EMERGING FUNCTIONS OF SEPTINS

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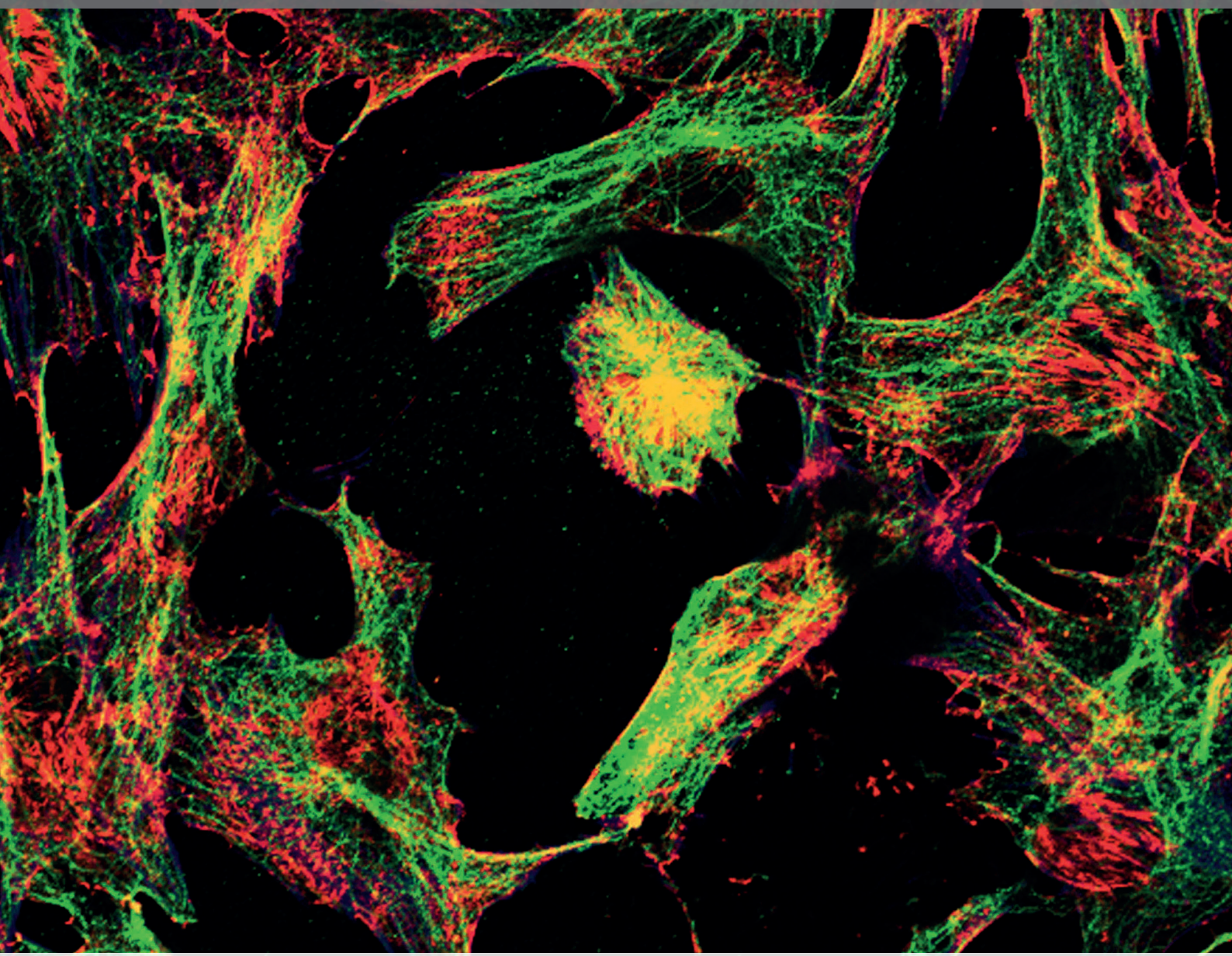

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EMERGING FUNCTIONS OF SEPTINS

Topic Editors:
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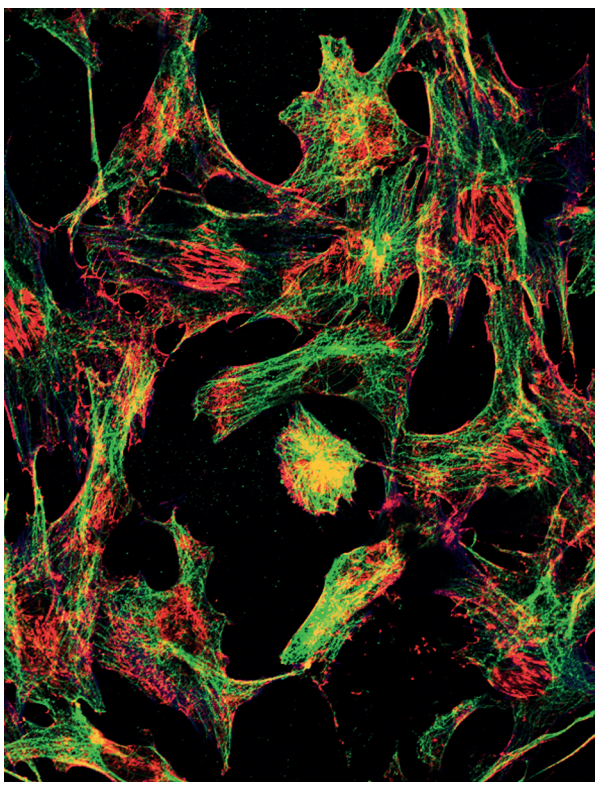

Confocal immunofluorescent image of fibroblasts co-stained for SEPT7 (red), tubulin (green) and actin (blue), depicting the septin, microtubule and the microfilament cytoskeletal networks respectively.

