-1 activation, ERK inactivation, and p53 downregulation (81). Microtubule destabilizing and stabilizing agents initiate apoptosis via JNK signaling through AP-1 dependent and AP-1 independent mechanisms, respectively

(82). The AP-1 dependent pathway leads to positive feedback of c-Jun levels and sustained JNK signaling (82), suggesting that microtubule–JNK interactions may constitute a feedback loop for the amplification and damping of signaling pathways to regulate stress response kinetics.

Extracellular regulated kinase also interacts with microtubules and phosphorylates MAPs to regulate their activity (83, 84). MAPK-mediated MAP phosphorylation is implicated in hypoxic stress responses (85). Differential induction of ERK signaling by TBAs may also mediate downstream effects independently of apoptosis induction (86).

It is well established that microtubules are involved in the translocation of messenger proteins between different cell compartments to enable efficient signal transduction. However, increasing evidence supports a role for microtubule dynamics, tubulin isotypes, and MAPs in specifically regulating the course, amplitude, and kinetics of MAPK signaling.

p53 AND MICROTUBULES

p53 is a key mediator of cellular stress responses and its activity heavily depends on microtubules (87). p53 is translocated to the nucleus along microtubule tracks by dynein proteins in a complex with heat shock protein 90 (Hsp90) and Hsp90 immunophilins (87–89). The binding of Hsp90 to p53 inhibits MDM2-mediated degradation of the protein by the ubiquitin–proteasome system (90).

Microtubule dynamics regulate p53 levels. p53 levels and its nuclear accumulation are increased by TBA treatment at doses that suppress microtubule dynamics but do not disrupt the structure of the microtubule network (87, 91). MAP1B also associates with p53, decreasing its activity and inhibiting doxorubicin-induced apoptosis in neuroblastoma cells (92). p53 signaling can influence microtubule dynamics and remodeling, as well as the expression of tubulin isotypes and MAPs (93). Taken together, by regulating p53 levels and translocation, microtubules significantly impact p53-mediated stress response signaling.

HYPOXIA

Rapid cell proliferation and poor vascular development leads to hypoxic regions within solid tumors. Hypoxia-inducible factor 1 (HIF1) is considered to be the master regulator of cellular adaptation to hypoxia and is upregulated in a large proportion of solid cancers (94).

In the absence of oxygen, HIF1α heterodimerizes to the constitutively active β subunit to initiate transcriptional changes [reviewed in Ref. (95)]. HIF1α stabilization is regulated by enzyme-mediated hydroxylation, which enables recognition of HIF1α for ubiquitinylation and degradation by proteins such as the von Hippel–Lindau (VHL) protein (96). Low oxygen levels inactivate the hydroxylases, leading to stabilization and nuclear translocation of the α subunit where the HIF1 heterodimer binds to hypoxia responsive elements in target gene promoters (95).

Dramatic microtubule remodeling occurs under hypoxic conditions. Decreased microtubule polymerization has been observed in response to anoxic conditions (0–2% O_2) (85, 97), while increased microtubule polymerization has been observed in physiological hypoxia (3% O_2) (98). Enhanced microtubule polymerization under these conditions is coupled with increased tubulin detyrosination and glycogen synthase kinase 3β (GSK3β) inhibition (98), while phosphorylation of the MAPs dynein light chain tctex-type 1 (DYNLT1), MAP4, and stathmin have each been associated with microtubule depolymerization (85). Discrepancies between these observations may be due to the differential effects of anoxia compared with physiological hypoxia, or alternatively may reflect the role of the GSK3β pathway and MAP interactions on microtubule remodeling (98). Hypoxic activation of the p38/MAPK pathway contributes to phosphorylation of MAP4 and stathmin (85). Microtubule remodeling in response to hypoxia may impact metastatic processes with increased microtubule polymerization influencing integrin trafficking and invasion in breast cancer cells (98).

MAP4 protects against microtubule disruption during hypoxia by enhancing tubulin polymerization and concomitant upregulation of tubulin expression (97). It also maintains ATP production under hypoxic conditions and prevents mitochondrial permeabilization (97). The non-phosphorylated form of DYNLT1 also protects against microtubule disruption and mitochondrial permeabilization and maintains the cellular energy status in hypoxia, with phosphorylation of DYNLT1 potentiating cell death through mitochondrial permeabilization (99). DYNLT1-mediated interactions between tubulin and Voltage Dependent Anion Channels (VDACs) may facilitate cross-talk between the microtubule cytoskeleton, intrinsic apoptotic pathway, and mitochondrial quality control system to influence cell survival in hypoxia (97).

Hypoxic adaptation may also be regulated by specific tubulin isotypes in cancer cells. For instance, βIII-tubulin (encoded by the TUBB3 gene) is induced under hypoxic conditions by direct binding of HIF1α to the E box motif within its 3′UTR (39). Hypoxic upregulation of this isotype appears to be cell type specific, depends on the epigenetic status of the TUBB3 3′UTR and is also influenced by the basal βIII-tubulin expression level (26, 39). The expression of this tubulin isotype is also regulated by HuR (40), which is involved in HIF1α stabilization (100). High βIII-tubulin expression is also detected in close proximity to necrotic tumor regions, further supporting a role for this protein in hypoxic adaptation (101).

Hypoxia-inducible factor 1α degradation is dependent on the short isoform of VHL, while the long isoform is a known regulator of microtubule dynamics (102). In renal cell carcinoma, where VHL mutations result in upregulated HIF expression, there is a loss of microtubule–HIF coupling, suggesting that VHL may be responsible for microtubule-mediated regulation of HIF signaling (103). However, the mechanisms underlying this observation and the functional consequences of this regulatory process are uncertain.

Hypoxia-inducible factor 1 activity depends upon its ability to translocate to the nucleus, and microtubules act as tracks for dynein-mediated HIF1 translocation (103). Suppression of microtubule dynamics decreases HIF1α levels by increasing HIF1α mRNA association with inactive ribosomal subunits and by targeting this mRNA to P-body components (104). Suppression of microtubule dynamics and HIF nuclear translocation prevents VEGF-mediated hypoxic adaptation in prostate and breast

cancer cells and decreases angiogenesis in a murine orthotopic breast tumor model (94). However in this study, microtubule dynamics regulated HIF1α levels to the same extent in both normoxic and hypoxic conditions; therefore, this mechanism may not be responsible for regulating HIF1α levels specifically in response to hypoxia. Recent evidence suggests that hypoxic adaptation also depends upon microtubule-mediated perinuclear mitochondrial clustering (105), and highlights the importance of organelle localization in cellular adaptation to hypoxia.

Overall, the hypoxic response is associated with dramatic microtubule remodeling, and altered MAP signaling to maintain bioenergetics and organelle function under hypoxic conditions. The microtubule network also regulates hypoxic adaptation by affecting HIF1α signaling and organelle localization, placing microtubules as a central player in the hypoxic stress response. While current evidence suggests that β-tubulins may function in an isotype-specific manner in this context, a more comprehensive analysis of the contributions of each individual isotype to hypoxic adaptation is required.

OXIDATIVE STRESS

Aberrant oxidative stress signaling has been reported in many cancers. The upregulation of enzymes responsible for redox homeostasis, metabolic reprograming, and exposure to extracellular inducers of intracellular oxidative species all contribute to aberrant oxidative conditions in cancer [reviewed in Ref. (106)]. Markers of oxidative stress correlate with chemotherapy response and upregulation of redox enzymes, such as glutathione peroxidases, have been observed in the acquisition of chemotherapy resistance and genomic instability [reviewed in Ref. (106)].

Tubulins interact with mediators of the oxidative stress response, with direct interactions between βIII-tubulin and glutathione *S*-transferase μ4 observed in ovarian cancer cells (107). βIII-tubulin and the DNA damage repair enzyme excision repair cross-complementation group-1 (ERCC1) act together to influence patient response to taxane and paclitaxel combination treatment (108); however, the mechanisms underlying this co-operative effect are unknown.

Specific tubulin isotypes may also alter oxidative stress responses by acting as redox switches (109). In particular, ser/ala124, which is a cysteine in βIII-, βV- and βVI-tubulins, and cys239, which is a serine in βIII-, βV-, and βVI-tubulins, have been specifically identified as potential sensors of oxidative stress (109). Cys239 is readily oxidized and its oxidation inhibits microtubule assembly and stability (109). Therefore, alterations in tubulin isotype composition may influence microtubule stability in an oxidative environment to maintain microtubule integrity and cell survival in these adverse conditions. Moreover, oxidative stress influences tubulin post-translational modifications. Nitrotyrosine is a common byproduct of nitrosyl radical production in oxidative stress and can be incorporated into microtubules through the tyrosination/detyrosination cycle (110). While nitrotyrosine incorporation does not affect microtubule assembly, architecture, or cell viability (111), it does increase the stability of neuronal microtubules (112). Furthermore, elevated levels of nitrosylated α-tubulins correlate with disease stage in gliomas (113).

Oxidative stress is also induced by TBAs, suggesting an involvement of microtubules in oxidative stress responses, and is an important mechanism of action for platinum-based chemotherapeutic agents (114). Paclitaxel treatment induces reactive oxygen species through activation of the JNK pathway in melanoma cells (115). TBA treatment also influences NADPH oxidase activity, increases ROS levels and induces bystander effects in breast cancer cells (116). This effect may be mediated by changes in microtubule dynamics and stability, with these factors regulating Rac1 translocation and subsequently, NADPH oxidase activity (117, 118).

Studies in neurons and endothelial cells indicate that the microtubule cytoskeleton undergoes remodeling in response to oxidative stress (119). Oxidative stress induces microtubule depolymerization, and increases the pool of soluble tubulin (120, 121). 4-Hydroxy-2-nonenal (4-HNE), a secondary product of lipid peroxidation and marker of oxidative stress, also causes microtubule depolymerization, together with tubulin crosslinking (122, 123). This depolymerization may be caused by preferential reaction of 4-HNE with soluble tubulin, thereby disrupting the soluble/polymer fractionation of tubulin subunits and subsequent microtubule assembly (124). Interactions between microtubules and MAPs protect microtubules from depolymerization in response to oxidative stress (122, 125), and alters cellular trafficking in oxidative conditions (126).

Collectively, there is growing evidence supporting a role for tubulin isotypes and the microtubule network in both sensing and responding to oxidative stress in cancer through direct structural changes and protein–protein interactions. This is supported by observations in neuronal models, however, the specific roles of tubulin isotypes and their accessory proteins in oxidative stress responses remain to be clarified.

METABOLIC STRESS

Metabolic stress occurs in cancer as a result of uncontrolled cell proliferation in the absence of adequate nutrients [reviewed in Ref. (127)]. Microtubules and tubulins are involved in responding to metabolic stress by sensing and modulating metabolic processes to maintain cellular energy levels. The microtubule network is hypothesized to play a critical role in the regulation of cellular metabolism (128).

Early studies suggested that microtubules may act as a sensor of the energy state of the cell (129) with ATP depletion causing instability of detyrosinated microtubule plus ends (130, 131). AMPK is a major sensor for the metabolic state of the cell and affects microtubule dynamics by phosphorylation of CLIP170 (132). CLIP170 alters paclitaxel sensitivity in breast cancer cells by enhancing the binding of the drug to tubulin (67). In neuronal cells, activation of AMPK in metabolic stress prevents growth of axonal microtubules (133), further supporting a role for microtubules in early metabolic stress signaling events. The main neuronal tubulin, βII-tubulin, was also identified as a downstream target of AMPK in murine brain extracts (134).

Metabolic modulation of microtubule dynamics and tubulin post-translational modifications may allow for rapid and widespread stress responses. For example, nutrient starvation induces hyperacetylation of tubulin, which may act in concert with

AMPK to induce autophagy in response to decreased ATP levels (77), thereby engaging multiple stress response pathways through microtubule-related signaling.

METABOLIC REGULATION

Tubulins and microtubules have been suspected to function as a key modulator of mitochondrial metabolism for some time (128). Recent studies have demonstrated that tubulin is capable of interacting with, and blocking the VDAC, thereby regulating ATP and metabolite compartmentalization and contributing to the Warburg effect (135–138). This interaction is mediated by the tubulin C-terminal tail (135), raising the possibility that post-translational modifications and different tubulin isotypes may differentially regulate VDAC dynamics to influence metabolic reprograming in cancer.

Tubulins, and in particular βIII-tubulin, associate with enzymes of the tricarboxylic acid cycle and glycolysis (107). *In vitro* studies in reduced systems showed that tubulin interacts with a variety of glycolytic enzymes including pyruvate kinase, phosphofructokinase, aldolase, hexokinase, GAPDH, and lactate dehydrogenase (139–144). Interactions with some of these enzymes may be isotype-specific, by interacting with the α-tubulin C-terminal tail (142) rather than the tubulin body (140).

Preferential interactions between glycolytic enzymes and either the soluble or polymerized tubulin pool may also influence metabolic activity and microtubule dynamics (139, 141, 144). GAPDH activity is differentially regulated by its interaction with either the soluble or polymerized tubulins (143), and this interaction influences microtubule dynamics (145). Interactions between metabolic enzymes and tubulins may therefore mediate bi-directional signaling events to sense and respond to metabolic stress. Indeed, mathematical modeling of metabolic pathways and tubulin's modulation of enzyme activity suggest that glycolytic flux is regulated by microtubule polymer levels (146), however, the mechanisms by which the microtubule network influences metabolic homeostasis and the importance of the soluble and polymerized tubulin fractions in these functions remain to be characterized experimentally.

The association between GAPDH and microtubules may also influence cellular trafficking, with a recent study finding that ATP generated from vesicular GAPDH activity fuels the energy consumption of motor proteins during vesicular transport (147). Furthermore, GAPDH is known to mediate membrane fusion, and its association with microtubules may co-regulate membrane trafficking during glycolytic stress (148). The presence of GAPDH on microtubules allows the recruitment of Rab2 protein to regulate membrane and ER–Golgi trafficking independently of its catalytic activity (145, 149). Given the importance of ER–Golgi trafficking in protein glycosylation, the interaction of GAPDH with microtubules may function as a point of communication between metabolic and protein modification pathways under a range of stresses. For example, in neuronal cells, GAPDH binds tubulin through the neuronal MAP1B protein but is relocalized upon oxidative stress (150).

Specific interactions between tubulin isotypes and glycolytic enzymes support the pro-survival effect of altered tubulin isotypes in cancer. Pyruvate kinase interacts with tubulin via the tubulin C-terminal tail and depolymerizes stabilized microtubules (140, 151). In particular, βIII-tubulin interacts with the mitochondrial-localized pyruvate kinase M2 (107), which is associated with the Warburg effect. Feedback from metabolic products also influences the association of pyruvate kinase with microtubules, as well as microtubule stability (151), further supporting a role for the microtubule cytoskeleton in the regulation of metabolic flux. Altered metabolic activity also influences microtubule architecture (152), raising the possibility that the microtubule system may communicate with metabolic networks in a bi-directional manner.

βIII-tubulin has been specifically implicated in glucose stress responses. Treatment of ovarian cancer cells with tunicamycin or wortmannin to block protein glycosylation and PI3K signaling, respectively, upregulates βIII-tubulin and alters the post-translational modifications of non-mitochondrial tubulins in cell lines with low basal βIII-tubulin expression (107). βIII-tubulin induction and decreased βI-tubulin expression have also been observed for ovarian cancer cells under glucose starvation (40). Upregulation of βIII-tubulin in these conditions correlates with HuR binding to the βIII-tubulin 3′UTR (40). This function of HuR is independent of its role in the nuclear export of mRNA; however, whether HuR is involved in the stabilization of βIII-tubulin mRNA under hypoglycemic conditions was not investigated. Correlations between increased HuR, βIII-tubulin expression, and poor survival in ovarian cancer samples further support a role for this mechanism in influencing cancer progression and patient outcome (40).

The current evidence strongly supports a role for the microtubules in regulating metabolic activity and metabolic reprograming in response to nutrient starvation. However, the mechanistic details underpinning these observations is lacking and the importance of specific tubulin isotypes, tubulin post-translational modifications, and associated proteins in regulating metabolic stress responses requires further characterization.

AUTOPHAGY

Macroautophagy (hereafter referred to as autophagy) can be induced in cells in response to diverse stresses, including metabolic and ER stress [reviewed in Ref. (153)]. Autophagy is a catabolic process that enables isolation and recycling of protein and organelle components by sequestering them into vacuoles for subsequent lysosomal degradation (154). It is also an important quality control process, allowing for the removal of damaged organelles and proteins, and protects cells from oxidative stress damage (155). Autophagic activity can support cells during ATP depletion, and thus is intrinsically linked with metabolic stress responses (154).

Recent evidence supports a role for autophagy in the survival and treatment sensitivity of cancer cells, and several recent reviews have been devoted to this topic (156–158). Microtubules have been known to play a critical role in autophagic flux for several decades (159), however our understanding of their importance in autophagy initiation, trafficking, and lysosomal fusion has been furthered in recent years.

Evidence for a microtubule role in autophagy regulation comes from the alteration of autophagic flux upon treatment with TBAs *in vitro* (160–163). Disruption of autophagic flux by

TBAs is important in the mechanism of action of, and resistance to, TBAs in cancer (4, 164). The influence of TBAs on autophagy may be mediated by inhibition of Akt/mammalian target of rapamycin (mTOR) signaling (165), or suppression of microtubule dynamics, and additional studies are required to characterize this mechanism.

Microtubule-associated protein-1 light chain 3 (MAP1LC3, also referred to as LC3), a critical member of the autophagy network, interacts directly with tubulin in both its free and phosphatidylethanolamine-conjugated form (77, 160). LC3 also interacts with microtubules through MAP1 proteins (166–168). The promotion of autophagy by MAP1S reduces genomic instability to suppress tumor development in hepatocarcinoma, and MAP1S may also co-ordinate mitochondrial dynamics and autophagy (155, 167). Other autophagy proteins also associate with microtubules, including ULK1, Beclin-1, WIPI1, autophagy related (Atg) protein 5, and Atg12, which are thought to be principally involved in autophagosome formation (77, 169, 170). In neuronal models derived from neuroblastoma cells, autophagy inhibition is associated with decreased β-tubulin levels and suppressed neurite outgrowth (171). However, links between altered tubulin expression and autophagy have not yet been reported in non-neuronal cancer cells.

Autophagy initiation involves activation of the master regulator mTOR and the formation of the mTOR-containing complexes. mTOR activity is regulated by lysosomal localization (172), with mTOR associating specifically with peripheral lysosomes (173). Peripherally localized mTOR is sensitive to nutrient starvation, which causes it to be released from lysosomes to form the mTORC1 complex and initiate autophagy (172). Microtubules control the peripheral localization of lysosomes, and therefore ensure the sensitivity of mTOR to nutrient starvation (172). Spatial partitioning of the microtubule-interacting kinesins KIF2A and KIF1B between peripheral or perinuclear lysosomes also influences mTOR activation and the initiation of autophagy (173).

Microtubules act as scaffolds and sequester proteins to regulate autophagy. Activating molecule in BECN1-regulated autophagy 1 (AMBRA1) acts as a linker protein between microtubules and the PI3K signaling complex responsible for autophagy induction (169). Starvation induces phosphorylation of AMBRA1 by ULK1, releasing the Beclin-1-PI3K complex from microtubules to the ER to initiate autophagosome formation (169). Beclin-1–Bcl-2 complexes are also sequestered on microtubules during periods of high nutrient availability. JNK1-mediated phosphorylation of Bcl-2 in response to nutrient starvation causes dissociation of Beclin-1 from this complex to initiate autophagosome signaling and influence apoptosis (174). Microtubules are also involved in the transport of several proteins whose localization is required for autophagosome formation (175).

Tubulin post-translational modifications also regulate autophagy initiation, as tubulin hyperacetylation occurs before autophagosome formation in response to nutrient starvation (77). Acetylation modifications signal kinesin recruitment to microtubules, with subsequent JNK activation, and release of Beclin-1 from Beclin-1–Bcl-2 complexes to initiate autophagy (77). Therefore, tubulins serve as interacting partners in the regulation of autophagy initiation.

During autophagy initiation autophagosome membranes are produced from existing intracellular membranes and microtubules are well positioned to act as carriers of these membrane components from existing organelles to sites of phagophor nucleation. Recent studies have shown that LC3 enrichment and autophagosome formation occur at contact sites between Parkin-tagged mitochondria and the ER (176). Microtubules mediate translocation of both these organelles (177, 178) and may critically regulate their co-localization to initiate autophagosome formation.

The role of microtubules in autophagosome formation is differentially regulated in basal and starvation conditions. Microtubule dynamics are required for autophagosome formation in response to nutrient starvation (77, 162) but not under basal conditions (162, 179, 180).

Once formed, autophagosomes are transported along microtubules in both anterograde and retrograde directions (77), where they are fused with lysosomes. The role of microtubules in mediating the fusion of autophagosomes with lysosomes remains controversial. Microtubule dynamics do not affect the co-localization and fusion of autophagosomes and lysosomes (162), which can occur in the absence of microtubules (160). However, Kimura et al. argue that more efficient fusion is enabled by active transport along microtubule (181). These contrary observations may be explained by the influence of pharmacological or RNA interference-based modulators on lysosomal behavior in addition to their effects on microtubule cytoskeleton. However, studies using tools that more selectively target the autophagy machinery are required to clarify the importance of microtubules in autophagosome–lysosome fusion in autophagy, and the mechanisms regulating these processes.

Overall, microtubules regulate autophagy through scaffolding functions and in the intracellular trafficking of autophagy components. While precise mechanistic details remain elusive, it is likely that tubulin alterations seen in cancer would influence autophagic function and the ability of cells to cope with microenvironmental and chemotherapeutic stressors that cause nutrient starvation and cellular damage.

PROTEIN FOLDING STRESS

Misfolded proteins may arise from protein damage, inadequate chaperone activity, and malfunction of protein processing systems. The ER is responsible for ensuring correct folding of membranous and secretory proteins and this organelle is highly sensitive to cellular conditions. Slight changes in any number of parameters can lead to accumulation of unfolded proteins in the ER lumen and initiation of the unfolded protein response (UPR) [reviewed in Ref. (182)]. The UPR involves the induction of the ER-associated degradation machinery that allows transport of unfolded proteins to cytoplasmic proteasomal systems, suppression of translation, and upregulation of chaperones in a concerted effort to reduce the burden of misfolded proteins (182). Initiation of the UPR leads to amelioration of ER stress, or the initiation of cell death (182). The UPR is upregulated in many cancers and is an important contributor to tumor development and maintenance (182–184). ER stress sensitizes cells to a broad range of chemotherapeutics including topoisomerase inhibitors (185),

temozolomide (186), platinum-based agents (187, 188), and TBAs (189).

Glucose regulated protein 78 (GRP78) is a member of the heat shock protein 70 (Hsp70) family and a master regulator of the ER stress response (190). Alterations in GRP78 expression and localization have been linked with tumor aggressiveness, migration, and invasion as well as chemoresistance, where it acts as a pro-survival factor (182). Taxanes and vinca alkaloids induce ER stress through upregulation of GRP78 in breast cancer cells (5). ER stress is also associated with JNK activation and apoptosis, which are inhibited upon GRP78 knockdown (5, 191). GRP78 interacts with βIII-tubulin (107), however, the functional consequences of this association are unknown. These observations suggest an intrinsic link between the microtubule cytoskeleton and the initiation of ER stress responses.

Tubulin-binding agent treatment also initiates mechanisms to repress translation and ameliorate misfolded protein accumulation. Treatment of cervical cancer cells with TBAs induces P-body formation, which are cytoplasmic regions where mRNA translation is inhibited (104). P-body targeting of miRNA and mRNA is also an important regulator of numerous stress responses, including the regulation of HIF1α levels in normoxic and hypoxic conditions (192). Microtubule dynamics are also critically involved in the association of mRNA with stress granules (193), which also regulate mRNA processing in response to stress (194).

Expansion of the ER network occurs during the UPR (195), where it acts to relieve ER stress (196). Microtubules are critically involved in regulating ER morphology, trafficking, and expansion of the organelle to the periphery of the cell by direct attachment of the ER to microtubules (197). Microtubule dynamics are tightly co-regulated with ER dynamics, which are suppressed by microtubule depolymerizing agents (178, 198). ER movement can occur by attachment to the microtubule plus ends (198), or kinesin-mediated ER sliding along microtubules (58, 199). While the former mechanism occurs on highly dynamic microtubules, ER sliding occurs on acetylated microtubules (58). Therefore, tubulin post-translational modifications may act as important regulators of ER expansion during the UPR. Mitochondria are also localized to acetylated microtubules, with this PTM potentially facilitating functional ER–mitochondrial interactions with diverse consequences for the cell, including autophagy induction (58, 176). Therefore, the microtubule network may co-ordinate whole cell reprograming in response to localized ER stress.

In neuronal neuroblastoma models, collapse of the microtubule network and evolution of ubiquitinated protein aggregates at the centrosome were observed in parallel with the initiation of ER stress (200). While this suggests that maintenance of a functional ER network relies heavily upon the microtubule cytoskeleton, similar observations are yet to be reported in non-neuronal cancer cells.

These observations suggest an intrinsic link between ER homeostasis, the initiation of ER stress responses and the microtubule network; however, the mechanisms co-regulating these systems remain elusive. Improved understanding of the role of microtubules in ER function, and the importance of this organelle in tumor development and cell survival may reveal strategies for more effective use of existing treatments in cancer.

TUBULIN AND MOLECULAR CHAPERONES OUTSIDE OF THE ER

Other chaperones outside of the ER system also interact with microtubules (201). The small heat shock protein (Hsp) α B-crystallin regulates microtubule dynamics (202) and tubulin polymerization (203) by associating with microtubules through interactions with MAPs (204). The association between α B-crystallin and tubulin may also prevent the aggregation of misfolded tubulin (202).

Heat shock protein 27 (Hsp27) associates with microtubules (205) and alters the microtubule structure by promoting microtubule nucleation distant to the centrosome (206). TBAs induce Hsp27 phosphorylation through the p38 signaling pathway in MCF-7 cells, with microtubule stabilizers and destabilizers inducing different phosphorylation patterns on this protein (207). However, the functional consequences of these phosphorylation sites are unclear. Hsp70 also associates with tubulin by interacting with the tubulin C-terminal tail, and this interaction may be mediated by MAP1B (208, 209). In particular, βIII-tubulin has been found to associate with mitochondria-localized Hsp70 (107). Hsp70 expression is induced by vinblastine treatment in melanoma cells (210). Furthermore, crosstalk between Hsp70 and oxidative stress enzymes (211) suggests that interactions between the microtubule network and these proteins could have profound implications for a variety of stress responses.

The Hsp90 family is the main cytosolic chaperones in basal and stressed conditions, where they mediate maturation of folded proteins (212). Hsp90 client proteins are diverse and include oncoproteins that promote survival in response to environmental stress [reviewed in Ref. (213)]. Hsp90 proteins have been found to associate with tubulin; however, this occurs in an ATP-independent manner, suggesting that tubulin–Hsp90 associations are not related to global tubulin re-folding or the targeting of tubulins to proteasome machinery (214, 215). The binding of Hsp90 to tubulins may instead ensure correct folding of nascent tubulin peptides, and prevent the formation of tubulin aggregates during cellular stress (214). The association between these proteins may also reflect the role of Hsp90 as a molecular chaperone for proteins translocating on microtubules (216).

Heat shock protein 90 recruitment to microtubules depends on acetylated tubulins, with HeLa cells having higher levels of acetylated tubulin and Hsp90 recruitment to microtubules compared with non-tumoral RPE1 cells (52). Tubulin acetylation is also associated with recruitment of the Hsp90 client proteins Akt and p53 to microtubules, with significant implications for downstream signaling events and chemosensitivity (52). Whether tubulin hyperacetylation is a widespread feature of cancers, or is specific to these cell types, is unclear, but these observations suggest that tubulin post-translational modifications may impact upon protein folding stress in cancer. Overall, interactions between tubulins and Hsp90 may act as an important link between tubulin PTMs, protein folding, and stress response signaling.

MITOCHONDRIAL FUNCTION

As integrators of cell state and mediators of apoptotic signaling, mitochondria play a critical role in determining cell fate in response to stress. There is growing evidence that tubulin, microtubules, and the microtubule network regulate

mitochondrial function in cancer (217). Microtubules are involved in mitochondrial trafficking and degradation, with these processes influencing microtubule stability and tubulin degradation (218). Tubulin is an integral component of mitochondrial membranes (136, 137, 219), and these membranes are enriched with βIII-tubulin (107, 137, 217). Mitochondria-associated βIII-tubulin is distinguished from the cytoplasmic tubulin pool by distinct post-translational modifications (107). Interactions between tubulin and VDAC discussed above, also support a role for tubulins in mitochondrial function.

Tubulin-binding agents are known to affect mitochondrial stress (115). Microtubule stabilizing and destabilizing TBAs cause changes in the mitochondrial membrane potential, which is critical for the maintenance of respiration and regulation of apoptosis (135, 220). It is currently unclear whether these effects are independent of the tubulin-targeted activity of these agents. Nevertheless, higher levels of soluble tubulin are associated with a lower mitochondrial membrane potential in cancer cells but not in non-transformed primary cells (220). Therefore, modulation of mitochondrial function by tubulin and microtubules may influence cell stress responses and cell survival signaling in cancer.

CELL DEATH SIGNALING

Failure of cellular stress responses to alleviate cellular dysfunction can result in the induction of cell death. Emerging evidence supports a role for tubulins and microtubules in the execution of cell death in response to stress. For instance, tubulins interact with regulators of mitochondrial membrane permeability and apoptosis. Interactions between tubulin, VDAC, and p53 (discussed above) may influence the mitochondrial permeability transition and regulate apoptosis induction (221). This is supported by evidence that TBAs mediate their apoptotic effects by directly compromising the mitochondrial outer membrane integrity (222), whether through interactions with their traditional target, tubulin, or with B-cell Lymphoma/Leukemia-2 (Bcl-2) (223).

Crosstalk between microtubules and apoptotic networks is also suggested by Bcl-2 involvement in TBA-mediated cell death. In leukemic cell lines, the overexpression of Bcl-2 suppresses the apoptotic response of TBAs independently of G2/M arrest and structural microtubule alterations (224–226). High Bcl-xL levels are protective against taxol-induced cell stress (225). These effects may be explained by direct interactions between Bcl-2 and tubulin (217, 227). Bcl-2 interacting mediator of cell death (Bim) is also sequestered on microtubules by binding to the dynein light chain, thereby preventing initiation of apoptotic signaling (227, 228). Once released from microtubules, Bim translocates to mitochondria, and interacts with Bcl-2, Bcl-xL, or Bax to promote apoptosis (228). Biophysical studies have also indicated that BH3-domain proteins, of which Bim is a member, can interact with tubulin through this domain (227). The pro-survival factors semaphorin 6A and survivin also associate with microtubules (107, 229, 230) with the latter affecting microtubule dynamics (229). Semaphorin 6A interacts directly with βIII-tubulin in ovarian cancer cell lines and its expression correlates with resistance to a broad range of chemotherapy agents (230). By interacting with apoptotic proteins, tubulin alterations may have a pro-survival effect by reducing the apoptotic potential of cancer cells.

Manipulation of the soluble and polymerized tubulin fractions may also modulate apoptotic potential. Bak associates with the polymerized fraction while Bid preferentially associates with the soluble fraction (227). This interaction is mediated by the β-tubulin C-terminal tail region (227), suggesting that tubulins may modulate apoptotic potential in an isotype-specific manner. However, this interaction, its tubulin isotype specificity and functional consequences are yet to be validated in the more complex cell environment.

Tubulin-binding agents are known to induce Bcl-2 phosphorylation, a state that inhibits the anti-apoptotic activity of this protein (231), suggesting that Bcl-2 activity may be regulated by microtubule integrity. However, Bcl-2 phosphorylation is elevated in cells undergoing G2/M arrest and this observation may reflect the action of TBAs on the cell cycle checkpoint, rather than apoptotic signaling (232).

Direct and indirect interactions between tubulins, apoptotic proteins, and mitochondria suggest that the microtubule network communicates with the apoptotic machinery to regulate the execution of the final stages of cell death signaling. While the precise mechanistic details of this cross-talk remain elusive, the current evidence supports a role for isotype-specific regulation of cell death by tubulins.

CONCLUSION

Tubulins, microtubules, and their interacting partners are increasingly recognized as central players in the maintenance of cell homeostasis and execution of cell stress responses. Emerging evidence suggests that the modulation of tubulin isotype composition, post-translational modifications and the expression of MAPs seen in cancer influence diverse cellular functions to promote cell survival under metabolic, protein, oxidative, and hypoxic stress. Microtubules and tubulins influence protein signaling networks through molecule and organelle transport, act as scaffolds for protein–protein interactions, modulate enzyme activity, and sequester stress response mediators. Developing a detailed spatiotemporal knowledge of the specific function of tubulin isotypes, their post translation modifications and the proteins they associate with presents a major challenge, and is a necessary foundation for understanding the role of the microtubule network in the regulation and execution of stress responses.

By influencing a variety of cell stress responses, microtubules are well positioned to act as coordinators of cell function in response to stress. Furthermore, crosstalk between different stress response signaling events means that microtubule involvement in this context may have profound implications on diverse cellular functions (**Figure 2**).

Improved understanding of the role of tubulins and microtubules in cell stress responses in cancer has appreciable clinical benefits. The identification of signaling pathways influenced by the microtubule cytoskeleton may offer a source of novel anticancer treatments. A firmer grasp on the role of the microtubule cytoskeleton in cell stress responses, and in particular in chemotherapeutic stress, should also enable more effective use of existing treatments. By profiling tubulin and microtubule aberrations in tumors, chemotherapeutic combinations known to induce

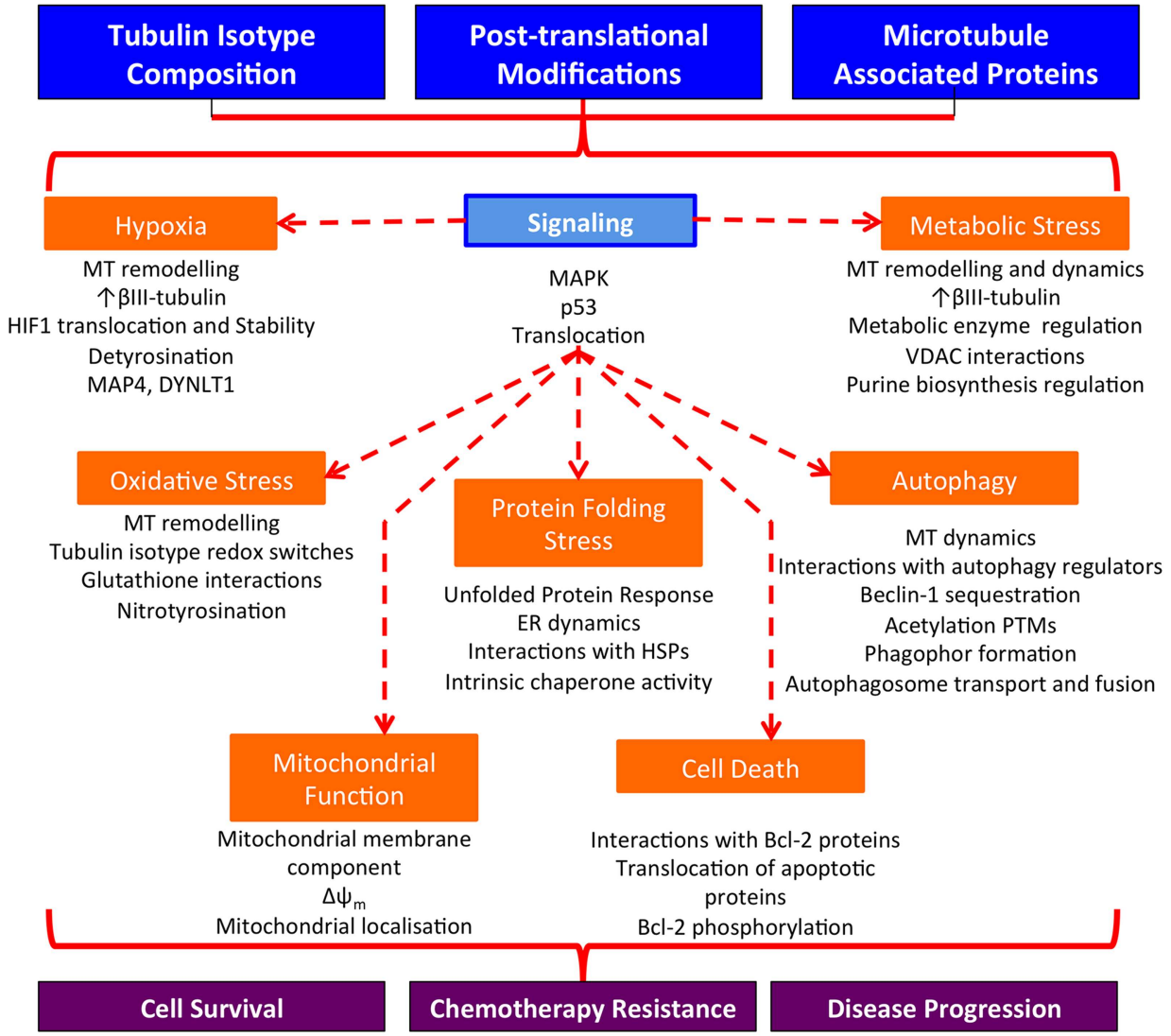

FIGURE 2 | Microtubules regulate and co-ordinate diverse cellular stress responses in cancer cells. Alterations in the expression of tubulin isotypes, tubulin post-translational modifications, and the interaction of microtubules with MAPs seen in cancer affect a wide range of homeostatic mechanisms in response to cellular stress. Microtubules may function to co-ordinate stress responses across the cell, resulting in enhanced cell survival in the harsh tumor microenvironment, resistance to chemotherapy treatment, and the development of more aggressive disease; MT, microtubules.

particular stress states could be selected to exploit altered stress response signaling in cancers. Through these avenues, a thorough understanding of the role of the microtubule cytoskeleton in stress responses has the potential to lead to larger therapeutic windows, reduced chemotherapy resistance, and more effective cancer treatment with reduced side effects.

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Stressing mitosis to death

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The final stage of cell division (mitosis), involves the compaction of the duplicated genome into chromatid pairs. Each pair is captured by microtubules emanating from opposite spindle poles, aligned at the metaphase plate, and then faithfully segregated to form two identical daughter cells. Chromatids that are not correctly attached to the spindle are detected by the constitutively active spindle assembly checkpoint (SAC). Any stress that prevents correct bipolar spindle attachment, blocks the satisfaction of the SAC, and induces a prolonged mitotic arrest, providing the cell time to obtain attachment and complete segregation correctly. Unfortunately, during mitosis repairing damage is not generally possible due to the compaction of DNA into chromosomes, and subsequent suppression of gene transcription and translation. Therefore, in the presence of significant damage cell death is instigated to ensure that genomic stability is maintained. While most stresses lead to an arrest in mitosis, some promote premature mitotic exit, allowing cells to bypass mitotic cell death. This mini-review will focus on the effects and outcomes that common stresses have on mitosis, and how this impacts on the efficacy of mitotic chemotherapies.

Keywords: mitosis, SAC, spindle, kinetochore, checkpoint, metaphase, DNA damage, Cdk1

INTRODUCTION

The cell cycle is driven by the activity of the cyclin dependent kinases (Cdk), and their associated regulatory cyclin subunits. Each cell cycle phase is dependent on the sequential activation and deactivation of unique cyclin and Cdk complexes, with mitosis dependent on cyclin B bound with Cdk1 (1). To ensure the cell division process occurs with absolute fidelity, cells have developed numerous cell cycle checkpoints that delay progression in the presence of a wide variety of cellular and environmental stresses. During interphase (G1, S, and G2) stress activates checkpoints, which block cell cycle progression by increasing the translation of Cdk inhibitory proteins and activation of checkpoint kinases (Chk) that phosphorylate and inhibit Cdk (2). However, in mitosis the situation is reversed, the spindle assembly checkpoint (SAC) is on by default, which maintains high Cdk activity, thereby preventing cells from exiting mitosis. The primary role of the SAC is to block the activity of the anaphase promoting complex (APC), an E3 ubiquitin ligase responsible for targeting cyclin B1 (and many other key mitotic proteins) for degradation by the proteasome (3). This inhibition is achieved by the recruitment of several SAC proteins to the kinetochores, a protein structure located on the centromere of each chromosome (**Figure 1**). This localization allows the formation of the mitotic checkpoint complex (MCC) consisting of Cdc20, Mad2, Bub3, and BubR1, which then binds to and potently inhibits the APC, blocking degradation and preventing cells from entering anaphase (4). Once each kinetochore is attached to the mitotic spindle, the SAC proteins are displaced, and Cdc20 is released, allowing the APC to target proteins for degradation. However, the SAC arrest can be overcome by premature degradation of cyclin B1 (5), or direct inhibition of Cdk1 activity (6, 7) (**Figure 1**). This process is referred to as mitotic slippage and results in aberrant segregation of chromosomes and failure of abscission during cytokinesis, which can drive polyploidy, chromosome instability, and cancer formation (8). Therefore, during mitosis it is critical that interphase checkpoint pathways are turned off to prevent the deleterious effects of premature Cdk1 inactivation.

INHIBITION OF INTERPHASE CHECKPOINTS

The inhibition of interphase checkpoints is achieved primarily by inhibition of transcription (9) and down regulation of the majority (60–80%) of protein translation (10). In addition, Cdk1 and other mitotic kinases phosphorylate and disable key effectors of interphase checkpoint pathways, providing a feedback loop that restricts this inhibition to mitosis (11).

Transcription

The inhibition of transcription is a critical mechanism for preventing the upregulation of Cdk inhibitor proteins, such as p21. The expression of p21 is strongly upregulated during interphase in response to a variety of cellular stresses. For example, during interphase, DNA single and double strand breaks induced by exposure to ultraviolet light (UV) or ionizing radiation (IR) respectively, results in the recruitment and activation of ataxia-telangiectasia mutated and related (ATM and ATR) kinases to the sites of damage. ATM/ATR then activate p53, which in concert with the transcription factor Sp1, increases p21 expression (12, 13). However, during mitosis the majority of proteins involved in transcription are removed from the DNA, inhibiting the production of new mRNA (14, 15). Surprisingly, transcription factors and other structural proteins can still gain access to the highly compacted chromosome structure (16), and are actively removed by mitotic kinases (17). For example, Cdk1 phosphorylates Sp1 and CUX1 resulting in their dissociation from chromatin

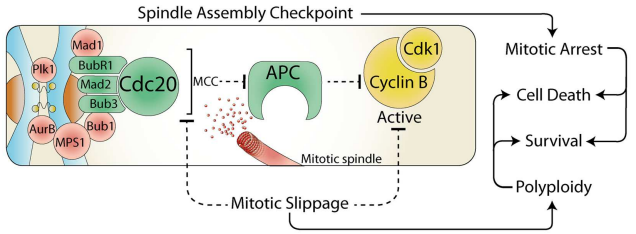

FIGURE 1 | The spindle assembly checkpoint and cell fate. During mitosis, the constitutively active spindle assembly checkpoint (SAC) delays anaphase until all chromosomes are attached to the mitotic spindle. Any stress that prevents satisfaction of the SAC results in a prolonged mitotic arrest, which often leads to cell death. However, the SAC can be over-come by the release of Cdc20 from the mitotic checkpoint complex (MCC) or by direct inhibition of Cdk1. This mitotic slippage can result in polyploidy, increased cell survival, and provides a potential mechanism for escaping mitotic cell death.

during mitosis (18, 19), thereby preventing upregulation of p21 (20).

Phosphorylation

During interphase, stress often triggers a kinase phosphorylation cascade, which culminates in the inhibitory phosphorylation of the interphase Cdk. To ensure that Cdk1 is not inhibited during mitosis, these checkpoint kinases (Chk) must be inhibited. Surprisingly, Cdk1 itself disables many of these, for example, it phosphorylates Chk1/2 preventing activation by ATM/ATR (21). Furthermore, Cdk1 phosphorylation of the DNA damage signaling and repair proteins 53BP1 and BRCA1, blocks their recruitment to sites of DNA damage (22). In addition, many of these interphase Chks are repurposed and required for normal progression through mitosis. For example, Chk2 localizes to kinetochores during mitosis, stabilizing MPS1 and phosphorylating Aurora B (23). Active Aurora B then phosphorylates ATM (24), which then phosphorylates γ-H2AX and Bub1 at kinetochores (24), promoting the accumulation of Mad2 and Cdc20 (25). Consequently, ATM activity is required to ensure correct centrosome and mitotic spindle formation (26, 27).

Translation

The translation of mRNA into proteins is actively inhibited during mitosis (10). During interphase, the majority of mRNA is guided to ribosomes by cap-dependent translation, however as cells enter mitosis this process is repressed (9) by phosphorylation of cap-binding proteins (28). As a result, translation switches from the cap-dependent system to mRNA that contains an internal ribosomal entry site (IRES) (29). The mRNA of several important mitotic proteins contain IRES sites (30, 31), which ensures their continued translation during mitosis. In addition, the mRNA of critical mitotic factors such as cyclin B, are restricted temporally to mitosis, and locally at the mitotic spindle, by polyadenylation (32, 33).

SAC AND THE RESPONSE TO STRESS IN MITOSIS

Any stress that directly or indirectly prevents the satisfaction of the SAC prevents cells from progressing past metaphase. However, some stresses are able to deactivate the SAC and induce mitotic slippage, therefore bypassing mitotic cell death. Interestingly, mitotic slippage has been suggested as a possible mechanism for resistance to mitotic chemotherapies, in particular the microtubule poison Taxol (34). Therefore, understanding exactly how common environmental and cellular stresses affect mitosis is critical for understanding how and why some cancer cells are sensitive and others are resistant to this important class of chemotherapies.

DNA damage

Attempting to repair DNA during mitosis is highly dangerous for cells and can result in the fusion of telomeres, failed separation of chromatids during anaphase, and the promotion of genomic instability and cancer (22). Therefore, some have suggested that the primary mitotic response to DNA damage is to mark sites of damage (with γ-H2AX), but not to arrest in mitosis (35). Instead, damaged cells are allowed to exit to the next G1 phase where repair or death can be triggered (36). However, many cells do arrest for varying amounts of time in response to an array of DNA damaging stresses. The length of arrest roughly correlates with the level of damage, with higher levels that disrupt kinetochore–microtubule function being more efficient at blocking mitotic exit in a SAC dependent manner (37). Furthermore, a prolonged arrest can itself damage telomeres (38), suggesting that mitotic cells damage their DNA on purpose. The point of this self inflicted damage is still unclear, but it may act as a backup pathway, ensuring even minor mitotic DNA damage is fully detected in the following G1 thereby preventing defects being passed on to subsequent generations.

DNA decatenation. During replication in interphase, sister chromatid pairs become interwound, and must be untangled prior to metaphase by decatenation, a process that requires topoisomerase II (Topo II). In addition, DNA decatenation is also required for correct chromatid and telomere separation during anaphase (39). Inhibition of Topo II during mitosis produces different mitotic responses, which are dependent on the inhibitor used, and specifically if DNA damage is produced. For example, doxorubicin creates significant levels of DNA damage (γ-H2AX foci), and consequently cells arrest in metaphase for up to 9 h (40). In contrast, ICRF-193 generally produces mild damage, and results in cells only delaying in mitosis for 1–2 h (37, 41) although ultrafine DNA bridges are formed during anaphase causing cells to fail abscission and form polyploid cells (41, 42). In all cases, the arrest during mitosis is dependent on the SAC, and is likely due to direct damage of kinetochore structure preventing stable microtubule attachments (**Figure 2**). For example, the delay induced by ICRF-193 requires inhibition of the APC by Mad2, but surprisingly Mad2 does not accumulate at kinetochores (35, 43). This may explain why this delay is short lived. Unfortunately, the inhibition of Topo II prior to mitosis blocks cells in G2 phase (44), consequently its use in combination with mitotic chemotherapies such as Taxol is often counter-intuitive as cells never enter mitosis and are resistant to Taxol induced death (45, 46).

Double strand breaks. Extensive double strand breaks during mitosis produce a strong SAC dependent arrest with cells delaying for more than 5 h over the normal 30–60 min transit time (37). Furthermore, extensive DNA damage has also been shown

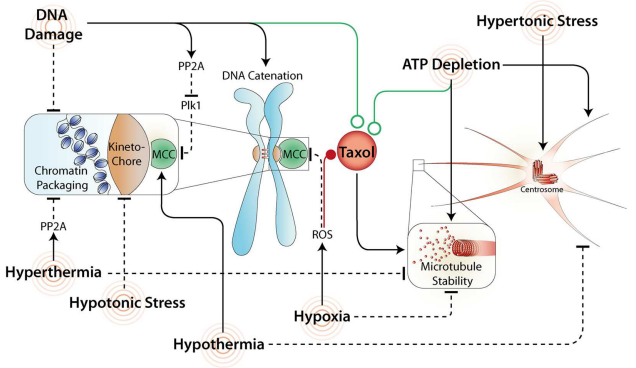

FIGURE 2 | Common stresses and their effects on mitosis and Taxol response. A variety of stresses affect mitosis by acting directly or indirectly on the SAC. Stresses that maintain the SAC and/or increase microtubule stability often synergize (green line) with Taxol. In contrast, stresses that inhibit the MCC and/or disrupt microtubule formation commonly antagonize (red line) Taxol induced mitotic arrest and promote mitotic slippage.

to inhibit the activity of Polo like kinase 1 (Plk1) (47), a mitotic kinase that plays a key role in mediating attachments between the kinetochore and mitotic spindle (48). This inhibition occurs independently of ATM (49), primarily through PP2A mediated dephosphorylation of Plk1 (50), and likely strengthens the mitotic arrest induced by extensive double strand breaks by preventing satisfaction of the SAC (**Figure 2**). Consequently, combining DNA damaging agents with Taxol, especially in p53 mutant cancer cells with dysfunctional interphase DNA damage checkpoints (51), may greatly enhance the amount of damage produced and promote a prolonged mitotic arrest resulting in increased levels of cell death. Accordingly, Taxol is commonly used in combination with platinum-based chemotherapeutics as a first line treatment for ovarian cancer (52), and is being trialed in combination with DNA damaging agents in several other cancer types including small cell lung cancer, melanoma, and pancreatic cancer (53–55).

Chromatin structure. Disruption of the mitotic chromosome architecture also produces a temporary mitotic delay. Treatment with histone deacetylase inhibitors (HDACi) prevents correct chromosome condensation and increases the access of transcription factors to the DNA, disrupting correct kinetochore formation (56). This delays the correct capture and alignment of chromosomes by the mitotic spindle, leading to SAC-dependent mitotic arrest (44, 57). However, if this damage is too severe, SAC proteins fail to remain at kinetochores, leading to silencing of the SAC and premature exit (slippage) from mitosis (58, 59). Interestingly, although HDACi have been highly successful in treatment of lymphoma, they have not been as successful with solid tumors, which could be due to SAC dysfunction (e.g., BubR1 mutation) in these cancers and an increased rate of mitotic slippage.

Hypoxia and oxidative stress

Reduced oxygen supply especially within the core of solid tumors results in a hypoxic environment within the tumor mass. Hypoxia is a poor prognostic factor, and correlates with resistance to radiation and many chemotherapeutic agents (60). Exposure to hypoxia during mitosis results in the rapid disruption and destabilization of microtubules (61), which delays mitotic progression. However, this arrest is unstable and cyclin B levels decrease rapidly (62), in turn inactivating Cdk1, and promoting mitotic slippage, providing an explanation for why hypoxia induces tetraploidization in melanoma (63). However, hypoxia induces a wide variety of intracellular responses including formation of reactive oxygen species (ROS), and a switch to anaerobic glycolysis resulting in decreased levels of ATP. The effects of hypoxia on mitosis are most likely due to the increased formation of ROS. In support, exposing mitotic cells to hydrogen peroxide (H_2O_2) to mimic ROS, induces mitotic slippage and the formation of hypertetraploid cells (64). The mechanism for this slippage is yet to be full elucidated, however, in yeast, H_2O_2 exposure depletes the SAC protein BubR1 from kinetochores, silencing the SAC allowing cells to exit mitosis prematurely (65). In addition, H_2O_2 also depolymerizes microtubules (66), which results in need for higher doses of Taxol to stabilize microtubules and induce cell death (67). These effects may explain why hypoxia reduces toxicity to Taxol in cancer cells (**Figure 2**). Consequently, reducing ROS with antioxidants has long been proposed as a co-treatment to enhance the effects of Taxol, with some limited success (68). The inconsistent results are likely due to the specific antioxidant used. For example, the popular dietary antioxidants Resveratrol and Fisetin (found in red wine), inhibit Cdks, induce a G2 arrest and prevent entry into mitosis (69, 70), providing an explanation for why they antagonize Taxol (71, 72). Therefore, finding methods that specifically reduce ROS without off target effects will be critical for the future success of co-treatment regimes.

ATP depletion

Hypoxia can also cause depletion of ATP pools, however the mitotic effects of ATP depletion are opposite to that of hypoxia and oxidative stress. Specific depletion of ATP pools with DNP, Azide, or AMP-PNP, results a rapid prolonged mitotic arrest in mammalian cells (73). ATP is needed for microtubule disassembly (74), and therefore depletion of ATP stabilizes microtubules (75). In addition, depletion of ATP activates AMP-activated protein kinase, which phosphorylates myosin regulatory light chain, and promotes astral microtubule growth (76) (**Figure 2**). Surprisingly, depletion of ATP also depletes Mad2 and BubR1 from kinetochores, with both proteins accumulating at spindle poles, however this does not appear to affect their ability to bind Cdc20 and inhibit the APC (77–79). Taken together, this area has significant potential for future novel therapeutic approaches, with some metabolic inhibitors already showing synergy with Taxol (80).

Thermal shock

Heat-shock (hyperthermia) has been commonly used as adjunctive cancer therapy to augment radiotherapy and chemotherapy, with varying levels of success (81). The initial mitotic response to acute (42°C) heat-shock is to arrest in mitosis (82). This delay is most likely SAC dependent due to effects on microtubules and centrosomes, which become permanently disorganized and destabilized upon exposure to heat (83). In addition, heat can increase the binding of the heat-shock transcription

factor 2 (HSF2) to DNA during mitosis (84). HSF2 binding attracts PP2A, which dephosphorylates condensin, thereby reducing the compaction of the chromosomes (85), and acting similar to HDACi treatment (**Figure 2**). Consequently, the mitotic delay is only temporary, and cells rapidly reform a nuclear envelope around chromosomes, and undergo mitotic slippage (86, 87), even in the presence of Taxol (88). Hyperthermia has been shown to both antagonize (89) and synergize (88) with Taxol, with the outcome dependent on the functionality of the apoptotic pathway (90, 91).

Interestingly, like hyperthermia, cold-shock (hypothermia) has also shown some success in synergizing with radiotherapy and a variety of chemotherapeutics (92, 93). Exposure to cold induces a transient mitotic delay in cells, however cells eventually complete mitosis and segregate their chromosomes normally (94). Hypothermia reversibly destabilizes non-kinetochore microtubules (95, 96), but this still allows chromosomes to be captured by kinetochore microtubules and positioned at the metaphase plate (94). However, a reduction in microtubule dynamics and loss of astral microtubules results in reduced tension at kinetochores leading to the retention of Bub1 and BubR1 at kinetochores and a SAC-dependent mitotic delay (97). The ability of cells to recover from hypothermia and complete mitosis may explain why cold-shock can reduce the number of mitotic defects induced by chemotherapies (98), and minimize side effects (e.g., hair loss) of Taxol in cancer patients (99). However, given that the delay is transient and reversible, it also explains why co-treatment regimes have not shown any significant synergy and are unlikely to be useful for enhancing the killing of cancer cells.

Mechanical stress

As cells enter mitosis they transform their architecture to create a spherical shape, which is driven by changes in the actin cytoskeleton (100), and by regulation of osmotic pressure (101). The small GTPase RhoA is critical for cortical retraction during mitotic cell rounding (102). During early prophase RhoA promotes remodeling of the actin cytoskeleton, increasing the mechanical stiffness of the cell (103). Cell rounding is achieved by combining RhoA-mediated cellular rigidity with increased hydrostatic pressure inside the cell. This occurs by increasing intracellular sodium levels resulting in an influx of water (101). Failure to round up, and/or disruption of the RhoA pathway prevents mitotic exit in a SAC-dependent manner by inducing spindle pole fragmentation (104), disruption of astral microtubule organization and spindle function (105, 106) (**Figure 2**). Interestingly, placing cells in hypertonic solution (preventing water influx) stably arrests cells in mitosis and was originally used in the 1970s as a method for enriching mammalian cells in mitosis (107). After several hours most arrested cells die, although some escape via mitotic slippage to form polyploid cells (108). Interestingly, in yeast, hypertonic stress can promote activation of Cdc14 phosphatase (109), which then dephosphorylates Cdk substrates driving cells out of mitosis, suggesting that phosphatases can drive slippage. However, in humans the role of Cdc14 is not conserved (110), and PP2A appears to be the primary phosphatase responsible for removing mitotic Cdk1 phosphorylations (111, 112). If PP2A is directly activated in response to hypertonic stress it could promote mitotic slippage in human cells, providing a rational for future research focusing on the effectiveness of PP2A inhibitors in combination with mitotic chemotherapies.

Exposure of mitotic cells to hypotonic conditions increases water influx, rising internal pressure and a swelling of mitotic cell size, with weak hypotonic solutions arresting cells in prometaphase (113). However, unlike hypertonic stress, this arrest is far less stable and cells rapidly undergo mitotic slippage, characterized by chromosome decondensation, disrupted kinetochore and spindle structure, and reformation of the nuclear envelope around un-segregated chromosomes (114, 115), which all promote chromosome aberrations and polyploidy (116). The effects of hypotonic stress in combination with Taxol have not been studied in detail, however, hypotonic solutions can increase the uptake of chemotherapies in cells (117), and have shown some promise in enhancing response to platinum-based treatments (118). Consequently, it is likely that similar to hyperthermia, local hypotonic conditions could be used to enhance Taxol response in tumors with a functional apoptotic pathway.

CONCLUSION/PERSPECTIVES

In summary, the ability of cells to arrest during mitosis in response cellular and environmental stresses is dependent on the presence of a functional SAC, the correct suppression of transcription and translation, and critically the maintenance of Cdk1 activity. Stress that prevents the satisfaction of the SAC results in a mitotic arrest, while those stresses that disrupt Cdk1 activity or directly disable the SAC force cells to prematurely exit mitosis. Future research on the role mitotic phosphatases, such as PP2A, play in stress response and slippage will be critical for fully elucidating the mechanisms of how a specific cancer will response or can be sensitized to mitotic chemotherapies such as Taxol.

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Size does matter: why polyploid tumor cells are critical drug targets in the war on cancer

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Tumor evolution presents a formidable obstacle that currently prevents the development of truly curative treatments for cancer. In this perspective, we advocate for the hypothesis that tumor cells with significantly elevated genomic content (polyploid tumor cells) facilitate rapid tumor evolution and the acquisition of therapy resistance in multiple incurable cancers. We appeal to studies conducted in yeast, cancer models, and cancer patients, which all converge on the hypothesis that polyploidy enables large phenotypic leaps, providing access to many different therapy-resistant phenotypes. We develop a flow-cytometry based method for quantifying the prevalence of polyploid tumor cells, and show the frequency of these cells in patient tumors may be higher than is generally appreciated. We then present recent studies identifying promising new therapeutic strategies that could be used to specifically target polyploid tumor cells in cancer patients. We argue that these therapeutic approaches should be incorporated into new treatment strategies aimed at blocking tumor evolution by killing the highly evolvable, therapy-resistant polyploid cell subpopulations, thus helping to maintain patient tumors in a drug sensitive state.

Keywords: polyploidy, hyperdiploidy, tumor evolution, therapy resistance, tumor initiation, cancer stem cell, aneuploidy, chromosomal instability

COMING TO TERMS WITH CANCER AS A RAPIDLY EVOLVING SYSTEM

It has long been appreciated that cancer is an evolutionary system (1). In this paradigm, individual cancer cells are the reproductive units within a tumor, with those cells that acquire a survival advantage through random genetic change being selected through multiple rounds of clonal expansion, during which they acquire further alterations that eventually combine to produce malignant phenotypes (1). The ability of a tumor to evolve solutions to selection pressures is a function of the *selectable heritable variation* that is present within the tumor, be it internal stressors such as low oxygen tumor micro-environments, or external stressors such as anti-cancer therapies (2–8). The paradigm of selectable heritable variation at the cellular level being a critical driver of cancer biology has been captured by the term *tumor heterogeneity*, and the emerging consensus is that tumor heterogeneity remains a fundamental obstacle preventing the development of truly curative anti-cancer therapies (2–8).

The introduction of efficacious targeted therapies highlighted the central role of evolution in cancer therapy failure. Patients with leukemia and lung cancer treated with specific inhibitors targeting oncogenic receptor tyrosine kinase (RTK) activity, eventually exhibit disease progression driven by point mutations within the oncogenic RTK that renders the tumor resistant to further therapy (9–11). Retrospective analysis revealed rare therapy-resistant mutants present in tumors prior to treatment initiation (12), confirming that in some cases targeted therapy selected for resistant clones that were already present within the tumor system. Melanoma offers a compelling case study of tumor evolution during targeted therapy. The identification of oncogenic mutations within the B-Raf kinase led to the development of specific inhibitors that initially display phenomenal clinical efficacy (13–16), which is swiftly followed by disease recurrence driven by rapidly evolving therapy resistance [reviewed in Ref. (17)].

Immunological based therapies are also vulnerable to therapy-resistance driven by tumor evolution, as revealed during a vaccine strategy trialed in adult patients with Glioblastoma. The vaccine therapy invokes a patient immune response that specifically targets the truncated, oncogenic EGFRvIII variant of the EGF receptor (18). The EGFRvIII variant is present in approximately one-third of Glioblastoma patients (19) and is an ideal target for anti-tumor immunotherapy because the constitutive activity of the EGFRvIII contributes to tumorigenicity, invasion and therapy resistance [reviewed in Ref. (18)]. Although the vaccine significantly increased overall survival time in treated patients whose tumors expressed the EGFRvIII receptor, disease recurrence occurred in all patients with most recurrent tumors losing EGFRvIII expression (18). EGFRvIII expression is typically heterogeneous in Glioblastoma tumors, and is only observed in a sub-population of tumor cells and rarely in the entire tumor (20, 21). The most plausible hypothesis is that the vaccine led to the immune-clearance of EGFRvIII expressing cells from patient tumors, but in the majority of cases it was the presence of viable EGFRvIII negative cells within the tumor that allowed immunological escape and rapid disease recurrence.

These and many other studies all converge on the hypothesis that long-term cancer patient survival requires the development of therapeutic strategies that actively suppress tumor evolution (2–8,

Box 1 | Definitions.

Polyploidy: An alteration of chromosomal number that is a multiple of the normal diploid (2n) complement.

Tetraploidy: A specific form of polyploidy that is a doubling of the normal diploid complement (i.e., 4n).

Aneuploidy: An alteration of chromosome number that is not a multiple of the diploid (2n) complement.

Hyperdiploidy: Having a chromosome number that is more than the diploid (2n) complement.

Around 90% of all solid tumors are aneuploid, and most aneuploidy tumors exhibit chromosomal gains and are therefore hyperdiploid (85). Many cancers have complex karyotypes [see for example Ref. (62)]. In this perspective, we have focused on subpopulations of cancer cells that have elevated genomic content relative to the tumor bulk, as a source of cells capable of rapid evolution. In the strict sense, these cells are grossly hyperdiploid relative to healthy, untransformed cells. However, we refer to them here simply as polyploid tumor cells (or pseudo-polyploid tumor cells) for the following reasons. (1) We are comparing these cells to the tumor bulk, and grossly hyperdiploid tumor sub-population of cells are typically polyploid (or close to polyploid) relative to the dominant aneuploid tumor karyotype, (2) many of the cited cell biology studies refer to this tumor cell sub-population as polyploid. We ask the reader to keep in mind that the "tumor polyploid cells" are in reality a genetically heterogeneous sub-population, which is composed of a variety of complex cancer karyotypes that are approximately polyploid relative to the dominant, aneuploid tumor cell population.

22). In this perspective, we propose that tumor cells containing an elevated genomic content (as described in **Box 1**) are key players in tumor evolution, and are therefore important therapeutic targets in preventing the acquisition of therapy resistance during treatment. We begin by summarizing seminal work conducted in yeast that characterizes how chromosomal gains facilitate rapid evolution under a wide variety of selection pressures. Next, we review recent work conducted in cancer, which show that chromosomal gains also underpin tumor initiation and the acquisition of therapy resistance in cancer patients. We then present an updated model of tumor evolution that highlights the central role of increasing ploidy in cancer initiation and disease progression.

We finish the perspective showcasing recent studies that identify anti-polyploid compounds that we hope will provide a foundation for the development of efficacious chemopreventative and evolutionary suppressing cancer therapies of the future. Our goal is to focus research efforts on the development and translation of such novel anti-polyploid therapies to prevent and treat incurable cancers.

HYPERDIPLOIDY AND POLYPLOIDY FACILITATES RAPID EVOLUTION: LESSONS FROM YEAST THAT ARE RELEVANT TO CANCER

HOW INCREASING GENOME SIZE FACILITATES RAPID EVOLUTION IN YEAST

Serious systemic fungal infections continue to endanger patients with immunocompromised immune systems (23, 24). Anti-fungal azole drugs are the most commonly used therapy against superficial and systemic fungal infections due to their efficacy and safety (23). Fluconazole is a widely used azole that is orally and intravenously available and effective against *Candida* infections, and is used clinically to treat oropharyngeal and esophageal *Candidas* in HIV patients, invasive candidiasis, as well as fungal infections in the urinary tract and central nervous system (23). Prior to the HIV pandemic, fluconazole resistance was rare. However, the widespread use of fluconazole to treat HIV/AIDS patients has increased the incidence of fluconazole-resistant *Candida* isolates (25). Generally, resistance develops after administration of sub-optimal doses of fluconazole over long periods of time, but in 1992, Bossche et al. isolated a resistant *Candida* strain in a patient after only 9 days of fluconazole treatment (26), revealing circumstances under which the evolution of fluconazole therapy-resistance occurs astonishingly quickly. In a follow-up study examining the mechanisms underlying the rapidly acquired fluconazole resistance, it was found that the resistant strain expressed more cytochrome P-450 14α-lanosterol demethylase (the target for azole antifungals) due to duplication of the entire chromosome containing the CYPO51 gene (27). Subsequent studies have confirmed that chromosome duplication is an effective and widely utilized mechanism to evolve drug-resistance in fungal infections (28–31).

Increasing chromosome numbers also provides fitness advantages in other contexts. A powerful example of rapid adaptation through increasing genomic content was provided by Rancati et al. (32) when they experimentally perturbed cytokinesis by deleting the MYO1 gene in the yeast *Saccharomyces cerevisiae*, and then selected for mutant strains that had evolved a solution to MYO1 deletion to restore functional cytokinesis. Strikingly, they found that most of the evolved strains, including the 10 fittest isolates, displayed an increase in DNA content. Further, diploid strains evolved much faster than haploid strains. Together, these data suggest that polyploidization facilitated the rapid evolution of cytokinesis rescue, a finding reminiscent of the rapid evolution of therapy-resistance driven by polyploidy described in immunocompromised patients treated with anti-fungal therapies described above.

Hyperosmotic stress occurs when an organism is exposed to higher solute concentration outside the cell, leading to water loss and subsequent increases in intracellular ion and metabolite concentrations [reviewed in Ref. (33)]. Hyperosmotic stress is a common environmental stressor, and yeast have evolved a hyperosmotic stress response that is mediated by the high osmolarity glycerol (HOG) pathway, which activates genes involved in salt tolerance and adaptation (34). Wagner and colleagues investigated how yeast could evolve adaptations to hyperosmolarity stress in long-term evolution experiments, where three replicate *Saccharomyces cerevisiae* yeast populations were exposed to high-salt conditions for 300 generations (33). All three populations evolved a faster growth rate under high-salt conditions after selection compared to their ancestral cultures (33). DNA content analysis revealed that all three evolved lines had an increase in ploidy,

suggesting that evolutionary adaptation to hyperosmotic stress is also facilitated through increasing genome size (33).

The Evolution Canyon originated in Israel 3–5 million years ago, and contains diverse micro-environments and has experienced minimal human disturbance, providing an excellent natural site to study evolutionary adaptations of many organisms (35). Chang et al. isolated and phenotypically characterized 14 diploid yeast strains collected from different micro-environments present within the Evolutionary Canyon (35). One of these strains was highly resistant to the metal copper. Strikingly, Chang et al. found that the copper-tolerant phenotype was the product of large-scale chromosomal rearrangements that increased the copy number of the CUP1 and CUP2, major genes involved in copper regulation (35). Additional copper-tolerance gene expression was up regulated by increased CUP2 copy number, showing that the increase in gene dosage both directly and indirectly contributes to the evolution of copper-tolerance. Surprisingly, when the tolerant strains were cultured in the absence of copper, a wild-type chromosome reappeared and was fixed within 300 generations. These findings reveal that "large-scale chromosomal rearrangements provide not only fast arising but also readily reversible sources of variation during early stages of adaptive evolution" (35).

Collectively, these studies reveal increasing chromosome content as a mechanism that facilitates the rapid evolution of yeast across many different selection pressures and environments. These include the rapid acquisition of therapy resistance in patients, rapid adaptation during experimental evolution, and the successful adaptation to selection pressures present in nature.

HOW INCREASING GENOME SIZE CHANGES YEAST PHENOTYPES

One important mechanism for rapid adaptation provided by chromosomal gain is increased gene expression due to elevated gene dosage. Multiple studies have confirmed that messenger RNA levels scale with chromosome copy number in aneuploid systems. Hughes et al. conducted expression profiling of yeast strains with characterized aneuploidy and showed that increased genomic content data "precisely mirrored the expression data in this region" (36), revealing that gene duplication leads to a commensurate increase in messenger expression (36). The second important finding from this study was that under experimental selection, large-scale gene duplications were shown to be the dominant adaptive response to loss-of-function deletions (36), providing early support for the hypothesis that increasing genomic content facilitates rapid adaptation.

In a later study, examining the effects of extra chromosomes on cell physiology and cell division in yeast, Torres et al. observed an approximate doubling of gene expression in duplicated chromosomes, with greater than 90% the amplified genes being expressed at a higher level (37). These data indicate that most genes are expressed in proportion to their gene copy number, and gene amplification results in a roughly proportional increase in gene expression (37).

The classic evolution study by Rancati et al. confirmed that on average there is a stoichiometric relationship between gene copy number and gene expression level, with gene expression levels from chromosomes roughly scaling with chromosome copy number (32). However, they noted that some gene expression levels deviated significantly from this trend, identifying outlier genes whose expression changed more than three standard deviations away from the stoichiometric trend (32). Further evidence suggests that the majority of outlier expression is caused by the increased expression of transcription factors (or their upstream regulators) caused by chromosomal copy number increase (32). Similarly, expression of the copper resistance gene CUP2 due to increase in gene dosage causes the expression of downstream genes, several of which also enhance resistance to copper (35). This reveals how a simple linear change in gene expression can generate a non-linear adaptive response through pathway amplification (35).

Changes in yeast chromosome numbers also increase protein expression levels in yeast cells. Pavelka et al. generated a panel of stable aneuploidy yeast strains to directly address this question (38). They found that chromosomal copy number changes in general caused proportional changes in gene expression and protein expression levels (38). Further, they found that yeast strains with similar karyotypes tend to display similar changes in global protein expression patterns (38). Interestingly, the Authors also identified outliers in gene and protein expression, however only a small fraction of the gene expression outliers overlapped with the protein expression outliers (38), revealing that gene dosage changes are likely to have complex effects on cellular phenotypes. The Authors applied a variety of selection pressures on euploid parent controls and aneuploid strains, revealing that aneuploid strains grew better under selection by generating rapid phenotypic variation, showing that aneuploidy that can provide fitness gains under diverse selection pressures (38).

Together, the data sets summarized above show that increasing DNA content modifies both gene and protein expression, in linear and non-linear ways, allowing cell populations to rapidly explore a wide range of heritable phenotypes. Thus, increasing ploidy enables yeast cells to experience large phenotypic leaps, which in turn facilitates rapid evolution to novel selection pressures (39).

HOW INCREASED GENOMIC CONTENT BUFFERS CELLS AGAINST DELETERIOUS MUTATIONS

The mutator hypothesis proposes that mutations that increase genomic instability (the mutator phenotype) drives tumorigenesis by allowing cells to rapidly acquire the necessary number of mutations required for cellular transformation (40). The mutator phenotype was first proposed by Loeb to explain how tumors can accumulate the number of mutations necessary for tumorigenesis despite the extremely high accuracy with which mammalian cells replicate the genome (41, 42). One primary criticism of the mutator hypothesis is that most mutations are deleterious and therefore the mutator phenotype will accelerate the accumulation of mutations that reduce fitness, leading to negative clonal selection [(3) and references therein]. Although experimental and theoretical counters to this criticism have been provided [recently reviewed in Ref. (43)], one potentially important phenomenon that has been overlooked in this debate is the role of genome amplification in buffering eukaryotic cells against the effects of deleterious mutations.

Using adaptation to different laboratory environments as their selection pressure, Thompson et al. (44) compared the relative fitness of mismatch repair defective (mutator) strains of yeast

within haploid and diploid yeast genetic backgrounds, with striking results. In the diploid genetic background, mutators displayed an advantage over non-mutators, and mutators that "win" adaptation experiments were on average fitter than non-mutator winners (44). In contrast mutators in the haploid background displayed no advantage when competed against haploid non-mutators and the haploid mutator winners were less fit than the haploid non-mutator controls (44). The most parsimonious explanation for this result is that most deleterious mutations are recessive, and are therefore buffered in the diploid yeast strain. Haploid yeast must bear the cost of deleterious mutations in full, which gives haploid yeast less time to accumulate beneficial mutations before the cumulative effects of deleterious mutations eliminates them from the population. An additional important observation from this study was the type of mutations that occurred with haploid versus diploid populations. The diploid mutators displayed a generalist class of beneficial mutation that provided a large selective advantage across a range of selection pressures (44). In contrast, haploid mutators displayed beneficial mutations whose advantage was limited to the specific stress they were selected under (44).

These results suggest two intriguing hypotheses. First, increases in ploidy may act co-operatively with a mutator phenotype by reducing the effect of deleterious mutations. Second, increased ploidy enables a mutator phenotype to generate "generalist" beneficial mutations that confer selective advantage across a wide range of stressors.

HOW ELEVATED PLOIDY DRIVES THE EVOLUTION OF CANCER

ANEUPLOIDY AND TUMORIGENESIS

The vast majority of cancers are aneuploidy, with around 90% of solid tumors and 75% of hematopoietic cancers having abnormal chromosome numbers (45). The high incidence of aneuploidy in cancer cells inspired Boveri over 100 years ago to propose the hypothesis that aneuploidy causes cancer (46). Consistent with this hypothesis, aneuploidy has been shown to precede transformation in a variety of cancers (47–54), and several studies provide both experimental and theoretical support for a fundamental role of aneuploidy during tumor initiation (55–59). Duesberg and colleagues have proposed that aneuploidy generates cancer causing karyotypes that are selected during the evolutionary process of tumor initiation and transformation (60–62). However, these ideas have been contested, in part because the aneuploidy model of tumor initiation is thought to downplay the established role of oncogenes in the process of transformation (63–65). Recent studies examining the role of polyploidy in tumor initiation may help incorporate the oncogenes and aneuploidy tumor initiation models into a single paradigm.

EVIDENCE SUPPORTING TETRAPLOID CELLS BEING THE CELL OF ORIGIN IN TUMOR INITIATION

Experimental evidence directly linking tetraploidy with tumor initiation was provided when Fujiwara et al. created a tetraploid cell population in p53-null mouse mammary epithelial cells (66). Tetraploid cells displayed a high level of tumorigenesis when injected into nude mice, in contrast to the diploid p53-null controls, which did not form tumors (66). Subsequent studies perturbing the mitotic spindle led to accumulation of tetraploid cells and a higher incidence of tumor formation, further supporting a central role of tetraploidy in tumor initiation (67, 68).

Tetraploidy potentially provides multiple beneficial functions during tumor initiation. First, a large body of evidence supports the hypothesis that tetraploidy acts as a gateway karyotype by inducing chromosomal instability (CIN), which leads to aneuploidy and the evolution of a transformed phenotype [reviewed in Ref. (69)]. Using several experimental models of telomere crisis, Davoli and de Lange recently demonstrated that endoreplication and mitotic failure created tetraploid cells during telomere crisis (70). Importantly, the resulting tetraploid cells displayed enhanced tumorigenic capacity relative to diploid controls in soft agar and mouse implantation assays (70). Finally, Davoli and de Lange then showed tumors that are initiated by tetraploid cells evolve more complex aneuploidy karyotypes *in vivo*, showing tetraploidy functions as a gateway mutation to aneuploidy (70).

Lv and colleagues used the spontaneous transformation of primary ovarian epithelial cells to provide compelling evidence for the role of tetraploidy as a gateway karyotype during tumorigenesis (71). Lv et al. generated primary cultures of mouse ovarian surface epithelial cells (MOSECs), which they continually subcultured for over 30 passages (71). Following ploidy status during culture revealed that the diploid cells underwent an intermediate tetraploid phase, and then evolved into aneuploid (near-tetraploid) cells (71). Tetraploidy was caused by cytokinesis failure in diploid cells, with the tetraploid cells subsequently experiencing chromosome mis-segregation during bipolar and multipolar mitosis to generate aneuploid progeny (71). When the lines were re-injected into mice, only late passage aneuploid cells formed tumors (71), showing that spontaneous transformation during long-term passaging likely involves a diploid–tetraploid–aneuploid transition caused by defects in mitosis.

Two recent studies have provided compelling support for the hypothesis that genome doubling facilitates the acquisition of a transformed phenotype in tumor initiation in human cancers. Examining neuroblastomas, Lundberg et al. combined karyotypic analyses of tumors with mathematical modeling and concluded that the loss of chromosomes from a tetraploid precursor cell was the most parsimonious hypothesis explaining the chromosomal numerical alterations present in neuroblastoma tumors (72). This conclusion was supported experimentally when it was shown that neuroblastoma lines displayed a high frequency of polyploidization events, and that clonal cultures with elevated genomic content generated aneuploid progeny with high frequency (72). Altogether these data suggest that polyploidy is a gateway cell state that facilitates the generation of aneuploidy and increases karyotypic complexity in neuroblastoma tumors (72).

More recently, Swanton and colleagues (73) systematically addressed the role of tetraploidy in colorectal cancer evolution (73). Colorectal cancers that had undergone genome doubling (i.e., tetraploid) displayed a significantly higher incidence of genomic instability than those cancers that began as diploids, with tetraploidization appearing to be an early event in the majority of colorectal cancers (73). Tetraploid clones were isolated from colorectal cancer lines, and these displayed a higher incidence of segregation errors during anaphase and increased chromosomal structural abnormalities relative to their cognate, diploid controls

(73). Strikingly, daughter cells derived from diploid clones that had undergone a segregation error during mitosis frequently died or underwent cell-cycle arrest, whereas daughter generated from tetraploid clones after segregation error died much less frequently and continued to proliferate (73). These data provide direct experimental support for the hypothesis that tetraploidy endows tumor precursor cells with an elevated tolerance to CIN, facilitating the generation of aneuploidy and the evolution of a complex karyotype (74). Consistent with this model, genome doubling is associated with poor prognosis, being significantly associated with disease relapse (73).

In addition to increasing tolerance to aneuploidy and facilitating the evolution of a transformed karyotype, tetraploidy also helps overcome oncogene induced senescence. Aberrant activation of oncogenes such as Ras, Raf, or PI3-kinase triggers cellular senescence, which functions as a tumor suppressor by permanently restricting the proliferative capacity of cells (75–77). Activation of DNA-damage response pathways plays an important role during oncogene induced senescence (78–80), as does activation of p53 pathways (76, 81–83). Exploring how malignant cells overcome the senescent barrier, Zheng et al. used a mouse model of tumorigenesis discovered that cells that overcame tumorigenesis barriers to drive long-term proliferation in culture all displayed near-polyploid levels of aneuploidy (84). These near-polyploid cells overexpressed DNA repair genes to reduce the DNA-damage response, as well as methylating p53 promoter regions to silence p53 expression (84). These results indicate that polyploid cells may be able to overcome the oncogene induced senescence by increasing DNA repair activity and epigenetic reprograming of p53 expression (84).

Altogether, these studies show that tetraploidy functions as a gateway phenotype that cooperates with oncogenes to induce cellular transformation in three ways. First, tetraploidy helps overcome oncogene induced senescence. Second, tetraploidy facilitates the acquisition of oncogenic karyotypes and phenotypes by inducing CIN leading to aneuploidy. Third tetraploidy buffers pre-malignant cells against the deleterious effects of chromosomal loss. Collectively, these findings go some way to explaining why the majority of human tumors contain a hyperdiploid karyotype (85).

POLYPLOIDY TUMOR CELLS AND THE EVOLUTION OF CANCER THERAPY RESISTANCE

HOW POLYPLOIDY OVERCOMES THERAPY-INDUCED SENESCENCE

Cancer cells can survive chemotherapy and radiotherapy by entering a reversible senescent state, called therapy-induced senescence (TIS), which is a senescent-like phenotype that displays many of the features of the normal physiological senescence phenotype (86). Even transformed cells lacking functional p53 and retinoblastoma protein (Rb) pathways retain the capacity to undergo TIS (87). TIS has been observed *in vivo* using both xenograft and transgenic cancer models (88, 89). Senescence markers have been observed from breast and lung cancer patient tumor specimens treated with chemotherapy, supporting the hypothesis that TIS is a clinically relevant cell fate in human cancer patients treated with cytotoxic therapies (90, 91).

Unfortunately TIS is not permanent, with rare cells being able to bypass TIS to re-enter the cell cycle and re-initiate tumor growth (90). One way cells overcome TIS is through the over-expression of the mitotic kinase CDK1, which phosphorylates the protein survivin to promote TIS escape and subsequent survival of cancer cells (92). In a follow-up study, Wang and colleagues went on to show that over-expression of CDK1 induced the formation of polyploid cells during TIS, and that these CDK expressing polyploid cells represent an important transition state through which escape from TIS preferentially occurs (92). Intriguingly, Wang et al. also reported that non-small cell lung cancer patients expressing markers of TIS following neo-adjuvant therapy had a significantly worse prognosis than patients who did not express TIS markers (92). Altogether, these data support a model whereby TIS provides an escape mechanism for tumor cells to avoid the toxic effects of chemotherapy to drive disease recurrence (92). Moreover, polyploid tumor cells are far more likely to overcome the TIS barrier, and polyploidy-mediated TIS escape represents an important new therapy-resistance mechanism in cancer patients undergoing a variety of chemotherapy regimes (92).

HOW POLYPLOIDY INDUCES INFREQUENT CELL CYCLE

Infrequent cell cycle is a well-established resistance mechanism against cytotoxic insult. Normal quiescent (G_0) hematopoietic stem cells (HSCs) are resistant to the anti-proliferative chemotherapeutic agent 5-fluoro-uracil (5-FU) (93, 94), and become sensitive to 5-FU treatment when they are forced into a proliferative state by treatment with IFNα (95). Healthy HSCs can be protected from the effects of irradiation by increasing the proportion of HSCs in G_0 through a variety of treatments *in vivo* (96–98). In cancer, the chemoprotective effect of cell-cycle-mediated drug-resistance is well-established (99). For example, Schmidt and colleagues demonstrated that colon adenocarcinoma cells arrested in G_1 by over-expression of $p27^{Kip1}$ are significantly more resistant to a variety of chemotherapeutic agents, including temozolomide (100). Using a mouse xenograft model, Naumov et al. showed that the DNA intercalating compound doxorubicin (DXR) effectively reduced the metastatic tumor burden but spared non-cycling tumor cells, which persisted during therapy and subsequently developed into metastases after DXR therapy was discontinued (101). More recently, label-retention has been used to phenotypically identify infrequently dividing cells that are resistant to chemotherapy from a variety of tumor types (102–105). Studies examining the cancer stem-cell phenotype have also shown that quiescence provides protection against cell death induced by DNA-damage agents (106, 107) and chemotherapy (108). Recently, a landmark study by Kreso et al. revealed how chemotherapy selects for minor, infrequently cycling subpopulations using lineage tracking in mouse models of cancer evolution (109). Collectively these studies provide strong support the hypothesis that infrequent cell cycle as a fundamental mechanism that contributes to the evolution of therapy resistance in cancer patients.

Recently, we identified a genetically diverse, polyploid tumor cell sub-population in Glioblastoma patients that is able to initiate and maintain tumor growth *in vivo*, and is resistant to cytotoxic therapy (110). Proliferation markers revealed that the polyploid tumor cell sub-population contain approximately three times more quiescent cells than the bulk near-diploid tumor population (110). Infrequently cycling cells retain the dye CFSE,

and CFSE label-retention has been used to enrich for therapy-resistant, tumor-initiating cells in several tumor types [reviewed in Ref. (111)]. Polyploid tumor cells accumulate within the label-retaining sub-population of cells, providing a functional confirmation of their infrequent cell cycle (110). Altogether, these data show that increasing chromosome numbers provides a mechanism to generate infrequently cycling tumor cells, providing a general resistance mechanism against cytotoxic chemotherapy treatments designed to target actively cycling cells (110).

Why do polyploid tumor cells cycle less frequently? Seminal studies conducted in yeast show that increased transcription and translation caused by elevated genomic content causes cell-cycle delays during G_1 (37). Murine embryonic fibroblasts (MEFs) containing extra chromosome copies also cycle less frequently (112, 113), likely due to changes in transcription and translation (85). In addition, polyploid tumor cells have a twofold larger cell volume compared to their diploid counterparts (110). Cell growth, cell size, and cell division are co-regulated to ensure cells are large enough to divide at mitosis (114). Studies in yeast reveal a size requirement for G_1-S transition, with smaller cells delaying in G_1 until a sufficient size was reached to maintain viable progeny after cell division (115, 116). Complementary studies in animal cells show that mammalian cells also delay in G_1 to allow an appropriate cell size to be achieved (117, 118). A plausible hypothesis that combines both these observations is that the larger polyploid tumor cells arrest during G_0/G_1 to allow for a sufficient growth to occur before committing to division, which is hampered due to the increased transcription and translational demands placed on polyploid tumor cells by their elevated and unbalanced chromosomal copy number.

Thus increased ploidy provides a general resistance mechanism (that of infrequent cell cycle) to tumor cells, which are well-positioned to contribute to the rapid evolution of patient tumors during conventional chemotherapy and radiotherapy regimes.