Image by Manoj B. Menon and Alexey Kotlyarov

Together with the microfilament, microtubule and intermediate-filament networks, septins constitute an integral part of the eukaryotic cytoskeleton. Historically identified as proteins critical for septum formation in the budding yeast *Saccharomyces cerevisiae*, septin family GTPases are expressed and participate in the process of cytokinesis in most eukaryotes except higher plants. More than a dozen septin genes in mammals, together with various splice variants displaying

tissue-specific expression patterns and flexible hetero-polymeric higher-order assembly achieve an unfathomable complexity superior to the other cytoskeletal components. Even though the initial studies in the septin field was restricted to their evolutionarily conserved role in cell division, strong expression of septins in the non-dividing cells of the brain generated great interest in understanding their role in neuronal morphogenesis and other aspects of cellular function. On one hand, recent developments indicate complex non-canonical roles for septins in diverse processes ranging from neuronal development to immune response and calcium signaling. On the other hand several lines of data including those from knockout models question the universal role for septins in animal cell cytokinesis. Mammalian hematopoietic cells seem to proliferate and efficiently undergo cytokinesis in the absence of pivotal septin proteins in a context-dependent manner. The lack of septin-dependence of hematopoiesis also opens the possibility of safely targeting septin-dependent cytokinesis for solid-tumor therapy. Thus the septin field is perfectly poised with novel roles for septins being discovered and the basic understanding on septin assembly and its canonical functions constantly revisited.

The objective of this research topic was to provide an exclusive platform for discussing these rapid advances in the septin field. With a mixture of reviews and research articles encompassing diverse areas of septin research, ranging from the humble yeast model to human cancer, this ebook will be an interesting reading material for both experts as well as new comers to the septin field.

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EDITORIAL
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Editorial: Emerging Functions of Septins

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Keywords: septin, cytokinesis, cytoskeleton, SEPT, GTPase, signaling

Editorial on the Research Topic

Emerging Functions of Septins

Septins are a family of conserved cytoskeletal GTPases with unique heteropolymerization properties and diverse physiological functions (reviewed in Mostowy and Cossart, 2012). Originally discovered in the budding yeast *Saccharomyces cerevisiae* as factors crucial for the mother-bud separation, septin genes were later identified in almost all eukaryotes except higher plants. Initial studies on septins were limited to their functions in yeast, with a major focus on cell division. However, much information has been gathered about mammalian septins in the present century and septins are gaining importance as a distinct fourth component of the mammalian cytoskeleton. The presence of multiple septin genes with possibly redundant roles and the lack of potent and specific septin inhibitors have hindered functional characterization of the septin cytoskeleton. These difficulties have been effectively tackled by the widespread use of RNAi technologies and at least 10 out of the 13 mouse septin genes have been successfully knocked out, aiding in the identification of several general and isoform-specific physiological functions of septins. This Frontiers Research topic on the emerging functions of septins compiles contributions from the diverse field of septin research dealing with complex issues from yeast cell division to human cancer, from cellular morphogenesis to protein stability and from plant pathogens to human infections.

Historically, the name "septin" originated from their role in "septa" formation in the budding yeast. Septins participate in crucial cell cycle phases in *S. cerevisiae* and the mini-review from Glomb and Gronemeyer summarizes our current understanding on septin functions in this model organism, with a special focus on the subcellular organization and septin assembly. There is a long-standing view that septins could act as signaling scaffolds facilitating localized regulatory events. Perez et al. discuss septins as kinase scaffolds explaining the relevance of these septin associated kinases in the regulation of different cell cycle phases in the budding yeast. They describe the salient features of septin-dependent cell-cycle checkpoints, which determine the precise time of mitotic entry, exit, and cytokinesis. In addition to the role of septin scaffolds in coupling cell morphology to cell cycle decisions, the different signaling events regulating septin assembly are also discussed here. The complexity in the regulatory role for septins in yeast budding is further addressed by McQuilken et al. by monitoring septin reorganization at the bud neck using advanced polarized fluorescence microscopy. By the systematic analysis of multiple mutants lacking septin interacting proteins, they further establish a complex role for kinases in septin remodeling at the bud-neck. While the above mentioned articles focus on the model organism *S. cerevisiae*, Momany and Talbot present a different view on how septins are essential for fungal pathogenesis. Taking examples from human and plant pathogenic fungi they discuss the importance of septin-driven morphological changes as a prerequisite for focusing invasive force in an efficient and directed manner to facilitate pathogenesis.

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Septins associate with membranes and can facilitate selective diffusion of components, a property of septin family contributing to processes including neuronal morphogenesis, ciliogenesis, and sperm motility. In a thought-provoking mini review article, Palander et al. discuss the current understanding and possible functions of septins in the biogenesis of cilia and sperm flagella. The research article by Kaplan et al. addresses the functional redundancy of SEPT2 family members in neuronal morphogenesis, concluding that SEPT2, SEPT4, and not SEPT1 can compensate for the loss of SEPT5 in dendritic branching. In the context of membrane association of septins, the mini review by Song et al. deals with the evidences which indicates a role for septins in endo-lysosomal membrane traffic. They discuss the role of septins in vesicular trafficking and autophagy, as indicated by multiple septin-interactors with essential roles in these processes. In a completely different approach, Neubauer and Zieger summarize the current status of the mammalian septin interactome with important additional insights into their previous findings on clinically relevant septin interactors of human platelets and endothelial cells. Septins are described here as key mediators of platelet degranulation and, accordingly, the authors also deliberate on the clinical relevance of the *SEPT5* gene locus in bleeding disorders.

Since septins are crucial player in multiple stages of cell division and septin gene translocations and fusions were detected in leukemia, septins have been implicated in cancer development (Cerveira et al., 2011). While a direct functional role for septin in tumorigenesis has not been established yet, there are several studies linking septins to diverse forms of human cancer, such as the use of *SEPT9* gene methylation as a biomarker for colon cancer (Warren et al., 2011). In a very unique contribution on this subject, Poüs et al. look at the cancer related functions of septins through the prism of their subcellular localization. This alternate view of localization-dependent septin functions in cancer may ignite the interest of researchers from diverse fields to investigate septin functions in tumorigenesis. This article is complemented by a thought-provoking review article of Angelis and Spiliotis who provide a comprehensive view of septin mutations in human cancers. By an approach coupling large scale sequence data analysis and basic septin biochemistry, the authors convince the readers that despite the complexity associated with multiple septin genes and diverse cancers, there are recurring mutations on conserved amino acids across the septin family. Interesting observations on the possible effects of these mutations on septin oligomerization and function are described. Being the most investigated septin gene in the field of cancer, the authors also give due importance to SEPT9, and separately discuss the mutations of the unique N-terminal domain of SEPT9.