THE ROLE OF GIANT POLYPLOID CELLS IN THERAPY RESISTANCE AND TUMOR REPOPULATION AFTER THERAPY

Giant polyploid cells are formed if DNA replication is uncoupled from mitosis (119). This process has been termed the endocycle and is a characteristic of p53-null cells (120), which is further increased by exposure to radiation (121). It was thought that the process of endocycles was irreversible and the resulting giant polyploid cells represent a reproductive dead end (122). However two back-to-back manuscripts suggested that giant polyploid cells may provide an escape mechanism from severe genotoxic damage. The first study followed p53-null cells after genotoxic insult, noting that after delaying at G2/M for several days the cells enter endoreplication cycles that generate giant polyploid cells (123). Although the majority of giant polyploid cells die, a small subset survive that are able to produce viable progeny cells as determined using sensitive clonogenic assays (123). Viable giant polyploid cells appear to follow a defined path of chromosome re-organization that involves reconstructing nuclei into polyploidy "bouquets," which subsequently return to an interphase state and separate into secondary nuclei (124). These secondary nuclei give rise to secondary cells in a manner reminiscent of the life-cycles of protozoa (124).

Looking at two forms of transformation, carcinogen-induced transformation of $p53^{+/+}$ cell lines and spontaneous transformation of $p53^{-/-}$ cell lines Sundaram et al. reported a transformation process that involved giant polyploid cell intermediates (125). Here, the giant polyploid cells undergo a novel type of cell division that involves nuclear budding within the giant polyploid cells followed by intracellular cytokinesis to produce mononuclear daughter cells that bud off the parental giant polyploid mother cells (125). These mononuclear daughter cells are transformed, displaying anchorage-independent growth (a classical hallmark of cellular transformation) (125). A series of follow-up studies provided strong support for the hypothesis that a subset giant polyploid cells undergo some form of reductive division to produce small cells with near-diploid chromosomes that are proliferative and competent to re-initiate tumor growth (reviewed in Ref. (126)]. Interestingly, irradiated giant polyploid cells activate key meiotic genes that are involved in metaphase arrest, genetic recombination, and reductive divisions that occur during meiosis, indicating that giant polyploidy reductive divisions are likely "meiosis-like" in nature (127–130).

Puig et al. undertook a systematic study using xenograft *in vivo* models and *in vitro* approaches to characterize the role of giant polyploid cells in therapy response to cisplatin (131). Cisplatin treated tumors initially undergo shrinkage, and are increasingly populated with giant non-proliferating tumor cells that maintain DNA synthesis (131). After several weeks of latency tumor growth recurs, driven by a small fraction of proliferating cells (131). Cells treated *in vitro* using clinically relevant cisplatin doses also generate giant polyploid cells, a subset of which are able to generate colonies of rapidly cycling small diploid cells. This recapitulated the *in vivo* disease recurrence and suggested that giant polyploid cells are active contributors to disease progression after therapy (131). Intriguingly, the proliferative diploid cells generated from giant polyploidy cells have altered karyotypes and display increased resistance to cytotoxic drugs (131), suggesting for the first time that giant polyploid cells actively contribute to the evolution of therapy resistance.

Very recent work studying ovarian cancer has underscored the importance of giant polyploid cells in cancer disease progression and therapy resistance (132). Zhang et al. purified giant polyploid cells from established ovarian cancer lines and patient tumors, and confirmed that these cells can initiate tumors *in vivo* and are resistant to cisplatin cytotoxic therapy (132). Like previous studies, Zhang et al. confirmed that giant polyploidy cells cycle infrequently and generate smaller near-diploid progeny through budding and bursting mechanisms (132). In this way, giant polyploid cells are posited to function in a manner analogous to spores in lower organisms, surviving harsh conditions to facilitate rapid repopulation after stressful conditions have subsided (132, 133).

POLYPLOIDY, EMT, AND THE CANCER STEM-CELL PHENOTYPE

Cells with a primitive, undifferentiated phenotype tend to cycle infrequently and display enhanced DNA repair, making them difficult to kill using cytotoxic and genotoxic therapies that preferentially target actively cycling cells (134–136). The underlying drivers leading to the generation of a primitive phenotype in patient tumors remain incompletely understood. It has been reported

that the frequency of CSC's increases after treatment with genotoxic therapies (137–139). Salmina et al. tested the hypothesis whether polyploidy, which allows cells to survive cytotoxic therapy to continue proliferation, is also capable of endowing cells with a primitive cell phenotype (140). They found that irradiated giant polyploidy cells caused up regulation of the self-renewal stem-cell genes OCT4 and NANOG, and that the NANOG, OCT4, and SOX2 proteins were concentrated onto nuclear foci in giant polyploidy cells (140). The giant polyploid cells resisted apoptosis, overcame TIS, and transmitted the NANOG-OCT4-SOX2 self-renewal program to their progeny (140). Subsequently Lagadec et al. reported that ionizing radiation reprogramed differentiated breast cancer cells toward an undifferentiated CSC state (141). Strikingly, CSC reprograming only occurred within polyploidy subpopulations, and involved re-expression of the transcription factors OCT4, NANOG, sex determining region Y-box 2 and Klf4 (141). More recently, Zhang et al. demonstrated that ovarian cancer giant polyploid cells displayed the CSC properties of $CD44^{+}/CD133^{+}$ expression, generation of spheroids under serum-free culture conditions, increased tumorigenicity, and elevated therapy resistance (132).

Cancer cells can also undergo epithelial to mesenchymal transition (EMT), where the cancer cells activate an evolutionarily conserved trans-differentiation program that is used during morphogenesis to convert differentiated epithelial cells into migratory mesenchymal cells [reviewed in Ref. (142)]. Cancer cells undergoing EMT not only adopt an invasive cell phenotype that can drive metastasis, but may also enter a drug refractory state due to epigenetic reprograming (142). Recent work has revealed that polyploidy facilitates EMT, with Zhang et al. showing that giant polyploid tumor cells gain a mesenchymal phenotype (132) that correlates with increased expression levels of EMT transcriptional factors (143). These data suggest that polyploidy can facilitate EMT, providing access to cell phenotype that is both invasive and resistant to a variety of therapies.

Together, these studies provide compelling support for the hypothesis that polyploidy drives the acquisition of undifferentiated, primitive cellular phenotypes in human cancer. These cell phenotypes can potentially increase therapy resistance, provide an elevated tumor initiation capacity, and increase both the invasive and metastatic potential of tumor cells.

PLOIDY-INDUCED ESCAPE FROM TARGETED ANTI-CANCER THERAPIES

Strong evidence supporting the role of polyploidy in evolving solutions to targeted therapy has come from mouse models of cancer. A defective spindle assembly checkpoint (SAC) results in "mitotic slippage," where cells exit mitosis without undergoing anaphase or cytokinesis to produce a tetraploid cell [reviewed in Ref. (69)]. As essential component of the SAC is Mad2, and Mad2 over-expression commonly occurs in many human cancers and is associated with poor prognosis [reviewed in Ref. (68)]. Over-expression of Mad2 increases the frequency of mitotic slippage and tetraploidy (68, 69), and promotes tumorigenesis in mice (69). In a doxycycline-inducible K-Ras model of cancer, Sotillo and colleagues explored how Mad2 over-expression determined the tumors ability to escape inhibition of the primary oncogenic driver K-Ras (144). In these experiments, Sotillo et al. allowed K-Ras tumors to form in the presence or absence of Mad2, revealing that the presence of Mad2 expression increased the aggressiveness of the K-Ras tumor, as indicated by increased invasion, elevated proliferative index, and a significant decrease in overall survival (144). When doxycycline was removed, K-Ras and Mad2 expression was lost, leading to tumor regression in all animals. K-Ras only tumors recurred rarely, however the tumors expressing both K-Ras and Mad2 displayed a marked increase in recurrence rate, driven by activation of a variety of compensating transforming pathways (144). This finding supports the hypothesis that CIN increases the probability of disease relapse during targeted therapy by facilitating alternate pathway activation that allows tumor cells to avoid the effects of targeted therapy (144). Further, this study highlights how aneuploidy and oncogenes can act synergistically during tumor initiation and cancer evolution.

The proteasome inhibitor, bortezomib, has forged new horizons in the treatment of multiple myeloma (MM) (145). Although efficacious, bortezomib is non-curative for MM because patients eventually evolve therapy resistance. However, the underlying resistance mechanisms remain poorly understood (146). To begin to characterize resistance mechanisms, Balsas et al. generated bortezomib-resistant MM lines that displayed five to sixfold increased resistance to bortezomib (147). Unexpectedly, the target of bortezomib (PSMβ5, the β5 subunit of the proteasome) was not mutated, but was instead significantly overexpressed at both the mRNA and protein levels within resistant cells (147). In addition, the bortezomib-resistant cells had evolved a near-tetraploid genomic content, which also displayed cross-resistance to other chemically unrelated proteasome inhibitors (147). Together, these data provide direct support for the hypothesis that, as for yeast, increasing genomic content allows cancer cells to circumvent targeted therapy through over-expression of the therapy target.

An interesting and unwelcome twist to the use of targeted therapy came from study of Sharma et al. (148). When Sharma et al. treated several breast cancer lines with the tyrosine kinase inhibitor BMS-777607, they noted that the surviving cell population displayed elevated levels of polyploidy due to an increase in the incidence of failed cytokinesis caused by off-target inhibition of Aurora kinase B (148). They tested the surviving polyploidy cells for sensitivity toward a range of chemotherapeutics (doxorubicin, bleomycin, cisplatin, methotrexate, and paclitaxel), and found that the therapy-induced polyploidy cells were resistant to all classes of chemotherapies tested (148). This finding is reminiscent of an evolutionary study undertaken in yeast, where transiently targeting the function of Hsp90 protein led the chromosomal gains and the rapid evolution of therapy-resistance toward unrelated cytotoxic compounds (149).

Together these studies reveal that tumor cell polyploidy generates resistance toward targeted therapy. Of concern is the finding that treating tumor cells with targeted therapies can elevate levels of polyploidy in tumor cell populations, which then increases the risk of developing multi-drug-resistance within clinical settings.

MEASURING THE PREVALENCE OF POLYPLOID TUMOR CELLS IN CANCER

How many polyploid cells are there in patient tumors? Quantitation of polyploidy in patient cell lines and primary tumors

is challenging due to the infrequent cell cycle of polyploid tumor cells. Cancer cell biologists have traditionally used a flow-cytometry approach, where they estimate the frequency of polyploidy by measuring the number of cells with greater than 4n DNA content. However, this approach can only detect polyploid cells that are actively cycling, because most of the polyploid tumor cells are tetraploid or near-tetraploid, and therefore remain indistinguishable from the G_2/M cells of the "diploid" tumor population. More sophisticated metaphase analyses [for example those in Ref. (62)] are also likely to underestimate the frequency of polyploid tumor cells, because their infrequent cell cycle means they will be under-represented using a metaphase-dependent karyotypic analysis.

Flow-cytometry screens can utilize the expression of Cyclin-B1 to discriminate between the cycling tumor "diploid" cells that are transitioning through the G_2/M phase of the cell cycle, from the polyploid tumor cells in that are in the G_0/G_1 phase of the cell cycle (**Figure 1**). The advantage of this assay is that it allows the assessment of large populations of cells (in this example, 100,000 single cells for each patient line were analyzed), and the inclusion of a control line in the same tube means that the ploidy levels of tumor cells relative to control cells can be estimated within the same assay under identical staining conditions, eliminating the confounding effects of cell number variation between tubes [**Figure 1**; Ref. (110)]. Here, we use the prodrug carboxyfluorescein diacetate succinimidyl ester (CFSE), which is converted by cellular esterase activity into a fluorescent compound covalently bound to proteins and retained within the cells (150). CFSE staining clearly delineates the CFSE-stained control from the unstained test cell populations, and the control and test cell populations are readily identified using standard flow-cytometry gating strategies (110). CFSE-stained control lines can be either healthy diploid cells to provide a more accurate estimate of DNA content [(110) and shown in **Figure 1**], or alternatively untreated tumor cells can be used to directly compare the effect of drug treatments on the prevalence of polyploidy during compound screening or pre-clinical testing. Because control cells are stained with CFSE immediately before fixation (110), the staining process has no effect on cell ploidy or viability and is therefore unlikely to generate Type I or Type II errors during compound screening.

Using this method, we assessed the prevalence of polyploidy in 10 low-passage primary patient glioblastoma lines (**Figure 1**), cultured under tumorsphere conditions, a culture method that preserves the genotype, and phenotype of the original tumor (151). In 10 primary patient tumor lines, the lowest frequency of tumor cell polyploidy was 1 in 20 cells (i.e., 5% of the total tumor cell population were polyploid). To put this into context, it is estimated that a 1 g solid tumor contains 10^8–10^9 tumor cells (74, 152). If the lowest polyploid estimate of 5% is applied, then between 5 and 50 million rapidly evolving, therapy-resistant polyploid tumor cells will be present in brain cancer patients that have tumor volumes of 1 cm^3.

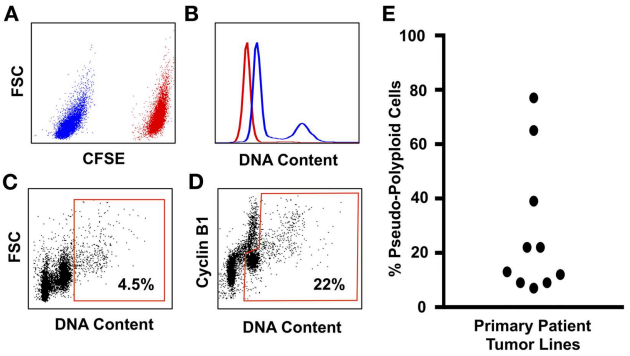

FIGURE 1 | An improved flow-cytometry assay for measuring the prevalence of polyploidy in tumors cell populations. **(A)** Tumor cell samples are spiked with carboxyfluorescein diacetate succinimidylester (CFSE) stained primary neonatal foreskin fibroblasts (NFF) diploid control. The CFSE-negative tumor cells shown in blue are readily gated from the CFSE-high NFF diploid controls, shown in red. **(B)** DNA content of the Glioblastoma tumor cells (blue histogram) overlayed onto the NFF diploid control histogram, shown in red. Most Glioblastoma cell lines that we have studied are aneuploid with a slightly hyperdiploid DNA content, and contain a small sub-population of cells that are near-tetraploid with respect to the tumor bulk population (i.e., pseudo-polyploid). **(C)** A typical polyploidy flow-cytometry assessment utilized by many cancer cell biologists, who use the proportion of live single cells with greater than 4n DNA (shown within the red gate) as being representative of the total pseudo-polyploid population. In this example, 4.5% of the total cells are classed as pseudo-polyploid. **(D)** The same tumor sample assessed for pseudo-polyploidy using Cyclin-B1 staining to discriminate between the G_2/M (the Cyclin-B1 *high* cells with a 4n DNA content) population of the pseudo-diploid bulk, from the pseudo-diploid G_0/G_1 population (the Cyclin-B1 *low* cells with a 4n DNA content). The pseudo-polyploid gate (shown in red) identifies both the cycling *and* the non-cycling pseudo-polyploid tumor cells, which make up approximately 22% of the total tumor cell population. **(E)** Ten low-passage primary patient glioblastoma cell lines, grown under serum-free tumorsphere conditions, assessed for pseudo-polyploidy using the Cyclin-B1 gating strategy from **(D)**. In eight lines, the dominant cell population was aneuploidy with a near-diploid DNA content, with a sub-population of pseudo-polyploid cells that made up 5–38% of the total cell population. In contrast, two glioblastoma lines were pre-dominantly pseudo-polyploid (65 and 78%), with a small of near-diploid sub-population. Detailed staining protocols are provided in Ref. (110).

AN INTEGRATED MODEL EXPLAINING HOW INCREASED GENOMIC CONTENT FACILITATES CANCER EVOLUTION

From the perspective of cancer as an evolutionary disease, we argue that the studies summarized above provide sufficient grounds for the development of an updated model of cancer that highlights a central role of polyploidy during tumorigenesis and disease progression (**Figure 2**). The hallmarks of cancer outlined by Hanahan and Weinberg (153) clearly highlight the selection pressures that must be overcome on the journey from pre-malignant lesion to full-blown cancer. The early selection pressures include apoptosis, senescence and terminal differentiation. We argue that pre-malignant polyploid cells are more likely to overcome these barriers than diploid pre-malignant cells. Polyploidy enables epigenetic silencing of p53 (84), reducing the probability of apoptosis and weakening the senescence barrier. Polyploidy also rewires the DNA-damage response (84), further subverting the senescence barrier and increasing the probability of pre-malignant polyploid cells re-entering the cell cycle (84). The vast majority of cells are terminally differentiated. Differentiated pre-malignant cells must somehow overcome the terminal differentiation program,

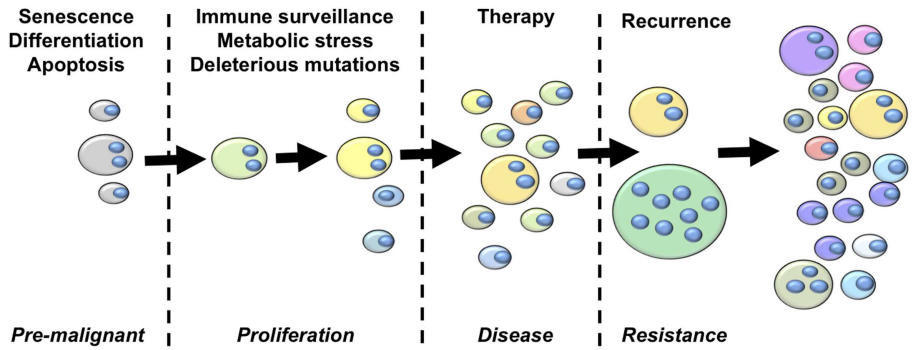

FIGURE 2 | An integrated model of tumor evolution highlighting potential roles of polyploidy during cellular transformation. Here, we present a simplified view of disease progression, highlighting the role of polyploidy in overcoming selection pressures to drive the evolution of cellular transformation. The fist selections pressures pre-malignant lesions must overcome are those of apoptosis, senescence and terminal differentiation. Polyploidy enables adaptation to these barriers by silencing p53 remove p53-dependent pro-apoptotic and senescence signaling, rewiring the DNA-damage response to suppress p53-independent senescence programs, and enabling acquisition of primitive stem-cell phenotypes. Once a proliferative state is reached, polyploidy increases the acquisition of transforming mutations by increasing chromosomal instability and buffering the proliferative cells against the effects of deleterious mutations. Polyploidy also increases glycolysis, enabling survival in low oxygen environments, and enables EMT and the generation of invasive and metastatic phenotypes. Polyploid cells provide multiple mechanisms of therapy resistance, buffer the cancer genome against deleterious mutation resulting from genotoxic therapies, and generate primitive tumor-initiating phenotypes that are capable of driving disease recurrence. Throughout this process, tumor heterogeneity and karyotypic complexity increases, which in turn increase the heterogeneity and evolutionary capacity of the tumor.

revert to an undifferentiated phenotype, and reclaim the unlimited proliferative capacity of multi-potent stem cells (154). We now know that polyploidy facilitates acquisition of a primitive, stem cell like phenotype (132, 140, 141), although the underlying mechanisms remain incompletely uncharacterized.

Once pre-malignant cells circumvent these initial selection pressures to generate a proliferative phenotype, they must then acquire further transforming mutations to overcome subsequent selection pressures such as immune-surveillance, metabolic stressors, and the effects of deleterious mutation (154). Polyploidy is likely to facilitate the rapid acquisition of new transforming mutations in two ways. First, elevated ploidy reduces the lethality of deleterious mutations and chromosome loss (44, 73, 74). Second, polyploidy increases CIN (70, 71, 73, 141, 155, 156), which elevates karyotypic variation within the tumor cell population through large-scale genetic change (74). Hence, polyploidy enables both CIN and mutator phenotypes, thereby greatly increasing the speed at which proliferating tumor precursor cells can acquire the portfolio of mutations and the oncogenic karyotypes necessary for full-blown transformation (3, 61). In addition, polyploidy can help overcome metabolic stress by contributing to metabolic reprograming, invasion, and metastasis. Polyploid tumor cells display elevated levels of anaerobic glycolysis (110, 157) and are highly resistant to oxygen deprivation (132). Polyploid tumor cells also increase the expression of metastasis-related proteins (143), which may enable the acquisition of metastatic phenotypes by driving EMT (132).

Once disease presents and treatment commences, polyploid, and hyperdiploid cells remain key players driving the ongoing evolution of the patient disease. Elevated ploidy provides cells with multiple therapy-resistance mechanisms including infrequent cell cycle (110, 132), acquisition of primitive, therapy-resistant cell phenotypes (132, 158), over-expression of therapeutic targets leading to resistance (147), alternate pathway activation leading to therapy escape (144), as well as facilitating acquisition of the dreaded multi-drug resistant phenotype (148, 149). Polyploid tumor cells are created by cytotoxic and targeted therapies (149, 158–160), therefore the frequency of tumor cells with elevated ploidy is likely to significantly increase during therapy. Further, many front-line therapies are genotoxic mutagens. In this scenario, the therapy itself imparts a mutator phenotype onto the tumor, with polyploidy functioning as a genetic buffer to reduce the effects of deleterious mutations, increasing the probability of beneficial mutations surviving within the polyploidy sub-population to drive disease recurrence.

The adaptive capacity inherent to polyploidy cells means that even a small sub-population of surviving polyploid tumor cells are able to drive disease recurrence (39, 161). The capacity of polyploid tumor cells to repopulate post-therapy is likely to be significantly enhanced due to the ploidy-driven acquisition of a primitive cell phenotype with an elevated tumor-initiating capacity (132, 141), combined with a greatly reduced competition for resources due to the competing non-resistant tumor cells being killed off during therapy (162).

For these reasons, we predict that polyploid tumor cells play an integral role in disease recurrence and the acquisition of a therapy-resistant, increasingly malignant disease in patients during therapy.

NEW THERAPEUTIC STRATEGIES THAT TARGET POLYPLOIDY AND HYPERDIPLOID TUMOR CELL SUBPOPULATIONS

Experiments in yeast and cancer model systems have shown that the presence of polyploidy generates points of fragility within cellular systems that can be targeted using specific therapeutics (163–168). These studies provide the critical proof-of-principle that polyploidy is in fact a druggable phenotype. However,

the strategies proposed in these pioneering studies target stress responses or mitotic machinery, which poses the risk of increasing polyploidy in surviving cells (148, 149). Fortunately, recent studies have identified new avenues for therapy development that could be used in conjunction with established therapies to inhibit the formation of polyploidy tumor cells and decrease the adaptive capacity of tumors *in vivo*.

TARGETING METABOLISM TO ATTACK POLYPLOIDY AND HYPERDIPLOID TUMOR CELLS

Cell size scales linearly with DNA content in Eukaryotes (169–172). Cancer polyploid cells are proportionally larger than the euploid bulk population (110, 147), with giant polyploid cells being much larger than the euploid population (124). One potential consequence of large genome size and increased cell volume is a heightened metabolic demand, as bigger cells require more energy to grow to a sufficient cell volume to allow for cell doubling (117, 118). Further, the increased mRNA and protein expression caused by increased ploidy also demands more energy consumption (37). Consistent with increased genome size cell volume, and elevated transcription and translation, we noted polyploid tumor cells displayed a higher metabolic rate than the euploid control population (110).

The large cell size and increased metabolism of polyploid tumor cells may represent a point of fragility specific to the polyploid sub-population that could be exploited therapeutically. To test this hypothesis, we treated parental euploid and polyploid clonal cultures with the 2-deoxy-D-glucose, an established inhibitor of glycolysis (173–176). We found that the brain tumor polyploid cells were significantly more sensitive to the effects of glycolysis inhibition then the euploid parent control (110).

Critically, this increased dependence of polyploidy cells on glycolysis has been reported across several types of cancer. In acute myeloid leukemia (AML), Liu et al. demonstrated that targeting aurora kinases with specific inhibitors increased the prevalence of polyploidy in AML cells, and that AML polyploidy cells displayed increased glycolysis as measured by increased glucose uptake and lactic acid production (157). AML polyploidy cells were sensitive to the effects of 2-DG, suggesting that targeting metabolism may preferentially kill polyploidy tumor cells (157). mTOR is a conserved serine/threonine kinase that links cell signal transduction with cell metabolism and growth (177). Specific mTOR inhibitors promoted apoptosis and autophagy in polyploidy tumor cells and increased the efficacy of Aurora kinase inhibitors, confirming tumor metabolism as a viable point of therapeutic intervention against AML polyploidy tumor cells (157). Using breast cancer cells, Sharma et al. used the tyrosine kinase inhibitor BMS-777607 to induce polyploidization in breast cancer lines, and confirmed that therapy-induced polyploidy cells were resistant to the effects of a variety of chemotherapies (148). They then performed drug screens looking for additional inhibitors that specifically targeted polyploidy tumor cells, and identified that an inhibition of mTOR signaling prevented the formation of therapy-induced polyploidy and maintained the sensitivity of breast cancer cells toward the effects of chemotherapies (148). These results indicate that reducing polyploidy tumor cell formation by targeting metabolism may delay the evolution of therapy resistance (148). More recently, the same group revealed that BMS-777607 induced polyploidization in pancreatic cancer cells, which displayed pan-resistance to a range of chemotherapeutic compounds (160). Targeting metabolism using mTOR inhibitors reduced the formation of therapy-resistant polyploidy cells and synergized with BMS-770607, showing that for pancreatic tumor cells targeting tumor metabolism prevents the emergence of therapy-resistant polyploidy tumor cells (160).

Together, these results support the hypothesis that in brain, breast, leukemia, and pancreatic cancers, polyploid tumor cells have a commensurately higher metabolic requirement than euploid tumor cells, and that inhibiting metabolism is an effective therapeutic strategy to specifically target polyploid tumor cells to maintain tumors in a drug sensitive state.

STIMULATING AMP KINASE ACTIVITY USING RESVERATROL AND ASPIRIN

Lissa et al. used an elegant high-throughput screening to screen a compound library for drugs that preferentially kill tetraploid cells, identifying resveratrol as an anti-tetraploid therapeutic agent (178). Resveratrol is an anti-fungal agent naturally occurring in grapes that has been reported to reduce tumor formation in a genetic mouse model of intestinal carcinoma when administered orally (179). Resveratrol stimulates AMPK activation by inhibiting phosphodiesterase 4 (PDE-4), allowing cAMP accumulation in cells and subsequent activation of protein kinase A (PKA) (180). Consistent with this being the primary mode of tetraploid killing, resveratrol treatment activated AMPK in tetraploid cells (180). Activation of AMPK using a separate PDE-4 inhibitor or overexpression of AMPK selectively killed tetraploid cells, whereas PKA inhibitors specifically blocked resveratrol killing (178). Aspirin (acetyl salicylate) and it is more active derivative salicylate also activate AMPK, and consistent with other AMPK activators were shown to selectively kill tetraploid cells (178). Using the mouse intestinal carcinoma cancer model, the Authors then confirmed that oral administration of either resveratrol or aspirin reduced the frequency of tetraploid intestinal epithelial cells (178), confirming that resveratrol and aspirin preferentially kill tetraploid cells *in vivo* using clinically relevant therapeutic doses (178).

Together, these data show that activating AMPK using the natural products resveratrol and aspirin can be used to specifically target tetraploid tumor cells *in vitro* and *in vivo*, potentially explaining the cancer-preventing effects of these two compounds reported using mouse models of tumor initiation (181). These findings support the exciting hypothesis that targeting tetraploid malignant precursor cells may become an effective chemopreventative strategy for humans.

CONCLUSION AND FUTURE DIRECTIONS

Cancer evolution has been intensely studied in recent years using cutting edge genomic approaches (182–185), and novel therapeutic strategies aimed at delaying tumor evolution are being developed using increasingly sophisticated and predictive computational models of tumor evolution (162, 186–189). Here, we have presented a complementary approach, which consists of identifying and therapeutically targeting the polyploid tumor cell subpopulations that are likely to facilitate rapid evolution. We have

based our argument on evidence derived from yeast and cancer model systems, as well as from primary patient tumor samples, all of which support the hypothesis that polyploidy facilitates rapid evolution and the acquisition of therapy-resistant phenotypes in cancer patients.

We have also presented recent studies that identify promising new anti-polyploid therapeutic approaches, which could potentially be used to target polyploid tumor cells in cancer patients. We predict that the therapies stemming from these pioneering studies will be successfully translated and incorporated into novel anti-evolution therapies designed using systems biology approaches, which will significantly increase cancer patient lifespan by slowing the emergence of therapy resistance, as well as being used as chemopreventative agents to reduce the incidence of cancer.

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Chemotherapeutic compounds targeting the DNA double-strand break repair pathways: the good, the bad, and the promising

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The repair of DNA double-strand breaks (DSBs) is a critical cellular mechanism that exists to ensure genomic stability. DNA DSBs are the most deleterious type of insult to a cell's genetic material and can lead to genomic instability, apoptosis, or senescence. Incorrectly repaired DNA DSBs have the potential to produce chromosomal translocations and genomic instability, potentially leading to cancer. The prevalence of DNA DSBs in cancer due to unregulated growth and errors in repair opens up a potential therapeutic window in the treatment of cancers. The cellular response to DNA DSBs is comprised of two pathways to ensure DNA breaks are repaired: homologous recombination and non-homologous end joining. Identifying chemotherapeutic compounds targeting proteins involved in these DNA repair pathways has shown promise as a cancer therapy for patients, either as a monotherapy or in combination with genotoxic drugs. From the beginning, there have been a number of chemotherapeutic compounds that have yielded successful responses in the clinic, a number that have failed (CGK-733 and iniparib), and a number of promising targets for future studies identified. This review looks in detail at how the cell responds to these DNA DSBs and investigates the chemotherapeutic avenues that have been and are currently being explored to target this repair process.

Keywords: DNA damage repair, chemotherapeutic compounds, DNA damage response, cancer, radiosensitize, radioprotective, DNA double-strand break

INTRODUCTION

Genomic stability at a cellular level requires precise, tightly coordinated pathways to detect DNA damage and either repair the damage or, if the damage is too great, ensure the cell dies via apoptosis or enters senescence. Organisms have evolved complex DNA damage response (DDR) pathways to respond to insults to the DNA either from endogenous (cellular metabolic pathways, reactive oxygen species, and errors in DNA replication) or exogenous sources [environmental factors including ionizing radiation (IR) and ultra violet radiation].

Cellular DNA damage that is not repaired correctly can lead to genomic instability, apoptosis, or senescence, which can greatly affect the organism's development and aging process and in addition can predispose the organism to immunodeficiency, neurological disorders, and cancer.

DDR AND REPAIR PATHWAYS

Following the initial work on the DDR in yeast, investigations into the DDR in mammals have yielded a highly conserved and elaborate process. This process mainly controls DNA repair (ensuring genomic stability) and cell cycle checkpoints, however it has also been shown to be involved in circadian rhythms (1), insulin signaling (2), and telomere maintenance (3).

The DDR pathway encompasses a set of tightly coordinated processes: detection of DNA damage, a protein cascade to enhance the signal, the accumulation of repair factors at the site of damage, and physical repair of the damage. The DDR also induces cell cycle checkpoints to ensure the damaged cells do not continue dividing until the DNA damage is repaired. To ensure genomic stability, the DDR must be able to recognize all types of DNA structural alterations, including nicks, gaps, stalled replication, and double-strand breaks (DSBs).

Depending on the type of DNA lesion, there are a number of DNA repair pathways available for the cell to repair the alteration, including homologous recombination (HR) and non-homologous end joining (NHEJ) for DNA DSBs; and mismatch repair (MMR), nucleotide excision repair (NER), and base excision repair (BER) for single DNA strand damage.

Highlighting the importance of the DDR, mutations in a number of repair proteins lead to human syndromes, which include multiple cancers, immunodeficiency, and genomic instability phenotypes. Ataxia telangiectasia mutated (ATM), a protein involved in the DDR is mutated in the syndrome ataxia telangiectasia (AT) (4). AT is a cancer-prone syndrome that also includes progressive cerebellar ataxia, telangiectasia's of the conjunctivae, and immunodeficiency. Consistent with ATM's role in the DDR, AT patients presented a high level of sensitivity to radiation (5). Nijmegen breakage syndrome (NBS) is another syndrome where the key cause of the disease is a mutation in a protein involved in the DDR, NBS1. NBS1 is involved in the detection of DSBs as part

of a complex of proteins including Mre11 and Rad50. NBS is a cancer-prone syndrome that is also characterized by progressive microcephaly, short stature, and progressive ovarian failure in females (6).

Current chemotherapeutic compounds development is largely focused in targeting proteins specific to pathways important to the development, growth, and progression of cancer. DSBs are the most deleterious lesion to cells, where unrepaired DSBs can lead to cell death and incorrectly repaired DSBs have the potential to produce chromosomal translocations and genomic instability, potentially leading to cancer. Targeting the repair proteins involved in the repair of DSBs with chemotherapeutic compounds has the potential for cancer therapies in conjunction with radiation therapy or as a monotherapy.

CHEMOTHERAPEUTIC COMPOUNDS

Double-strand breaks are highly cytotoxic and this fact is exploited in conventional cancer treatment, with radiation therapy and chemotherapeutic drugs treatments generating vast amounts of DSBs. These include chemotherapeutic drugs that induce DNA cross-links or function as topoisomerase inhibitors, inducing the generation of DSB's in all cells. However, cancer cells are much more susceptible to these drugs, as they are rapidly dividing and often have inactivated components of their DNA repair machinery and deregulated cell cycle checkpoints (7).

However, these chemotherapeutic drugs will also target normal proliferating cells that are dividing as part of their normal processes. These naturally regenerating tissues include bone marrow, gastrointestinal tract, liver, and hair follicles. The formation of secondary hematologic and solid tumors after DNA-damaging therapies is a potential issue for patients undergoing treatment (8).

A number of chemotherapeutic compounds are used in conjunction with radiotherapy or in combination with other chemotherapeutic agents to produce a synergistic effect. The use of radiosensitizing agents that increase the cytotoxic effects of radiation on cancer cells and radioprotective agents that decrease the adverse effects of radiation on normal cells (by increasing their radioresistance) is common. The use of radiosensitizing agents can greatly enhance the efficacy of radiotherapy and genotoxic drugs. Recently, chemotherapeutic compounds have been studied that may also be useful as a monotherapy, where the chemotherapeutic compound achieves what is termed as "synthetic lethality." Synthetic lethality exploits the fact that many cancer cells acquire defects in DNA repair pathways and become dependent on a compensatory mechanism in order to survive (9, 10). Inhibition of the complementary DNA repair pathway selectively kills cancer cells that have a defect in a particular DNA repair pathway.

The safety, tolerability, pharmacokinetics, and efficacy of potential chemotherapeutic compounds have to be carefully validated before they may enter clinical trials to determine the benefits for cancer therapy. This means there is a significant delay between the initial discovery of a potential chemotherapeutic compound in the laboratory, to an actual clinical outcome for patients, however this delay ensures patient safety.

This review will focus on the pathways responsible for the repair of DSBs, namely HR and NHEJ, and the current chemotherapeutic compounds that are being investigated that target these repair pathways.

THE DDR TARGETS AND CHEMOTHERAPEUTIC COMPOUNDS

DNA DSBs are considered the most cytotoxic of DNA lesions. Cells are estimated to accumulate around 50 endogenous DSBs per day, mostly induced by reactive oxygen species (11). In response to DSBs, the DDR utilizes two main pathways to repair the damage. During late S-phase and the G2 phase, cells have a sister chromatid available as a template for targeted HR, which allows for error-free repair of the DNA damage. However, DSBs that occur when there is no sister chromatid available are repaired via NHEJ, which is more error-prone than HR. NHEJ is also active in S and G2 phases of the cell cycle and remains the predominant pathway by which cells repair DSBs. In NHEJ, the two ends of the break are joined together (ligated), though this can involve resection with the consequent loss of genetic material (12). Cancer therapy agents induce DSBs including IR and topoisomerase II poisons, and also indirectly via single-stranded DNA (ssDNA) lesions which induce replication forks collapse, leading to DSB formation (13).

HOMOLOGOUS RECOMBINATION AND CHEMOTHERAPEUTIC COMPOUNDS TARGETS

Following the induction of a DSB, the Mre11/Rad50/NBS1 (MRN) complex is recruited to the break site by human single-stranded binding protein 1 (hSSB1) (14–16). MRN binds to the DNA surrounding the lesion and resects the DNA around the break in a 5′–3′ dependent direction. This acts as a signal to recruit other DDR proteins. This resection by the MRN complex is stimulated in the early stages of HR by an interaction with CtIP (17, 18). Following initiation of resection by Mre11, Exo1 performs more extensive resection to expose long stretches of ssDNA (19–21). Replication protein A (RPA), a ssDNA binding heteromeric complex, binds to the exposed ssDNA and is retained at the lesion site by BRCA1 (22). The binding of RPA to the ssDNA substrate ensures that secondary structures are not formed in the DNA and protects the ssDNA from nucleases. RPA is displaced from the DNA by the recombinase Rad51, which is loaded by BRCA2. Rad51 forms a nucleoprotein filament along the ssDNA and functions to allow strand invasion of the sister chromatid (23). Once the DSB is resolved, the DNA is ligated together, completing the process of HR (24). There are a number of key proteins involved in HR that are currently therapeutic targets or have been identified as potential targets (see **Figure 1**).

THE MRN COMPLEX

Human single-stranded binding protein 1 serves as the primary sensor of DSBs and is also involved in the early steps of HR through the recruitment of the MRN complex (14–16). Once recruited, the MRN complex specifically functions in the resection of DNA ends, activates the ATM kinase, and subsequently activates the cell cycle checkpoints (25). The human syndromes that result from mutations in each component in the MRN complex highlight the requirement of the MRN complex for the maintenance of genomic stability: NBS (26), AT-like disorder (AT-LD) (27), and NBS-like NBS disorder (28) result from NBS1, Mre11, and Rad50 mutations, respectively. The MRN complex is also indispensible in

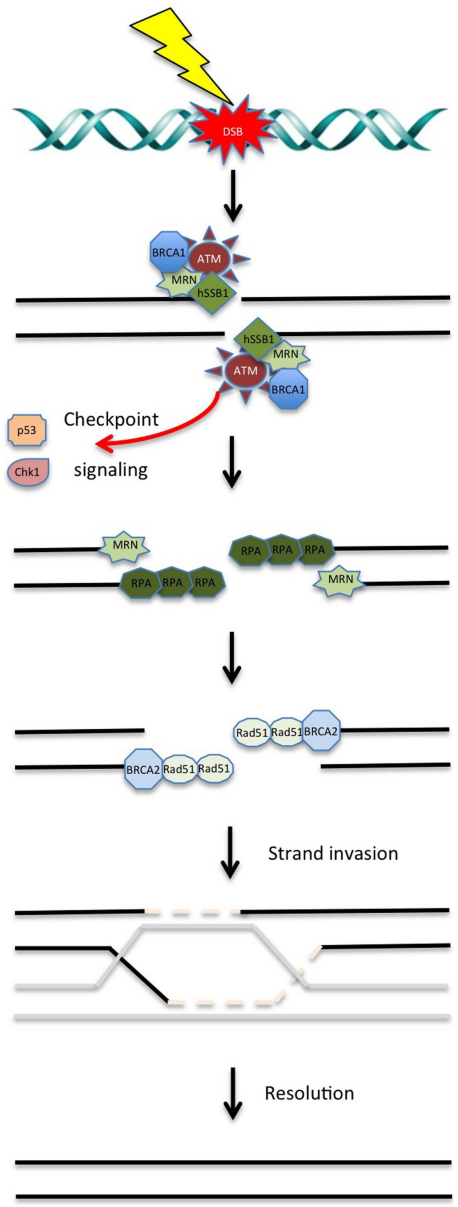

FIGURE 1 | DNA double-strand break repair via homologous recombination. During S and G2 phases of the cell cycle, DSBs can be repaired via HR using a sister chromatid. Targeting of these proteins involved in HR with chemotherapeutic compounds shows promise in the clinical setting. See text for details.

development, as null-mutations of these genes cause embryonic lethality in mice (29–31).

A study using a forward genetic screen identified a specific small molecule inhibitor of the MRN complex, dubbed Mirin. Mirin inhibits MRN-dependent ATM activation and disrupts the endonuclease activity of Mre11, which leads to the failure of the G2/M checkpoint and HR repair (32). More recently, Mirin was also shown to effect DSB repair via NHEJ (33). Despite the promise shown in early studies, at the time of writing this review, Mirin has not progressed to assessment in the clinic.

The use of retroviral gene therapy using recombinant adenovirus with mutant forms of the individual proteins in the MRN complex has shown promising results *in vitro* and *in vivo*. In human head and neck squamous cell carcinoma cell lines, expression of adenoviral mutant NBS1 significantly increases cisplatin-induced DSBs and cytotoxicity. This suggests that tumor chemosensitization occurred through the increase of DSBs, as the MRN complex was not able to detect the breaks (34). A novel dominant-negative adenoviral vector containing a mutant Rad50 gene significantly down regulated MRN expression and markedly disrupted MRN function in human squamous cell carcinoma cells. A combination of cisplatin and mutant Rad50 gene therapy produced significant tumor cytotoxicity *in vitro*, with a corresponding increase in DNA damage and telomere shortening. In cisplatin-resistant human squamous cell cancer xenografts, this combination therapy caused dramatic tumor regression with increased apoptosis (35). Further studies have shown this method is effective *in vivo*, however clinical trials using this method of radiosensitizing have not progressed at present.

Telomelysin is a type 5-adenovirus in which the genes have been modified to be able to selectively replicate in cancer cells. The replication of telomelysin is controlled by the human telomerase reverse transcriptase promoter and has been shown to be effective in sensitizing cells to IR (36). The radiosensitivity was due to inhibition of the MRN complex *in vivo* (37). It was found that the expression of the adenoviral E1B55 kDa protein lead to the degradation of the MRN complex (38). A Phase I clinical trial studying telomelysin demonstrated it was effective in various solid tumors and was well tolerated without any adverse effects to patients (39). A Phase I/II trial for the effects of telomelysin on esophageal cancer has commenced in Japan and a Phase I/II clinical trial on liver cancer is planned in the near future.

Resveratrol is a naturally occurring polyphenol that is present in more than 72 plant species. Resveratrol has been shown to arrest the cell cycle (40), promote cellular differentiation (41), and induce apoptosis (42). However, the precise mechanism for these effects remains to be elucidated. A recent gene expression analysis of breast cancer cells treated with resveratrol identified decreased expression of Mre11 and NBS1, key components of the MRN complex. A number of other proteins involved in HR were also down regulated, including BRCA2 and Rad51, whereas Rad52 was up-regulated (43). This suggests resveratrol may function through a number of mechanisms including the MRN complex. *In vivo* studies showing positive, neutral, as well as negative outcomes depending on dose, administration method, and cancer type (44). There have been 76 clinical trials using resveratrol listed at clinicaltrials.gov. Further studies need to be performed to determine if resveratrol can be used for human cancer prevention or therapy and also determine the exact mechanism of the radiosensitizing properties of resveratrol.

ATAXIA TELANGIECTASIA MUTATED

As discussed above, the MRN complex is responsible for the activation of ATM, a major kinase in the DDR. ATM is a member of the phosphoinositide 3-kinase-related kinase (PIKK) family, which

also includes ataxia telangiectasia and Rad3-related (ATR) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs). The MRN complex activates ATM in response to DSBs by recruiting it to the sites of damage (45). Activated ATM is responsible for the induction of the G1/S, intra-S, and G2/M checkpoints, via the phosphorylation of a number of down-stream effector kinases and transcription factors, including p53 and p21 (46). The activation of the cell cycle checkpoints is critical in the DDR to allow for DNA repair to occur before the cell divides ensuring genomic stability.

A study showed that ATM is responsible for hundreds of phosphorylation events in the cell in response to DNA damage, highlighting the key role this kinase plays in the DDR (47). ATM is also required for the full activation of Akt (also known as protein kinase B) in response to insulin in the cytoplasm (48). ATM has also shown to be involved in the regulation of the expression and stability of ribonucleotide reductase and the mitochondrial homeostasis through the control of mitochondrial DNA (mtDNA) copy number dynamics and expression (49). This link with ATM and the regulation of mtDNA may be involved in the resistance of genotoxic stress, highlighted by the potential role of the nuclear co-activators peroxisome proliferator-activated receptor gamma co-activator-1β in DNA damage repair (50). These key roles in the DDR have ensured that ATM has been a prime candidate for inhibition in cancer treatment and further investigations into synthetic lethality for AT patients may show promise.