The immune-related functions of septins are also often linked to their membrane association and interplay with the cortical actin cytoskeleton. Despite normal development of hematopoietic lineages on pan-septin depletion in the *Sept7* knockout mouse model (Menon et al., 2014), septins regulate lymphocyte migration (Tooley et al., 2009) and associate with macrophage phagosomes (Huang et al., 2008), indicating a clear participation in the immune response. A significant role for septins in host cell entry and invasiveness of intracellular bacterial pathogens in non-phagocytic cells was one of the more recent discoveries in the field (Mostowy et al., 2010). In their mini review Torraca and Mostowy discuss this role of septins in bacterial infection together with its interplay with the autophagy pathway. The diverse methods employed by bacterial pathogens to take advantage of the host cytoskeleton to gain entry into cells and the counter mechanisms in place are nicely described by the authors here. In the context of bacterial pathogenesis, it was recently shown that the intracellular longevity of *Clostridium botulinum* neurotoxin Bont/A arise from their association with septins (Vagin et al., 2014). Vagin and Beenhouwer discuss this concept in their mini review dealing with the role of septins as regulators of protein stability. They broadly look into the role played by septins in regulating the stability of pathogenic toxins as well as other signaling molecules, which could be relevant to cancer as well as immunity.

One of the latest discovered functions of septins concerns its involvement in the store operated calcium entry (SOCE). While an siRNA screen identified SEPT2 family members as mediators of calcium signaling in HeLa cells (Sharma et al., 2013), T cell calcium signaling was not affected in a conditional *Sept7* knockout mouse model (Mujal et al., 2016). Studies in the Drosophila model also suggests an isoform specific role for septins in regulating SOCE (Deb et al., 2016). A perspective article by Deb and Hasan discuss the intricacies associated with these findings and their possible relevance to mammalian neuronal function.

Our understanding on the canonical functions of septins in cell division is constantly being revisited. In addition, the foot print is spreading far and wide as more and more physiologically relevant functions emerge for the septin family proteins. Collectively, the articles in this research topic including original research, perspectives and reviews on diverse aspects of septin research comprehensively summarizes the advances in the field. The contributions also provide ideas of promising directions for future investigations.

AUTHOR CONTRIBUTIONS

Both authors equally contributed to the drafting and revision of the editorial and approved it for publication.

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MINI REVIEW
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Septin Organization and Functions in Budding Yeast

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The septins are a conserved family of GTP-binding proteins present in all eukaryotic cells except plants. They were originally discovered in the baker's yeast *Saccharomyces cerevisiae* that serves until today as an important model organism for septin research. In yeast, the septins assemble into a highly ordered array of filaments at the mother bud neck. The septins are regulators of spatial compartmentalization in yeast and act as key players in cytokinesis. This minireview summarizes the recent findings about structural features and cell biology of the yeast septins.

Keywords: septins, budding yeast, GTPase, crystal structure, cytokinesis

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The classical Hartwell screen revealed genes involved in cell cycle regulation of the budding yeast *Saccharomyces cerevisiae* (Hartwell, 1971). Beside many others, the screen showed mutations in the four genes CDC3, CDC10, CDC11, and CDC12 (**c**ell **d**ivision **c**ycle). These were later classified as members of the septins, a new family of cytoskeletal proteins in the budding yeast (Sanders and Field, 1994; Pringle, 2008). Another mitotic septin, Shs1 (**s**eventh **h**omolog of **s**eptin), was identified more than two decades after the initial screen (Mino et al., 1998). In meiosis, Cdc11 and Cdc12 are replaced by two other septins, Spr28 and Spr3, respectively (Ozsarac et al., 1995; De Virgilio et al., 1996). These two septins are expressed under an own promoter and the resulting meiosis-specific septin complexes exhibit distinct functionalities than their mitotic counterparts (Garcia et al., 2016). In this review we will focus on the mitotic septins.

The mitotic yeast septin proteins can be recombinantly expressed and purified from *E. coli* (Bertin et al., 2008; Renz et al., 2013). Analysis of mutated or truncated recombinant septin preparations uncovered the organization of the individual subunits: They assemble into hetero-oligomers, called rods, with the order Cdc11-Cdc12-Cdc3-Cdc10-Cdc10-Cdc3-Cdc12-Cdc11 with Shs1 sometimes replacing the terminal subunit Cdc11 (Bertin et al., 2008; Garcia et al., 2011). These rods appear *in vitro* as short filaments of 32 nm length under high salt conditions (Kaplan et al., 2015). Septin rods can be induced to form long septin filaments *in vitro* by lowering the salt concentration of a buffer that otherwise keeps the rods stable in solution (Bertin et al., 2008; Renz et al., 2013). These filaments are about 1.5 μM long and exhibit a thickness within the range observed for microtubuli (Kaplan et al., 2015). Hexameric rods lacking the terminal subunit Cdc11 fail to form filaments suggesting that filament formation occurs end-over-end via the terminal subunit Cdc11 (Bertin et al., 2010). This observation was further fostered by applying single molecule localization microscopy to rods with their central and terminal subunits labeled with fluorescent dyes (Kaplan et al., 2015). Another study using septin preparations labeled with suitable FRET donor and acceptor dyes confirmed the end-over-end assembly via Cdc11 and showed that this interaction has a high affinity with a K_D in the nano-molar range (Booth et al., 2015). Alternative modes for filament formation via other subunit contacts could be excluded by evaluation of different combinations of subunits labeled with the FRET compatible dyes (Booth et al., 2015).

Septin octamers containing Shs1 instead of Cdc11 as terminal subunit do not form linear filaments *in vitro* but rather curved bundles that assemble into closed rings (Garcia et al., 2011) suggesting that Shs1 is necessary to form also curved structures *in vivo*. Recombinant septin rods exclusively capped with Shs1 do not assemble end-over-end into filaments whereas Shs1-Cdc11 contacts are formed *in vitro* (Booth et al., 2015).

Crystal structures of the human subunits SEPT2 and SEPT7 provided invaluable insight into the architecture of subunit contacts and filament formation (Sirajuddin et al., 2007, 2009; Zent et al., 2011). Structurally, septins are GTP-binding proteins sharing similarities with the small GTPases of the Ras family (Leipe et al., 2002). A central conserved G-domain is flanked by variable N- and C-terminal extensions. The G-domain core resembles the conserved Ras structure with six β-strands and five α-helices. Structural elements from Ras like the switch I and switch II motifs and the P-loop (Schweins and Wittinghofer, 1994) classify the septins as *bona fide* GTPases. Besides these canonical structural elements, the septin G-domain harbors a septin unique element at its C-terminus that forms a distinct α-helix, α6 following the domain nomenclature from Ras, which points away from the G-domain in a 90° angle. Other septin specific features are the presence of a short N-terminal helix, α0, and a distinct loop with two antiparallel strands β7 and β8.

The crystal structure of a septin filament consisting of SEPT7, SEPT6, and SEPT2 revealed the nature of the binding interface of the septin subunits within the filament (Sirajuddin et al., 2007): Interactions between two adjacent G-domains (called G-interface) alternate with interactions of two adjacent N- and C-termini of two subunits (called NC-interface). The filament subunit is a hexamer with the order SEPT7-SEPT6-SEPT2-SEPT2-SEPT6-SEPT7. Here SEPT7 forms a G-interace with SEPT6, SEPT6, and SEPT2 form a NC-interface, SEPT2 and SEPT2 a G-interface and so on. The α6 helix is the prominent element of the NC-interface. C-terminal extensions like predicted coiled-coils do not seem to contribute to the NC-interface (Sirajuddin et al., 2007). The G-interface is composed of a complex network of interactions between different amino acid residues of the respective opposite subunit. Especially the interacting Val, His, and Trp residues located in the β7 and β8 sheets are highly conserved among human and fungal septins. Based on the high sequence homology between fungal and human septins one would assume that the architecture of the G-interface is shaped similarly in yeast septins. However, in the recently solved crytsal structure of Cdc11 (Brausemann et al., 2016, **Figure 1**), the terminal subunit of the *S. cerevisiae* septin rod, the loop containing the β7 and β8 sheets is not structured and the base of the loop points away from the presumed G-interface in an approximetaly 90° angle (**Figure 1**). This, together with the finding that Cdc11 dimers are maintained via the C-terminal coiled-coil extensions of the Cdc11 monomers and not via a NC- or G-interface, suggests that at least some yeast septins have interaction interfaces that might differ from the "classic" NC- and G-interfaces from the human septins.

Cdc11 exhibits one more unique feature: The crystal structure is from the apo form and does consequently not contain a nucleotide (Brausemann et al., 2016). All yeast and human septins harbor an absolute conserved lysine residue in the canonical P-loop sequence GXXXXGKS/T. The P-loop is usually in contact with the β-phosphate of the bound GTP or GDP. Instead of the conserved lysine, Cdc11 has an arginine in this position of the P-loop whose side chain would collide with the β-phosphate of any bound nucleotide (**Figure 1**). Is Cdc11 not capable of binding a nucleotide? This assumption is fostered by the finding that cells expressing *cdc11* alleles that contain mutants in the P-loop show wild type like phenotype at 30°C (Casamayor and Snyder, 2003). Furthermore, only Cdc10 and Cdc12 have been shown to bind and hydrolyze GTP whereas GTPase activity could not be shown for Cdc3 and Cdc11 (Versele and Thorner, 2004).

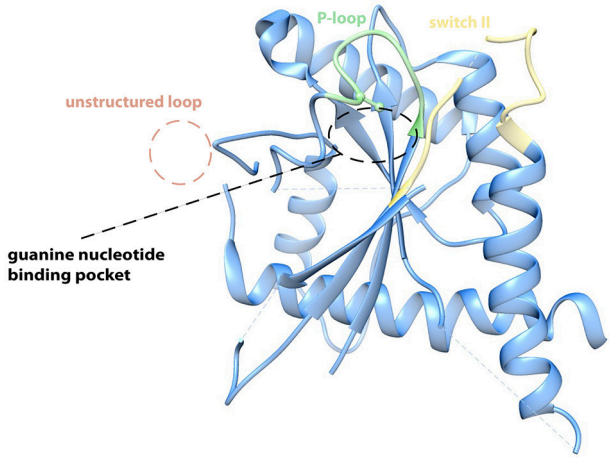

FIGURE 1 | Crystal structure of Cdc11$_{20–298}$ (PDB-ID 5AR1). The protein crystallized in its apo form. The P-loop, the switch II loop and the anti-parallel ß7–ß8 sheets contribute to the G-binding interface in the human SEPT2 and SEPT7 (Sirajuddin et al., 2007; Zent et al., 2011). In the Cdc11 crystal the ß7–ß8 hairpin is unstructured and points away from the binding interface in a 90° angle, whereas P-loop (green) and switch II region (yellow) show high similarities. An arginine within the P-loop of Cdc11 possibly hinders effective binding of a nucleotide and thereby possibly explains why Cdc11 is incapable of binding a nucleotide (Brausemann et al., 2016). Figure created with Chimera vers. 1.10.2. from PDB structure 5AR1.

In the living yeast cell, the septin filaments assemble at the bud neck in an organized array, the so-called septin ring. This ring undergoes different cell cycle dependent architectural transitions (**Figure 2**). In early G_1-phase, the septins are recruited to and accumulate at the presumptive bud site in a patch-like structure. Shortly before bud emergence, the septins assemble in a Cdc42 dependent manner into a ring marking the future site of bud growth and cytokinesis (Gladfelter et al., 2002; Caviston et al., 2003; Iwase et al., 2006). This initial recruitment of the septins to the future bud site depends besides Cdc42 on its effectors Gic1 and Gic2, and the action of the cyclin-dependent kinases Cdc28 and Pho85 (Tang and Reed, 2002; Iwase et al., 2006; Egelhofer et al., 2008; Okada et al., 2013). Evaluation of the mechanisms underlying the annealing of recombinant septin rods and formation of filaments on lipid bilayers suggests that septin filaments are formed *in vivo* at the plasma membrane from small complexes that diffuse in two dimensions (Bridges et al., 2014). Initial recruitment to the plasma membrane is supposed to be mediated by phospholipids. In an *in vitro* lipid