Caffeine and wortmannin were the first ATM inhibitors identified in the lab and were shown to increase sensitivity to radiation and chemotherapeutic compounds (51, 52). Both caffeine and wortmannin were later shown to be non-specific inhibitors of ATM and also inhibited the other PIKK, ATR, and DNA-PKcs and the potency of these drugs rendered them unsuitable for use in a clinical setting.

The flavonoid quercetin was identified as an inhibitor of phosphoinositide 3-kinase (PI3K) with an IC_{50} of 3.8 μM (53). Analogs of quercetin were synthesized and investigated for their inhibition of PI3K, which led to the discovery of LY294002, an ATP-competitive inhibitor (54). LY294002 was later found to also inhibit ATM (55) and DNA-PKcs (56). However, in the high doses required to inhibit these proteins (>10 μM), LY294002 targeted several unrelated proteins including calcium channels and the estrogen receptor (57, 58).

Despite its lack of specificity, LY294002 was used as a research tool to identify more specific PIKK inhibitors. A study screening a drug-library based on LY294002 identified KU-55933, a small molecule ATP-competitive inhibitor, which was specific to ATM (55). KU-55933 is a potent inhibitor of ATM with an IC_{50} of 0.013 μM and is highly specific to ATM compared to other PI3K and PIKK's. This compound effectively sensitized tumor cells to radiation and DSBs inducing chemotherapeutic compounds, such as camptothecin and etoposide, and initial work has shown this compound may be used as a potential clinical treatment (55).

KU-60019, an improved analog of KU-55933 has been shown to inhibit ATM with an IC_{50} of 0.0063 μM and also inhibits migration and effectively radiosensitizes human glioma cells (59). Further studies on KU-60019 are currently being performed, specifically as a radiosensitizer with standard chemotherapy regimes on glioblastoma in preparation for clinical trials (60).

Another ATM specific inhibitor, CP466722, was identified in a targeted compound library screen, looking for inhibitors of ATM-dependent phosphorylation events *in vitro*. *In vivo* treatment with CP466722 resulted in transient inhibition of ATM and sensitized cells to IR however, upon removal, ATM kinase activity and the subsequent phosphorylation of down-stream targets was completely restored (61). The clinical implications of this transient inhibition of ATM, requires further study.

KU59403 is the latest of the ATM inhibitors that have been studied and one that shows the most promise for clinical trials in patients. KU59403 increased the cytotoxicity of the topoisomerase I and II poisons camptothecin, etoposide, and doxorubicin, *in vitro*, in a non-p53-dependent manner. Importantly, upon injection, KU59403 was seen to be distributed to tissues in mice at concentrations required to inhibit ATM activity, these were shown to be maintained for at least 4 h in colon cancer tumor xenografts and enhanced the anti-tumor activity of topoisomerase poisons. This chemosensitization was both dose and schedule-dependent and provided the first proof-of-principle pre-clinical data to support future clinical development of ATM inhibitors (62). However, at present, there are no reports of ATM inhibitors in use in clinical trials.

It should be noted that CGK-733, a small molecule that was initially reported to inhibit both ATM and ATR kinase activities and block checkpoint signaling with great selectivity, was later retracted (63). Further studies were completed showing that CGK-733 has no specific inhibitory effect on ATM or ATR (64). Unfortunately, the compound is still being marketed as an ATM/ATR inhibitor.

hSSB1/2

Human single-stranded binding protein 1 is required for the activation of ATM through the recruitment of the MRN complex to the break site (14–16). Until the identification of hSSB1 and hSSB2, as a DNA single strand-binding proteins, RPA was the only known eukaryote member of the single strand-binding protein family (SSB) to be involved in DNA repair (14). As mentioned above, hSSB1 is required for the efficient recruitment of the MRN complex to sites of DSBs and for the efficient initiation of ATM-dependent signaling (15, 16). hSSB1 binds directly to the MRN complex through NBS1 and functions to also stimulate its nuclease activity. Identification of specific chemotherapeutic compounds to target hSSB1 and hSSB2 will enable the sensitization of cancer cells to radiotherapy and these are currently being explored by our laboratory and by industry.

Chk1/2

The checkpoint kinases, Chk1 and Chk2, are critical for cell cycle activation following the induction of DSBs and serve to maintain the genomic integrity of cells (65). This cell cycle checkpoint activation is achieved through maintaining or augmenting the inhibitory phosphorylation of the cyclin-dependent kinases (CDKs) by inhibiting the CDC25 phosphatases. Specifically, phosphorylation of CDC25A is required for the initiation of the S-phase checkpoint and phosphorylation of CDC25C for the G2/M checkpoint (66). Both Chk1 and Chk2 are also required for the activation

or inhibition of a number of other cell cycle checkpoint proteins and tumor suppressor proteins, including p53 (67).

Of these checkpoint kinases, Chk1 is critical for the induction of HR, as inhibition of Chk1 *in vitro* lead to persistent unrepaired DSBs and cell death (68). Rad51 was also shown to be recruited to DSBs at replication forks in a Chk1-dependent manner, therefore Chk1 is essential for HR at stalled replication forks. Highlighting this critical role, Chk1 null mice were found to be embryonically lethal (69). Due to its critical role in HR, this review will focus on Chk1 inhibitors only, however a number of these Chk1 inhibitors are also known to target Chk2.

UCN-01, a staurosporine inhibitor was the first inhibitor identified for both Chk1 and Chk2 and treatment with this compound lead to G2/M checkpoint deficiencies in IR-treated p53-deficient tumor cells (70). However, due to the broad spectrum of targets for UCN-01, including protein kinase C, CDK, and CDK2, the use of UCN-01 presented challenges in the clinical setting (71). Since the discovery of UCN-01, increasingly specific inhibitors for Chk1 have been identified.

XL-844, also known as EXEL-9844, is a potent, ATP-competitive inhibitor of Chk1 and Chk2. *In vitro*, XL-844 showed limited ability as a monotherapy but substantially enhanced gemcitabine-induced cell killing. XL-844 increased the gemcitabine-induced DNA damage, blocked CDC25A phosphorylation, abrogated the gemcitabine-induced S-phase checkpoint, and induced premature mitotic entry. Interestingly, XL-844 also induced phosphorylation of Chk1 (72). However, a further *in vitro* study showed that, in response to IR, Chk2, rather than Chk1 appeared to be activated by irradiation and this activation was suppressed by XL-844 (73). A Phase I clinical trial of XL-844 as a monotherapy or in conjunction with gemcitabine in patients with advanced malignancies was discontinued before completion (clinicaltrials.gov – NCT00475917).

AZD7762 is a potent ATP-competitive checkpoint kinase inhibitor that was identified via a compound library screen using Chk1. AZD7762 was found to inhibit both Chk1 and Chk2 with *in vitro* and *in vivo* studies confirming the abrogation of the checkpoint response to gemcitabine (74). *In vivo* studies involving human breast cancer xenografts in mice demonstrated that AZD7762, in combination with irinotecan, improved host survival and reduced tumor growth selectively in p53 mutant tumors (75). A Phase I clinical trial to evaluate the safety, tolerability, and pharmacokinetics of AZD7762, as a monotherapy or in conjunction with gemcitabine, in patients with advanced solid malignancies has just been completed, however no published data have been released as yet (clinicaltrials.gov – NCT00413686). However, interestingly two Phase I trial of AZD7762 in conjunction with irinotecan (clinicaltrials.gov – NCT00473616) and gemcitabine (clinicaltrials.gov – NCT00937664) were terminated early. Trial NCT00937664 was terminated due to incidence of cardiac toxicities reported in the overall Phase I development program (76). This result may affect further clinical development of AZD7762.

PF-00477736 is a potent, selective ATP-competitive small molecule inhibitor of Chk1 and was shown to abrogate cell cycle arrest induced by DNA damage and enhanced the cytotoxicity of clinically important chemotherapeutic agents, including gemcitabine and carboplatin both *in vitro* and *in vivo* in mouse xenografts (77). In combination with docetaxel, PF-00477736 was found to abrogate the DNA damage checkpoints and resulted in sensitization to docetaxel (78). The only clinical trial to date with PF-00477736 looked at the effects in combination with gemcitabine in advanced solid tumors, however this study was prematurely terminated (clinicaltrials.gov – NCT00437203).

SCH900776, also known as MK-8776, was identified as a highly potent Chk1 inhibitor using a high-content, cell-based screen for γ-H2AX induction (γ-H2AX is a surrogate marker for double-strand DNA breaks). SCH900776 also enhanced the anti-tumor effects of gemcitabine *in vivo* (79). SCH900776 was also shown to inhibit CDC25C degradation, abrogates S-phase arrest, and induces DNA damage (80). When SCH900776 was combined with low concentrations of hydroxyurea, both p53-deficient and p53-proficient cell lines were sensitive to the combination (81). It was also demonstrated, *in vitro*, that some cell lines were highly sensitive to SCH900776 alone. *In vivo* models with a human pancreas tumor xenografts mouse model combined SCH900776 with gemcitabine, this showed a significantly delayed tumor growth compared to either drug alone (82). A Phase I clinical trial was undertaken using SCH900776 in combination with cytarabine in patients with acute leukemia. This trial indicated that SCH900776 was tolerated by patients and progressed to a Phase II clinical trial (83). A randomized Phase II clinical trial is currently recruiting to study how well cytarabine, with or without SCH900776, works in treating adult patients with relapsed acute myeloid leukemia (clinicaltrials.gov – NCT01870596).

LY2603618, a pyrazinyl-urea compound, was identified as a Chk1 inhibitor via *in vitro* kinase assays. LY2603618 is currently being investigated in a number of Phase I and II clinical trials and pre-clinical data on their effects *in vitro* and *in vivo* have been recently released. LY2603618 was shown *in vitro* to produce a cellular phenotype similar to that reported for depletion of Chk1 by siRNA and impaired DNA synthesis, elevated H2AX phosphorylation, which is indicative of DNA damage, and caused premature entry into mitosis. *In vivo* treatment of human mutant p53 lung cancer cell xenografts in mice, with gemcitabine, resulted in a stimulation of Chk1 kinase activity that was inhibited by co-administration of LY2603618 (84). In a Phase 1 dose escalation clinical trial of LY2603618 combined with pemetrexed, 9 out of 31 patients achieved stable disease and 1 pancreatic cancer patient had a partial response (85). Two other clinical trials have been completed using LY2603618, in conjunction with gemcitabine, in patients with pancreatic cancer (clinicaltrials.gov – NCT00839332); and an open-label study in patients with advanced and/or metastatic solid tumors (clinicaltrials.gov – NCT01296568), however no results have been published on these clinical trials. There are currently two active clinical trials currently underway studying LY2603618: the first is studying the safety and tolerability of LY2603618 in combination with gemcitabine in patients with solid advanced or metastatic tumors (clinicaltrials.gov – NCT01341457) and the second is investigating the safe dose of LY2603618 that can be combined with pemetrexed and cisplatin and to test if this triplet offers a significant improvement in progression-free survival in participants with Stage IV non-squamous non-small cell lung cancer (clinicaltrials.gov – NCT01139775).

LY2606368 has been identified as a Chk1 inhibitor by Eli Lilly and a Phase I clinical trial is currently recruiting to investigate the effects of LY2606368 in patients with advanced solid tumors (clinicaltrials.gov – NCT01115790), however no pre-clinical data are available for this inhibitor.

Two recently identified Chk1 inhibitors, GDC-0425 and GDC-0575, are currently undergoing clinical trials, however again no pre-clinical data are available. Both compounds are currently being evaluated for the safety, tolerability, and pharmacokinetics administered as a monotherapy or in combination with gemcitabine in patients with refractory solid tumors or lymphoma: GDC-0425 (clinicaltrials.gov – NCT01359696); and GDC-0575 (clinicaltrials.gov – NCT01564251).

SAR-020106 is a novel, selective, and potent ATP-competitive inhibitor of Chk1. SAR-020106 has been shown to abrogate the etoposide-induced G2 arrest and significantly enhances the cell killing of gemcitabine *in vitro* and in a p53-dependent fashion. *In vivo*, it was found that irinotecan, gemcitabine, and radiation activity was enhanced by SAR-020106 with minimal toxicity (86, 87). Whilst SAR-020106 has not undergone any clinical trials at present, this pre-clinical data suggest it is a prime candidate for investigation in p53-defective tumors.

p53

The tumor suppressor protein p53 is a transcriptional regulator of a number of genes involved in DNA repair, cell cycle progression, and apoptosis. Highlighting this function, p53 was found to be mutated in approximately 50% of cancers (88). p53 is activated in response to DNA damage by phosphorylation by the ATM, ATR, Chk2, and Chk1 kinases and these phosphorylation events allow for the stabilization of p53 and activate its transcriptional functions allowing the regulation of a number of genes responsible for cell cycle progression and apoptosis (89).

Initial work on p53-mutated cancers has investigated restoring the function of p53, thus leading to effective apoptosis in response to the chemotherapy. Two main mechanisms have been investigated – restoring p53 to cancer cells using a recombinant adenovirus encoding p53 or using small compounds or short peptides to restore the activity of p53.

Pre-clinical *in vitro* and *in vivo* studies of adenovirus-mediated p53 (Ad-p53) cancer gene therapy showed promising results with advexin (90), gendicine (91), and SCH-58500 (92). Initial clinical trials with these Ad-p53 vectors showed that administration was a safe, feasible, and effective strategy against many types of cancers, however, the anti-tumor efficacy has been limited in some cancer patients. These Ad-p53 vectors have also been used in combination with conventional DNA-damaging treatments, indicating the induction of the apoptotic pathway via Ad-p53 can restore the sensitivity to radiation and chemotherapy in some resistant tumors.

However, issues exist with the low transduction of p53 into cancer cells via these Ad-p53 vectors, to overcome this replication, competent oncolytic adenoviruses have been developed. The CRAd-p53 vector has been used where the promoters of cancer-related genes are used to regulate virus expression in a tumor-dependent manner. Recent work has focused on AdDelta24-p53 (93), SG600-p53 (94), and OBP-700 (95). Initial *in vitro* and *in vivo* studies have shown these CRAd-p53 vectors are a safe and effective therapy for inducing anti-tumor effects and have been shown to induce higher p53 expression and stronger anti-tumor effects than the Ad-53 vectors, highlighting their potential in future clinical trials.

A number of small compounds and peptides have been shown to be effective in restoring the function of p53 in tumor cells, including CP-31398 (96), PRIMA-1 (97), CDB3 (98), peptide 46 (99), and SCH529074 (100). These small compounds and peptides act to stabilize p53 in its active biological conformation, thus restoring its transcriptional activity. Initial *in vitro* work on these small compounds and peptides have shown promising results and further *in vivo* studies are required to determine their efficacy before clinical trials can commence.

REPLICATION PROTEIN A

Due to its key role in DNA replication and repair, via HR and NER, RPA has been the subject of a number of studies to identify potential inhibitors. RPA is over-expressed in a number of cancers, including colon (101), esophageal (102), and breast (103). RPA is a heterotrimeric protein, consisting of RPA1 (p70), RPA2 (p32), and RPA3 (p14) subunits. RPA protects ssDNA from nucleolytic attack, prevents DNA hairpin formation, and blocks DNA reannealing by binding directly to the ssDNA through four OB-folds. After DNA damage, RPA coats ssDNA and enhances the capacity of Rad51 oligomer formation at sites of damage (104).

Initial work has investigated the disruption of the DNA binding capacity of RPA and also inhibition of its protein partner interactions using small molecule inhibitors. Selective inhibition of both the protein binding and DNA binding capacity of RPA has the potential to inhibit the DDR and to sensitize cancer cells to DNA-damaging agents.

TDRL-505, a novel small molecule inhibitor, has recently been shown to inhibit the RPA–DNA interaction, thereby preventing cell cycle progression, induces cytotoxicity, and increases the efficacy of the chemotherapeutic DNA-damaging agent, cisplatin, *in vitro* (105). TDRL-505 inhibits the DNA binding capacity of RPA by blocking the OB-folds of RPA1. Further studies need to be completed in mouse models to determine the efficacy of this compound.

Isobornyl derivatives have also been shown to be RPA inhibitors in a screen of the National Cancer Institute library, with CheSS19 shown to interact irreversibly with the OB-folds of RPA1 (106). MCI13E, a haloester modified form of CheSS19, decreased cell viability and induced apoptosis, showing synergistic effects with cisplatin in lung cancer cells (107). However, this compound did not affect the DNA binding capacity of RPA, but instead may act through the alkylation of cysteine residues of RPA. Further studies, both *in vitro* and *in vivo* are required to fully understand the mechanisms of RPA inhibition by MCI13E prior to clinical studies being undertaken.

The initiation of the DDR by RPA is also mediated by protein–protein interactions involving the N-terminal domain of the p70 subunit with partner proteins, including the MRN complex (108), Rad51 (109), and BRCA2 (110). Inhibition of these interactions increases sensitivity toward DNA damage and replication stress and may therefore be a potential strategy for cancer drug discovery.

Combining RPA inhibition with radiation therapy could lead to increased cytotoxicity in tumor cells via inhibition of DNA DSB repair via NHEJ or HR, both of which have been shown to require RPA.

Rad51

Rad51 plays an important role in maintaining genome stability through the HR pathway in response to DNA damage. This is highlighted by the fact that Rad51 knockout mice show early embryonic lethality (111). Rad51 is a DNA recombinase and polymerizes onto resected DNA ends to form a nucleoprotein filament that promote strand invasion and exchange between homologous DNA duplexes (112). It is suggested the improper regulation of Rad51 may affect tumorigenesis, as Rad51 has been shown to be over-expressed in a number of cancer phenotypes, including esophageal, pancreatic, lung, leukemia, and head and neck cancers. Conversely, Rad51 is also under-expressed in a number of cancer cells. This variable expression of Rad51 has been shown to promote the resistance of tumors to chemotherapy (113). Using antisense RNA or RNAi to deplete the levels of Rad51 *in vitro* has been shown to sensitize tumor cells to chemotherapy agents, including cisplatin (114). These effects of Rad51 depletion demonstrate the potential of Rad51 inhibitors in cancer therapy.

The first identification of a Rad51 inhibitor was a small peptide, homologous to the BRC-motif of the BRCA2 protein, which was found to bind Rad51, thereby preventing its DNA binding capacity (115). This peptide inhibited the formation of Rad51 nuclear foci and disrupted HR *in vitro*. Using an *in silico* approach on the BRC domains of BRCA2, a chimeric peptide with an efficiency 10 times higher than the original peptide was identified (116). This new peptide inhibited Rad51 DNA binding and DNA strand exchange activity however, although these peptides are currently being used as a research tool they have not yet found clinical applicability. Further investigation of peptides and peptidomimics inhibiting Rad51 function may elucidate novel inhibitors targeting the HR pathway in tumors. However, this approach still holds drawbacks mainly due to the pharmacokinetics of peptide-based inhibitors and administration of these agents may not be optimal in a clinical setting.

More recently, a DNA strand exchange assay was performed and used to identify Rad51 inhibitors by high-throughput screening of the NIH small molecule repository. This study identified 17 potential inhibitors, of which 3 were studied further. Compound B02 was identified that specifically inhibited human Rad51 with two other compounds, A03 and A10, which inhibited both Rad51 and RecA, but not the structurally unrelated Rad54 protein. B02 directly interacts with Rad51 and disrupts its binding to DNA and nucleoprotein filament formation. The interaction of B02 with Rad51 disrupted DSB-induced HR and enhanced the sensitivity of cells to cisplatin (117). Further work on these compounds, both *in vitro* and *in vivo*, are required before they can be introduced into clinical trials.

A small molecule inhibitor to Rad51 was recently identified through a high-throughput screen of a library of 10,000 small molecules (118). The molecule RI-1 covalently binds to Rad51, thereby inhibiting its ability to form filaments on ssDNA. RI-1 inhibits the nuclear foci of Rad51 at sites of DNA damage and sensitizes various cancer cell types to cross-linking chemotherapy, but did not affect Rad51 protein levels. There are limits to the development of RI-1 in pre-clinical *in vivo* models due to its short half-life in tissue culture media and aqueous buffers. RI-2, a homolog of RI-1, was created that mitigated these effects (119). RI-2 was shown to bind Rad51 and inhibit the nuclear foci of Rad51 at sites of DNA damage. RI-2 is currently the subject of further *in vitro* and *in vivo* studies and is being used to identify third generation analogs that inhibit the function of Rad51.

A further screen using a yeast-2 hybrid system identified a phenylsulfonyl indolyl isoquinoline compound, IBR2, as a Rad51 inhibitor. IBR2 functions to block Rad51 multimerization, accelerating proteasome-mediated Rad51 protein degradation, and thus impairing IR-induced Rad51 foci formation in the nucleus and HR activity. IBR2 inhibited cancer cell growth and induced apoptosis (120). A synergistic cell-killing effect was produced with a combination of IBR2 and imatinib *in vitro*. *In vivo* studies involving breast cancer xenografts in nude mice showed significantly inhibited tumor growth with no apparent secondary physiological abnormalities. Further studies on the effects of IBR2 are required before moving into a clinical trial.

BRCA1/2

The breast cancer susceptibility proteins, BRCA1 and BRCA2, have a key role in efficient HR response to DSBs. Mutations in these genes greatly increase the susceptibility to cancer, especially breast, ovarian, and prostate. Mutations in the BRCA genes are responsible for the increased risk of breast cancer, specifically 59–87 and 38–80% for BRCA1 and BRCA2 mutations respectively. BRCA1 functions in both checkpoint activation and also in the early steps of HR, by controlling DNA resection (121). BRCA2 functions in Rad51 transport and loading (122). Both BRCA1 and BRCA2 are required for normal embryonic development in mice (123, 124).

Direct inhibition of BRCA1 and BRCA2 in tumors is generally problematic due to the wide expression of these proteins in most tissues and inhibition may lead to other issues, including cancer development in healthy tissue. One approach is the possible up regulation of the BRCA1 and BRCA2 proteins, however there is no data to suggest that up regulation blocks tumorigenesis.

POLY ADP-RIBOSE POLYMERASE 1

Most of the work in the BRCA therapeutic research area has focused on tumors that are known to have mutations in BRCA1 or BRCA2. Using synthetic lethality, these studies have focused on disrupting complimentary pathways to repair DNA damage, with the most interesting results coming from poly ADP-ribose polymerase 1 (PARP1) inhibitors. PARP1 is involved in DNA repair, replication, transcriptional regulation, chromatin modification, and apoptosis (125, 126). In regards to DNA repair, PARP1 is involved in BER which repairs DNA damage due to reactive oxygen species and alkylation (127). Inhibition of this pathway, taken together with a loss of HR due to BRCA mutations, creates a synthetic lethality, which can be exacerbated when used in conjunction with chemotherapy agents.

However, PARP1 inhibition and the subsequent synthetic lethality can be used on other cancers that do not have mutations in BRCA1 or BRCA2 but that have a defect in the HR

pathway, including mutations in ATM, Chk2, Rad51, and NBS1. However, NHEJ could compensate for the loss of HR in these cells. Further studies have identified that PARP1 normally functions to promote HR by suppressing various components of the NHEJ pathway (128). Inhibition of PARP1 would therefore lead to increased NHEJ, a more error-prone repair mechanism than HR, this would increase chromosome instability in response to chemotherapy and during S-phase of the cell cycle at stalled DNA replication forks.

Poly ADP-ribose polymerase catalyzes the cleavage of NAD+ to nuclear acceptor proteins, leading to the formation of ADP-ribose polymers, realizing nicotinamide in the process. Nicotinamide was the first PARP inhibitor identified, although it was not potent (129). Analogs of nicotinamide, including 3-aminobenzamide, were the first generation of PARP inhibitors (130).

The first clinical trial targeting PARP1 in BRCA populations was with the oral drug olaparib, also known as AZD2281, as a monotherapy (131). Olaparib achieved encouraging response rates of 41 and 33% in patients with BRCA1 or BRCA2 mutations, respectively. Olaparib was also used in a combined therapy with carboplatin *in vivo* and showed a profound decrease in tumor growth and increase in patient survival (132). A Phase II trial with olaparib was conducted on patients with advanced BRCA1/2 mutant breast cancers (133) and ovarian cancers (134). Both of these studies showed a dose-dependent effect of olaparib. Currently, there is a Phase III olaparib trial being undertaken by AstraZeneca. This trial aims to determine the benefit, by progression-free survival, of olaparib as a maintenance monotherapy, in BRCA mutated ovarian cancer patients, who are in complete or partial response following platinum-based chemotherapy.

Another PARP inhibitor is veliparib. An *in vivo* study of veliparib, also known as ABT-888, confirmed the PARP inhibitory effects in paired tumor biopsies and peripheral mononuclear cells (135). A number of Phase I trials have been conducted with veliparib in combination, including topotecan (136) amongst others. Many of these trials have shown promising results, however myelosuppression, where bone marrow activity is decreased, has been shown as the most common adverse event observed.

Iniparib, also known as BSI-201, was the first PARP inhibitor to undergo Phase III clinical trials after showing promising results in randomized Phase II trials in patients with triple-negative breast cancer (137). The results of the subsequent Phase III clinical trial were not as expected, missing the co-primary endpoints of overall survival and progression-free survival. However, very little pre-clinical data on the effects of iniparib were published before clinical trials began and iniparib was shown not be related to other PARP inhibition and showed very low PARP inhibition *in vitro* (138, 139).

Rucaparib, also known as AG014699, was used in a Phase I clinical trial in combination with temozolomide (140). Rucaparib was well tolerated in patients and showed promising results for assumed HR-deficient tumors (based on tumor type). Rucaparib was also used as a monotherapy in a Phase I/II clinical trial with patients with solid tumors. Rucaparib was well tolerated in this trial and showed promising results (clinicaltrials.gov – NCT01482715). A subsequent Phase II clinical trial, with patients with melanoma, was conducted in combination with temozolomide, and showed an objective response rate with 17.4 and 36% of patients remaining progression-free after 6 months (141).

A therapeutic index-based strategy was used to identify CEP-8983, a novel 4-methoxy-carbazole inhibitor of PARP1 and PARP2 (enzyme IC_{50} values of 20 and 6 nmol/L, respectively). CEP-8983 was found to cause significant sensitization of chemotherapy-resistant tumor cell lines to the effects of temozolomide and camptothecin *in vitro*. Administration of CEP-8983, delivered orally in the form of CEP-9722, attenuated *in vivo* PARP activity and resulted in significant chemosensitization of temozolomide and irinotecan in chemotherapy-resistant tumor xenografts (142). A Phase I clinical trial with CEP-9722, used as a monotherapy or in conjunction with temozolomide, was recently completed with patients with advanced solid tumors (clinicaltrials.gov – NCT00920595). A Phase I/II clinical trial using CEP-9722 on solid tumors is currently underway (clinicaltrials.gov – NCT01311713).

MK-4827, also known as niraparib, is a novel 2-phenyl-2H-indazole-7-carboxamide PARP inhibitor that displayed anti-proliferation activities against BRCA1- and BRCA2-deficient cancer cells *in vitro*. MK-4827 was found to be well tolerated *in vivo* and demonstrated efficacy as a single agent in a xenograft model of BRCA1-deficient cancer (143). A Phase I clinical trial of patients with solid tumors using MK-4827 was shown to have favorable pharmacokinetics, inhibited PARP activity effectively, is well tolerated and has anti-tumor activity in carriers of BRCA1 and BRCA2 mutations and patients with sporadic cancers (144).

BMN 673, an inhibitor of PARP catalytic activity, has exhibited selective anti-tumor activity at much lower concentrations ($IC_{50} = 0.57$ nM) than the earlier generation of PARP inhibitors, including olaparib, rucaparib, and veliparib. BMN 673 is readily orally bioavailable and *in vivo* studies with xenograft tumors carry defects in BRCA1/2 or PTEN were sensitive to BMN 673. Synergistic effects were observed when BMN 673 was combined with temozolomide, SN38, or platinum drugs (145). A number of pre-clinical studies and Phase 1, Phase II, and Phase III clinical trials utilizing BMN 673 as a monotherapy or in conjunction with various drugs, are currently underway.

It is important to note that not all breast cancer patients with BRCA mutations responded to PARP inhibition (131) and a substantial number of patients with advanced BRCA1-mutant cancers are resistant to these agents. Further studies on PARP inhibitors, along with the current clinical trials, are needed to assess the efficacy of PARP inhibition in BRCA mutant and other HR-defective cancers in conjunction with chemotherapy or as a monotherapy.

A list of all chemotherapeutic compounds targeting the HR pathway is provided in **Table 1**.

NON-HOMOLOGOUS END JOINING AND CHEMOTHERAPEUTIC COMPOUNDS TARGETS

CLASSICAL-NHEJ

The classical-NHEJ (C-NHEJ) pathway is the major pathway of DSB repair [reviewed in Ref. (146)], estimated to rapidly repair up to 85% of IR-induced DSBs (147). In straightforward terms, this pathway involves simply ligating the two DNA ends back together. Due to the resection of DNA overhangs surrounding the DSB, NHEJ is sometimes considered the error-prone pathway of DNA

Table 1 | Chemotherapeutic compounds targeting the homologous recombination DNA repair pathway.

Compound	Class	Clinical phase	Combination
Mirin	MRN complex	–	–
Adenoviral mutant NBS1	NBS1	–	Cisplatin
Adenoviral mutant Rad50	Rad50	–	Cisplatin
Telomelysin	MRN complex	I/II	Monotherapy
Resveratrol	MRN complex	–	–
Caffeine	PIKK	–	–
Wortmannin	PIKK	–	–
Quercetin	PI3K	–	–
LY294002	ATM and DNA-PKcs	–	–
KU-55933	ATM	–	–
KU-60019	ATM	–	–
CP466722	ATM	–	–
KU59403	ATM	–	–
UCN-01	Chk1 and Chk2	I	Monotherapy or topotecan or cisplatin
XL-844	Chk1 and Chk2	I	Monotherapy or gemcitabine
AZD7762	Chk1 and Chk2	I	Monotherapy or gemcitabine or irinotecan
PF-00477736	Chk1	I	Gemcitabine
SCH900776	Chk1	I/II	Monotherapy or cytarabine or gemcitabine
LY2603618	Chk1	I/II	Gemcitabine or pemetrexed and cisplatin
LY2606368	Chk1	I	Monotherapy
GDC-0425	Chk1	I	Monotherapy or gemcitabine
GDC-0575	Chk1	I	Monotherapy or gemcitabine
SAR-020106	Chk1	–	–
Advexin	p53	I/II	Monotherapy and chemotherapy drugs
SCH-58500	p53	I/II	Monotherapy and chemotherapy drugs
AdDelta24-p53	p53	–	–
SG600-p53	p53	–	–
OBP-700	p53	–	–
CP-31398	Stabilizes p53	–	–
PRIMA-1	Stabilizes p53	–	–
CDB3	Stabilizes p53	–	–
Peptide 46	Stabilizes p53	–	–
SCH529074	Stabilizes p53	–	–
TDRL-505	RPA	–	–
CheSS19	RPA	–	–
MCI13E	RPA	–	–
B02	Rad51	–	–
A03	Rad51 and RecA	–	–
AI-10	Rad51 and RecA	–	–
RI-1	Rad51	–	–
RI-2	Rad51	–	–
IBR2	Rad51	–	–
3-Aminobenzamide	PARP1	–	–
Olaparib	PARP1	I/II/III	Monotherapy
Veliparib	PARP1	I	Topotecan or carboplatin or doxorubicin or irinotecan
Rucaparib	PARP1	I/II	Monotherapy or temozolomide
CEP-9722	PARP1 and PARP2	I/II	Monotherapy or temozolomide
MK-4827	PARP1	I	Monotherapy or temozolomide
BMN 673	PARP1 and PARP2	I/II/III	Monotherapy or temozolomide or irinotecan

(Continued)

DSB repair. NHEJ is active in all stages of the cell cycle, with activity peaking in G0 and G1 (12). The major proteins involved in NHEJ include the DNA-PKcs and the Ku70/80 heterodimer. Other core NHEJ proteins include artemis, XRCC4-XLF, and ligase IV. DNA-PKcs and Ku70/80 initially bind to the two ends of the DSB. The DNA ends are then processed by artemis, ligated by ligase IV and stabilized by XRCC4 and XLF.

There are a number of key proteins involved in NHEJ that are targets of chemotherapeutic compounds (see **Figure 2**).

DNA-DEPENDENT PROTEIN KINASE CATALYTIC SUBUNIT

The DNA-PK holoenzyme plays a major role in NHEJ and is involved in tethering the DNA ends at DSBs, allowing recruitment of other repair proteins. It also has serine/threonine kinase activity and can phosphorylate down-stream DNA repair proteins,

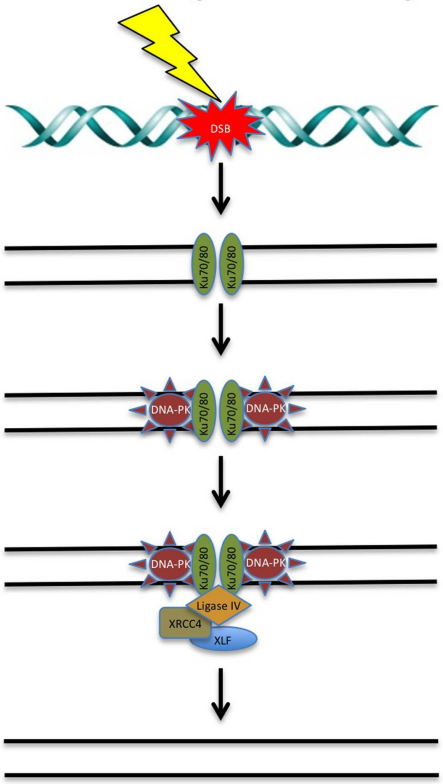

FIGURE 2 | DNA double-strand break repair via non-homologous end joining. DSBs can be repaired via NHEJ throughout the cell cycle. Targeting of these proteins involved in NHEJ with chemotherapeutic compounds shows promise in the clinical setting. See text for details.

leading to their activation. DNA-PKcs has been reported to be up-regulated in tumors or radiation-resistant cell lines, indicating that it is likely to have a role in tumor growth and survival (148, 149). In addition, B-cell chronic lymphocytic leukemia cells have been shown to escape apoptosis via the NHEJ pathway (150). Moreover, DNA-PKcs mutations have been detected in colorectal cancer cells (151). In light of the above and due to its pivotal role in NHEJ, DNA-PKcs is a protein of interest in developing new cancer treatments [reviewed in Ref. (152)].

Several inhibitors of DNA-PKcs have been identified, the most efficient of which target the ATP-binding pocket of the DNA-PKcs kinase domain (153). Compound library studies have identified several specific inhibitors of DNA-PKcs, but their development as cancer therapies has been restricted by weak pharmacokinetics.

Wortmannin, a metabolite of the fungus *Penicillium funiculosum* was one of the first DNA-PKcs inhibitors and has been widely used to study DNA-PKcs experimentally. This drug was used in the first studies that showed that inhibition of DNA-PKcs inhibited DNA DSB repair and enhanced the tumor-killing properties of agents that induce DNA damage, such as etoposide and IR. Although it can efficiently inhibit DNA-PKcs at an IC_{50} of 5 nM, its poor solubility, lack of specificity, and *in vivo* toxicity, have ensured that wortmannin has little clinical application (154).

As discussed earlier, another non-specific DNA-PKcs inhibitor utilized in several studies is LY294002, the morpholine derivative of the plant flavonoid quercetin. This inhibitor binds the kinase domain of DNA-PKcs with an IC_{50} of 1.4 μM (154). Like wortmannin, the clinical use of this inhibitor is limited by its lack of specificity and *in vivo* toxicity. In addition, LY294002 also displays rapid metabolic clearance in 1 h. Despite its limitations, LY294002 has proved useful as a foundation for biochemical modification, leading to several, more specific, clinically viable compounds (155).

An example of a compound developed from LY294002 is NU7026, which possesses 50-fold more selective inhibition of DNA-PKcs than other PI3Ks, with an IC_{50} of 0.23 μM against DNA-PKcs (156). This compound was found to enhance the tumor growth inhibition of several chemotherapy drugs, including daunorubicin, idarubicin, doxorubicin, and etoposide (157). However, due to metabolic instability, it is unlikely that high enough concentrations of NU7026 could be achieved in tumors to allow treatment in conjunction with chemotherapy or radiation treatment. Like other DNA-PK inhibitors, NU7026 also displays poor solubility in saline solutions (158).

Another DNA-PKcs inhibitor that resulted from the modification of LY294002 is NU7441, which strongly inhibits DNA-PKcs and has an IC_{50} of 0.3 μM. Treatment of cells with this drug led to an increase in HR and the persistence of IR- and doxorubicin-induced DSBs (159). Cellular treatment with NU7441 was also shown to delay the repair of IR- and etoposide-induced DSBs, in turn enhancing the tumor cell-killing properties of these treatments (160). In animal models, xenograft studies showed that NU7441 could increase the tumor growth inhibition of etoposide twofold with no increased toxicity. NU7441 has recently been shown to inhibit the multidrug-resistance 1 (MDR1) protein, in addition to DNA-PK, which may increase its therapeutic potential when combined with MDR substrates (161).

Two other inhibitors of DNA-PK have also been shown to sensitize cells to DNA-damaging agents, SU11752 and OK-1035 (162, 163). Unfortunately, both compounds displayed weak pharmacokinetic properties making them unsuitable for further clinical development.

Another agent, NK314 was already used in the clinic as a topoisomerase II alpha (TIIa) inhibitor and was also found to promote the degradation of DNA-PKcs, leading to defective DNA DSB repair. DNA-PKcs is highly expressed in adult T-cell leukemia–lymphoma (ATL), so NK314 may potentially be used as a dual targeting anticancer agent for treatment of ATL (164). A clinical trial for the use of NK314 in ATL patients is currently underway.

CC-115 is a DNA-PKcs inhibitor that is also undergoing early clinical evaluation. CC-115 is a dual inhibitor for DNA-PKcs and mTOR and the first clinical trial aims to assess its safety and action in patients with advanced solid tumors and hematologic malignancies that are unresponsive to standard therapies.

In summary, due to the role DNA-PKcs plays in DNA DSB repair via C-NHEJ and its overexpression in many cancers it was implicated as a suitable target for inhibition. Although several DNA-PKcs inhibitors have reached the pre-clinical evaluation stage, their use in patients have been limited by inadequate pharmacokinetics; as they are generally metabolically unstable, a high

cellular concentration is unable to be achieved and therefore they are not clinically viable to potentiate other forms of cancer therapy. The use of antibody and oligonucleotide approaches to target DNA-PKcs may overcome the pharmacokinetic restrictions of small molecule inhibitors. However, there is still hope for this area of treatment as the crystallographic structure of DNA-PKcs was recently reported, allowing more efficient small molecule inhibitors of DNA-PKcs to be developed (165, 166). Computer-modeled compound design will allow targeting of the DNA-PK auto-phosphorylation sites or the DNA-PK/Ku80 interface, which are predicted to be more efficient than current DNA-PKcs inhibitors.

Ku70/Ku80

The levels of the regulatory subunit of the DNA-PKcs holoenzyme, Ku70/80, like DNA-PKcs, are also increased in many tumors, which suggests that tumors may rely on Ku70/80 for survival (149). Indeed, it was shown that depletion of Ku70 or Ku80 using shRNA inhibited the growth of pancreatic tumor cells (167). Ku70- or 80-depletion also sensitized pancreatic cells to IR, suggesting that it may be a potential target for inhibition in cancer therapy in the future, although to date specific inhibitors have not been identified.

DNA LIGASE IV

DNA ligase IV is an ATP-dependent DNA ligase that catalyzes the ligation step in NHEJ. Together with XRCC4 and XLF, DNA ligase IV forms a functional complex that is central to NHEJ (168–171). All DNA ligases catalyze the formation of the DNA phosphodiester bond in a three-step process. The initial hydrolysis of ATP leads to the covalent linkage of an AMP moiety to a specific lysine residue within the active site of DNA ligase and the subsequent release of pyrophosphate. A DNA adenylate intermediate is formed through the transfer of the AMP moiety from the adenylated ligase to the 5′ terminus of a DNA nick with a 5′ phosphate and 3′ hydroxyl terminus. Finally, the non-adenylated DNA ligase interacts with the DNA adenylate and the termini are linked together via a phosphodiester bond, with the final release of AMP (172).

Inhibiting the activity of DNA ligase IV has become an attractive approach to increase the sensitization of cancer cells to DNA damage. As DNA ligation is required during DNA repair and replication, cells deficient in DNA ligases have been shown to be sensitive to a variety of DNA-damaging agents (173). To date, there are two described DNA ligase IV inhibitors, L189 and SCR7.

A computer-aided drug design approach, based on the structure of human DNA ligase I complexed with nicked DNA, was performed to identify low molecular weight inhibitors of DNA ligases that specifically abrogate functional interactions between the ligase and nicked DNA (174). L189 was 1 of a 192 potential candidate inhibitors chosen from this rational approach. L189 was further characterized *in vitro*, and shown to inhibit DNA ligase I, III, and IV in DNA joining assays using purified protein and in DNA replication, BER, and NHEJ in cell extract assays. Specifically, L189 inhibited the ligase reaction by >90%, however, only had a minimal effect on T4 DNA ligase. In cell culture, L189 was found to be cytotoxic, using colony-forming assays. Furthermore, L189 significantly increased the cytotoxicity of the DNA-damaging agents MMS and IR in three cancer cell lines (breast, cervical, and colon) but not in a normal breast epithelial cell line. Hence, *in vitro* data suggest that L189 is a potential lead compound for the development of chemotherapeutics (174). However, *in vivo* data and subsequent clinical trials are required to further substantiate these results.

SCR7 is a L189 derivative that was identified by an *in silico* docking approach, as a specific inhibitor of DNA ligase IV. SCR7 disrupts the sealing of DSBs by ligase IV by interfering with its binding to DNA. *In vitro*, SCR7 inhibits NHEJ in a ligase IV-dependent manner, leading to the accumulation of DSBs and subsequent cytotoxicity. SCR7 was used on four different mouse models to determine tumor progression. Three of the four mouse models were responsive and SCR7 was found to significantly reduce tumor progression and increase lifespan, relative to the control. SCR7 slowed the progression of the tumor by activating the p53-mediated apoptotic pathway and hence increasing lifespan. Additionally, when SCR7 was co-administered with IR and etoposide in mouse models, it significantly increased the sensitivity of tumors (175). This study demonstrates that inhibitors of DNA repair, in combination with existing chemo and radiotherapy, may lead to a better efficacy of treatment.

XRCC4

The initial step in NHEJ is the recognition and binding of the Ku70/80 heterodimer to the DSB (176). After Ku70/80 is bound to DSB ends, it recruits other NHEJ factors such as XRCC4 to the site of damage (177). Ku70 and XRCC4 directly interact with each other and XRCC4 may act as a flexible tether between Ku70/80 and DNA ligase IV (176). XRCC4 has no known enzymatic activity, but may function as an additional NHEJ scaffolding protein, responsible for the recruitment of other NHEJ factors to the site of the damage (177).

In mice, XRCC4 deficiency has been shown to cause late embryonic lethality (178) and mouse Xrcc4 was found to stimulate adenylation of DNA ligase IV *in vitro*, the first chemical step in DNA ligation (179).

Since XRCC4 plays a central role in the repair of DSB by NHEJ (177), the presence of active XRCC4 in cells may decrease DSB-mediated apoptosis in cancer cells during radiotherapy. Therefore, the use of potent XRCC4 inhibitors has the potential to enhance radiotherapy outcomes in patients.