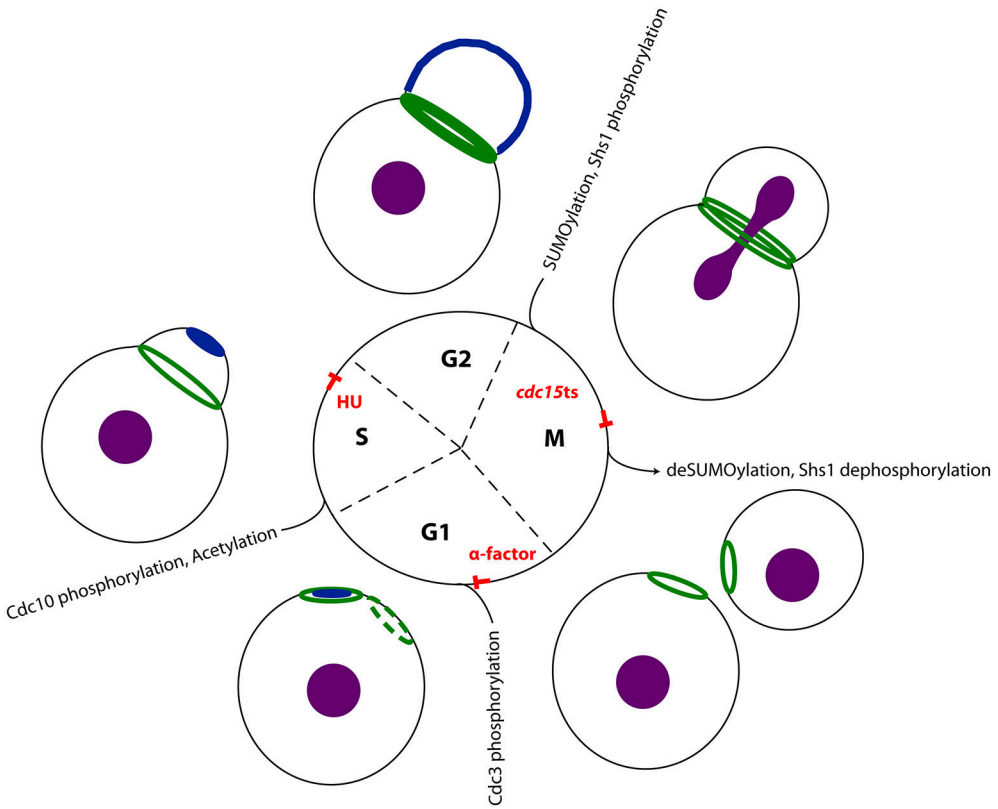

FIGURE 2 | Septins in the cell cycle of *S. cerevisiae*. The septins undergo cell cycle depending transitions: In G1 phase active Cdc42 (dark blue) polarizes at the plasma membrane, defining the presumptive bud site. The septins (dark green) are recruited to this site and remain as a ring at the neck upon bud emergence. This ring expands into a stable hourglass-shaped collar until the onset of mitosis. In late anaphase the septin collar splits into two distinct rings. In a recent study, Renz et al. detected interaction partners systematically at different stages of the cell cycle and could thereby reconstitute a time-resolved interactome of the septin rod (Renz et al., 2016). Treatment of the yeast cells with alpha factor arrested cells in early G1 phase, where no septin structure was visible. Using hydroxyurea arrested the cells in S-phase, where a stable septin ring was visible at the bud neck. Finally, a temperature shift in cells carrying a *cdc15-1* allele, allowed determining interaction partners with split septin rings. The respective cell cylce blocks and major known post translational modifications (Hernández-Rodríguez and Momany, 2012) of the respecive cell cycle state are indicated. The nucleus is colored in purple.

monolayer model PIP2 enhanced the filament assembly rate of septin hetero-octamers (Bertin et al., 2010).

After bud formation, the septin ring expands into a stable hourglass-shaped collar that is present at the bud neck until the onset of mitosis (Vrabioiu and Mitchison, 2006; Oh and Bi, 2011; Ong et al., 2014). Before cytokinesis, the septin collar splits into two distinct rings, one located at the mother and one at the daughter site of the bud neck. The contractile actomyosin ring (AMR), which powers the ingression of the cleavage furrow and septum formation, is assembled between the two septin rings (Wloka and Bi, 2012). Myo1, one essential constituent of the AMR, is recruited to the site of cytokinesis via the septin interacting protein Bni5 (Fang et al., 2010; Schneider et al., 2013). Bni5 in turn associates with the C-terminal extensions of the septin subunits Cdc11 and Shs1 (Finnigan et al., 2015).

After completion of cell separation, the old septin rings are disassembled and septin subunits are partially replaced and recycled for the next round of the cell cycle (McMurray and Thorner, 2008).

Taken together, the septins act mainly as scaffold for other proteins that are recruited to the bud neck or the site of cytokinesis, respectively. Additionally, the septins function as a diffusion barrier for proteins and organelles at the cortex of the bud neck (Luedeke et al., 2005; Shcheprova et al., 2008; Caudron and Barral, 2009; Orlando et al., 2011).

The transitions that the septins undergo throughout the cell cycle are supposed to be regulated by posttranslational modifications: Transition of the septin ring into a stable septin collar after bud emergence is associated with the phosphorylation and acetylation of certain subunits (Mitchell et al., 2011; Hernández-Rodríguez and Momany, 2012). For example, the p21-activated kinase Cla4 phosphorylates Cdc10 after bud emergence and a deletion of *CLA4* strongly affects the timely formation of septin structures (Dobbelaere et al., 2003; Kadota et al., 2004; Versele and Thorner, 2004). The splitting of the septin collar at the onset of cytokinesis is supposed to be initiated by a collective switch in the orientation of the septin filaments from parallel to perpendicular to the growth axis of the cell (DeMay et al., 2011). The switch is accompanied by at least two different

modifications. First, the bud neck kinase Gin4 phosphorylates Shs1 at residues different from those being modified in G_1-phase (Mortensen et al., 2002). Gin4 is recruited together with the septins to the presumptive bud site, co-localizes with the septins at the bud neck for the complete cell cycle and disappears from the bud neck after splitting of the septin collar (Longtine et al., 1998; Mortensen et al., 2002; Au Yong et al., 2016). Second, the small ubiquitin-like modifier (SUMO) Smt3 is covalently attached to Cdc3, Cdc11, and Shs1 at the mother site of the bud (Johnson and Blobel, 1999). Phosphorylation events play apparently an important role in septin structure transitions and septin organization and several more kinases have been identified to interact with the septins: Gin4 (Dobbelaere et al., 2003), Kcc4 (Okuzaki and Nojima, 2001; Kozubowski et al., 2005), Ste20 (Ptacek et al., 2005), Hsl1 (Finnigan et al., 2016), Elm1 (Kang et al., 2016), and Kin2, the ortholog of animal MARK/PAR-1 kinase (Yuan et al., 2016), were all identified as septin interactors.

However, for most of these regulatory proteins the exact timing of the interaction with the septins remains unknown and one can speculate that much more interactions exist but are not identified so far. Recently, Renz et al., performed a systematic screen for septin interaction partners throughout the cell cycle by combining cell cycle synchronization at stages with a distinct septin structure (**Figure 2**) with quantitative mass spectrometry to timely resolve the septin interactome (Renz et al., 2016). Yeast cells were synchronized in G1 phase with alpha factor, in S phase with hydroxyurea and in late anaphase with the help of a tempertaure sensitive *cdc15* allele. This strategy allowed to link interaction partners to specific transition states of septin structures. Distinct sets of regulatory proteins were found to interact with the septins at certain stages of the cell cycle: Gin4 and the anillin-like protein Bud4, a known septin interactor (Kang et al., 2013), were identified both in S-phase and anaphase. The kinases Hsl1, Mck1, and Prk1 are specifically associated with the septins in S-phase cells. Ste20 and yeast SUMO (Smt3) were only identified as specific septin interactors in cells with split septin rings. An activity of Mck1 and Prk1 on the septins has not been reported and remains to be confirmed by supplementary studies. This screen strengthened also evidence that septins play a role in endocytosis: Syp1 is a negative regulator of the WASP-Arp23 complex and involved in endocytic site formation, but was also classified as septin organizing protein (Qiu et al., 2008; Merlini et al., 2015). It interacts with the septins in anaphase (Renz et al., 2016). In S-phase, the septins interact with Sla2, an adaptor protein that links actin to clathrin and endocytosis (Skruzny et al., 2015). In alpha facor arrested cells, Vps1 interacts with the septins (Renz et al., 2016). Vps1 is a dynamin related protein that functions at several stages of membrane trafficking including endocytic scission (Smaczynska-de Rooij et al., 2010, 2015).

The septins influence the composition of the ER at the bud neck and thereby render diffusion of ER membrane proteins from mother to bud. Septins, septin dependent kinases, the membrane protein Bud6 and sphingolipids were shown to be required for the ER diffusion barrier (Luedeke et al., 2005; Clay et al., 2014). Interestingly, the published septin interactome contains a hit for Erp1 (Renz et al., 2016), a member of the p24 complex that was reported to be involved in ER retention and ER-Golgi transport (Copic et al., 2009). Another constituent of COPII vesicles that interacts with p24 family members, Sfb3/Lpt1 (Manzano-Lopez et al., 2015), was additionally identified in the MS screen. These findings support the previsously published model of a septin dependent ER diffusion barrier.

Recapitulatory, the past few years have witnessed a considerable increase of published results concerning yeast septins, the majority of these in budding yeast. Efforts were made in all areas of septin biology, from structural investigations using EM, light microscopy and X-ray diffraction to reconstruction of septin assembly in complex *in vitro* systems and to elucidation of septin related processes in the living cell. All these studies together establish and/or approve the budding yeast as a prime model organism for septin research. While structural information of several of the human septin subunits is now avaliable (the PDB provides crystal structures for SEPT2, SEPT7, SEPT9, SEPT3 and the septin trimer 2/6/7), only one structure from a yeast septin was solved (Cdc11). Structure determination, the evaluation of the role of posttranslational modifications of the septins and the uncovering of so far unknown septin related processes in the living cell will represent the challenges for septin biologists for the next decade.

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Septin-Associated Protein Kinases in the Yeast *Saccharomyces cerevisiae*

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Septins are a family of eukaryotic GTP-binding proteins that associate into linear rods, which, in turn, polymerize end-on-end into filaments, and further assemble into other, more elaborate super-structures at discrete subcellular locations. Hence, septin-based ensembles are considered elements of the cytoskeleton. One function of these structures that has been well-documented in studies conducted in budding yeast *Saccharomyces cerevisiae* is to serve as a scaffold that recruits regulatory proteins, which dictate the spatial and temporal control of certain aspects of the cell division cycle. In particular, septin-associated protein kinases couple cell cycle progression with cellular morphogenesis. Thus, septin-containing structures serve as signaling platforms that integrate a multitude of signals and coordinate key downstream networks required for cell cycle passage. This review summarizes what we currently understand about how the action of septin-associated protein kinases and their substrates control information flow to drive the cell cycle into and out of mitosis, to regulate bud growth, and especially to direct timely and efficient execution of cytokinesis and cell abscission. Thus, septin structures represent a regulatory node at the intersection of many signaling pathways. In addition, and importantly, the activities of certain septin-associated protein kinases also regulate the state of organization of the septins themselves, creating a complex feedback loop.

Keywords: cell cycle, cell signaling, cytoskeletal element, morphology, protein phosphorylation

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INTRODUCTION

Septins are a conserved family of GTPases (Pan et al., 2007; Peterson and Petty, 2010; Nishihama et al., 2011) that serve multiple biological functions in diverse cell types (Weirich et al., 2008; Hall and Russell, 2012; Hernandez-Rodriguez and Momany, 2012; Mostowy and Cossart, 2012; Fung et al., 2014). All eukaryotes examined to date (except higher plants) encode multiple septin genes, ranging from just two in the nematode *Caenorhabditis elegans* (John et al., 2007), to five in *Drosophila melangaster* (O'Neill and Clark, 2016), to seven in *Saccharomyces cerevisiae* (Garcia et al., 2016), to 13 in humans (Peterson and Petty, 2010; Hall and Russell, 2012; Fung et al., 2014).