Salvianolic acid B, lithospermic acid, and 2-*O*-feruloyl tartaric acid were identified as potent agents for interrupting XRCC4-mediated DNA repair, from a screen involving 20,000 compounds from the traditional Chinese medicine (TCM) database (180). The compounds were modeled for their binding affinities to the DNA ligase IV binding region on XRCC4 and for all three inhibitors, the protein–ligand interactions were focused at Lys188 on chain A and Lys187 on chain B of XRCC4. From this study, salvianolic acid B, lithospermic acid, and 2-*O*-feruloyl tartaric acid are potential enhancers of radiotherapy and furthermore, may have characterized the key binding elements for inhibiting XRCC4 activity (180). While this study is promising, the efficacy of these inhibitors has yet to be tested using *in vitro* and *in vivo* models.

Inhibiting the XRCC4/DNA ligase IV complex formation could also provide a novel strategy for inhibiting NHEJ. The minimal

inhibitory fragment of the XRCC4-interacting region (XIR) capable of abolishing XRCC4/XIR complex was recently identified (181). The key interfaces of ligase IV necessary for interaction with XRCC4 were identified by the development a competitive displacement assay using ESI-MS/MS. The results suggest that by targeting the interface of helix 2 of DNA ligase IV, modulators that inhibit the XRCC4/DNA ligase IV complex may be identified. In addition, adjuvant compounds to further block the XRCC4/DNA ligase IV complex may be discovered by further targeting helix 1 and the loop regions of the helix–loop–helix clamp, which offer a secondary target surface (181). While this study has the potential to identify inhibitors of XRCC4, to date, inhibitors that have been tested *in vivo* and in clinical trials have not been described.

XCRCC4-LIKE FACTOR

XCRCC4-like factor/cernunnos (XLF/cer) is a recently discovered XRCC4 interaction partner. XLF directly interacts with the XRCC4-ligase IV complex both *in vitro* and *in vivo*. Furthermore, siRNA knockdown of XLF in mammalian cells gives rise to radiosensitivity and impaired NHEJ and the re-introduction of wild-type XLF into defective cells corrects the observed defects (171). Data suggest that following DSBs, XLF accumulates at DNA damage sites via constitutive interaction of the XRCC4 head domains and XLF globular head domains in the XRCC4–DNA ligase IV complex and dependent components of the DNA-PK complex. Following this, XLF stimulates the ligation of complementary and non-complementary DNA ends via XRCC4 and DNA ligase IV. XLF in summary ensures the accuracy of the joining of DSBs during NHEJ and V(D)J recombination (182).

While there are no inhibitors of XLF in current use, inhibitors that abrogate the formation of the XRCC4/XLF/DNA ligase IV functional complex that is central to NHEJ may provide a novel strategy to improve radiotherapy outcomes in patients.

p53-BINDING PROTEIN 1

p53-binding protein 1 (53BP1) is a human BRCT protein that was initially identified by a yeast 2-hybrid screen as a p53-interacting protein (183). 53BP1 binds to p53 and enhances p53-mediated transcriptional activation. 53BP1 is a central regulator of DNA DSB repair and functions to promote the end joining of distal DNA ends induced during V(D)J and class switch recombination. Additionally, 53BP1 is involved in the fusion of unprotected telomeres (184, 185). 53BP1 is an ATM substrate that forms nuclear foci in response to DNA damage (186) and promotes NHEJ while preventing HR. Recent evidence suggests that 53BP1 recruitment requires the direct recognition of a DSB-specific histone code and the choice of NHEJ vs. HR is dependent on BRCA1 (185).

The identification of specific chemotherapeutic compounds targeting 53BP1 and thereby sensitizing cancer cells to radiotherapy is an approach that requires further investigation.

ALTERNATIVE NHEJ

Recent studies have identified another DSB repair pathway, termed alternative NHEJ (A-NHEJ). This pathway comprises another simple end joining process that is normally suppressed by the C-NHEJ pathway and only operates when C-NHEJ and HR pathways are compromised. Therefore, A-NHEJ is generally considered a backup repair pathway and is implicated to be highly error-prone (187). It has been suggested that A-NHEJ may actually be comprised of several pathways due to the functional diversity of the A-NHEJ proteins identified so far. However, it has also been suggested that A-NHEJ results from the initiation and failure of C-NHEJ or HR, resulting in C-NHEJ or HR proteins already being present at the DSB. When the initiation of A-NHEJ follows unsuccessful C-NHEJ, C-NHEJ factors are already at the DSB, but instead of DNA ligase IV performing the ligation step, this is performed by DNA ligase 3 or 1 (188–190). It has also been suggested that A-NHEJ may also function to join DNA ends that have been processed by HR factors such as the MRN complex, CTIP, and BRCA1 (190–192). The A-NHEJ pathway has been implicated as enabling tumor cells that have disrupted HR or C-NHEJ pathways to survive, making it an attractive target for inhibition.

DNA LIGASE 3α

A recent study demonstrated that KRAS mutated leukemic cells have increased levels of components of the A-NHEJ pathway, including DNA ligase 3α, PARP1, and XRCC1 and that these cells rely on the A-NHEJ for survival (193). In addition, it was also shown that depletion of DNA ligase 3α using RNAi sensitized the KRAS-mutant leukemic cells to chemotherapy. This suggests that targeting the A-NHEJ pathway may be a promising avenue for inducing synthetic lethality in combination with DNA-damaging agents in cells bearing KRAS mutations, for which there is currently no reliable treatment.

A list of all chemotherapeutic compounds targeting the NHEJ pathway is provided at **Table 2**.

CONCLUSION

Human solid tumors have frequently been found to have pronounced genetic and gene expression heterogeneity, of both cancerous and the normal cells within the tumor. This diversity of cell populations within the tumor may explain why cancer is so resistant to therapy, including more targeted therapeutic approaches. The increased proliferation of cancer cells also places stress on the genome, with the fastest growing cell populations having an advantage in the environment. To increase growth rates and

Table 2 | Chemotherapeutic compounds targeting the non-homologous end joining DNA repair pathway.

Compound	Class	Clinical phase	Combination
Wortmannin	DNA-PKcs and other PIKKs	–	–
LY294002	DNA-PKcs	–	–
NU7026	DNA-PKcs	–	–
NU7441	DNA-PKcs	–	–
SU11752	DNA-PKcs	–	–
OK-1035	DNA-PKcs	–	–
NK314	DNA-PKcs and topoisomerase II alpha	I	Monotherapy
CC-115	DNA-PKcs and mTOR	I	Monotherapy
L189	DNA ligase IV	–	–
SCR7	DNA ligase IV	–	–

remove normal restrictions on growth, cancer cells evolve to have defects in the DNA repair pathways and in checkpoint signaling and apoptosis. As a result of these defects and increased metabolic activity, cancer cells are genomically unstable with the most aggressive cancers showing the most genetic instability. However, this instability differentiates the cancer cells from normal cells, potentially opening up therapeutic windows. The DSB repair pathway is the most promising of these therapeutic windows, as defects in this pathway are commonly associated with diseases such as cancer.

The disruption of the DSB repair mechanisms HR and NHEJ, via chemotherapeutic compounds used as either a monotherapy or in conjunction with radiotherapy, has shown promise in the clinical setting for the treatment of various cancers. The targeting of these processes can be further exploited as further investigations into the HR and NHEJ pathway lead to the identification of new potential targets. However, complete inhibition of HR and NHEJ for any extended time period is likely to be lethal to all dividing cells, therefore targeted or temporary inhibition is likely to be useful in conjunction with radiotherapy. Also, complete inhibition of NHEJ may lead to further genomic instability in normal cells, as it is the only pathway for repairing DSBs in non-dividing cells.

Further investigation into synthetic lethality, beyond the identified PARP/BRCA lethality, may lead to additional avenues to be targeted and exploited by the use of chemotherapeutic compounds. Promising targets to expand on PARP inhibition using synthetic lethality are other proteins involved in the HR pathway, including cancers with mutations in ATM, p53, Chk2, Rad51, and NBS1. Inhibition of other proteins involved in the DDR response has also shown promise when combined with BRCA mutations. *In vitro* depletion of Rad52 in BRCA2-deficient cells showed synthetic lethality when compared with BRCA2-competent cells (194). Recently, synthetic-lethal relationships in chromatin-regulating genes have been identified, including chromatin remodeling factors (195) and methyltransferases (196). The concept of synthetic lethality could allow the exploitation of differences between tumor cells and normal cells that have previously been considered to be intractable and it has been shown to be a promising means of selectively killing tumor cells.

Whilst there have been a number of good and promising results using chemotherapeutic compounds, there have been a number of failed studies. Ensuring that there are sufficient investigations completed both *in vitro* and *in vivo* confirming the specificity and pharmacokinetics of these chemotherapeutic compounds before introduction in the clinical setting is critical. As the response to chemotherapeutic compounds becomes more predictable and with the identification of specific tumor biomarkers, this will allow for targeted, more efficient cancer treatments. Inhibition of these DSB repair proteins holds great promise for the future of cancer therapy in the future.

AUTHOR CONTRIBUTIONS

All authors were involved in the conception of the manuscript, the drafting and/or critically reviewing of the manuscript, have approved the final version for publishing, and have agreed to be accountable for the work.

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Intratumoral heterogeneity, its contribution to therapy resistance and methodological caveats to assessment

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Cancer is one of the most urgent health issues of today. According to WHO, the number of cancer cases is expected to increase by 75% in the next two decades (1). Despite some remarkable achievements in the fields of cancer prevention and early detection, the goal of developing effective anti-cancer therapies still remains unmet. Tumor recurrence due to treatment resistance is the most common cause of death from cancer. Delineating cellular and molecular mechanisms underlying tumor recurrence is of prime importance for the ability to improve the efficacy of existing therapies and develop new strategies to cancer treatment.

The aim of any anti-cancer treatment is to selectively kill cancer cells by targeting key biological properties essential for the maintenance of tumorigenicity and malignant progression (2). Currently, cytotoxic therapies are still a mainstay of cancer treatment that relies heavily on radiation treatment and chemotherapy. Even though cytotoxic treatments can be effective in some types of cancers, the clinical experience accumulated over the past few decades indicates that conventional cytotoxic therapy may not suffice to achieve a satisfactory level of the therapeutic efficacy. A conceptual framework for cytotoxic therapies derives from the observation that there is a direct relationship between proliferation rate and cytotoxic sensitivity, implying that rapidly dividing cancer cells rather than largely quiescent normal cells should be preferentially targeted by cytotoxic agents. However, proliferation rates of tumor cells can vary in a broad range between and within tumors. This is thought to be one of the reasons for insufficient efficacy of cytotoxic therapies (3). Furthermore, cancer cells can neutralize the effects of cytotoxic treatments by utilizing a plethora of often overlapping mechanisms that include aberrant DNA repair and cell death pathways, drug efflux, hypoxia-induced apoptosis resistance and invasion, alterations in drug metabolism, unfolded protein response, and autophagy [reviewed in Ref. (4, 5)].

A newer type of anti-cancer therapy generally called molecularly targeted therapy relies on rationally designed agents to target, with a high degree of specificity, well-defined molecules or pathways that operate in cancer cells to maintain their malignant potential. Although both cytotoxic and molecularly targeted therapeutic approaches generally exploit differences between neoplastic and normal cells, only targeted therapies enable the so-called precision medicine. Recent advances in the field of molecular profiling have opened up a real possibility to make better informed treatment decisions based on the data from personalized tumor profiling [reviewed in Ref. (6, 7)]. However, despite some remarkable successes of targeted therapies (8, 9), their utility in advanced cancers has so far been limited due to an almost inevitable tumor recurrence even after successful initial response [reviewed in Ref. (6, 10, 11)]. The escape mechanisms underlying the inherent and acquired resistance to targeted therapies include feedback activation of signaling pathways with redundant functions (12), co-occurrence of mutations in other genes involved in synergistic interactions with the target gene (13), or emergence of subclones with secondary mutations coding for resistant versions of drug targets (14). Global profiling of cancer genomes has enabled the stratification of major cellular pathways involved in the development of therapeutic resistance in different types of cancer. Providing a molecular explanation of the limited efficacy of targeted monotherapies, cancer genomics studies reveal a high degree of functional redundancy between oncogenic driving events [reviewed in Ref. (7, 15)].

New generation sequencing methodologies while enabling to identify genomic alterations associated with different types of cancer with an unprecedented completeness also revealed the high degree of genetic diversity existing not only between different types of cancer but also between individual tumors of the same histotype [reviewed in Ref. (16, 17)]. A broad range of phenomena encompassed in the term "tumor heterogeneity" include (epi) genetic, phenotypic, and gene expression pattern diversity across different types of cancer, between different tumors of the same histotype (interpatient heterogeneity), between different tumors from the same patient (primary tumor or metastasis), or within the same tumor (intratumor heterogeneity). Intratumor heterogeneity manifests in spatial and temporal patterns of genetic, phenotypic, and functional diversity (18). There is a growing evidence of intratumor heterogeneity in different types of cancers including breast cancer (19), renal carcinomas (20, 21), and glioblastomas (22). Mechanisms underlying intratumor heterogeneity can be broadly divided into those that are

powered by genomic instability or non-mutational mechanisms. The latter include stochastic variations in cellular responses between genetically identical tumor cells, modulation of cellular responses by tumor microenvironment, and/or phenotypic and functional plasticity contributed by cancer stem cells (CSCs) [reviewed in Ref. (17, 23)]. Genomic instability defined as progressive mutagenic process accompanying neoplastic growth is the major mechanism of generating new mutations. Less well-characterized mechanisms include genome doubling (24) and rare cataclysmic genomic rearrangements resulting in massive genomic rearrangements (25). According to the clonal evolution model, persistent changes in tumor genomes generate genetically and functionally distinct clones that may occupy different geographic territories within the tumor. There are many lines of evidence for the spatial patterns of intratumor heterogeneity in advanced cancers. In glioblastomas (glioblastoma multiforme, GBM), distinct patterns of genomic alterations and gene expression signatures can be found in different regions within the same tumor (22). Strikingly, molecular signatures that were previously thought to be associated with clinically distinct subtypes of GBM (26, 27) were found to co-exist within the same tumor (22). Similarly, more than 60% of all somatic mutations identified through a multi-region genetic analysis in renal carcinoma were found spatially separated within the same tumor and not detectable in every tumor region analyzed (20). These findings indicate that different sampling strategies can strongly impact the interpretation of molecular profiling data obtained with single tumor samples and emphasize the need for suitable methodologies that would take into account the spatiotemporal patterns of intratumor heterogeneity.

These considerations are of particular relevance in the context of the CSC hypothesis, which postulates that CSCs constitute only a minor fraction of tumor cells capable of initiating tumor growth [reviewed in Ref. (1, 28)]. In light of the findings that different types of tumor cells can be geographically separated within the tumor (20, 22), it is possible that CSCs may be unevenly distributed throughout the tumor. It should be noted that in many studies, the tumorigenic potential is compared between CSCs and non-CSC tumor cells isolated from a single tumor region. Thus, the relative proportion of CSCs may vary not only between different tumor types (CSC-derived malignancies vs. non-CSC tumors) but also within the tumors that comply with the CSC paradigm, depending on the tumor region analyzed. The CSC hypothesis postulates that CSC is the only type of tumor cells (in CSC-derived tumors) that possesses the propensity to initiate and maintain tumor growth (29, 30). However, in the light of consideration that a single tumor region may not be representative of the whole tumor (20, 22), it cannot be excluded that highly tumorigenic non-CSC may have been missed in analyses using single tumor specimens. In such a case, the conclusion that non-CSCs have generally lower tumorigenicity compared to CSCs would have been misleading due to a sampling bias.

The fact that genetically (and functionally) heterogeneous types of cancer cells can be separated spatially within a tumor raises several important questions concerning the identity of tumor clones that are capable of escaping from anti-cancer treatments and repopulating the tumor. There is some evidence that exposure to therapy may influence the dynamics of clonal repopulation and lead to the alternation of clonal dominance as a consequence of treatment. For example, by applying next generation sequencing to compare somatic mutations in matched pairs of *de novo* and recurrent AMLs, it was established that a minor AML clone underrepresented in the primary tumor became dominant in recurrent tumors as a consequence of chemotherapy (31). Similarly, cytogenetics and gene expression analyses in a series of sequential samples of multiple myeloma from the same patient treated with different chemotherapy regimens have revealed that tumor relapse was associated with the preferential outgrowth of a minor clone (32). In the emerging scenario, the dominance of clone A in untreated tumors can be lost during anti-cancer therapy (provided that clone A fulfils the criteria for the target cell) whereas clone B lacking the molecular target can become dominant even if it was underrepresented in untreated tumors.

The realization that intratumor heterogeneity poses one of the major challenges to overcome resistance to anti-cancer therapy raises a number of questions: are there common molecular denominators underlying resistance to different types of therapy? Is there an interaction between different populations of cancer cells residing in the same or different geographic regions of the same tumor? What is the impact of different types of anti-cancer therapy in the emergence of resistant clones? To address these issues, there is a need of suitable methodologies that would take into account the spatiotemporal patterns of intratumoral diversity. It has been proposed that multiple sampling analyses of multiple regions from matched pairs of untreated and recurrent tumors would be required to assess the impacts of intratumoral diversity on the development of resistance to anti-cancer therapies (22). Such an approach may have limited applicability in those tumors for which serial sampling is difficult to achieve. For example, serial tumor sampling in post-operated GBM is likely to be a challenge considering that repeat surgery, as a treatment option, is possible only for 15–45% of patients depending on age, neurologic performance, and extent of resection during the first operation (33). Considering that multisampling is a much more realistic task during the first surgery, a combined approach based on establishing heterogeneous primary cultures from multisampled untreated tumors and selecting from them therapy-resistant clones *in vitro* might be more feasible. Such an approach has the advantage of reducing the variability in treatment conditions and dissecting the effects of single and combined treatments. By comparing treatment responses in different types of cancer cells from the same tumor should allow to improve predictions on the efficacy of a particular treatment scheme in a particular tumor.

It should be noted that the degree of intratumoral heterogeneity may not necessarily reflect an enhanced malignant potential. It is believed that a considerable portion of new mutations arising in the course of tumor evolution are passenger mutations (7). In such a case, the number of clinically relevant oncogenic driver mutations may still be within the range attackable by combinatorial treatment regimens using different therapies applied either simultaneously or sequentially. Also, the growing realization that tumor growth

before, during, or after treatment can be driven by molecularly distinct populations of cells (31, 32) may have important implications for the rational design of combinatorial therapy regimens that would match the dynamically changing cellular and molecular composition of the tumor. Unfortunately, increased toxicity poses a general problem impeding the benefits of combined therapies. In this regard, alternating targeted therapies using agents with non-overlapping toxicity profiles may provide a means to achieve additive anti-tumor effects without increasing overall toxicity. The efficacy of alternating therapies guided by "real-time" molecular assessments has been demonstrated for metastatic lung tumor originating from adenocarcinoma of the tongue (34). In this study, a clinical benefit could be reached by applying alternating treatments with different therapeutic agents whose effectiveness was inferred by comparing whole-genome and RNA profiles of untreated and recurrent tumors.

The emerging scenario of recurrent tumor growth reveals key roles of intratumoral heterogeneity in intrinsic and acquired resistance to cytotoxic and targeted therapies. Understanding spatiotemporal patterns and dynamics of intratumoral heterogeneity before and during therapy is crucial for the ability to design individual-tailored treatment regimens best suited to a particular molecular context.

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Dihydroartemisinin is a hypoxia-active anti-cancer drug in colorectal carcinoma cells

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Tumor hypoxia is one main biological factor that drives resistance to chemotherapy and radiotherapy. To develop a novel strategy for overcoming hypoxia-induced therapy resistance, we examined the anti-neoplastic activity of the reactive oxygen donor dihydroartemisinin (DHA) in human colon cancer cell lines in normoxia and severe hypoxia. In addition, we analyzed the involvement of the intrinsic apoptosis pathway for DHA-mediated cytotoxicity in HCT116 cells in short-term and long-term *in vitro* assays. When applied at lower concentrations (≤25 μM), DHA induced apoptosis in Colo205, HCT15, and HCT116 cells, whereas necrotic cell death was increased when cells were treated with higher DHA concentrations (50 μM). However, no preference for DHA-induced apoptosis or necrosis could be detected between the treatment under normoxic or hypoxic conditions. Moreover, DHA potently reduced clonogenic survival of HCT116 cells in normoxia and hypoxia. Treatment of HCT116 cells with 25 μM DHA resulted in activation of Bax under normoxic and hypoxic conditions. Interestingly, cytochrome *c* release from the mitochondria and caspase-activation were observed only under normoxic conditions, whereas, under hypoxic conditions DHA induced a caspase-independent apoptosis-like cell death. However, under both conditions, generation of reactive oxygen species was an important mediator of DHA-induced toxicity. Further molecular analysis suggests that DHA-mediated cell death involves different sets of pro-apoptotic Bcl-2 family members. The pronounced cytotoxic activity of DHA in severe hypoxia as well as normoxia offers new perspectives for targeting the hypoxic tumor cell fraction to improve treatment outcome for cancer patients.

Keywords: therapy resistance, hypoxia, dihydroartemisinin, apoptosis, autophagy, Bim, Puma, BNIP3

INTRODUCTION

Tumor cell resistance to classical chemotherapy and radiotherapy remains a major obstacle in the treatment of solid human tumors (1). Genetic or epigenetic alterations in the tumor cells that affect cellular death and survival signaling can allow the tumor cells to escape the cytotoxic action of standard genotoxic therapies and molecularly targeted agents. Moreover, specific conditions within the tumor microenvironment and the intimate dialog between tumor cells and their surrounding stroma by soluble growth- and survival-promoting factors provide a multitude of mechanisms for therapy resistance. Therefore, current research activities focus on the identification of drugs that are active under adverse environmental conditions to counteract environment-mediated resistance to therapies.

Tumor hypoxia is a common characteristic of solid human tumors and is mostly associated with poor prognosis (2). Tumor hypoxia plays a central role in tumor progression and is one main biological factor that drives therapy resistance at multiple levels: reduced availability of molecular oxygen during acute hypoxia reduces the burst of reactive oxygen species (ROS) induced by genotoxic treatments, e.g., radiotherapy, and thus hampers the manifestation of DNA-damage and the generation of pro-death signals. Moreover, acute hypoxia also profoundly reorganizes cell signaling to increase the death threshold [overview in Ref. (3)]. Finally, chronic intermittent tumor hypoxia promotes selection of hypoxia-tolerant cells that are characterized by diminished apoptotic potential, increased therapy resistance, and worse prognosis (4–6).

At present, researchers follow diverse approaches to overcome hypoxia-mediated therapy resistance: (i) reduce the hypoxic fraction of the tumor by increasing the blood oxygen tension, (ii) specifically kill hypoxic cells by using bioreductive drugs, and (iii) reduce the tolerance of hypoxic cells by using signal transduction inhibitors targeting pathways that are essential for the survival of hypoxic cells (2, 7).

Here, we propose a novel strategy to overcome therapy resistance under conditions of acute hypoxia by using the cyclic endoperoxide dihydroartemisinin (DHA). DHA belongs to a family of compounds derived from the natural sesquiterpene

Abbreviations: Acc, *N*-acetylcysteine; CCCP, protonophore carbonyl cyanide m-chlorophenylhydrazone; DHA, dihydroartemisinin; ΔΨm, mitochondrial transmembrane potential; Hx, hypoxia; LC3B, light chain 3B; Nx, normoxia; PARP, poly (ADP-ribose) polymerase; PE, plating efficiency; ROS, reactive oxygen species; TMRE, tetramethylrhodamine ethyl ester perchlorate; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone.

lactone artemisinin (Coartem®/Riamet®) that are known to generate ROS-like superoxide anions and hydroxyl radicals as well as carbon-centered radicals upon activation of their endoperoxide bridge in the presence of ferrous iron (8). Artemisinin and DHA are already in clinical use for antimalaria treatment (9). However, both drugs also exert potent anti-neoplastic effects on human tumor cells in preclinical *in vitro* and *in vivo* investigations (10–12). Earlier studies revealed that the generation of ROS and carbon-centered radicals is critical for the toxic effects of artemisinin and derivatives on malaria parasites (13, 14). These reactive molecules also contribute to the potent anticancer activity of these compounds through alkylation of essential proteins and induction of oxidative damage to membrane lipids and DNA and subsequent ROS-dependent apoptosis that includes the activation of pro-apoptotic Bcl-2 family member Bax, and caspase-activation (11, 15, 16).

Though anti-neoplastic activity of artemisinin and derivatives is well-documented for standard treatment conditions in normoxia, the potential of these drugs to kill cancer cells under conditions of acute hypoxia and the involved molecular pathways have not yet been studied. On the basis of their potential to generate ROS and further reactive molecular species, we hypothesized that treatment with compounds of the Artemisinin drug family may be a promising approach to efficiently attack hypoxic cancer cells and overcome therapy resistance induced by acute hypoxia. To verify our hypothesis, we compared the anti-neoplastic activity of DHA under normoxic and hypoxic conditions using three different colorectal cancer cell lines as experimental model. We demonstrate for the first time that DHA is a hypoxia-active drug that efficiently kills colon cancer cells even in presence of very low oxygen levels. When treated at lower DHA concentrations ($\leq$25 μM), colon cancer cells mainly underwent apoptosis, whereas necrosis was increased when higher doses of DHA (50 μM) were applied.

Further molecular analysis of DHA-mediated cytotoxicity in HCT116 cells revealed that DHA induced the canonical mitochondrial apoptosis pathway that includes the activation of Bax, cytochrome *c* release from mitochondria into the cytosol, caspase-activation, dissipation of the mitochondrial transmembrane potential ($\Delta\Psi$m) and DNA-fragmentation. Although Bax-activation occurred to similar extent when HCT116 cells were treated under normoxic conditions, release of cytochrome *c* and caspase-activation were almost abrogated. However, a high amount of cells with fragmented or condensed DNA was observed even in the absence of caspase-activation suggesting the induction of caspase-independent apoptotic cell death by DHA in severely hypoxic cancer cells. Moreover, under both conditions DHA-induced ROS production mediated the cytotoxic effect since blocking the ROS production resulted in reduced DNA-fragmentation. In addition, hypoxic HCT116 cells induced a different set of regulatory BH3-only proteins in response to DHA compared to normoxic cells suggesting that different BH3-only proteins might contribute to the canonical and non-canonical apoptosis in normoxia and hypoxia by inhibiting anti-apoptotic Bcl-2 family members and facilitating the activation of the Bax.

MATERIALS AND METHODS

CHEMICALS AND DRUGS

Dihydroartemisinin [(3,5,6,8,9,10,12R,12aR)-decahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin-10-ol, $C_{15}H_{24}O_5$)] and propidium iodide (PI) were obtained from Sigma-Aldrich (Deisenhofen, Germany). Hoechst 33342 was purchased from Calbiochem (Bad Soden, Germany). The pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-fmk) was obtained from Bachem (Bubendorf, Switzerland). Tetramethylrhodamine ethyl ester perchlorate (TMRE) and dihydroethidium (DHE) were from Molecular Probes (MoBiTec, Goettingen, Germany).

Antibodies specific for full length and cleaved poly (ADP-ribose) polymerase (PARP), caspase-3, light chain 3B (LC3B), Bax, Bak, Bcl-xL, and Puma were obtained from Cell Signaling (Frankfurt, Germany). Bcl-2 antibody was purchased from Santa Cruz Biotechnology (Heidelberg, Germany), Bim antibody was purchased from Epitomics (Biomol, Hamburg, Germany). The antibody specifically recognizing the active conformation of Bax (Bax NT) was from Upstate (Hamburg, Germany). Moreover, we used antibodies specific for Noxa (Calbiochem, Darmstadt, Germany), cytochrome *c* (Pharmingen, Hamburg, Germany), or β-actin (Sigma-Aldrich, Deisenhofen, Germany) as well as HRP-conjugated and Cy2-conjugated secondary antibodies (Amersham-Biosciences, Freiburg, Germany).

All other chemicals and drugs were from Sigma-Aldrich if not otherwise specified.

CELL CULTURE

Colon cancer cell lines HCT15, Colo205, and HCT116 were obtained from ATCC (Bethesda, MD, USA). Morphology and phenotype of the distinct cell lines were routinely tested before and during data acquisition.

Cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (Gibco Life Technologies, Eggenstein, Germany) and maintained in a humidified incubator at 37°C and 5% CO_2 (normoxic conditions). Hypoxic cells were grown in a humidified hypoxia work station (In vivo 400, Ruskinn Technology Ltd., IUL Instruments GmbH, Königswinter, Germany) at 37°C, 0.2% O_2, and 5% CO_2.

DRUG TREATMENT

Cells were treated 24 h after seeding with 0–50 μM DHA. For treatment under hypoxic conditions, cells were transferred to the hypoxic chamber 2 h before drug treatment. For all experiments, 0.1% ethanol was used as solvent control.

QUANTIFICATION OF CELL VIABILITY AND CELL PROLIFERATION

The number of living cells was determined upon staining of the cells with the vital dye trypan blue. For this, cells were harvested with Trypsin-EDTA, re-suspended in fresh medium, diluted with trypan blue, and counted employing a Neubauer chamber.

In addition, metabolic activity of the cells was determined as an indirect measure of cell viability using the WST-1 proliferation assay according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany). Reduction of the

water-soluble tetrazolium salt WST-1 to formazan was determined using an ELISA reader (Bio-Tek, Bad Friedrichshall, Germany; 480 and 680 nm).

COLONY FORMATION ASSAY

For this long-term assay, 200–1600 cells/well were plated in 6-well plates, incubated in normoxia for 24 h, and then treated with DHA in normoxia or severe hypoxia. Plates seeded for treatment under hypoxic conditions were transferred into the hypoxic chamber 2 h prior to DHA treatment until 24 h after treatment and subsequently incubated in normoxia. Plates were incubated for a total of 10 days to allow growth of single colonies. Cells were then fixed in 3.7% formaldehyde and 70% ethanol and subsequently stained with 0.05% Coomassie Brilliant Blue. Colonies ($\geq$50 cells/colony) were counted under the microscope at fivefold magnification. The survival curves were established by plotting the log of the surviving fraction against the treatment dose. Fitting of the curves was performed using Excel software.

ANALYSIS OF APOPTOTIC AND NECROTIC CELL DEATH BY FLUORESCENCE MICROSCOPY

Changes in nuclear morphology indicative for apoptosis and necrosis were analyzed by fluorescence microscopy (Zeiss Axiovert 200, Carl Zeiss, Göttingen, Germany; G365/FT395/LP420 filter set) upon cell staining with 1.5 μM Hoechst 33342 and 2.5 μg/ml PI. Apoptotic and necrotic cells were quantified by counting the relative amount of fragmented blue or red nuclei (early or late apoptosis, respectively) and non-fragmented red nuclei (necrosis). At least 100 cells were quantified in three independent fields per well.

FLOW CYTOMETRY ANALYSES

For quantification of apoptotic DNA-fragmentation (sub-G1 population), cells were incubated for 60 min with a staining solution containing 0.1% (w/v) sodium citrate, 50 μg/ml PI, and 0.05% (v/v) Triton X-100 (v/v) and subsequently analyzed by flow cytometry (FACS Calibur, Becton Dickinson, Heidelberg, Germany; FL-2).

Dissipation of the $\Delta\Psi$m was measured using the $\Delta\Psi$m-specific dye TMRE (FL-2) as described elsewhere (17). To ensure a complete dissipation of $\Delta\Psi$m, cells were treated with 100 μM protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) before incubation with TMRE.

To quantify ROS production, cells were stained for 15 min at 37°C with 5 μM of the ROS-sensitive dye DHE (Molecular Probes, MoBiTec, Göttingen, Germany) and washed subsequently once with PBS. ROS-positive cells were detected by flow cytometry (FL-2). Treatment with 250 μM H_2O_2 (2 h) was used as positive control for ROS formation. As additional control, cells were pretreated with 2 mM *N*-acetylcysteine (Acc) to block ROS formation.

Activation of Bax was determined using the activation-specific anti-Bax NT antibody. In brief, cells were fixed for 20 min on ice in 2.5% (w/v) PFA/PBS, washed in 1% (v/v) FCS/PBS, permeabilized for 30 min on ice with 0.1% (v/v) Triton X-100/PBS, washed with PBS, and then incubated for 15 min at room temperature (RT) with a blocking solution (10% FCS/PBS). Cells were stained for 30 min with the activation-specific anti-Bax NT antibody or the respective isotype control, washed, incubated for 30 min with a cy2-conjugated anti-rabbit secondary antibody (Amersham, Freiburg, Germany), washed again, and then suspended in blocking buffer for flow cytometric analysis (FL-1).

RELEASE OF CYTOCHROME *C*

Cells were plated on cover slips, washed with PBS, and fixed for 15 min with 3% (v/v) paraformaldehyde in PBS at RT. Subsequently, cells were permeabilized for 10 min with 0.2% (v/v) Triton X-100 in PBS, washed with PBS, blocked for 30 min in 1% (v/v) fetal calf serum, and subsequently incubated for 1 h at RT with the anti-cytochrome *c* primary antibody (Pharmingen, Becton Dickinson). After repeated washing, cells were incubated for 45 min at RT in the dark with the secondary Cy2-conjugated anti-mouse antibody (Amersham, Freiburg, Germany). Finally, the cover slips were mounted with Fluorescence Mounting Medium (Dako, Hamburg, Germany). Cytochrome *c* release was visualized by fluorescence microscopy using a Zeiss Axiovert 200 microscope equipped with an Apotome with an 63× oil objective and GFP filter set (Carl Zeiss, Jena, Germany). Analysis of green fluorescence and overlay were performed with AxioVision software (Carl Zeiss, Jena, Germany).

WESTERN BLOT ANALYSIS

Cells were lysed for 10 min at 99°C in 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 50 mM dithiothreitol, and 0.01% (w/v) bromophenol blue. Proteins were separated by SDS-PAGE and blotted onto PVDF-membranes (Roth, Karlsruhe, Germany). After blocking with 5% (w/v) non-fat dry milk, membranes were incubated at 4°C over night with the respective primary antibody (1:20,000 for β-actin, 1:1000 for all other antibodies). After washing, the membranes were incubated for 1 h at RT with the secondary antibody (anti-IgG-HRP 1:2000, Amersham-Biosciences, Freiburg, Germany), washed again, and developed using enhanced chemiluminescence staining (ECL western blotting analysis system, Amersham-Biosciences, Freiburg, Germany). We indicated that protein levels were quantified by densitometry using ImageJ software (ImageJ 1.40g, NIH, USA). The respective protein levels were normalized to β-actin levels.

STATISTICS

Data represent mean values of at least three independent experiments ± standard deviation (SD). Specific values represent normalization to respective solvent controls [% treated cells − % solvent control]. Data analysis was performed by two-tail unpaired *t*-test (Prism5™ software, GraphPad Inc., La Jolla, CA, USA) or two-way ANOVA test using parametric methods and employing Bonferroni multiple comparison post-test where appropriate. *P*-values $\leq$0.05 were considered as significant.

RESULTS

DIHYDROARTEMISININ EXERTS POTENT ANTI-NEOPLASTIC EFFECTS UNDER NORMOXIC AND HYPOXIC CONDITIONS

To characterize the anti-neoplastic potential of DHA in severely hypoxic cancer cells, we first compared tumor cell survival after treatment with DHA under normoxic and under severely hypoxic conditions. For this, we treated three different human colon cancer cells (HCT15, Colo205, and HCT116 cells) with 0–80 μM DHA

for 48 h in the presence of normal (21% O_2) or greatly reduced oxygen tensions (0.2% O_2). DHA significantly reduced the number of viable HCT15, HCT116, and Colo 205 colon cancer cells in a concentration-dependent manner under normoxic (**Figure 1**, upper panel) as well as under hypoxic conditions (**Figure 1**, lower panel). The response of the three cell lines to DHA differed slightly depending on the drug concentration and the oxygen levels. Whereas HCT15 cells turned out to be highly sensitive in normoxia and hypoxia with almost 50% reduction in the number of viable cells upon treatment with 10 μM DHA under both conditions, the sensitivity of HCT116 cells was higher in hypoxia (almost 50% reduction in the number of viable cells with 10 μM DHA compared to 20% in normoxia). In contrast, the number of viable cells decreased only by around 20% in Colo205 upon treatment with 10 μM DHA under both, normoxic and hypoxic conditions. Nevertheless, the results indicate that DHA is highly active in normoxic and hypoxic colorectal cancer cells.

To characterize the mechanism of cytotoxicity more closely, the mode of cell death induced by DHA was analyzed. For this purpose, the three colon cancer cell lines were treated with 0–50 μM DHA under normoxic and hypoxic conditions. Forty-eight hours later, the cells were co-stained with the membrane permeable DNA dye Hoechst 33342 and PI, a DNA dye that cannot enter cells with intact plasma membrane integrity, to distinguish between apoptotic and necrotic cell death (**Figure 2A**). PI-negative and PI-positive cells with condensed DNA were mainly observed after treatment with 25 μM DHA under normoxia or hypoxia indicating that at this applied concentration the dominant mode of cell death was apoptosis (**Figures 2B–D**, left panels). In contrast, treatment with 50 μM DHA yielded elevated levels of PI-positive cells without DNA condensation suggesting that necrotic cell death was increased in response to treatment with higher DHA concentrations (**Figures 2B–D**, right panels). However, the impact of normoxia or hypoxia on DHA-induced necrosis is not clear. Whereas DHA-induced necrosis was much higher in normoxic than in hypoxic HCT15 and HCT116 cells, Colo205 cells showed more necrosis upon DHA treatment in hypoxia compared to normoxia.

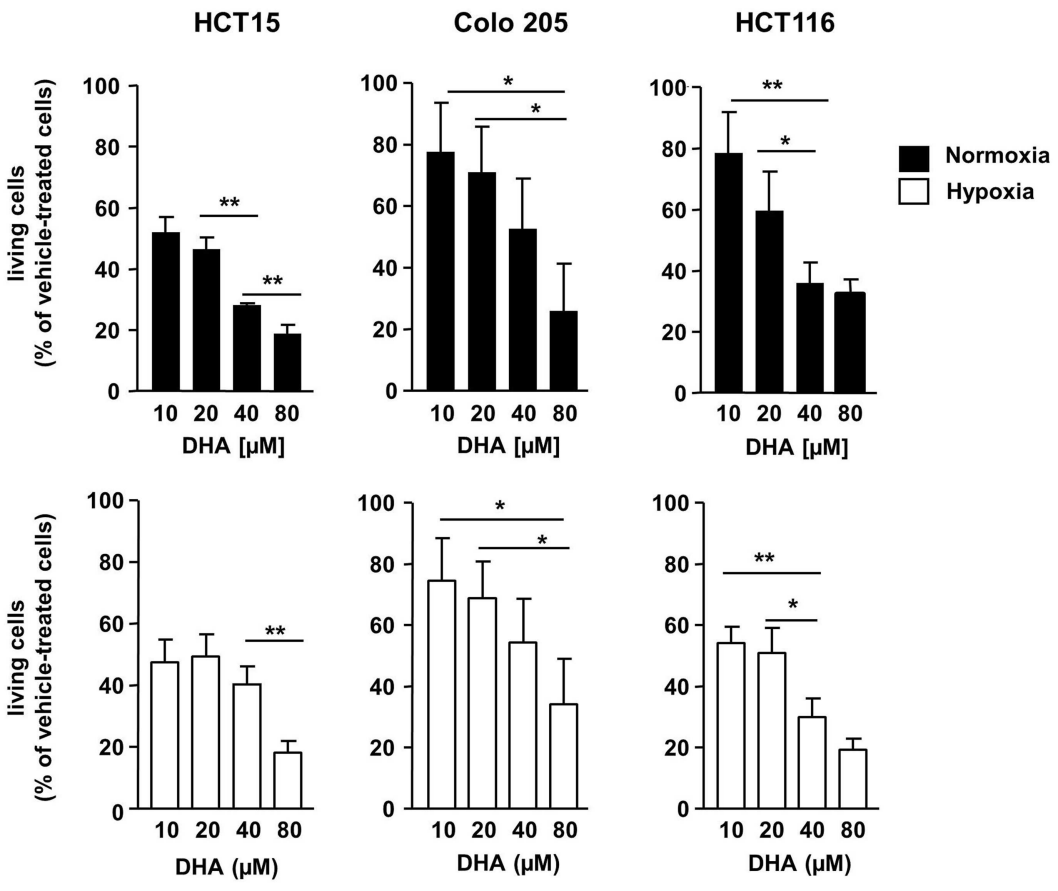


FIGURE 1 | DHA exerts potent anti-neoplastic effects on colon cancer cells in normoxia and severe hypoxia. HCT15, Colo205, and HCT116 colon cancer cells were treated for 48 h with various concentrations of DHA (0–80 μM) under normoxic (21% O_2; black bars) or severely hypoxic conditions (0.2% O_2; white bars) as indicated. Cell proliferation and survival were monitored 48 h after treatment by using the WST-1 assay that quantifies the metabolic activity of viable cells. Ethanol (0.1%) was used as solvent control. DHA reduces the number of viable HCT15, HCT116, and Colo 205 cells in a concentration-dependent manner in normoxia (upper panel) and hypoxia (lower panel). DHA displays almost similar anti-neoplastic activity in normoxia and hypoxia. Data represent means ± SD ($n = 3$). Values were normalized to the ethanol control. *P*-values were calculated applying unpaired two-tailed *t*-test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Nx, normoxia; Hx, hypoxia.

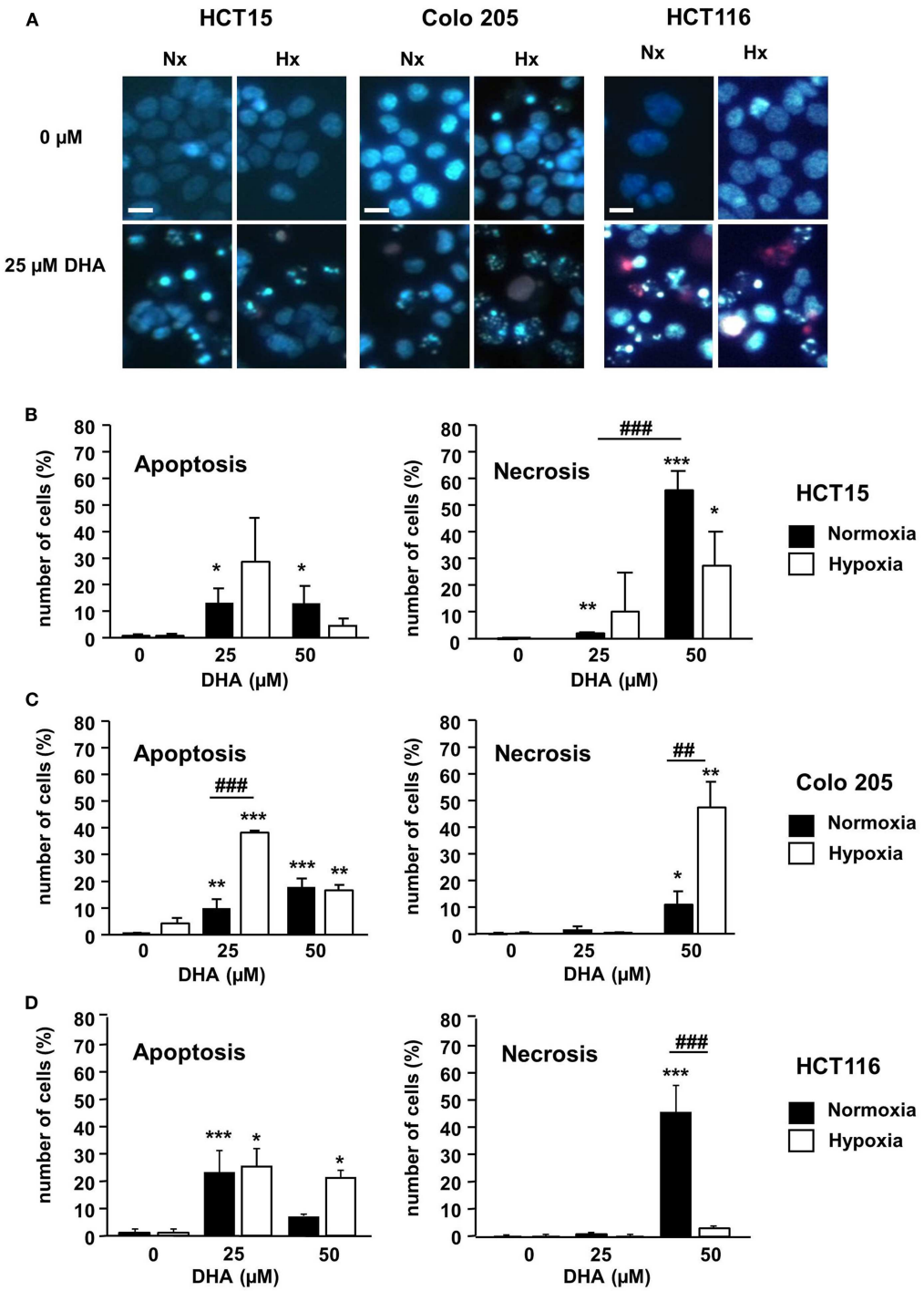


FIGURE 2 | DHA induces apoptotic and necrotic cell death in colon cancer cells in normoxia and hypoxia. HCT15, Colo 205, and HCT116 cells were treated for 24 h with solvent (Ethanol, 0 μM DHA) or DHA (25, 50 μM) under normoxic (21% O_2; black bars) or under severely hypoxic conditions (0.2% O_2; white bars). To visualize apoptotic and necrotic cell death, cells were stained with Hoechst (blue) and PI (red) and submitted to fluorescence microscopy 24 h post treatment. **(A)** Representative fluorescence microscopy pictures from one of three independent experiments are shown. Scale bar corresponds to 100 μm. Pictures were taken using 10-fold magnification. **(B–D)** At concentrations of 25 μM and below, DHA preferentially induced apoptosis, whereas at 50 μM DHA necrosis became more prominent. The percentage of apoptotic and necrotic cells in **(B)** HCT15 cells, **(C)** Colo 205 cells, and **(D)** HCT116 cells was quantified by counting the fraction of cells with normal, apoptotic, and necrotic nuclei using fluorescence microscopy (DAPI channel). At least 100 cells per field were quantified from three independent areas per condition. Data represent means from three independent experiments ± SD. Statistical analysis was performed according to unpaired two-tailed *t*-test. $^{*}P < 0.05$; $^{**,\#\#}P < 0.01$, $^{***,\#\#\#}P < 0.001$. *Indicates the significance between the solvent treated control cells and DHA-treated cells either in normoxia or hypoxia, #indicates the significance between normoxic and hypoxic cells treated with the same concentration of DHA.

Taken together, our results indicate that DHA displays cytotoxic activity in colon cancer cells under normoxic and hypoxic conditions.

EFFECT OF DIHYDROARTEMISININ ON SHORT-TERM AND LONG-TERM CELL VIABILITY IN HCT116 CELLS

Although DHA induced cell death in colon cancer cells, the drug also might affect the cell proliferation rate. Thus, we analyzed the amount of viable HCT116 cells in a short-term assay measuring the number of viable cells 48 h after treatment with DHA (**Figure 3A**) as well as in a long-term assay measuring the surviving fraction in response to DHA (**Figure 3B**) under normoxic or hypoxic conditions. Under normoxia, the number of living cells was significantly reduced 48 h after treatment with 12.5 or 25 μM DHA. In hypoxia, the number of living cells was already slightly decreased without treatment and was further lowered by treatment with DHA, however, not to the same extent as under normoxic conditions. On the other hand, the long-term assay showed that the number of surviving cells able to regrow and form a colony declines with increasing DHA concentration. Surprisingly, DHA treatment reduced the surviving fraction even slightly more efficiently under hypoxic conditions when compared to normoxic conditions. Our results indicate that the long-term toxicity of DHA is slightly improved under hypoxia as compared to normoxia, though the number of surviving cells was slightly higher after short-term treatment under hypoxic conditions. The results suggest that DHA exerts pronounced long-term anti-neoplastic effects that enhance clonogenic cell death particularly under hypoxic conditions.

DEPRIVATION OF OXYGEN RESULTS IN A SWITCH FROM CASPASE-DEPENDENT APOPTOSIS TO CASPASE-INDEPENDENT APOPTOSIS IN RESPONSE TO DIHYDROARTEMISININ

Earlier investigations in Jurkat T-lymphoma cells had demonstrated that, under standard normoxic culture conditions, DHA activates the intrinsic apoptosis pathway to trigger ROS-dependent cell death (11). Intrinsic apoptosis is characterized by the activation of the Bcl-2 effector proteins Bax and/or Bak which, in turn, induce the permeabilization of the outer mitochondrial membrane and cytochrome *c* release from the mitochondrial intermembrane space into the cytosol, which acts as a co-factor to activate the caspase cascade [for review see Ref. (18)]. This ultimately results in dissipation of the $\Delta\Psi$m and DNA-fragmentation. To gain insight into the regulation of DHA-induced cell death in HCT116 cells under normoxic and hypoxic conditions, we compared the canonical steps of the intrinsic apoptosis pathway in HCT116 cells in response to DHA at 21 and 0.2% O_2 (**Figure 4**). In normoxia, DHA readily induced Bax-activation (**Figure 4A**), cytochrome *c* release into the cytosol (**Figure 4B**), and cleavage of caspase-3 and of the caspase-3 substrate PARP (**Figure 4C**), dissipation of $\Delta\Psi$m (**Figure 4F**), and DNA-fragmentation (**Figure 4G**), respectively. Though Bax-activation was detected to a similar extent upon DHA treatment under hypoxic conditions, cytochrome *c* release into the cytosol, caspase-3 activation, and PARP-cleavage were abrogated under these conditions. In addition, blocking caspase-activation by co-treatment with pan-caspase inhibitor zVAD-fmk and DHA under normoxia prevented cleavage of caspase-3 and PARP (**Figure 4D**) as well as DNA-fragmentation (**Figure 4E**), suggesting that the canonical apoptosis pathway is activated in response to treatment with DHA under normoxic conditions.

Interestingly, mitochondrial dissipation (**Figure 4F**) and DNA-fragmentation (**Figure 4G**) were only slightly reduced in hypoxic cells in response to treatment with 25 μM DHA when compared to treatment under normoxic conditions. Thus, our results suggest that, in response to DHA, a caspase-independent apoptotic cell death occurred when cells were treated under hypoxic conditions.

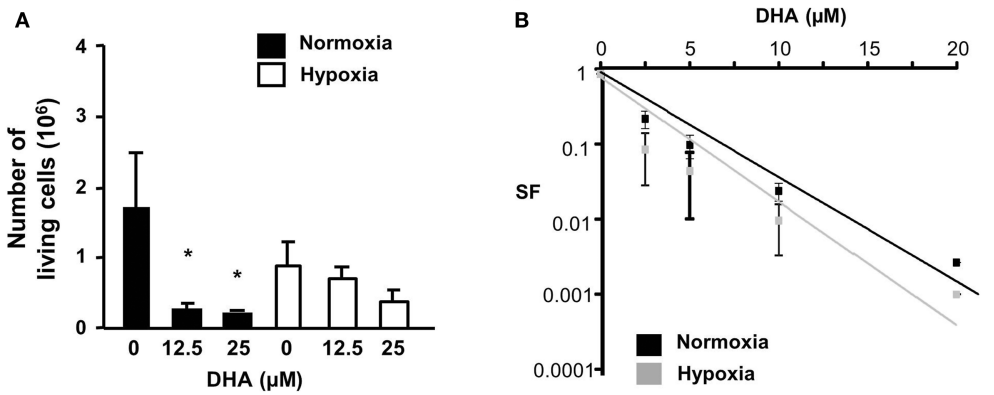


FIGURE 3 | DHA reduces cell survival and eradicates clonogenic tumor cells in normoxia and hypoxia. **(A)** HCT116 cell were treated with DHA (12.5, 25 μM) or with ethanol (solvent control, 0 μM DHA). Forty-eight hours later, living cells were counted using trypan blue exclusion dye. *P*-values were calculated applying unpaired two-tailed *t*-test; $*P < 0.05$. DHA significantly reduced the number of viable cells under normoxic conditions, but only slightly decreased the number of viable cells under hypoxic conditions. **(B)** HCT116 cells were plated for colony formation assay, treated 24 h later for 24 h with 0–20 μM DHA under normoxic (21% O_2) or hypoxic conditions (0.2% O_2) and subsequently further incubated at 21% O_2 for additional 10 days. Values were normalized to the plating efficiency of the cells treated with ethanol (0 μM DHA). Data show the surviving fractions (SF) from three independent experiments (means ± SD). DHA-induced eradication of clonogenic tumor cells was slightly better under hypoxic than under normoxic conditions.

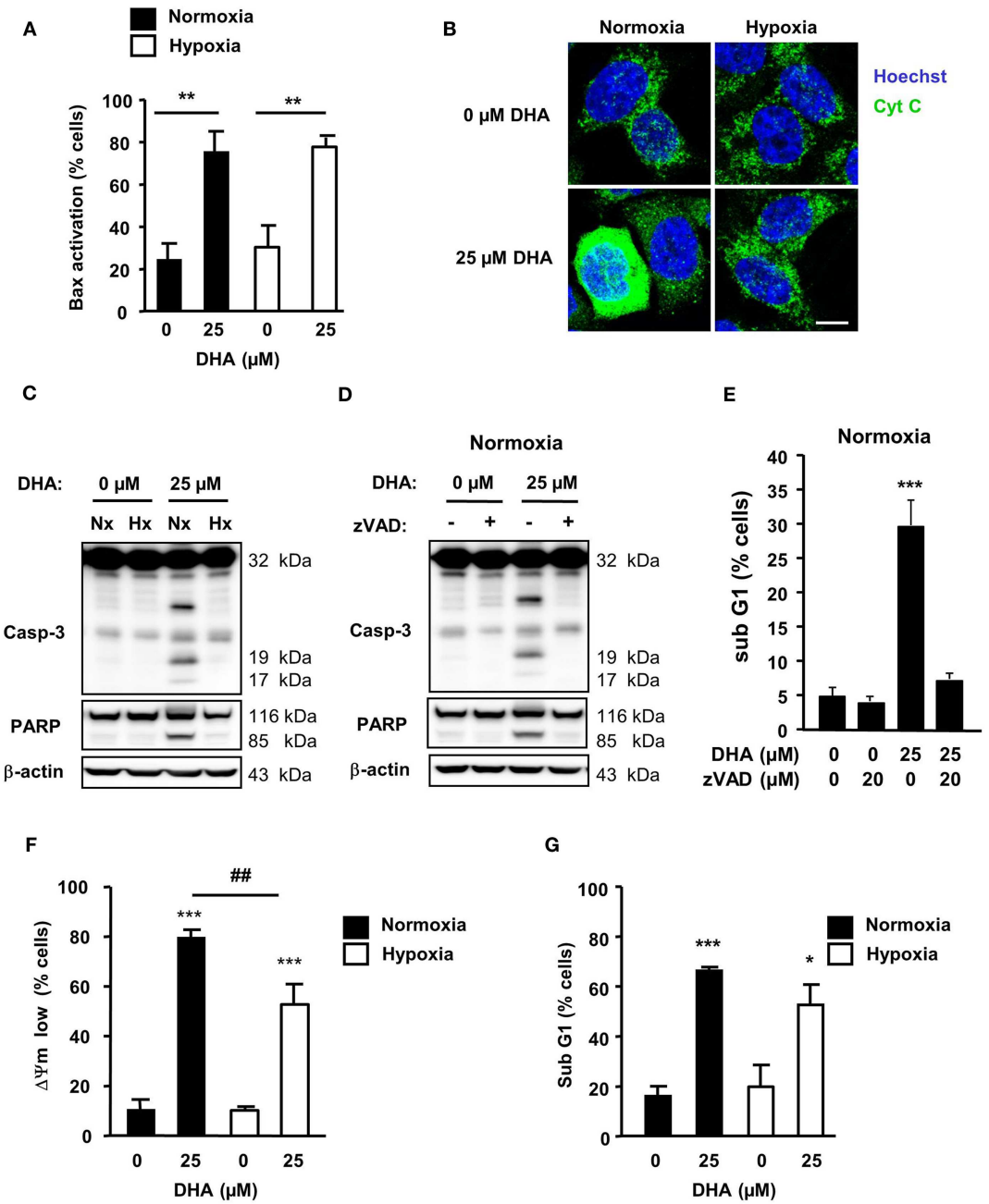


FIGURE 4 | DHA induces caspase-dependent apoptosis in normoxia but a caspase-independent apoptotic cell death in hypoxia. HCT116 cells were treated for 12–48 h under normoxic and hypoxic conditions with solvent control (0 μM), 12.5 or 25 μM DHA as indicated. **(A)** DHA-induced Bax-activation is similar in normoxia and hypoxia. Bax-activation was monitored by flow cytometry 12 h after treatment using an activation-specific anti-Bax antibody. **(B)** DHA induces significant release of cytochrome *c* only in normoxia. Cytochrome *c* release was visualized 12 h after DHA treatment by immunofluorescence (Zeiss Cell Observer fluorescence microscope with Apotome; Axiocam MRm camera; GFP filter). Scale bar corresponds to 10 μm. Data show representative pictures from three independent experiments. **(C,D)** Activation of caspase-3 and cleavage of caspase-3 substrate PARP were analyzed by western blotting at 24 h after treatment with DHA. Data are representative blots out of three independent experiments. **(C)** DHA-induced caspase-activation is abrogated in hypoxia. Caspase-3 activation **(D)** and DNA-fragmentation **(E)** can be blocked by co-treatment with the pan-caspase inhibitor zVAD-fmk under normoxic conditions. DHA induces depolarization of the mitochondrial membrane potential [$\Delta\Psi$m, **(F)**] and DNA-fragmentation [sub-G1, **(G)**] in normoxia and hypoxia. Depolarization of $\Delta\Psi$m **(F)** and DNA-fragmentation **(G)** were determined at 24 h after treatment by flow cytometry. **(A,E–G)** Data represent means ± SD from at least three independent experiments. *P*-values were calculated applying unpaired two-tailed *t*-test; $^{*}P < 0.05$; $^{**,\#\#}P < 0.01$; $^{***}P < 0.001$. *Indicates the significance between the solvent-treated control cells and DHA-treated cells either in normoxia or hypoxia, #indicates the significance between normoxic and hypoxic cells treated with the same concentration of DHA.

DIHYDROARTEMISININ-INDUCED ROS PRODUCTION CONTRIBUTES TO THE CYTOTOXIC EFFECTS UNDER NORMOXIC AND HYPOXIC CONDITIONS

Earlier reports including own data pointed to ROS-dependent induction of apoptosis by DHA and related compounds (11, 15, 16). Therefore, we next examined whether treatment with DHA would increase cellular ROS levels in HCT116 cells and whether DHA-induced apoptosis was ROS-dependent. As shown in **Figure 5A**, the amount of ROS-positive cells increased in response to treatment with DHA in a concentration-dependent manner under both, normoxic and hypoxic conditions. However, ROS production was significantly lower when treatment was performed in severe hypoxia. Moreover, pre-treatment with the radical scavenger Acc not only decreased DHA-induced ROS production (**Figure 5A**), but concurrently decreased DHA-induced DNA-fragmentation under normoxic and hypoxic conditions (**Figure 5B**). These observations demonstrate that the induction of ROS is essential for the cytotoxic action of DHA in both treatment conditions.

NORMOXIC AND HYPOXIC HCT116 CELLS UP-REGULATE DIFFERENT SETS OF BH3-ONLY PROTEINS UNDER NORMOXIC AND HYPOXIC CONDITIONS

Since activation of Bax seems to be an important step during DHA-induced cell death under normoxic and hypoxic conditions, a western blot analysis was performed to examine whether a change in the expression of other Bcl-2 family members could contribute to apoptosis induction (**Figure 6**). HCT116 cells express higher levels of Bax than the closely related Bak as well as higher levels of the anti-apoptotic Bcl-xL than the closely related protective Bcl-2. However, the expression of these four proteins was not affected by DHA. In contrast, treatment with DHA resulted in slightly increased levels of the anti-apoptotic Mcl-1, although Mcl-1 levels were lower when cells were cultured in hypoxic condition as compared to normoxic conditions. Interestingly, the pro-apoptotic BH3-only protein Noxa showed similar regulation like its interacting partner Mcl-1.

In contrast, levels of the BH3-only proteins Bim and Puma were only elevated in response to treatment with DHA when treatment was performed under normoxic conditions. Instead, expression of the BH3-only protein BNIP3 was up-regulated when HCT116 cells were exposed to severe hypoxia and its levels further increased after DHA treatment though BNIP3 was not detected in normoxia.

Taken together, our data suggest that different sets of BH3-only proteins are up-regulated in response to DHA under normoxic and hypoxic conditions.

BNIP3 is known to interact with Bcl-2 thereby counteracting Bcl-2's anti-apoptotic activity or preventing Bcl-2 from binding to Beclin-1. The former interaction results in apoptosis induction whereas the latter one results in autophagy induction. In addition, autophagy-associated cell death might also contribute to clonogenic cell death. Therefore, we examined the induction of autophagy by analyzing LC3B processing to a 14 kDa form in HCT116 cells. Interestingly, an increase of the 14 kDa LC3B band was observed under normoxic conditions when BNIP3 expression was hardly detectable. In contrast, under hypoxic conditions, no increase of processed LC3B could be detected although BNIP3 levels were dramatically increased. Thus, our results suggest that BNIP3 does not regulate autophagy but rather apoptosis in hypoxic HCT116 cells.

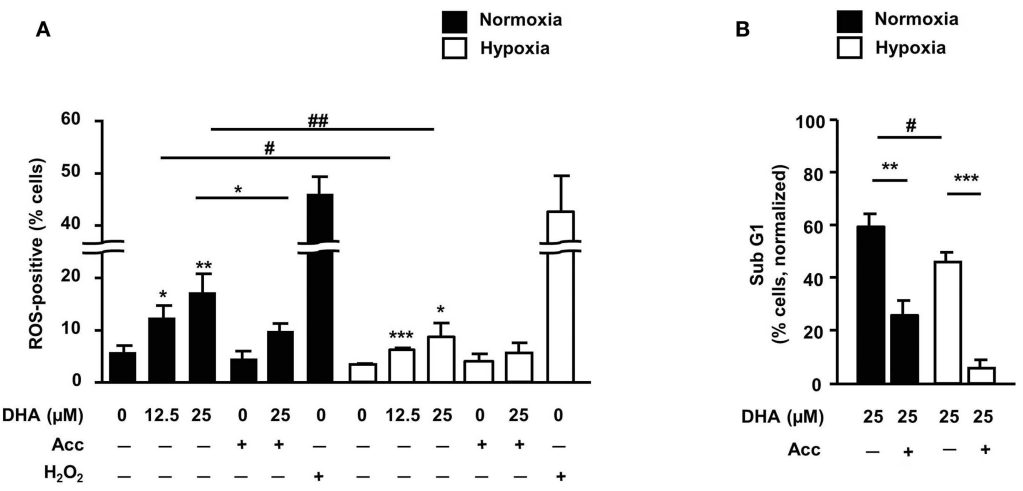
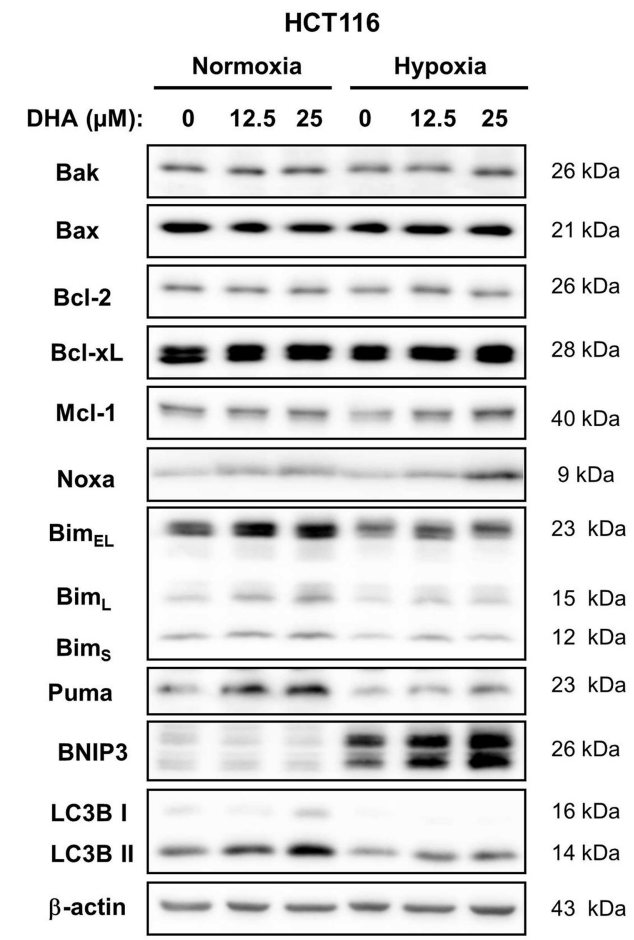


FIGURE 5 | ROS are important mediators of DHA-induced cytotoxicity in both, normoxia and hypoxia. **(A,B)** HCT116 cells were pre-incubated for 30 min in the absence or presence of the radical scavenger *N*-acetylcystein (Acc; 2 mM) and then treated for 24 h **(A)** or 48 h **(B)** with DHA (0–25 μM) under normoxic and hypoxic conditions as indicated. **(A)** DHA induces ROS under normoxic and hypoxic conditions in a concentration-dependent manner. Treatment with 250 μM H_2O_2 for 2 h was applied as a positive control for ROS formation. Pre-treatment with the ROS scavenger *N*-acetylcystein (Acc) was used to intercept and neutralize ROS. ROS formation was detected by flow cytometry upon loading of the cells with the oxidation-sensitive dye DHE. **(B)** DHA-induced DNA-fragmentation is ROS-dependent in normoxia and hypoxia. DNA-fragmentation (sub-G1) was analyzed by flow cytometry after staining the cells with PI. Data represent means ± SD from three independent experiments. *P*-values were calculated applying unpaired two-tailed *t*-test. $^{*,\#}P < 0.05$; $^{**,\#\#}P < 0.01$; $^{***}P < 0.001$. *Indicates the significance between the solvent-treated control cells and DHA-treated cells either in normoxia or hypoxia, #indicates the significance between normoxic and hypoxic cells treated with the same concentration of DHA.



Mcl1 (%):	100	93	87	64	72	90
Noxa (%):	100	162	164	107	119	221
Bim_{EL} (%):	100	135	116	67	66	59
Bim_{L} (%):	100	168	188	65	83	78
Bim_{S} (%):	100	116	118	44	61	48
Puma (%):	100	215	202	48	52	73
BNIP3 (%):	100	91	79	307	332	346
LC3B II (%):	100	141	167	54	62	63

FIGURE 6 | BH3-only proteins Bim, Puma, Noxa, and BNIP3 are differentially regulated in response DHA in normoxia and hypoxia. HCT116 cells were treated with 0, 12.5, or 25 μM DHA under normoxic or under hypoxic conditions as indicated. Twenty-four hours later, whole cell lysates were made. Levels of anti-apoptotic and pro-apoptotic proteins of the Bcl-2 family were analyzed by western blot. Induction of autophagy was accessed by western blot using an antibody against LC3B and a subsequent analysis of processed LC3B form (LC3B II, 14 kDa). Levels of anti-apoptotic Bcl-2 and Bcl-xL and pro-apoptotic Bax and Bak did not change in response to treatment with DHA under normoxic and hypoxic conditions. Levels of BH3-only protein Noxa were increased in response to DHA in normoxia as well as in hypoxia. Levels of Bim and Puma were only increased by DHA in normoxia, whereas levels of BNIP3 were elevated by DHA only in hypoxia. Induction of autophagy in response to DHA was observed only under normoxic conditions. Protein levels were analyzed by densitometry and normalized to β-actin levels. Relative protein levels are shown below the respective blots. Data show representative blots from at least two independent experiments.

DISCUSSION

Hypoxia is a major biological factor that limits the success of anti-cancer treatment. Here, we demonstrate for the first time that the anti-neoplastic cyclic endoperoxide DHA is a hypoxia-active anti-cancer drug that efficiently eradicates colon cancer cells not only under normoxic conditions but also when treatment is performed in a severely hypoxic microenvironment.

ANTI-NEOPLASTIC ACTIVITY IN NORMOXIA AND HYPOXIA

Under normoxic conditions, DHA potently induced cell death, particularly apoptosis, in colon cancer cells whereas necrotic cell death was only induced to a substantial extent when rather high drug concentrations were used.

The main mode of DHA-induced cell death in colon cancer cells under hypoxic conditions also resembled apoptosis. This assumption is based on the findings that dissipation of the mitochondrial membrane potential as well as DNA-fragmentation were still observed in severe hypoxia, though caspase-3 activation was greatly reduced. This is reminiscent of a caspase-independent apoptosis with DNA-fragmentation that was described before (19). In this context, previous publications suggested a translocation of apoptosis-inducing factor (AIF) and endonuclease G (EndoG) from mitochondria to the nucleus, where both proteins are able to initiate DNA-degradation independent of caspase-activation (20, 21). The pronounced sensitivity to DHA-induced apoptosis observed in short-term assays strongly correlated with efficient eradication of clonogenic HCT116 cells in long-term colony formation assays. Notably, the drug displayed even slightly higher activity in HCT116 cells treated under severely hypoxic conditions.

POTENTIAL OF DHA IN ANTI-CANCER THERAPIES

A hypoxic microenvironment renders cancer cells resistant to most standard anti-cancer therapies and, thus, constitutes a major obstacle in the treatment of cancer patients (2). The molecular mechanisms leading to the increased resistance of hypoxic cancer cells to standard chemotherapeutic drugs or ionizing radiation are not fully understood. However, previous publications have demonstrated that hypoxic tumor cells stabilize the hypoxia-inducing factor-1α (HIF-1α) leading to the HIF-1α-dependent cell protection, increased transcription of vascular endothelial factor (VEGF), and tumor vessel formation (3, 22). There is increasing evidence that DHA interferes with HIF-1α activation, VEGF expression, and angiogenesis. On the one hand, DHA treatment reduced hypoxia-induced HIF-1α activation and VEGF expression in multiple myeloma and C6 rat glioma cells resulting in reduced tumor cell growth and angiogenesis, respectively (23, 24). On the other hand, DHA enhanced the toxicity of cisplatinum in lung adenocarcinoma cells *in vivo* and this effect was accompanied by reduced expression of HIF-1α and VEGF and reduced tumor microvessel density (25). Interestingly, this anti-angiogenic effect of DHA was attributed to growth inhibition of endothelial cells and depended on the level of tissue oxygenation and the drug concentration (26). Thus, the anti-angiogenic effects described above differ from our findings where DHA was almost similarly active under normoxic and hypoxic conditions in all three colon cancer cell lines examined (see **Figure 1**). The pronounced activity of DHA in severe hypoxia suggests that the drug has a great

advantage over many standard anti-cancer drugs and radiotherapy whose cytotoxic effects strongly rely on adequate oxygen tensions as well as on intact cell death pathways (7, 27).

Moreover, although not yet examined in clinical trials, DHA is able to improve treatment efficacy in combination with ionizing radiation or cisplatin in preclinical models (25, 28, 29) and thus shows great potential as anti-neoplastic drug.

Of note, drug concentrations used in the present study correspond to DHA plasma levels achievable in humans. Upon repeated intravenous application of 8 mg/kg artesunate, peak plasma concentrations up to 5.8 mg/l (around 20 μM) of its principal active metabolite DHA were measured in healthy volunteers (30).

GENERATION OF ROS IN RESPONSE TO DHA

Up to now, preclinical studies about the anti-neoplastic activity of artemisinin and derivatives had been mostly restricted to experiments conducted in normoxia. These studies demonstrated that the cytotoxic action of these compounds involves the formation of ROS and membrane oxidation upstream of apoptosis-associated changes in mitochondrial function (11, 31, 32). Moreover, chronic treatment of LN-229 glioblastoma cells with artesunate led to a continuous increase in ROS and in oxidative DNA-damage resulting in continuously increasing DNA double-strand breaks and finally tumor cell death (16).

Our present data show for the first time that DHA can induce ROS formation in hypoxia, though at slightly reduced levels. The generated ROS were important mediators of DHA-induced cytotoxicity under normoxic as well as under hypoxic conditions since blocking ROS formation by Acc resulted in abrogation cell death under normoxic or hypoxic conditions. Although DHA-induced oxidative DNA-damage and the resulting DNA-damage response have not been studied here, it is highly likely that treatment with DHA under normoxic as well as under hypoxic conditions will also result in the generation of DNA double-strand breaks and, finally, in cell death due to the inability of the cells to repair the damaged DNA.

Taken together, our novel data indicate that the generation of ROS is a hallmark of DHA-induced cell death under normoxic as well as under hypoxic conditions.

ROLE OF BCL-2 FAMILY MEMBERS FOR THE REGULATION OF DHA-INDUCED CLONOGENIC DEATH IN HYPOXIA

The effector proteins of the Bcl-2 protein family, Bax and Bak, are essential for apoptosis induction. Both of them become activated in response to many apoptotic stimuli (11, 33–35). In addition, up-regulation of Bax levels was detected in response to cerebral ischemia or following exposure to ionizing radiation in a p53-dependent way (36–38). Although the levels of pro-apoptotic proteins Bax and Bak were not changed in response to treatment with DHA, their activation might be essential for DHA-induced cell death. Indeed, Bax was activated in most HCT116 cells after treatment with DHA under normoxia and hypoxia. Thus, the results suggest, that at least Bax might be a very important mediator of DHA-induced cytotoxicity under normoxic as well as under hypoxic conditions.

Furthermore, anti-apoptotic proteins of the Bcl-2 family participate in the regulation of the balance between apoptosis and autophagy in response to cell stress, including hypoxia (39, 40). They associate either with Bax or Bak to prevent outer mitochondrial membrane permeabilization and apoptosis induction or with Beclin-1 preventing this molecule from induction of autophagy (18, 41–45). Both, apoptosis and autophagy could contribute to clonogenic cell death induced by DHA. However, Bcl-2 or Bcl-xL levels did not change in response to DHA treatment under normoxic or hypoxic conditions. Only Mcl-1 levels were decreased when cells were grown under hypoxia. Treatment with DHA slightly increased Mcl-1 levels under hypoxia, but had no effect under normoxia. Yet, the three protective proteins can be released from a complex formed with Bax/Bak or Beclin-1 by a competitive interaction with BH3-only proteins such as Bim, Puma, Noxa, and BNIP3. Interestingly, Noxa levels were increased in response to DHA under normoxia as well as hypoxia. In contrast, Bim and Puma levels were up-regulated only under normoxic conditions, whereas BNIP3 levels were increased only under hypoxic conditions after treatment with DHA. Furthermore, previous publications have shown that BNIP3 up-regulation was observed preferentially in hypoxic cells and was often associated with autophagy induction due to the release of Beclin-1 from interaction with Bcl-2 or Bcl-xL by replacement (46). However, BNIP3 is also able to induce apoptosis by binding to Bcl-2 and Bcl-xL (47). Surprisingly, autophagy induction as measured by LC3B processing was detected only in normoxic cells in response to DHA treatment in the absence of BNIP3. In contrast, under hypoxia, when BNIP3 levels were greatly increased, processing of LC3B could not be detected. Thus, our data suggest that BNIP3 regulates rather apoptosis than autophagy under hypoxic conditions. However, up-regulation of the BH3-only proteins, Bim and Puma could result in an enhanced interaction with Bcl-2 and Bcl-xL to induce both, autophagy and apoptosis, under normoxic conditions. Taken together, our data clearly demonstrate a differential expression of BH3-only proteins under normoxic and hypoxic conditions in response to treatment with DHA in HCT116 colon cancer cells to induce cancer cell death.

Given that we did not analyze the protein complexes in more detail, we cannot state whether the discussed BH3-only proteins associate with the anti-apoptotic Bcl-2 members to release Beclin-1 and induce autophagy or to activate Bax and Bak to induce apoptosis. A further analysis of Bcl-2, Bcl-xL, and Mcl-1 as well as their interacting partners will clarify the molecular mechanisms by which DHA induces apoptosis and autophagy under normoxic and hypoxic conditions.

CONCLUSION

In contrast to many genotoxic drugs and radiotherapy, which are generally less efficient in hypoxic tumor cells, DHA exerts pronounced anti-neoplastic effects under severely hypoxic conditions. While the canonical intrinsic apoptosis pathway seemed to be predominantly activated by DHA in oxygenated cells, DHA induced a caspase-independent apoptosis-like cell death in severe hypoxia. Since DHA targets normoxic as well as hypoxic cells with equal potency, the drug might be a promising tool to improve treatment outcome, particularly in hypoxic human tumors resistant to conventional therapies.

AUTHOR CONTRIBUTIONS

Verena Jendrossek designed the study. Teona Ontikatze, Justine Rudner, and René Handrick performed the experiments and analyzed the data. Justine Rudner and Verena Jendrossek interpreted the data, drafted and revised the manuscript. All authors read the manuscript and gave the final approval for publication. The authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

ACKNOWLEDGMENTS

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Estrogen signaling and the DNA damage response in hormone dependent breast cancers

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Estrogen is necessary for the normal growth and development of breast tissue, but high levels of estrogen are a major risk factor for breast cancer. One mechanism by which estrogen could contribute to breast cancer is via the induction of DNA damage. This perspective discusses the mechanisms by which estrogen alters the DNA damage response (DDR) and DNA repair through the regulation of key effector proteins including ATM, ATR, CHK1, BRCA1, and p53 and the feedback on estrogen receptor signaling from these proteins. We put forward the hypothesis that estrogen receptor signaling converges to suppress effective DNA repair and apoptosis in favor of proliferation. This is important in hormone-dependent breast cancer as it will affect processing of estrogen-induced DNA damage, as well as other genotoxic insults. DDR and DNA repair proteins are frequently mutated or altered in estrogen responsive breast cancer, which will further change the processing of DNA damage. Finally, the action of estrogen signaling on DNA damage is also relevant to the therapeutic setting as the suppression of a DDR by estrogen has the potential to alter the response of cancers to anti-hormone treatment or chemotherapy that induces DNA damage.

Keywords: estrogen receptor, DNA damage response, breast cancer, p53, BRCA1, DNA repair, tamoxifen, DDR

DNA DAMAGE INDUCED BY ESTROGEN

Lifetime exposure to estrogen is a major risk factor for breast cancer. Elevated serum levels of estrogen are associated with a 2–2.5× greater risk of breast cancer development (1) and high levels of estrogen in the breast of postmenopausal women are associated with increased cancer risk (2). Estrogen signaling drives proliferation in the 60–70% of breast cancers that express the estrogen receptor, and adjuvant anti-estrogen therapy is prescribed to the majority of these patients to prevent breast cancer recurrence.

Estrogen signals through its two receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ). Only ERα is essential for breast development and activates pro-proliferative signaling in the normal breast and breast cancer, whereas ERβ generally antagonizes ERα in the breast (3). Upon estrogen binding ERα acts by parallel pathways to alter gene expression. ERα translocates to the nucleus to activate gene targets directly or in cooperation with co-activator proteins, or it can transactivate growth receptors to boost receptor tyrosine kinase signaling. These pathways converge to promote growth and proliferation and suppress apoptosis (3).

Despite the risks associated with estrogen exposure the exact mechanisms by which estrogen contributes to the initiation and progression of breast cancer remains elusive. However, a major mechanism is potentially the induction of DNA damage as estrogen treatment leads to double stranded DNA breaks and genomic instability (1, 4, 5). Early breast cancer lesions exhibit chromosomal instability and aneuploidy (6), and in rat models this is linked to estrogen exposure (7). Estrogen can induce DNA damage via the production of oxidative metabolites that cause DNA adducts, or other oxidative DNA damage, and this is supported by *in vitro* and animal model studies (1). The second explanation for estrogen-induced DNA damage is that hyperactivated estrogen signaling provokes excessive proliferation when pathways become dysregulated, and this theory has strong support from *in vitro* modeling and gene signatures in breast cancer (3). Excessive proliferation promotes DNA damage accumulation due to insufficient timely repair leading to replication fork stalling and possibly even double stranded DNA breaks (8). It is likely that both carcinogenic estrogen metabolites and deregulated estrogen signaling contribute to estrogen-induced DNA damage. In this perspective a third possibility is raised, that estrogen signaling suppresses the DNA damage response and DNA repair to allow the accumulation of genomic change conducive to tumorigenesis.

DNA DAMAGE RESPONSE AND DNA REPAIR PATHWAYS ALTERED BY ESTROGEN SIGNALING

DNA damage is recognized and processed by series of pathways called the "DNA damage response (DDR)". The DDR assesses the scope and severity of DNA damage to initiate cell cycle arrest, senescence, repair, or in the case of irreparable damage, apoptosis. If repair is activated then a number of different repair mechanisms can be engaged [reviewed in Ref. (9)]. Small lesions of damaged or incorrectly inserted nucleotides are repaired by base excision repair (BER), nucleotide excision repair (NER), or mismatch repair (MMR). The more catastrophic double stranded breaks are repaired via non-homologous end-joining (NHEJ) or homologous recombination (HR). Small distorting lesions are extremely common so the pathways that repair these defects (BER, NER, and

MMR) are also activated by constant genome surveillance, and repair is coupled to transcription and DNA replication.

The DDR signals through three main effector kinases, ATM, ATR, and DNA-PK. ATM and DNA-PK recognize double stranded breaks whereas ATR responds to single stranded regions that occur at stalled replication forks and double stranded break overhangs. The signaling pathways downstream of ATM, ATR, and DNA-PK involve a myriad of proteins, however there are a number of key effector proteins that include CHK1, CHK2, BRCA1, 53BP1, and MDC1 which signal to DNA repair coordinators such as BRCA2, PALB2 and to cell cycle checkpoints and the apoptotic machinery. The major tumor suppressor protein, p53, is activated downstream of ATM/ATR, and acts as a genome guardian to determine whether cells should arrest or apoptose. There is significant cross-talk between the various pathways depending on the nature and severity of the DNA damage.

The DDR is important to estrogen carcinogenesis as it dictates how estrogen-mediated damage is processed by breast cells. In prior genome wide studies of estrogen action, the major regulatory nodes of the ERα transcriptional program have included proliferation, growth, and apoptosis, but not the DDR or DNA repair (3). However, there is a growing body of literature, which identifies estrogen signaling as regulating key effector DDR proteins such as ATM, ATR, p53, BRCA1, and BRCA2, as well as direct interactions with the DNA repair machinery. This is significant not only for estrogen carcinogenesis, but also for the processing of any genotoxic insults by estrogen-responsive tissues. Described below are the most important interactions between ERα, the DDR, and DNA repair pathways (**Figure 1**). ERβ is not discussed in this perspective, but it should be noted that ERβ has opposing effects to ERα in many contexts (10), and this is also true of regulation of the DDR and DNA repair (11–13).

REGULATION OF EFFECTOR KINASES ATM, ATR, AND DNA-PK

ATM and ATR are key initiators of the DDR, and both are negatively regulated by ERα. ERα downregulates transcription of ATM via the activation of *miR-18a* and *miR-106a* (11). The ATR/CHK1 signal transduction cascade is suppressed by ERα-transactivated AKT phosphorylation of TOPBP1 to prevent an interaction with ATR at sites of DNA damage (15). AKT also phosphorylates CHK1 to prevent its interaction with co-activator CLASPIN (15). The downregulation of ATM and ATR by ERα interferes with the induction of cell cycle checkpoints so that cells continue to progress through the cell cycle after DNA damage, and DNA repair is delayed or not engaged (15, 16). Estrogen activity does not, however, preclude activation of the DDR. γ-H2Aχ foci form in response to estrogen-induced DNA damage, and the co-localization of Rad51 to these foci suggests the activation of HR (4).

While ERα negatively regulates both ATM and ATR, it is possible that ERα positively regulates DNA-PK mediated repair based on recent findings of DNA-PK regulation by the androgen receptor (AR). AR regulation of DNA-PK catalytic subunit (DNA-PKcs) promotes the repair of DNA double stranded breaks and resistance to DNA damage and DNA-PKcs likewise potentiates the function of AR (17). Like AR, ERα is in a complex with DNA-PK (18) and ERα is stabilized and its transcriptional function potentiated by DNA-PK (19), and by analogy to AR, ERα may also transactivate DNA-PK.

If ERα does positively regulate DNA-PK, ERα may suppress DNA repair processes of higher fidelity (ATM- and ATR-mediated) in preference for DNA-PK-mediated NHEJ. This is consistent with observations of ERα activity leading to the accumulation of DNA damage (1) as it would sustain proliferation by not engaging the ATM/ATR pathways, while promoting DNA-PK-mediated NHEJ to maintain genome integrity. Toillon et al. found that estrogen treatment of irradiated breast cancer cells led to their sustained proliferation without any increase in p53 activation or apoptosis (20). This is consistent with a failure to activate ATM or ATR but the repair of DNA by DNA-PK mediated NHEJ.

BRCA1

BRCA1 is a downstream effector of the DDR that is recruited to sites of DNA damage, functions directly in HR, but also influences cell cycle arrest and other DNA repair pathways. There is strong evidence that BRCA1 limits estrogen-mediated tumorigenesis: *Brca1* knockout mice show an enhanced proliferative response to estrogen treatment and accelerated development of preneoplastic mammary lesions (21), and the reduction of serum estrogen levels by oophorectomy protects carriers of the *BRCA1* mutation against breast cancer (22). Indeed, BRCA1 has a negative effect on ERα, through direct binding to inhibit ERα-mediated gene transcription (23, 24), downregulation of ERα co-activator, p300 (25), reduced cross-talk from growth factor signaling (26), and potentially monoubiquitination (25, 27). These effects are antagonized by cyclin D1, a direct transcriptional target of ERα that is instrumental in estrogen-induced proliferation (28).