Budding yeast (*S. cerevisiae*) has served as a path-finding model eukaryote in which to explore the structure, function, and regulation of septins and septin-associated proteins. The products of the yeast septin genes assemble into linear, apolar hetero-oligomeric rods that are the fundamental building block of all septin-based structures (Bertin et al., 2008; Bertin and Nogales, 2012), as has now also been shown for other organisms. These rods can self-associate end-to-end to form filaments and can, depending in their subunit composition, also interact in other modes to form

more elaborate super-structures, such as spirals, rings, braids, and gauze-like lattices (Rodal et al., 2005; Garcia et al., 2011; Oh and Bi, 2011; Bertin et al., 2012; Ong et al., 2014). Other factors nucleate the assembly of septin structures at discrete subcellular locations (Chen et al., 2011; Bi and Park, 2012) where they serve as both scaffolds (Shulewitz et al., 1999; Sakchaisri et al., 2004; Wloka et al., 2011; Bridges and Gladfelter, 2015) and diffusion barriers (Takizawa et al., 2000; Caudron and Barral, 2009) and thereby dictate, via direct and indirect interactions, the subcellular distribution of numerous other proteins (Gladfelter et al., 2001; McMurray and Thorner, 2009; Finnigan et al., 2016a). In particular, as discussed here, septin-based structures recruit, and thereby localize (and, in some cases, regulate the activity of) a multiplicity of protein kinases that integrate multiple inputs into signaling pathways and ultimately initiate ensuing biological responses (**Figure 1**).

Septins were first discovered in *S. cerevisiae* as cell division cycle (*cdc*) mutants whose compromised function resulted in abnormal growth patterns and the inability of cells to execute cytokinesis (Hartwell, 1971; Hartwell et al., 1974). Electron microscopy revealed a prominent set of filaments that encircle the yeast bud neck (Byers and Goetsch, 1976) and, later, antibody staining demonstrated that septins were constituents of those filaments (Haarer and Pringle, 1987; Kim et al., 1991). The advent of GFP and other genetically encoding fluorescent protein tags to follow protein dynamics in live cells in real time revealed further that septin organization undergoes dramatic changes during cell cycle progression. Septins first accumulate as a patch at the incipient site of bud emergence that rapidly resolves into a filamentous ring (Kozubowski et al., 2005; Okada et al., 2013), which then expands, concomitant with bud growth, into an hourglass-shaped tube or collar composed of at least 30–40 gyres of circumferential filaments at the bud neck (Byers and Goetsch, 1976; McMurray et al., 2011; Finnigan et al., 2015b; Patasi et al., 2015). At the onset of cytokinesis, the collar splits into two fllamentous bands of roughly equal size with a prominent gap in between (Dobbelaere et al., 2003; Dobbelaere and Barral, 2004) wherein factors needed for actomyosin contractile ring assembly and new plasma membrane (PM) and cell wall (CW) synthesis accumulate (Bi et al., 1998; Nishihama et al., 2009). After cytokinesis and cell separation, each daughter cell disassembles the half of the collar it inherited (Johnson and Blobel, 1999; Tang and Reed, 2002) before constructing a new septin-based site for the next bud to emerge. An N-terminal F-BAR domain—and C-terminal Muniscin/Mu homology domain (MHD)-containing protein Syp1 localizes prominently at the bud neck and has been implicated in the processes required for disassembly of the septin ring (Qiu et al., 2008). Syp1 is a highly phosphorylated protein (Albuquerque et al., 2008; Soulard et al., 2010; Swaney et al., 2013). In this regard, it is interesting that eight of the phospho-sites detected in Syp1 fit the -T/S-P- consensus for the protein kinase Cdk1/Cdc28 that is the major cell cycle driver (Verma et al., 1997; Mok et al., 2010) and five of them fit the consensus sequence (-R-x-x-S/T-) determined for the bud neck-localized protein kinase Gin4 (Mok et al., 2010; Roelants et al., 2015). However, Syp1 also localizes prominently to cortical puncta and functions as an endocytic adaptor that is involved in cargo selection and negative regulation of Las17 (yeast WASp)-Arp2/3 complex activity (Boettner et al., 2009; Reider et al., 2009) during the early stages of endocytic patch formation (Stimpson et al., 2009). Although eukaryotic proteins with such apparently disparate functions certainly exist, it is nonetheless a little hard to reconcile mechanistically these two rather distinct roles attributed to Syp1.

In *S. cerevisiae*, five (*CDC3*, *CDC10*, *CDC11*, *CDC12*, and *SHS1*) of its seven septin genes are expressed in mitotically-dividing haploid and diploid cells (Versele et al., 2004; Versele and Thorner, 2004, 2005), whereas the remaining two septin genes (*SPR3* and *SPR28*) are expressed only in diploid cells undergoing meiosis and sporulation (Garcia et al., 2016). The mitotic septins assemble into two types of hetero-octameric rods that differ only in their terminal subunit: Cdc11-Cdc12-Cdc3-Cdc10-Cdc10-Cdc3-Cdc12-Cdc11 (Bertin et al., 2008) and Shs1-Cdc12-Cdc3-Cdc10-Cdc10-Cdc3-Cdc12-Shs1 (Garcia et al., 2011). *In vitro* the former can associate end-to-end into paired filaments in low-salt solution and into sheets of paired filaments on the surface of a lipid monolayer containing phosphatidylinositol-4,5-*bis*phosphate (Bertin et al., 2010). Mutagenesis studies have shown that the residues needed for these characteristic *in vitro* behaviors are also essential for viability *in vivo* (Bertin et al., 2008; McMurray et al., 2011; Finnigan et al., 2015b). The Shs1-containing hetero-octamers associate laterally in a staggered fashion, rather than end-on-end, forming curved bundles, rings, and bird's nest-like structures *in vitro* (Garcia et al., 2011). Although cells lacking Shs1 are viable, the septin structures formed in its absence are aberrant (Garcia et al., 2011), most likely because, as observed *in vitro* (Booth et al., 2015), Cdc11-capped rods and Shs1-capped rods are able to form heterotypic end-on-end junctions, and likely also do so *in vivo* (Finnigan et al., 2015a,b). The dynamic interplay between these two types of hetero-octamers may facilitate the massive reorganizations of septin architecture that occur over the course of the cell cycle (Vrabioiu and Mitchison, 2006; Ong et al., 2014).