While BRCA1 suppresses ERα, ERα regulation of BRCA1 enhances BRCA1 function. Estrogen promotes transcription of *BRCA1* via binding of an ERα/p300 complex (29), and stimulates the formation of a complex between ERα, CBP, and BRCA1 that facilitates double stranded break repair (30). Surprisingly, BRCA1 induces the transcription of *ESR1* which encodes ERα, and the positive feedback between BRCA1 and ERα provides a rational explanation for why many BRCA1 negative cancers are ERα negative (31).

p53

Estrogen receptor α and p53 have a bi-directional relationship affecting both expression and function. The *TP53* gene is transcriptionally activated by ERα (32, 33) and downstream of ERα-target, c-MYC (34), and ERα stabilizes the p53 protein (35). Despite ERα inducing higher levels of p53 it may not be active: in breast cancer cell lines estrogen induces cytoplasmic redistribution of p53 to reduce its transcriptional function (12, 36). ERα alters the p53 transcriptional program to reverse transcriptional activation and repression by p53, including downregulation of the p53-mediated apoptotic response induced by DNA damage (37). ERα represses p53-mediated transcription either through the recruitment of co-repressors (38) or via independent targeting and repression of p53 target gene sets (39). A separate subset of target genes for p53 activation is enhanced by ERα activity (37).

p53 and ERα exist in complex with MDM2, and this complex modulates the activity of p53 and ERα. MDM2 is a negative

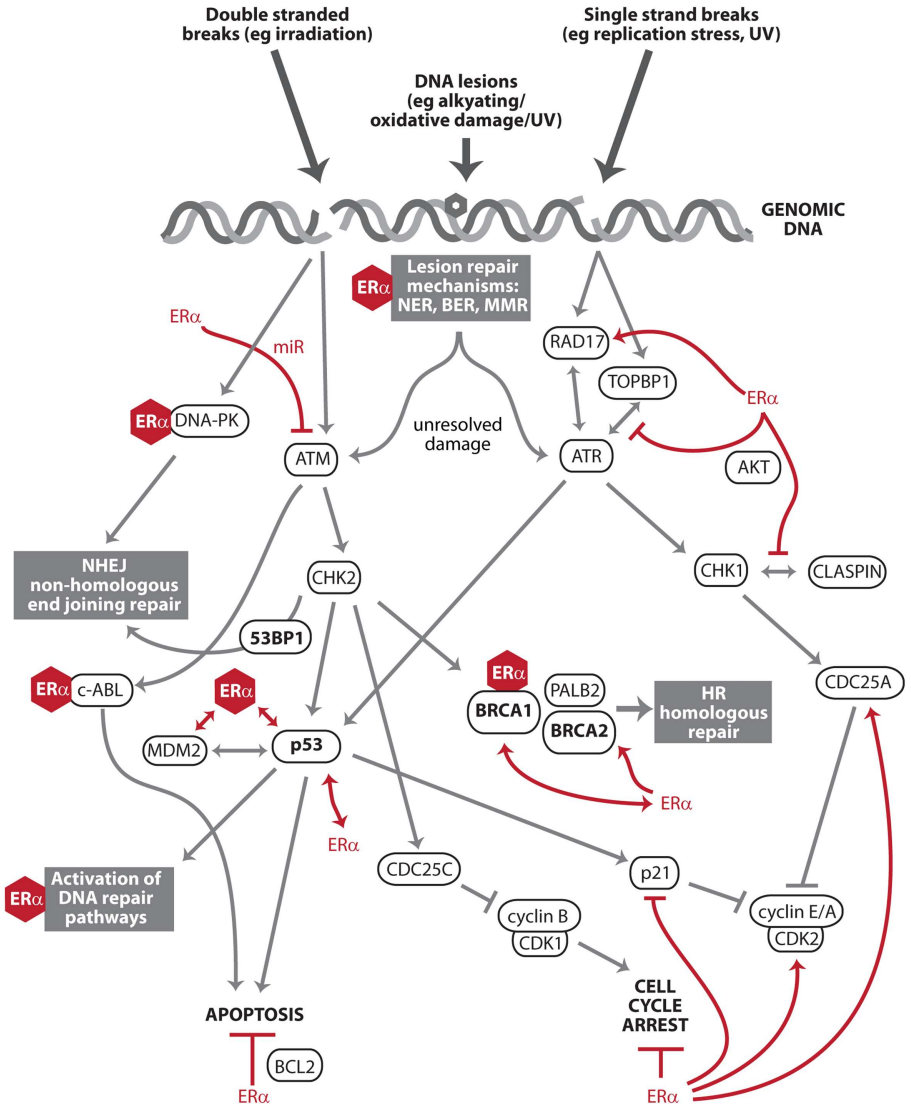

FIGURE 1 | Key effectors of the DNA damage response and DNA repair that intersect with estrogen receptor α signaling. The DNA damage response (DDR) is a series of pathways that recognize and process DNA damage. After DNA damage recognition, signals are transduced and amplified through kinase activation (ATM, ATR, DNA-PK, CHK1, and CHK2) to downstream effectors (e.g., p53 and BRCA1) that facilitate DNA repair, apoptosis, and cell cycle arrest. Estrogen receptor α (ERα) exists in complex with multiple members of the DDR and DNA repair pathways (e.g., DNA-PK, BRCA1, p53, and MDM2). These protein:protein interactions are denoted by ERα represented as a hexagon. This includes c-Abl, a multi-functional regulator of the DDR and its downstream pathways (14). ERα also transcriptionally regulates or is regulated by other members of these pathways (e.g., ATM, ATR, CHK1, BRCA2, and DNA damage checkpoint protein Rad17), denoted by red lines. ERα signaling antagonizes two major endpoints of DDR action: apoptosis and cell cycle arrest (red lines).

feedback regulator of p53 (40), whereas MDM2 positively regulates ERα transcriptional activity, most probably through direct MDM2:ERα interaction (41, 42). Conversely, the MDM2/p53/ERα ternary complex downregulates the activity of ERα by monoubiquitination, probably via the ubiquitin ligase activity of MDM2 (43). MDM2 may also downregulate ERα independently of p53 (43). In the presence of cellular stress, including UV-mediated DNA damage, p53 dissociates from MDM2 and this is associated with an increase in ERα levels and block of the estrogen-dependent downregulation of ERα (43). Paradoxically, while ERα represses p53-mediated transcription, ERα also protects p53 from repression by MDM2 (40), and estrogen treatment is necessary for a p53 response to be mounted in the mouse mammary gland against ionizing radiation (44).

p53 upregulates the expression of *ESR1*, but alters the transcriptional functions of ERα. p53 induction of *ESR1* occurs following DNA damage such as irradiation (45). Like ERα modulation of p53 function, p53 alters the transcriptional program of ERα to repress certain estrogen responsive genes such as *BRCA2*, *c-JUN*, and *BCL2* (37, 46). Indeed it appears that the combination of ERα

and p53 induces a distinct transcriptional program compared to either ERα or p53 alone (47).

Overall this body of work suggests that estrogen and ERα upregulate but sequester p53, such that the DDR and DNA repair are suppressed in the presence of active estrogen signaling, but there is still some safeguard via p53. When estrogen treated breast cancer cells are irradiated there is partial activation of p53 and its downstream pathways, but the pro-proliferative effects of estrogen override any checkpoint-mediated cell cycle arrest (20). Conversely, in mouse models, p53 provides protection from lymph node hyperplasia and ductal carcinoma *in situ* (DCIS) induced by deregulated estrogen signaling (48).

DNA REPAIR MACHINERY

Estrogen receptor α interacts directly with DNA repair proteins with varying impact on DNA repair mechanisms and ERα function. This includes FEN1, MPG, APE1, and TDG of the BER pathway (49, 50), *O*(6)-methylguanine-DNA methyltransferase, which corrects mutagenic DNA lesion *O*(6)-methylguanine back to guanine (51), NHEJ repair proteins Ku70 and Ku86 in the context of gene transcription (18) and MSH2 of the MMR pathway (52). The binding of ERα to MPG enhances BER (53), while estrogen treatment upregulates or downregulates NER, depending on cell type (54, 55). The binding of repair proteins has different outcomes on ERα: MPG inhibits ERα-induced transcription and transactivation of signaling pathways (53), MSH2 and TDG transactivate ERα (50, 52), and the binding of FEN1 and APE1 to ERα has distinct effects on different ERα target genes (56, 57).

Estrogen receptor α interacts with other core DNA damage processing proteins, although the consequence for DNA repair or ERα action is unknown. Estrogen treatment upregulates *BRCA2* (58) of the HR pathway, and through phosphorylation protects BRCA2 from degradation (59). ERα also directly interacts with DNA repair signaling and processing protein PARP-1 in the context of ERα-mediated gene transcription (18), which potentially affects ERα-regulated gene networks.

CELL CYCLE CHECKPOINTS AND APOPTOSIS

One of the most important functions of the DDR is to halt proliferation via the activation of cell cycle checkpoints or induce apoptosis. The effector proteins of these responses are not only targets of the DDR but as a set are antagonized by pro-proliferative ERα signaling. The DDR induces a G_1/S phase arrest downstream of ATR via CDC25A inhibition of cyclin A/E/CDK2 complexes, and downstream of p53 via p21 inhibition of cyclin D/CDK4/6 and cyclin E/CDK2 complexes. A G_2/M arrest is induced downstream of Chk1/Chk2 via activation of CDC25 phosphatases to inhibit cyclin B/Cdk1 complexes (60). ERα antagonizes cell cycle arrest by upregulating *CCND1* (cyclin D1), *CCNE2* (cyclin E2), and *CDC25A*, and downregulating *CIP1* (p21) downstream of c-MYC (61–63). Likewise, p53 induces apoptosis by induction of *FAS-R*, *BAX*, *PUMA*, and *NOXA* (64), but ERα induces an anti-apoptotic signal including upregulation of *BCL2* (65).

Consequently, active ERα signaling will antagonize the anti-proliferative and pro-apoptotic signals of the DRR. The outcome will be dictated by the strength of each signal, but ERα signaling is able to sustain proliferation in situations where otherwise DNA damage would have induced a cell cycle arrest and apoptosis (15, 20, 66).

DISRUPTION OF DDR AND DNA REPAIR PATHWAYS IN BREAST CANCER, AND THEIR ASSOCIATION WITH ERα STATUS AND PROGNOSIS

DNA damage pathways are altered in breast cancer by mutation, changes in expression, amplification, and methylation, and as a class the DDR and DNA repair proteins are frequently altered in cancer and associated with poor prognosis. A survey of the literature shows that DDR pathways differ significantly between ERα positive and ERα negative breast cancer (**Table 1**). At least part of this change may be due to loss of ERα signaling, and certainly changes to p53, ATM, and *TIMELESS* (which functions in the ATR pathway) are consistent with the loss of ERα regulation of these genes/proteins. However, given that changes to DNA damage processing are a hallmark of cancer that contributes to tumor initiation, some of the changes no doubt precede loss of ERα, and may in fact contribute to its loss. This is exemplified in cancers with low BRCA1 and ERα, and BRCA1 loss is hypothesized to lead to ERα downregulation in breast cancer (31). Nevertheless, the presence or absence of DDR/DNA repair proteins will affect DNA repair in hormone-responsive cancers and the bi-directional regulation of the DDR/DNA repair and ERα. Likewise, the loss of ERα will affect the DDR/DNA repair in ERα negative cancers.

PERSPECTIVES

Estrogen receptor signaling is not typically thought to influence DNA repair as the literature has focused on its classic nodes of action of proliferation, growth, and apoptosis. The evidence, however, is overwhelming that ERα signaling has an impact on DNA damage processing through its regulation of ATM, ATR, DNA-PK, p53, BRCA1, BRCA2, and the DNA repair machinery. Given that estrogen can *cause* DNA damage, this raises a vital question of how estrogen receptor signaling processes the DNA damage caused by estrogen action. For example, does it dampen damage responses in favor of continuing proliferation, or does it act as a sentinel against DNA damage? Overall, estrogen receptor activity appears to downplay the response to DNA damage while simultaneously promoting proliferation. Consequently sustained ERα signaling may be permissive of the accumulation of genomic change from low level DNA damage that contributes to tumor initiation. Some of the major effectors of the DDR (e.g., p53 and BRCA1) do have negative feedback on the estrogen receptor, as does active DNA repair. Thus in the face of serious DNA damage ERα signaling is downregulated to protect the cell from continuing proliferation, and potentially allow full engagement in the DDR.

Several critical experiments will clarify whether active ERα signaling overrides the DDR. These include co-treatment with estrogen and different DNA damaging agents to determine the extent to which the DDR is activated and how ERα promoter binding is affected by DNA damage. This should incorporate the titration of doses of DNA damage to determine if there is a tipping point between sustained proliferation due to ERα action, and engagement of the DDR and DNA repair. Since ERα has cross-talk with both BRCA1 and p53, the combinatorial effects

Table 1 | DNA damage response and DNA repair genes altered in breast cancer and relationship to ERα status.

Gene/ protein	Interaction with ERα	Alteration and relationship to ERα status in breast cancer	Prognosis	Reference
ATM	ERα downregulates *miR-18a* and *miR-106a* to downregulate ATM protein expression, and *miR-18a* directly binds to the ATM-3′-UTR	ATM protein is higher in ER negative breast cancers	High ATM protein is correlated with recurrence in breast cancer	(11, 16, 67)
ATR	ATR is functionally downregulated by ERα transactivated AKT signaling, which suppresses the DNA damage induced association between ATR:TOPBP1	–	–	(15)
BRCA1	The BRCA1:Oct1 complex directly binds the *ESR1* promoter to drive ERα transcription; BRCA1 suppresses ERα-mediated transcription through direct binding and co-activators; ERα promotes *BRCA1* transcription via an ERα/p300 transcriptional complex	Low *BRCA1*/BRCA1 (by mutation, methylation, or low mRNA) is associated with ER negative breast cancers	Oophorectomy (resulting in reduced estrogen levels) is protective against breast cancer in *BRCA1* familial breast cancers	(22–26, 29, 31)
BRCA2	*BRCA2* is upregulated by estrogen treatment, possibly as an indirect target rather than via ERα	*BRCA2* is higher in ER negative breast cancers	High *BRCA2* predicts poor disease-free survival	(68, 69)
c-ABL	c-ABL enhances estrogen receptor ERα transcriptional activity through its ERα stabilization by phosphorylation	Expression of c-ABL and ERα are not correlated	Co-expression of c-ABL and ERα is associated with advanced tumor stage and lymph node involvement	(70, 71)
CHEK2	–	*CHEK2* mutated breast cancers tend to be ERα positive	In ER positive breast cancers, *CHEK2* mutation is associated with increased risk of death and second breast cancers, but not in ER negative cancers	(72, 73)
CHK1	CHK1 is phosphorylated via ERα transactivated AKT signaling, which suppresses the DNA damage induced CLASPIN:CHK1 interaction	*CHK1* mRNA and protein are highly expressed in ER negative	*CHK1* not prognostic for outcome metastasis in breast cancer	(15, 74)
CLASPIN	CHK1 is phosphorylated via ERα transactivated AKT signaling, which suppresses the DNA damage induced CLASPIN:CHK1 interaction	*CLASPIN* mRNA and CLASPIN protein are highly expressed in ER negative breast cancers	*CLASPIN* mRNA is not prognostic for metastasis	(15, 74)
DNA-PK	The DNA-PK:ERα protein complex increases ERα phosphorylation and reduces ERα turnover. The DNA-PK:ERα complex binds to ERα responsive gene promoters, an effect that is not dependent on DNA damage	–	–	(19)
FANCD2	–	FANCD2 protein is higher in ER negative breast cancers	–	(75)
MDM2	MDM2 interacts with ERα in a ternary complex with p53. MDM2 positively regulates ERα transcriptional activity, but downregulates overall activity through ERα monoubiquitination	High MDM2 protein is correlated with ER positive breast cancers	Low MDM2 protein is correlated with high nuclear grade and lymph node involvement	(41–43, 76)

(Continued)

Table 1 | Continued

Gene/ protein	Interaction with ERα	Alteration and relationship to ERα status in breast cancer	Prognosis	Reference
p53	ERα upregulates *TP53* and stabilizes p53, but generally suppresses p53 transcriptional function. p53 upregulates *ESR1*, but also modulates ERα induced transcription	p53 is generally wild-type and expressed in ER positive breast cancer	*TP53* mutation or p53 mutated gene signature is prognostic for poor disease-free survival	(12, 32, 33, 35–39, 45–47, 77)
PCNA	PCNA interacts directly with ERα to modulate its transcriptional function in normally proliferating cells	–	–	(78)
RAD17	*RAD17* mRNA is upregulated by estrogen in an ERα dependent manner	*RAD17* mRNA often high in breast cancer; high RAD17 protein correlated with ER negative; *RAD17* sometimes lost in ER negative, but due to loss of 5q11 locus	High *RAD17* mRNA prognostic of increased lymph node metastasis	(79–81)
TIMELESS	*TIMELESS* is upregulated by estrogen, probably via ERα, and downregulated by anti-estrogens	*TIMELESS* mRNA is high in ER+ patients who have relapsed for endocrine therapy	High levels of *TIMELESS* mRNA prognostic of poor relapse-free survival for ER+ breast cancers	(82)
TOPBP1	TOPBP1 is regulated downstream of ERα transactivated AKT signaling, which suppresses the DNA damage induced association between ATR:TOPBP1	TOPBP1 expression has no relationship to ERα status	Low *TOPBP1* mRNA and high TOPBP1 protein are both associated with increased breast cancer grade	(15, 83, 84)

–, no relationship reported.

should be considered by simultaneously activating ERα signaling and treating with DNA damage in the context of BRCA1 and p53 ablation. Finally, it is a priority to investigate the effect of ERα on its binding partners DNA-PK, PCNA, and PARP-1 in the context of DNA damage.

The role of ERα in modulating DNA damage has important clinical implications. Anti-estrogen treatment is the mainstay of adjuvant therapy for breast cancer, but the most common therapy, Tamoxifen, is itself a source of DNA damage (85), and this damage has been detected in patients and is implicated in endometrial cancer (86). Tamoxifen has agonist effects through ERα in the endometrium (87) so it is interesting to speculate that Tamoxifen therapy induces DNA damage and disturbs a balance between estrogen signaling and the DDR in the endometrium to detrimental effect. Chemotherapies and radiation therapy induce DNA damage, so ERα may suppress the DDR to reduce the efficacy of these treatments. Indeed, patients with ER positive breast cancers have significantly lower response rates to chemotherapy than those with ER negative cancers (88), and *in vitro* studies suggest this is dependent on ERα action (89–91). Co-administration of anti-estrogens and radiation therapy or chemotherapy appears to enhance therapy cytotoxicity and a likely explanation is that anti-estrogen treatment prevents pro-proliferative bypass of cytotoxicity by estrogen (66, 90). Conversely, estrogen receptor action is needed for sustained p53 expression to allow the induction of apoptosis by chemotherapeutic doxorubicin (92), and good prognosis ERα positive breast cancers generally express p53. Consequently, the pro-apoptotic arm of the DDR appears compromised in some circumstances by the complete inhibition of ERα signaling. Further understanding of the cross-talk between ERα and DNA damage processing will provide crucial information to guide drug, radiation therapy, and hormone combination treatment of breast cancer patients.

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Aberrant proliferation of differentiating alveolar cells induces hyperplasia in resting mammary glands of SV40-TAg transgenic mice

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WAP-T1 transgenic mice express SV40-TAg under control of the whey acidic protein (WAP) promoter, which directs activity of this strong viral oncogene to luminal cells of the mammary gland. Resting uniparous WAP-T1 glands develop hyperplasia composed of TAg positive cells prior to appearance of advanced tumor stages. We show that cells in hyperplasia display markers of alveolar differentiation, suggesting that TAg targets differentiating cells of the alveolar compartment. The glands show significant expression of *Elf5* and milk genes (*Lalba*, *Csn2*, and *Wap*). TAg expressing cells largely co-stain with antibodies to Elf5, lack the epithelial marker Sca1, and are hormone receptor negative. High expression levels of *Elf5* but not of milk genes are also seen in resting glands of normal BALB/c mice. This indicates that expression of Elf5 in resting WAP-T1 glands is not specifically induced by TAg. CK6a positive luminal cells lack TAg. These cells co-express the markers prominin-1, CK6a, and Sca1, and are positive for hormone receptors. These hormone sensitive cells localize to ducts and seem not to be targeted by TAg. Despite reaching an advanced stage in alveolar differentiation, the cells in hyperplasia do not exit the cell cycle. Thus, expression of TAg in conjunction with regular morphogenetic processes of alveologenesis seem to provide the basis for a hormone independent, unscheduled proliferation of differentiating cells in resting glands of WAP-T1 transgenic mice, leading to the formation of hyperplastic lesions.

Keywords: SV40-TAg, hyperplasia, alveologenesis, mammary gland, tumorigenesis

INTRODUCTION

Breast cancer, a progressive disease of mammary gland epithelia, is marked by consecutive appearance of different alterations, namely hyperplastic lesions, carcinoma *in situ*, and invasive, malignant adenocarcinomas. The mutational signature of human breast cancer supports the view of a direct evolutionary relationship between pre-neoplastic and malignant lesions (1–5). Mutations that inactivate functions of tumor suppressor proteins and thereby decrease genomic stability seem to be basic to initiation of breast cancer. Transgenic mouse strains were developed to mimic this process at an experimental level and to decipher the mechanisms initiating breast cancer and promoting progression to malignant stages. Major questions concern the cell types targeted by oncogenes in these models as this might determine the phenotype of tumor cells appearing at advanced stages. Here, we asked whether non-malignant, hyperplastic lesions appearing in resting glands of WAP-T1 transgenic mice (6) reflect selection of a distinct epithelial cell type or whether these lesions already display a heterogeneous cell composition as is seen in advanced tumor stages.

WAP-T1 mice express SV40-T-antigen (TAg) under control of the whey acidic protein (WAP) promoter, which directs activity of this strong viral oncogene specifically to epithelial cells of the gland. The transgene encodes both, the large (LT) and small (t) T-antigen. They cooperate to inactivate the tumor suppressor proteins RB and p53, alter expression of cell cycle regulating genes, promote unscheduled transition through G1/S, and impair DNA-damage response mechanisms, events that are known to decrease genomic stability (7–9).

The WAP promoter responds to lactotrophic hormones and thus is inactive in glands of virgin mice, active during lactation in differentiated luminal epithelial cells of the lobulo-alveolar complex, and at a lower level also active in the resting gland during estrous cycle (10–14). In accordance, epithelial cells of glands in virgin mice lack TAg whereas differentiated epithelial cells of the lactating gland show high levels of this protein. TAg positive cells of the lactating gland disappear with regression of lobulo-alveolar structures during involution (15–17). Sections of resting glands derived from uniparous mice show re-appearance of T-antigen positive epithelial cells at about 30 days post-weaning (p.w.). They constitute small hyperplastic lesions, which gradually increase in size and completely pervade the gland at 100 days p.w. Similar to human breast cancer, these hyperplastic lesions develop prior to appearance of carcinoma *in situ* and advanced tumor stages WAP-T1 (2–6). Malignant tumors develop late at low frequency in glands of WAP-T1 mice. They reveal a gene expression profile that recapitulates the phenotype of aggressive human cancers (18).

Abbreviations: CSN2, casein; ER, estrogen receptor; IF, immunofluorescence; LALBA, lactalbumin; n.s., not significant; PR, progesterone receptor; SV40, Simian virus 40; Tag, T-antigen; WAP, whey acidic protein.

The data suggest that carcinogenesis in resting glands of WAP-T1 is largely delayed or halted at the stage of hyperplastic lesions. The cellular composition of hyperplasia and the status of TAg expressing epithelial cells in these lesions compared to lactating glands and advanced tumor stages are not defined. It was speculated that TAg expression in WAP-T1 selects for certain epithelial cell types. A gene expression analysis showed that WAP-T1 tumor samples are enriched in transcription factors relevant for embryonic stem cell maintenance. It led one to assume that TAg expression may favor survival and proliferation of cells displaying features of epithelial stem or progenitor cells (19). But not only stem or progenitor cells but also cells at advanced stages of differentiation have been proposed to generate hyperplastic lesions in transgenic mouse models (20–24).

Epithelia of the mouse mammary gland reveal a complex composition, marked by stem and progenitor cells, terminally differentiated cells, and regulatory units, such as hormone sensing cells (25). They rapidly change composition and functional status of the layer in dependence of developmental stages and environmental signals. This raises the question whether oncogenic activity of TAg in WAP-T1 mice at the early stage of hyperplasia randomly targets epithelial cells or promotes selection of a distinct cell type. Gene expression analysis of advanced WAP-T1 tumors identified at least two different tumor entities, which completely differ in marker expression: (i) low grade tumors, exhibiting a basal-like and morphologically differentiated phenotype with loss of chromosomes 2 and 19 and (ii) high grade tumors marked by strong expression of the *Met* gene and by co-expression of keratin 8/18, keratin 6, and the mesenchymal marker vimentin (26). But, a heterogeneous cell composition of advanced tumors does not necessarily contradict the idea that TAg selects for a distinct epithelial cell type. Data obtained with a tumor cell line derived from WAP-T1 glands showed that tumor cells are equipped with phenotypic plasticity, which for instance allows these cells to acquire a mesenchymal or an epithelial phenotype depending on the tumor environment (27).

Our data show that hyperplasia in resting glands of WAP-T1 mice are uniformly composed of cells differentiating along the alveolar lineage. The results suggest that expression of the viral oncogene in luminal epithelial cells pre-disposed to alveologenesis induces unscheduled proliferation of differentiating cells and thereby causes formation of hyperplasia.

MATERIALS AND METHODS

MICE

Inbred BALB/c and the transgenic WAP-SV40 early region mouse line T1 (6) were housed under SPF conditions in accordance with official regulations for care and use of laboratory animals (UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia) and approved by Hamburg's Authority for Health (Nr. 24/96).

PREPARATION OF MOUSE MAMMARY GLANDS AND ISOLATION OF LUMINAL CELL SUBPOPULATIONS

Mammary glands were collected at indicated time points from virgin mice, lactating mice, and uniparous mice of the BALB/c or WAP-T1 strains, respectively. Lymph nodes and tumors sometimes present at late stages in WAP-T1 mice were removed. Mammary glands to be used for RNA extraction were snap-frozen in liquid nitrogen. Glands intended for immunofluorescence were embedded in Shandon Cryomatrix (Thermo Scientific) and frozen at −80°C. To extract cells for subsequent FACS-sorting all mammary glands from one mouse were pooled in L15 Medium (Sigma-Aldrich), transferred to a sterile Petri dish and minced with scalpels. The organoid suspension was digested in serum-free L15 Medium with 3 mg/ml Collagenase Type I (Life Technologies) and 1.5 mg/ml trypsin (Sigma-Aldrich) for 1 h at 37°C. Cells were collected by centrifugation at 300 × *g* for 5 min and washed once in L15 + 10% fetal calf serum (FCS). Red blood cells were lysed by two rounds of incubation with red blood cell lysis buffer (Sigma-Aldrich) and after two washes with PBS/0.02% EDTA cells were incubated for 15 min at 37°C in SMEM Medium (Life Technologies). Cells were collected by centrifugation and incubated for 2 min at 37°C in 2 ml of 0.25% trypsin/0.2% EDTA in HANKS balanced salt solution (Sigma-Aldrich); Cells were resuspended in 5 ml L15 medium, 400 U/ml Dnase I (Sigma-Aldrich) were added, and the cells were incubated for another 5 min at 37°C before the reaction was stopped by addition of 5 ml L15 + 10% FCS. To obtain a single cell suspension, the cells were passed through a 30 μm filter (Miltenyi Biotech). Lineage depletion was performed using a mouse Lineage Cell Depletion Kit (Miltenyi Biotech), which selects for CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7-4, and Ter-119 positive cells and permits subsequent isolation of Sca1 positive cells.

FACS-SORTING

After lineage depletion cells extracted from mammary glands were stained with FITC hamster anti-mouse CD61 (BD Biosciences; 1 μg/10^6 cells), PE hamster anti-mouse/rat CD29 (BioLegend; 0.4 μg/10^6 cells), and AlexaFluor647 rat anti-mouse Ly-6A/E (Sca1) (BioLegend; 0.5 μg/10^6 cells). Respective isotype controls were included. The cells were sorted on a FACS Aria (BD Biosciences). After sorting, aliquots of cells for immunofluorescence were plated on poly-lysine coated cover slips. Cells for RNA extraction were collected by centrifugation.

Sizes of subpopulations were derived from independent FACS experiments (BALB/c $n = 10$; WAP-T1 $n = 23$). In each experiment, the percentage of total cells was determined for each subpopulation and mean values ± SEM were calculated from all experiments. Statistical significance was assessed using Student's t-test and p-values <0.05 were considered as statistically significant.

RNA EXTRACTION AND QUANTITATIVE REAL TIME PCR

Total RNA was purified using peqGOLD TriFast (Peqlab) according to the manufacturer's protocol. FACS-sorted cells were lysed directly in TRIfast, frozen mouse tissue was carefully cut into small pieces on dry ice, transferred to a lysing matrix tube (MP Biomedicals), and lysed in TRIfast by two cycles of 5 s in a FastPrep instrument (MP Biomedicals). DNA was removed by DNAse I digestion (QIAGEN) followed by another round of RNA extraction using TRIfast. Reverse transcription was performed with the High Capacity RT kit (Applied Biosystems) according to the manufacturer's protocol. PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems)

in a standard program (10 min 95°C; 15 s 95°C, 1 min 60°C; 40 cycles) running in an ABI 7900HT Fast Thermal Cycler (Applied Biosystems). PCR reactions for each sample were repeated in triplicates. The integrity of the amplified products was confirmed by melting-curve analysis. PCR primers (Table S1 in Supplementary Material) were designed using the Primer3 web tool (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). PCR efficiency was measured for each primer pair using serial dilutions of cDNA. 18S rRNA or HSC70 were used as endogenous controls and relative quantitation of transcript levels was performed based on the 2^{-ddCt} method.

For the gene expression analysis in whole mammary glands (**Figure 5**), 10 BALB/c and 10 WAP-T1 mammary glands from five mice each were analyzed and fold changes relative to a BALB/c mammary gland were calculated. Mean values and SEM were calculated from the 10 single values for BALB/c and WAP-T1 mice. Statistical significance was assessed using Student's *t*-test and *p*-values <0.05 were considered statistically significant.

For the gene expression analysis in sorted subpopulations (**Figures 6C**, **7** and **9**) data was derived from at least three independent sorting experiments (**Figure 6C**: BALB/c and T1 $n=5$; **Figures 7** and **9**: BALB/c $n=4$, T1 $n=5$, BALB/c vir and T1 vir $n=3$). Fold changes relative to BALB/c subpopulations (**Figure 6C**: CD29hi; **Figures 7** and **9**: $CD61^{+}Sca^{+}$) were calculated and data from single experiments were summarized as mean values ± SEM. Statistical significance was assessed using Student's *t*-test and *p*-values <0.05 were considered as statistically significant.

IMMUNOHISTOCHEMISTRY

Tissue specimens were fixed overnight with 4% formaldehyde and 1% acetic acid, and stored in 4% formaldehyde at 4°C. Fixed tissue was embedded in Paraplast X-TRA (Sherwood Medical). Deparaffinated sections were stained with H&E (Sigma) according to standard laboratory protocols. For labeling with antibodies deparaffinated sections were treated with an antigen retrieval solution (Citra Plus, Biogenex). Sections were blocked with normal serum for 1 h at room temperature and stained with primary antibodies at appropriate dilutions overnight at 4°C. Bound antibodies were detected using alkaline phosphatase-, respectively peroxidase-labeled anti Ig detection systems (Envision, DakoCytomation). Alkaline phospatase activity was visualized using naphthol AS-BI-phosphate and New Fuchsin (Fuchsin plus substrate-chromogene, DakoCytomation) as substrate; peroxidase activity was identified using diaminobenzidine (DAB) as substrate. Sections were counterstained with hemalum and embedded in Mowiol. Photographs were taken with a LEICA DMI6000B.

For preparation of cryosections glands embedded in cryomatrix were sectioned in a LEICA CM 3050 cryotome at −30°C. Sections were attached to glass cover slips and stored at −20°C. Sections attached to glass coverslips were incubated with −20°C acetone for 10 min, acetone was evaporated at room temperature for 5 min, and sections were rehydrated in PBS for 10 min at room temperature. Staining was done at room temperature in a dark chamber. Sections were blocked with 5% normal serum for 45 min, incubated with the primary antibody for 1 h, washed with PBS three times for 10 min each, stained with fluorochrome labeled secondary antibody for 1 h, and washed again with PBS three times 10 min each. Nuclei were visualized by counterstaining with DAPI or DRAQ5. Stained sections were embedded in Mowiol. Photographs were taken with a Zeiss LSM 501 confocal microscope or with epifluorescence microscopes (LEICA DMI6000B and LEICA DMRA).

ANTIBODIES

Primary antibodies directed to SV40-TAg were derived from guinea pig or rabbit. Commercial antibodies used were CK6a (PRB-169P-100, Covance), CK8/18 (AcrisBP5007), CK14 (Acris BP5009), Mcm2 (N-19 sc-9839, Santa Cruz), Ki67 (H-300, sc-15402, Santa Cruz), ELF5 (N-20, sc9645, Santa Cruz), PR (C-18 sc-538, Santa Cruz), ER (M-20 sc542, Santa Cruz), CD 133 (clone 13A4, eBioscience 14-1331-82), Ly-6A/E (Sca1) (BioLegend), and phospho-histone H3 (Ser10) (Cell Signaling 9701). Antibodies conjugated with Alexa Fluor dyes (Alexa 488, Alexa 555, Alexa 633) (Molecular Probes) were used as secondary antibodies.

RESULTS

RESTING UNIPAROUS WAP-T1 GLANDS SHOW FEATURES OF ALVEOLOGENESIS

Immunohistochemical studies performed on paraffin sections of resting uniparous WAP-T1 glands showed local clusters of TAg positive luminal epithelial cells in ducts (**Figure 1A**). These clusters were often associated with processes of side bud formation (**Figure 1B**, arrow) Condensing chromatin in individual TAg positive cells (see **Figure 1C**, arrows) suggested mitotic activity in these lesions. Reaction of individual TAg positive cells with antibodies to phospho-histone H3, a marker of condensing chromosomes in mitosis, corroborates this suggestion (**Figure 1D**). The data suggest that TAg expressing cells are involved in morphogenetic processes inducing lobulo-alveolar structures.

To characterize TAg positive cells in relation to functional stages of luminal epithelia we performed an IF double labeling study with antibodies to Sca1 and SV40-TAg on cryosections of WAP-T1 glands. Sca1 is a GPI-anchored protein that was originally identified in hematopoietic stem cells. In the mouse mammary gland, it was reported to mark luminal epithelial cells in ducts and bipotent luminal progenitor cells of the lobulo-alveolar compartment, but not terminally differentiated alveolar cells (28–30). In our labeling study, we included glands from virgin mice, lactating mice, and uniparous mice (120 days p.w.). As shown in **Figure 2A**, luminal epithelia of virgin WAP-T1 glands contained Sca1 positive and negative cells, but were generally devoid of TAg. Luminal epithelia of lactating glands were TAg positive but Sca1 negative (**Figure 2B**). In similar, cells in hyperplastic lesions of resting uniparous WAP-T1 glands which stained positively for TAg were also Sca1 negative (**Figures 2C,D**). Sca1 positive epithelial cells in glands were confined to luminal epithelia of ducts and did not stain with antibodies to TAg. These findings suggest that TAg expression is confined to Sca1 negative luminal epithelial cells.

To substantiate a relationship of TAg expression with alveologenesis, we assayed resting uniparous WAP-T1 glands (120 days p.w.) for expression of ELF5, a transcription factor known as master regulator of alveologenesis (31). Immunofluorescence staining on cryosections demonstrated that TAg positive cells co-expressed

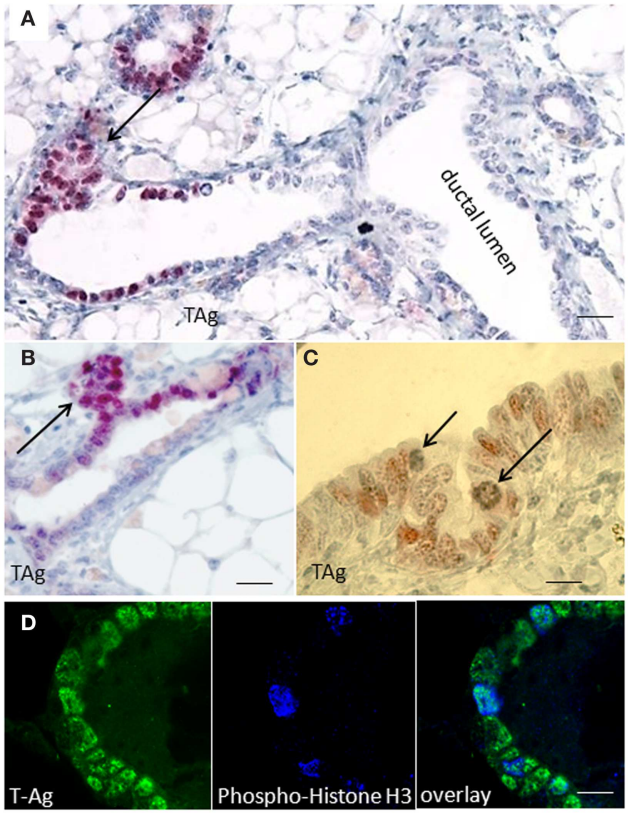

FIGURE 1 | SV40-TAg positive epithelial cells cluster at sites of bud formation. **(A–C)** TAg labeling on sections of paraffin embedded WAP-T1 glands isolated 60 days post-weaning (p.w.); alkaline phosphatase **(A,B)** or peroxidase **(C)** conjugated antibodies were used as secondary antibodies; TAg expressing cells cluster at sites of bud formation [arrows in **(A,B)**]; individual TAg positive epithelial cells show condensing chromatin [arrows in **(C)**]; **(D)** IF double labeling with antibodies to TAg (green) and phospho-histone H3 (blue) on cryosections of resting uniparous WAP-T1 glands; phospho-histone H3 staining points to mitotic activity in WAP-T1 hyperplastic lesions. Bars: **(A)** = 50 μm; **(B)** = 15 μm; **(C,D)** = 10 μm.

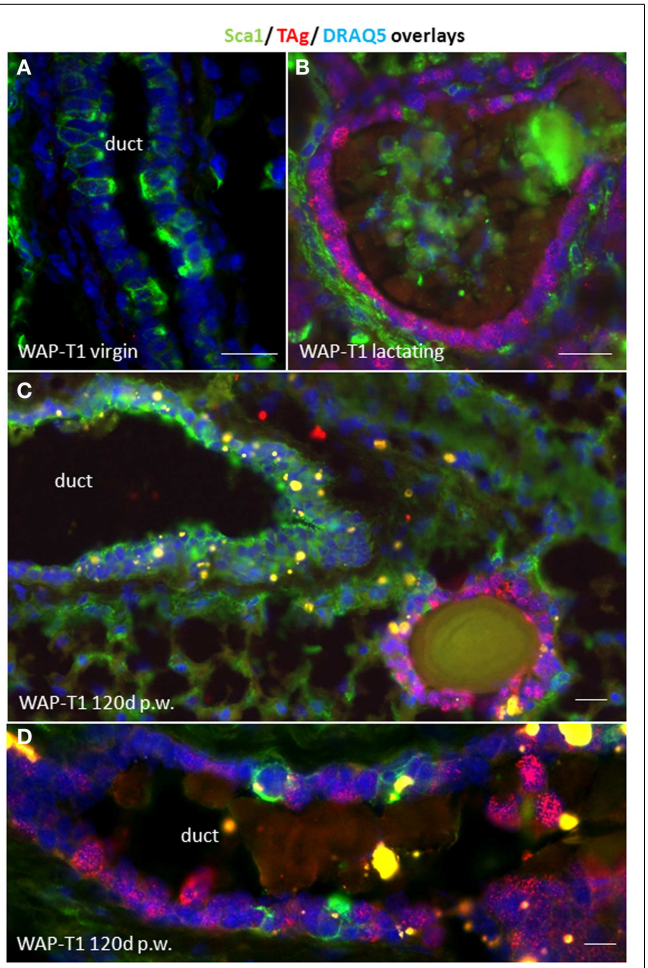

FIGURE 2 | TAg is expressed in Sca1 negative luminal epithelial cells. **(A,B)** IF double labeling with antibodies to Sca1 (green) and TAg (red) on cryosections of WAP-T1 glands; staining of DNA with DAPI (blue); **(A)** luminal epithelia in virgin WAP-T1 glands are TAg negative and reveal a heterogenous Sca1 staining pattern; **(B)** luminal epithelia in lactating WAP-T1 glands are TAg positive and Sca1 negative; **(C,D)** TAg positive cells in epithelial compartments of resting uniparous WAP-T1 glands (120 days p.w.) are also Sca1 negative. Bars: **(A–C)** = 50 μm; **(D)** = 5 μm.

ELF5 (**Figure 3**). But, expression of TAg and ELF5 in epithelia of hyperplasia did not totally overlap. A proportion of TAg positive cells showed only weak staining or was ELF5 negative (**Figure 3**).

The data suggest that TAg and Sca1 mark different functional entities of cells in luminal epithelia of WAP-T1 glands: $TAg^{+}/Sca1^{-}$ cells representing the differentiating lobulo-alveolar compartment, and $TAg^{-}/Sca1^{+}$ identifying cells of the ductal epithelium.

To determine whether TAg expressing cells in hyperplasia of WAP-T1 glands proliferate, we labeled cryosections with antibodies to Mcm2 and to Ki67, both of which are nuclear markers that are expressed in cycling cells. Mcm2 is a member of the licensing protein family, which facilitates coordinated transition from G1 into S-phase and is down-regulated, when cells exit the cell cycle (32). Human mammary gland epithelia show Mcm2 in nuclei of differentiating but not in terminally differentiated cells (33). Ki67 is expressed in G1, S, G2, and M-phase of the cell cycle but is absent from cells resting in G0. Thus, Ki67 is used to identify the proliferating cell compartment in tissue (34). Hyperplasia in resting uniparous WAP-T1 glands exhibited prominent Mcm2 staining as shown by immunoperoxidase labeling on paraffin sections (**Figure 4A**). IF double labeling on cryosections revealed coincident labeling of TAg and Mcm2 in epithelial cells (**Figure 4B**). TAg positive cells also co-stained with antibodies to Ki67 (**Figure 4C**). These results indicate that TAg expressing epithelial cells that accumulate in hyperplasia of resting WAP-T1 glands do not exit the cell cycle.

RESTING UNIPAROUS WAP-T1 GLANDS SHOW LACTOGENIC ACTIVITY

Next, we asked whether formation of lobulo-alveolar structures in resting WAP-T1 glands correlated with lactogenic activity, which is marked by expression of the milk genes *WAP*, *Csn2* (beta-casein), and *Lalba* (lactalbumin). For comparison, we included in our analysis one gland from each, a lactating WAP-T1 mouse, a BALB/c

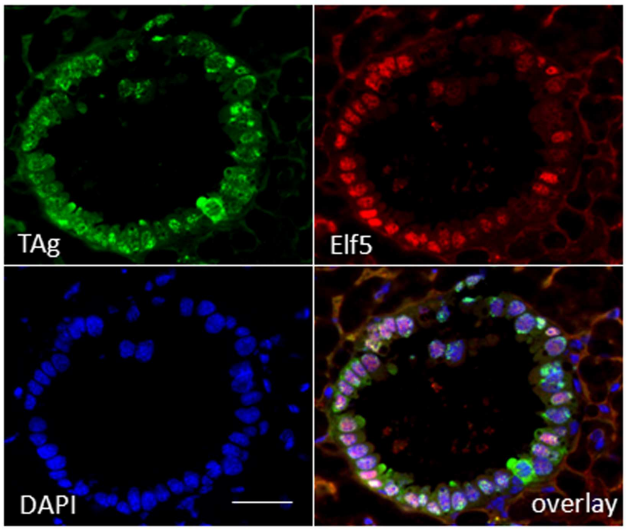

FIGURE 3 | TAg positive cells in hyperplasia co-stain with antibodies to ELF5. IF double labeling on cryosections of resting uniparous WAP-T1 glands (120 days p.w.) with antibodies to TAg (green) and ELF5 (red); staining of DNA with DAPI; individual TAg positive cells lack ELF staining signal. Bar = 50 μm.

virgin mouse, and a WAP-T1 virgin mouse. qRT-PCR analysis performed with whole glands (summarized in **Figure 5**) revealed high expression levels of *ELF5* and milk genes in glands of lactating WAP-T1 mouse (T1 Lak, **Figures 5A–D**), and low levels in glands from virgin mice (T1vir and BALB/c vir, **Figures 5A–D**). Resting glands of WAP-T1 (T1 120 pw) showed prominent *Elf5* expression (**Figure 5A**), clearly exceeding the level in resting BALB/c glands (BALBc 120 pw, **Figure 5A**) by 1.7-fold ($p < 0.01$). Milk gene expression was detectable in all glands, but showed clear differences between WAP-T1 and BALB/c. While *Csn2* expression was only slightly (~twofold, n.s.) upregulated in resting WAP-T1 glands compared to resting BALB/c glands (see BALB/c 120 pw and T1 120 pw in **Figure 5B**), *Lalba* and *Wap* expression levels were increased 40- and 10-fold, respectively ($p < 0.001$ and $p < 1.00E-05$) (**Figures 5C,D**). These data suggest enhanced alveologenesis in resting uniparous WAP-T1 glands.

TAg EXPRESSING CELLS REACH AN ADVANCED STAGE IN ALVEOLAR DIFFERENTIATION

Based on our *in situ* analysis, we asked if we could use Sca1 as marker to isolate luminal cell populations from WAP-T1 glands in order to define their status by RT-PCR. Single cell populations obtained after proteolytic digestion of glands and lineage depletion were fractionated into subpopulations applying fluorescent activated cell sorting. A first approach, trying to isolate the bulk of epithelial cells directly by use of CD24 did not reveal clear subpopulations in samples from WAP-T1 glands. Thus, we selected CD29 as marker to separate luminal ($CD29^{low}$) from basal epithelial cell populations ($CD29^{high}$). The $CD29^{low}$ population was then fractionated into $Sca1^+$ and $Sca1^-$ subpopulations. In addition, we included CD61 as marker in our sorting strategy (**Figure 6A**). CD61 was described to mark luminal progenitor cells, endowed with the potential to differentiate into alveolar or ductal cells (35).Our sorting strategy is similar to that previously described by Shehata et al. (36), who also used Sca1 to isolate luminal cell subpopulations. But in difference to our approach they isolated epithelial cells directly by use of Epcam and substituted CD61 for CD49b.

Sorting yielded four different luminal subpopulations (**Figure 6B**), which were assayed for gene expression by qRT-PCR: the subpopulations $CD29^{low}/CD61^+/Sca1^+$ and $CD29^{low}/CD61^-/Sca1^+$, which we expected to be enriched with TAg negative cells, and the subpopulations $CD29^{low}/CD61^+/Sca1^-$ and $CD29^{low}/CD61^-/Sca1^-$, which we expected to be enriched with TAg positive cells differentiating along the alveolar lineage. qRT-PCR (**Figure 6C**) verified that cells expressing the myoepithelial marker *CK14* clearly separated with the $CD29^{hi}$ subpopulation. Thus, basal epithelial cells were separated efficiently from luminal cell subpopulations in this approach. In accordance, immunofluorescence staining showed CK14 positive cells only in $CD29^{hi}$ subpopulations (data not shown). The relative proportion of $CD61^+$ cells in samples isolated from resting glands of BALB/c and WAP-T1 mice was rather identical (**Figure 6D**). But compared to BALB/c, the proportion of $CD61^-/Sca1^+$ cells was significantly decreased (15.6 vs. 27.9%, $p < 0.05$) and that of $CD61^-/Sca1^-$ cells was significantly increased (54.2 vs. 39.2%, $p < 0.05$) in WAP-T1 samples. This suggests a relative shift in population sizes to differentiating alveolar cells in WAP-T1 glands.

qRT-PCR analysis of *TAg* expression in luminal cell subpopulations isolated from resting uniparous WAP-T1 glands (T1 in **Figure 7D**) showed high and nearly identical levels in the Sca1 negative subpopulations $CD29^{low}/CD61^+/Sca1^-$ (further named $CD61^+/Sca1^-$) and $CD29^{low}/CD61^-/Sca1^-$ (further named $CD61^-/Sca1^-$). *TAg* levels were fourfold lower in the Sca1 positive subpopulations $CD29^{low}/CD61^+/Sca1^+$ (further named $CD61^+/Sca1^+$) and $CD29^{low}/CD61^-/Sca1^+$ (further named $CD61^-/Sca1^+$). Sca1 negative subpopulations from resting WAP-T1 glands also displayed high expression levels of *Elf5*, *Wap*, and *Lalba* (T1 in **Figures 7A–C**). Expression levels of these genes were significantly lower in $Sca1^+$ subpopulations.

Then, we asked if *ELF5* and milk gene expression observed in Sca^- subpopulations from resting WAP-T1 glands (T1 in **Figures 7A–C**) is also detectable in luminal cell subpopulations from resting BALB/c glands. qRT-PCR analysis revealed significant expression of *Elf5*, *Lalba*, and *Wap* in $Sca1^-$ subpopulations from BALB/c but only basal expression levels in $Sca1^+$ subpopulations (BALB/c in **Figures 7A–C**). Remarkably, *Elf5* expression (**Figure 7A**) in the $Sca1^-/CD61^+$ subpopulation reached the same high level as in WAP-T1, but was decreased 2.5-fold ($p < 0.01$) in the $Sca1^-/CD61^-$ subpopulation compared to WAP-T1. This indicates that Elf5 expression in differentiating alveolar cells of BALB/c does not reach the same high level as in WAP-T1. Overall expression levels of milk genes (**Figures 7B,C**) in $Sca1^-$ subpopulations from BALB/c were low compared to WAP-T1: *Lalba* expression was reduced by ~twofold (n.s.) in $CD61^+/Sca1^-$ and ~10-fold ($p < 0.01$) in $CD61^-/Sca1^-$ subpopulations; *Wap* expression was reduced ~70- and 100-fold ($p < 0.05$ and $p < 0.01$), respectively.

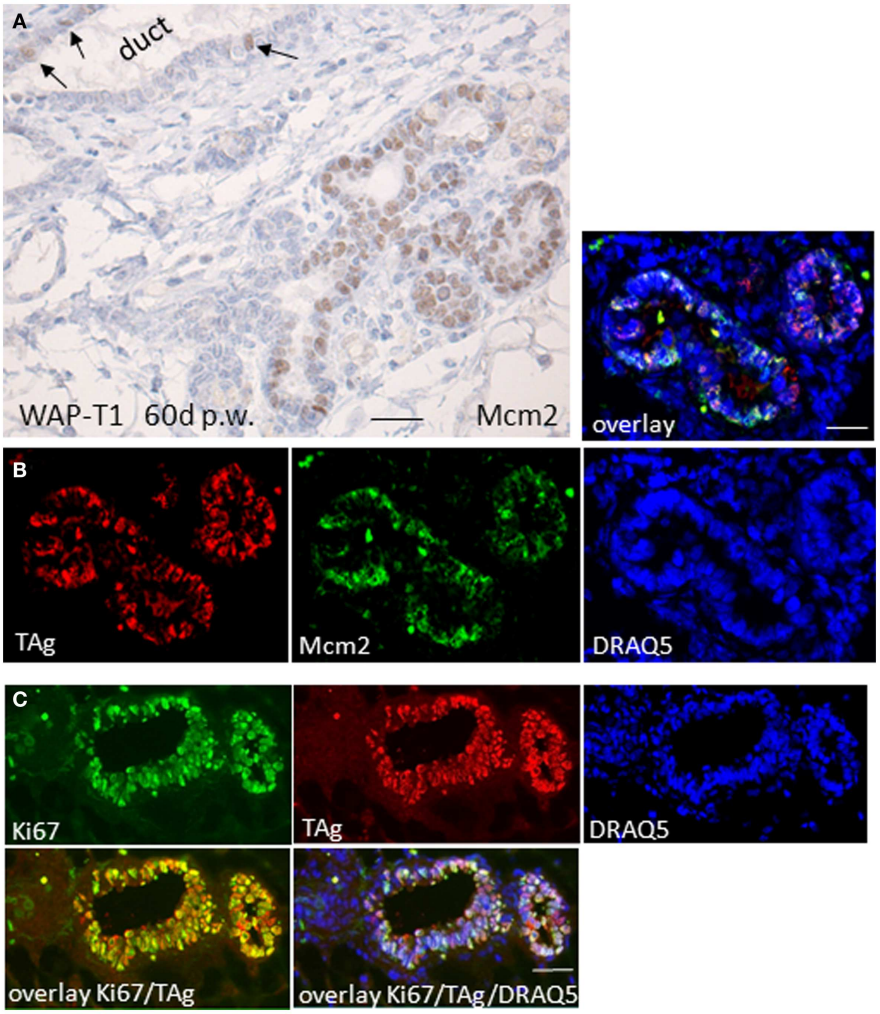

FIGURE 4 | TAg positive luminal epithelial cells co-stain with antibodies to proliferation markers. **(A)** Immunoperoxidase labeling with Mcm2 antibodies on sections of paraffin embedded resting uniparous WAP-T1 glands (60 days p.w.) shows strong nuclear staining of epithelia in hyperplasia; in ductal epithelia only individual cells stain positively for Mcm2 (arrows); **(B,C)** IF double labeling on cryosections from resting uniparous WAP-T1 glands (120 days p.w.) shows coincident labeling of TAg (red) and Mcm2 (green) **(B)**, respectively TAg (red) and Ki67 (green) **(C)**; DNA staining with DRAQ5 (blue) Bars: **(A,B)** = 20 μm.

The data indicate that alveolar differentiation marked by Elf5 also takes place in resting uniparous BALB/c glands and thus is not a specific feature of resting uniparous WAP-T1 glands. But, *ELF5* expression in subpopulations enriched with differentiated alveolar cells ($CD61^{-}/Sca1^{-}$) was significantly higher in WAP-T1 than in BALB/c mice. Furthermore, Sca1 negative subpopulations from WAP-T, but not from BALB/c showed significant expression of milk genes. This suggests that alveolar cells in resting uniparous WAP-T1 glands (120 days p.w.) reach a more advanced stage of differentiation marked by enhanced lactogenic activity.

TAg DOES NOT TARGET CK6a POSITIVE LUMINAL EPITHELIAL CELLS

Luminal epithelia of the mouse mammary gland contain CK6a positive cells, which were repeatedly discussed to represent putative progenitor cells of the ductal and alveolar lineage (20, 37). Furthermore, they were described as potential targets of activated oncogenes in transgenic mice giving rise to mammary gland tumors (22, 23). High grade tumors in WAP-T1 mice showed significant expression of *CK6* as assayed by gene expression analysis (26). Thus, we asked whether TAg targets CK6a positive cells in resting uniparous WAP-T1 glands.

IF studies on cryosections of resting uniparous WAP-T1 glands indicated that CK6a positive cells are present in luminal epithelia of ducts, but absent from hyperplasia composed of TAg positive cells (**Figure 8A**).There was no evidence for CK6a positive cells co-expressing TAg. In ducts, Ck6a positive luminal cells were often seen in close proximity to TAg expressing cells (**Figure 8B**). These data suggest that TAg does not target CK6a positive cells.

TAg EXPRESSING CELLS ARE ESTROGEN AND PROGESTERONE RECEPTOR NEGATIVE

It is well-known that proliferation and differentiation of luminal epithelia into lobulo-alveolar structures are under control of hormone receptors (38–41). Thus, we asked whether high

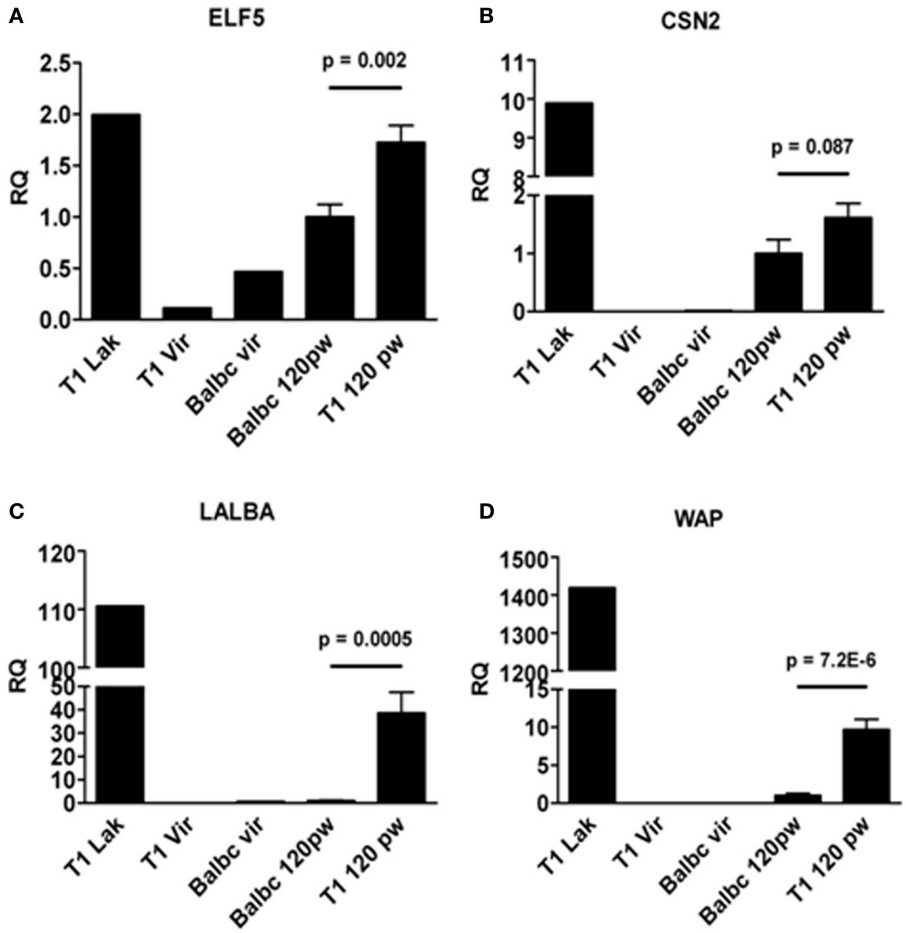

FIGURE 5 | qRT-PCR analysis of ELF5 and milk gene expression in whole glands. *ELF5* **(A)** and milk genes (*CSN2*, *LALBA*, *WAP*) **(B–D)** are expressed in resting glands (120 days p.w.) from BALB/c and WAP-T1 mice ($n = 10$ from five mice); *ELF5*, **(A)**, *LALBA* **(C)**, and *WAP* **(D)** levels are significantly higher in WAP-T1 than in BALB/c glands; individual glands from lactating WAP-T1 mice (T1 Lak), WAP-T1 virgin mice (T1Vir), and BALB/c virgin mice (Balbc vir) are included for comparison; data presented as mean ± SEM.

TAg levels in luminal cell subpopulations derived from resting uniparous WAP-T1 glands correlated with high expression of either the estrogen (Esr1) or progesterone receptor (Pgr). qRT-PCR analysis showed prominent *Esr1* expression in Sca1$^+$ subpopulations and a ~sevenfold lower expression in Sca1$^-$ subpopulations from WAP-T1 (T1 in **Figures 9A,B**). A similar pattern was observed in subpopulations from resting uniparous BALB/c glands (BALB/c in **Figures 9A,B**). But, compared to WAP-T1, *Esr1* receptor expression was significantly higher (1.6-fold, $p < 0.01$ for CD61$^+$/Sca1$^+$; twofold, $p < 0.01$ for CD61$^-$/Sca1$^+$). Interestingly, WAP-T1 subpopulations with high *Esr1* levels showed low expression of *TAg*, *Elf5*, and milk genes (compare with **Figure 7**), suggesting that TAg and estrogen receptor mark different cells. In accordance by immunofluorescence, we found no overlap between estrogen receptor (ER) and TAg staining in luminal epithelia of glands (see **Figure 10A**).

Pgr expression levels in luminal cell subpopulations from BALB/c glands generally exceeded those in subpopulations from WAP-T1 glands (BALB/c and T1 in **Figure 9B**). *Pgr* levels were most prominent in subpopulations enriched in CD61$^-$/Sca1$^+$ cells with a tendency to be higher (~twofold, n.s.) in BALB/c compared to WAP-T1. Sca1$^-$ subpopulations of BALB/c and WAP-T1 mice exhibited strongly reduced expression levels of *Pgr*. Thus, *Pgr* expression also shows a negative correlation with expression of *TAg*, *Elf5*, and milk genes as observed for *Esr1*. *In situ*, progesterone (PR) positive cells were present in luminal epithelia of ducts and generally showed no overlapping staining with antibodies to TAg (**Figures 10B,B′**). Taken together, these data indicate that TAg expressing luminal epithelial cells are estrogen and progesterone receptor negative.

HORMONE RECEPTOR POSITIVE CELLS LOCALIZE TO DUCTAL EPITHELIA AND EXPRESS CK6a, PROMININ-1, AND Sca1

Previous studies of others on adult virgin mice showed that estrogen and progesterone receptor expression are confined to "hormone sensing cells" located in the ductal epithelium; these cells were shown to express prominin-1 (CD133) and Sca1 and revealed a relatively differentiated phenotype (42). In line with

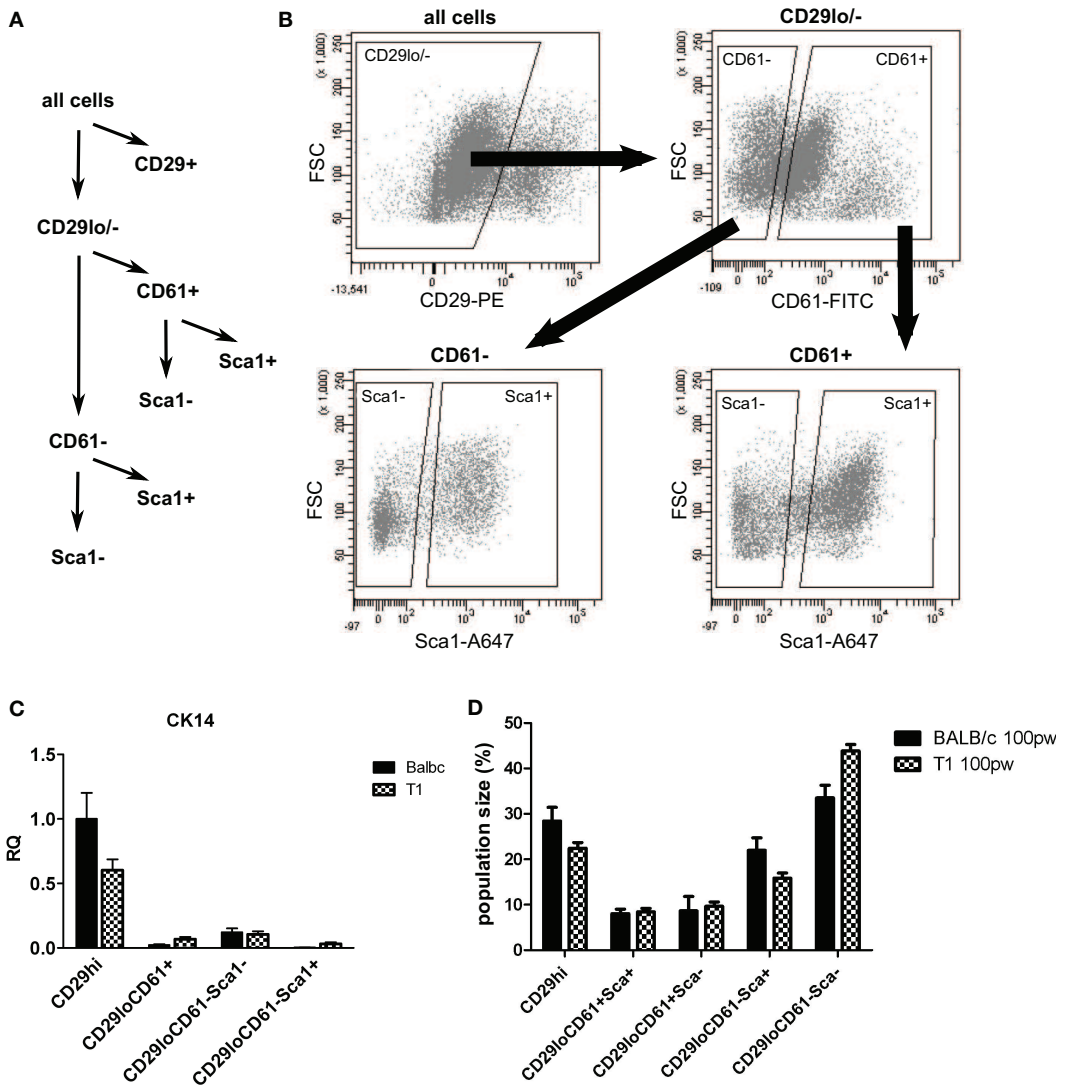

FIGURE 6 | Sorting strategy for isolation of luminal epithelial cell populations. After lineage depletion, basal, myoepithelial cells ($CD29^{+}$) were separated from luminal epithelial cells ($CD29^{lo/-}$). Luminal epithelial cells were separated into undifferentiated ($CD61^{+}$) and differentiated ($CD61^{-}$) subpopulations and further separated into $Sca1^{-}$ and $Sca1^{+}$ subpopulations **(A,B)**. **(C)** CK14, a marker of myoepithelial cells, is enriched in the basal CD29hi subpopulation ($n = 5$). **(D)** Sizes of subpopulations from BALB/c ($n = 10$) and WAP-T1 ($n = 23$) mice as percent total cells; data presented as mean ± SEM. Population size of the $CD61^{-}/Sca1^{+}$ population was significantly decreased in WAP-T1 mice compared to BALB/c (15.6 vs. 27.9%, $p < 0.05$) while $CD61^{-}/Sca1^{-}$ population size was significantly increased (54.2 vs. 39.2%, $p < 0.05$).

these data, our qRT-PCR analysis showed high expression of *Prom1* specifically in the $CD61^{-}/Sca^{+}$ subpopulation both, from WAP-T1 and BALB/c, and this population also exhibited high expression levels of *Esr1* and *Pgr* (BALB/c and T1 in **Figures 9C,D**). *Krt6a* expression was also most prominent in this subpopulation. Therefore, we asked whether CK6a and prominin-1 localized to identical epithelial cells. IF studies performed on cryosections of resting uniparous WAP-T1 glands demonstrated that CD133 (prominin-1) and CK6a antibodies marked the same cells in luminal epithelia of ducts (**Figure 11A**). CD133 positive cells were also positive for the estrogen (**Figure 11B**), respectively progesterone receptor (**Figure 11C**), but definitely negative for TAg. Our data indicate that CK6a, prominin-1, and Sca1 are common markers of hormone receptor positive cells in luminal epithelia of ducts. These cells proved to be absent from hyperplasia in resting uniparous WAP-T1 glands, but in ducts they often localized in close proximity to TAg expressing cells.

RESTING GLANDS OF AGED WAP-T1 VIRGIN MICE ARE NOT SENSITIZED TO ALVEOLOGENESIS

At this point, we asked whether WAP-T1 specific changes seen in resting glands of parous animals are already pre-determined at the virgin stage. We assumed that significant alterations should accumulate with time and thus be most prominent in aged virgin mice. Therefore, we extended our gene expression analysis to

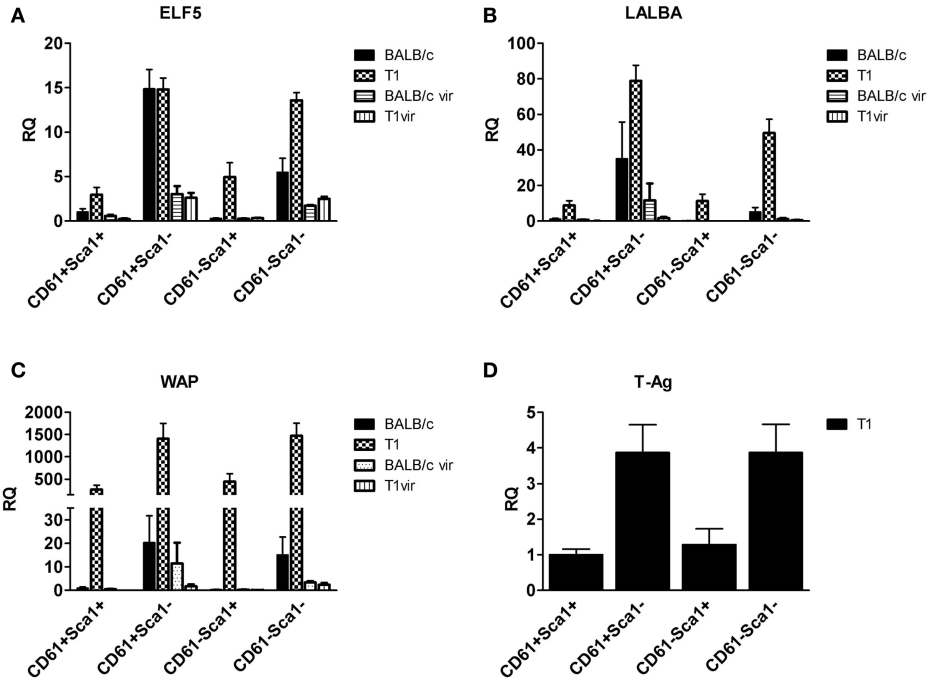

FIGURE 7 | qRT-PCR analysis of ELF5, milk gene (LALBA, WAP), and TAg expression in luminal epithelial cell subpopulations from resting (120 days p.w.) glands of uniparous mice (BALB/c, T1) and glands from virgin mice (BALB/c vir, T1 vir). ELF5 **(A)**, LALBA **(B)**, WAP **(C)**, and TAg **(D)** expression are most prominent in Sca1 negative cells; note equivalent ELF5 levels in undifferentiated ($CD61^+Sca1^-$) cells of BALB/c and T1, but different levels in differentiated ($CD61^-Sca1^-$) cells from both mouse strains; virgin mice show only basal expression of ELF5 and milk genes (BALB/c $n=4$, T1 $n=5$, BALB/c vir $n=3$, T1 vir $n=3$); ata presented as mean ± SEM.

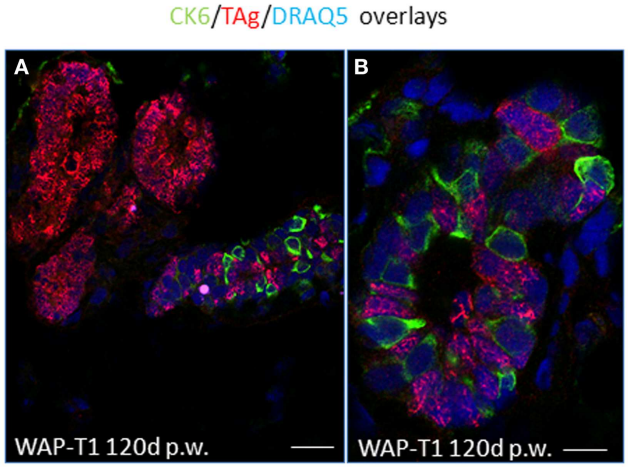

FIGURE 8 | TAg does not target CK6a positive luminal epithelial cells in resting uniparous WAP-T1 glands. IF double labeling with antibodies to CK6a (green) and TAg (red) on cryosections from resting uniparous WAP-T1 glands (120 days p.w.); DNA staining with DRAQ5 (blue); TAg positive lesions lack CK6a positive cells **(A)**; CK6a and TAg mark different luminal epithelial cells in ductal compartments **(A,B)**. Bars: **(A)** = 30 μm; **(B)** = 5 μm.

aged WAP-T1 and BALB/c virgin mice and isolated luminal cell subpopulations at 160 days post-partum (p.p.). We combined the qRT-PCR results shown in **Figures 7** and **9** with the data obtained with resting glands. qRT-PCR of aged virgin mice showed low and nearly identical *Elf5* levels in $Sca1^-$ subpopulations of both strains (see BALB/c vir and T1 vir in **Figure 7A**).The milk genes *Wap* and *Lalba* were barely expressed (see BALB/c vir and T1 vir in **Figure 7B**). *Esr1* and *Pgr* levels in luminal cell subpopulations of aged virgins were virtually identical in both mouse strains (see BALB/c vir and T1 vir in **Figures 9A,B**) and similar to the level in resting (120 days p.w.) glands of normal BALB/c mice (see BALB/c in **Figures 9A,B**). They reached the highest levels in $CD61^-/Sca1^+$ subpopulations, and like in resting glands these populations also showed highest expression of *Prom1* and *Krt6a* (BALB/c vir and T1 vir in **Figures 9C,D**).

These results demonstrate that glands of aged virgin mice display a hormone receptor status similar to that of resting glands. However, pathways leading to expression of ELF5 and milk genes seem not to be active at the virgin stage in both mouse strains. Therefore, changes observed in resting uniparous WAP-T1 glands are not already pre-determined in virgin mice. The data indicate that passage through pregnancy, lactation, and involution sensitizes luminal cell populations in resting glands of parous BALB/c and WAP-T1 mice to pathways of alveologenesis.

DISCUSSION

Our data show that hyperplasia composed of TAg expressing epithelial cells in resting uniparous WAP-T1 glands display features of lobulo-alveolar cells. The cells not only express ELF5, a transcription factor known to specify secretory alveolar cell fate

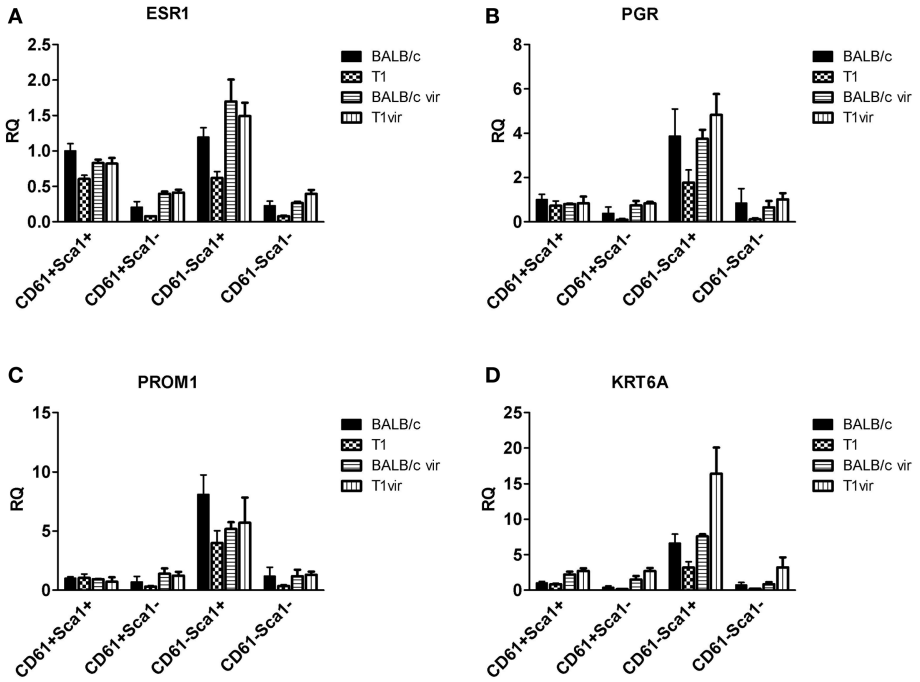

FIGURE 9 | qRT-PCR analysis of hormone receptor, prominin-1, and Ck6a expression in luminal cell subpopulations. Expression of the estrogen receptor (ESR1) **(A)** and progesterone receptor (PGR) **(B)** in the $CD61^{-}Sca1^{+}$ ductal cell subpopulation coincides with expression of prominin-1 (PROM1) **(C)** and CK6a (KRT6A) **(D)**; subpopulations from virgin mice (BALB/c vir $n = 3$; T1vir $n = 3$); subpopulations from uniparous mice 120 days p.w. (BALB/c $n = 4$; T1 $n = 5$); data presented as mean ± SEM.

of $CD61^{+}$ precursors in the mature alveolar epithelium (43, 44), but are secretory as indicated by expression of milk genes. This suggests they reach an advanced stage in alveolar differentiation.

As shown by immunofluorescence, TAg positive cells in lactating WAP-T1 glands and in hyperplasia of resting uniparous WAP-T1 glands did not express Sca1; Sca1 positive cells were clearly confined to ductal epithelia. It suggests that Sca1 is a marker which separates ductal cells from cells with an alveolar cell fate in the mouse mammary gland. In accordance, *ELF5* and milk gene expression clearly separated with Sca1 negative subpopulations in our RT-PCR analysis.

TAg was expressed at a high level not only in $CD61^{-}/Sca1^{-}$, but also in $CD61^{+}/Sca1^{-}$ cells. This led us to assume that TAg targets luminal epithelial cells early during alveolar differentiation. Possibly, TAg expression is initiated with the onset of ELF5 expression, as this master regulator of alveologenesis was shown to induce WAP expression (43). Additional markers are needed to precisely decipher alveolar differentiation of luminal cells in relation to TAg expression. Progress is being made in this area as a recent study identified hitherto unknown epithelial cell lineages that regulate spatial placement of tertiary branches as well as formation of alveolar clusters in ducts of mammary glands (45). It would be interesting to see if the onset of TAg expression coincides with early priming to the alveolar lineage.

Elf5 expression was not only seen in resting uniparous WAP-T1 glands, but also in resting uniparous BALB/c glands. We show that $CD61^{+}/Sca1^{-}$ subpopulations from WAP-T1 and BALB/c glands exhibited nearly identical, high levels of *Elf5*. Thus, alveologenesis seen in resting WAP-T1 glands is not specifically induced by TAg. Alveolar differentiation and formation of lobulo-alveolar structures are initiated regularly in rodent mammary glands during estrous cycle (46). Normally, these structures regress during diestrus. Thus, we assume that development of hyperplasia in WAP-T1 mice is initiated by mechanisms normally inducing transient alveologenesis in parous mice. Considering that ELF5 induces the *Wap* promoter (43), it is conceivable that ELF5 sustains TAg expression in differentiating alveolar cells. Although the cells show lactogenic activity, they seem not to reach a terminal stage of alveolar differentiation, as they stay in cell cycle which is indicated by positive reaction with antibodies to Mcm2 and Ki67. Both of these markers are not expressed in differentiated cells (33, 34).

It has been well-established that differentiation into alveolar, secretory active cells is regulated by a complex regulatory network comprising hormone receptors and transcription factors (43, 44, 47, 48). Apparently, estrogen (ER) and progesterone receptor (PR) positive cells compose a hormone sensing compartment within the luminal epithelium of ducts that induces differentiation and proliferation of hormone receptor negative cells through paracrine mechanisms. In addition, proliferation of hormone sensitive cells seems to be stimulated directly by progesterone and estrogen (42, 49). Recently, a relatively differentiated subpopulation of luminal cells has been identified in glands of adult virgin mice displaying the markers $CD24^{high}/Sca1^{+}$/prominin-1^{+}(CD133)/$CK18^{+}$ and expressing estrogen, progesterone, and prolactin receptors. These

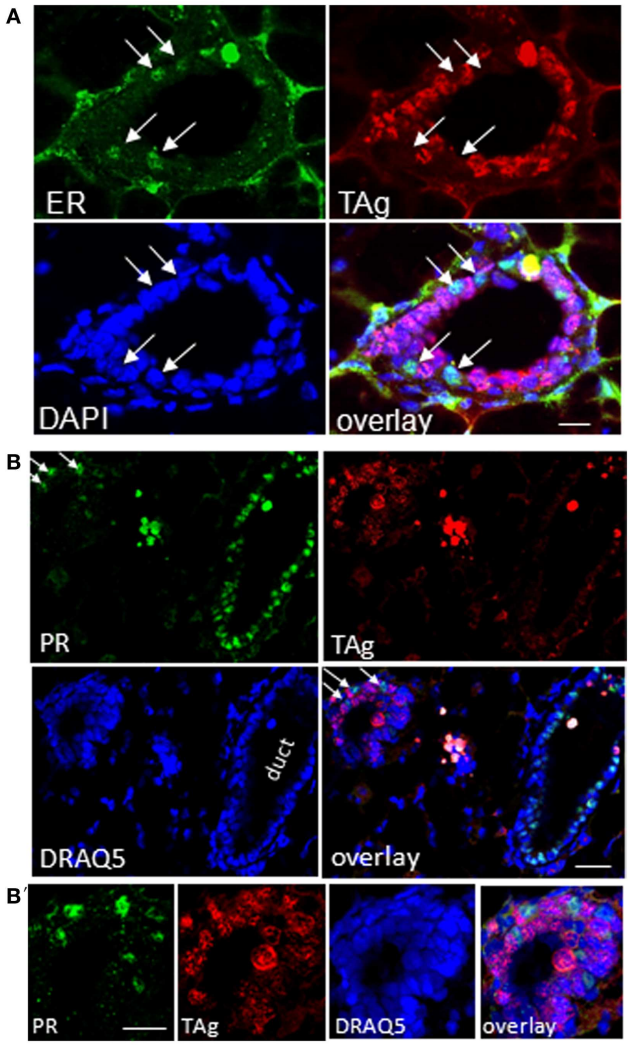

FIGURE 10 | TAg positive epithelia in resting uniparous WAP-T1 glands are negative for estrogen receptor (ER) and progesterone receptor (PR). **(A–B′)**: IF double labeling on cryosections from resting uniparous WAP-T1 glands; **(A)** Double labeling with antibodies to estrogen receptor (ER) (green) and TAg (red); staining of DNA with DAPI (blue); ER positive cells are TAg negative [arrows in **(A)**]; **(B,B′)**: double labeling with antibodies to progesterone receptor (PR) (green) and TAg (red); DNA staining with DAPI (blue); note the high number of PR positive but TAg negative cells in epithelia of ducts; no coincident labeling of PR [arrows in **(B)**] and TAg. Bars: **(A)** = 20 μm; **(B,B′)** = 50 μm.

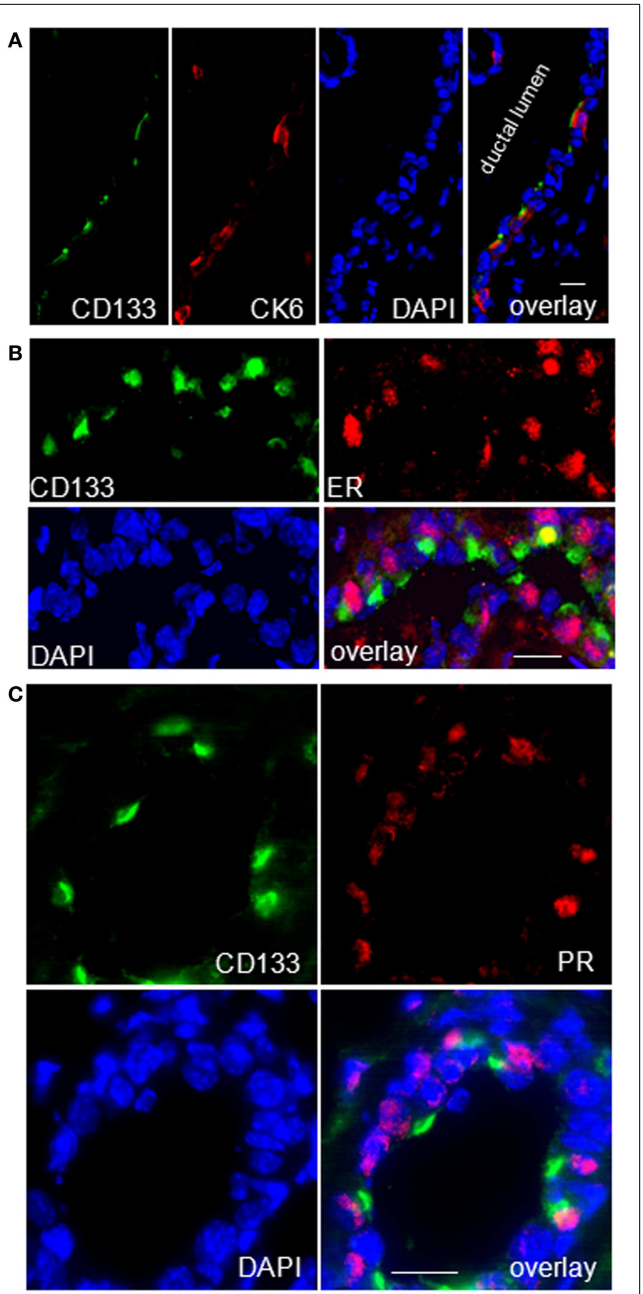

FIGURE 11 | CK6a marks CD133 (prominin-1) and hormone receptor positive cells in luminal epithelia of WAP-T1 glands. **(A–C)** IF double labeling on cryosections of resting uniparous WAP-T1 glands (120 days p.w.); DNA staining with DAPI (blue); **(A)** Coincident staining of luminal epithelial cells in ducts with antibodies to CD133 (green) and CK6 (red); **(B)** Coincident labeling of luminal cells with antibodies to CD133 (green) and estrogen receptor (red); **(C)** Coincident staining of luminal epithelial cells with antibodies to CD133 and progesterone receptor (PR); note the cap-like staining of CD133 at the luminal side of epithelial cells in **(A–C)**. Bars: **(A)** = 20 μm; **(B,C)** = 50 μm.

cells represent little stem cell activity and show no proliferative activity, suggesting they constitute a hormone sensitive compartment (42). Here, we demonstrate for WAP-T1 glands that these cells localize to luminal epithelia of ducts, are TAg negative, but in close proximity to TAg positive cells. Furthermore, we show for the first time that ductal cells positive for hormone receptors and prominin-1 (CD133) are identical with those expressing cytokeratin CK6a. CK6a has been discussed to mark a population of luminal mammary progenitor cells (30, 50, 51), which lack repopulation activity (37). A progenitor cell function is compatible with changes in the relative proportion of cells during mammary gland development. These cells are found at significant quantity in mammary ducts of virgin mice, reduced in number during pregnancy,

apparently absent from epithelia of lactating glands, and reappear in resting glands after involution (37).

Our qRT-PCR data further corroborate a relationship between expression of *CK6a*, *prominin-1*, and hormone receptor genes. *Prom1* and *Krt6a* expression were prominent in the same Sca1 positive subpopulation of luminal cells. This subpopulation also proved to be unique with regard to expression of the progesterone receptor, a hormone receptor known to drive mammary secretory differentiation via induction of ELF5 in luminal progenitors (47). Thus, we speculate that $CK6a^+$/$prominin^+$/$Sca1^+$/ER^+/PR^+ luminal cells have a unique function in induction of TAg and ELF5 leading to the formation of TAg positive, estrogen, and progesterone receptor negative hyperplasia. Crossing of WAP-T1 mice with conditional *progesterone receptor* knock-out mice would be an interesting approach to investigate this hypothesis by testing for the requirement of this hormone receptor for tumor formation.

Features described here for WAP-T1 mice may also apply to other transgenic mouse models of mammary carcinogenesis that display hyperplasia with an alveolar phenotype. Similar to WAP-T1, C3(1)/SV40-T transgenic mice showed TAg expression in terminal duct lobular units (TDLU), an increased number of TDLU proliferative lesions and side ducts, and at later stages expansion of cells into the ductal lumen with multistage progression to carcinoma (52). Mice transgenic for the polyomavirus middle T-antigen (PyV-mT) under control of the MMTV promoter showed focal pre-malignant lesions and an enhanced number of abortive side buds with lumen that was positive for milk proteins (WAP and OPN) (53). Mice transgenic for *Wnt-1*, *Int-2*, *Cyclin D*, or *TGF*α under control of mammary specific promoters (MMTV, WAP, ß-lactoglobulin) also developed alveolar hyperplasia (54–56). In MMTV-neu transgenic mice, parity induced epithelial cells endowed with the potential to differentiate into alveolar or ductal cells were identified as targets for induction of tumorigenesis (24). Thus, hormone dependent activation of an oncogene in differentiating luminal epithelial cells of the mammary gland could generally be a crucial step to induction of aberrant proliferative activity leading to the formation of hyperplastic lesions.

AUTHOR CONTRIBUTIONS

Timo Quante, Florian Wegwitz, Wolfgang Deppert, and Wolfgang Bohn conceived and designed the experiments. Timo Quante and Florian Wegwitz designed and performed FACS and qRT-PCR analysis; Julia Abe and Alessandra Rossi were responsible for animal work and immunofluorescence analysis; Timo Quante, Wolfgang Deppert, and Wolfgang Bohn wrote the paper. The authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fonc.2014.00168/abstract

Table S1 | Primer XS.

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