Diverse protein kinases are associated with septin structures at various points throughout progression through the cell division cycle. However, it is still not completely clear how many of these enzymes contribute directly to installing post-translational modifications on septins and/or septin-associated proteins that drive the observed dynamic changes in septin structure during cell cycle progression and how many of these enzymes are recruited to septin structures as "readers" of the status of septin assembly to phosphorylate other substrates and thereby drive subsequent downstream events. Here, we highlight key regulatory pathways that use the septin cytoskeleton as a signaling platform to direct other orchestrated events required for successful passage through the cell cycle.

A MORPHOGENESIS CHECKPOINT

Eukaryotes have evolved quality control mechanisms, collectively dubbed checkpoints (Hartwell and Weinert, 1989; Paulovich et al., 1997; Ibrahim, 2015), by which to ensure that the events required for successful cell division are only executed

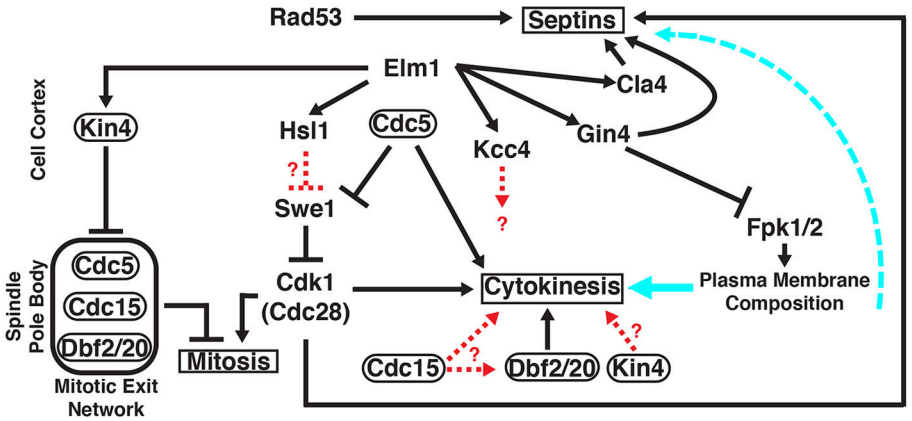

FIGURE 1 | Roles of multiple protein kinases in septin-mediated signaling networks in *S. cerevisiae*. Unless otherwise indicated, all of the gene products shown (Cdc5, Cdc15, Cdk1/Cdc28, Cla4, Dbf2, Dbf20, Elm1, Fpk1, Fpk2, Gin4, Hsl1, Kcc4, Kin4, Rad53, and Swe1) are protein kinases that co-localize with the septin collar at the bud neck at specific stages of the cell cycle, either very transiently or for a prolonged time period. Protein kinases that, in addition, localize at other sites are encircled by ovals. Signaling outputs of the indicated protein kinases at their non-septin locations are depicted on the left side of the panel; signaling events emanating from the septin collar itself are diagrammed on the right side of the panel. *Solid black arrows*, regulation by direct substrate phosphorylation; *dashed red arrows*, regulation exerted by unknown mechanisms; *solid cyan arrow*, influence of the plasma membrane lipid composition on the execution of cytokinesis; *dashed cyan arrow*, influence of the plasma membrane lipid composition on septin filament assembly and structural organization. See text for further details.

at the proper time and in the proper order. Virtually all recognized checkpoint mechanisms involve regulation by reversible protein phosphorylation mediated by protein kinases and phosphoprotein phosphatases (Domingo-Sananes et al., 2011; Rhind and Russell, 2012). In yeast, a checkpoint delays the decision to pass from G2 to M phase if there is some incompleteness (or abnormality) in cell morphogenesis that needs time to be finished (or repaired). It was initially thought that this control was exerted in response to defects in actin cytoskeleton assembly and/or function (McMillan et al., 1998). However, it was demonstrated shortly thereafter that it is the state of septin collar assembly that is monitored by this checkpoint (Shulewitz et al., 1999), a conclusion that was amply confirmed subsequently (Lew, 2003; Howell and Lew, 2012). In essence, when the septin collar is properly formed, it recruits and thereby serves as a congregation point for information exchange among the regulatory factors required to release the cell from cell cycle blockade (**Figure 2A**, *upper*). This arrangement provides a feedback circuit by which the state of septin organization is temporally and spatially integrated with other processes necessary for cell cycle progression. Because chromosome segregation and cytokinesis cannot proceed productively if the bud neck is occluded, this checkpoint delays initiation of the G2-M transition until the septin collar has been erected properly.

In brief, the cyclin B (Clb2)-bound form of protein kinase Cdk1 (Cdc28), which is the primary driver of mitosis, is held in check by inhibitory phosphorylation on Tyr19 in its P-loop, a modification installed by protein kinase Swe1 (the *S. cerevisiae* ortholog of mammalian Wee1) (Booher et al., 1993). This phosphorylation is reversed, in part, by the action of phosphoprotein phosphatase Mih1 (yeast ortholog of mammalian Cdc25) (Russell et al., 1989). However, to lift Swe1-mediated inhibition of Cdk1 completely, Swe1 is also degraded in a highly regulated manner, as follows. In conjunction with the joint actions of the protein-arginine N-methyltransferase Hsl7 (Cid et al., 2001; Sayegh and Clarke, 2008) and the protein kinase Hsl1 (Ma et al., 1996; Barral et al., 1999; Shulewitz et al., 1999) (closest mammalian orthologs are the AMPK-related protein kinase family members, BRSK1, and BRSK2), Swe1 is exported from the nucleus (Keaton et al., 2008), captured at the bud neck, and marked there for timely cyclosome/anaphase-promoting complex (APC) protein-ubiquitin ligase-dependent ubiquitinylation (Simpson-Lavy and Brandeis, 2010; Lianga et al., 2013) and proteasome-mediated destruction via phosphorylation by protein kinase Cla4 (yeast ortholog of mammalian PAK1) and especially by protein kinase Cdc5 (yeast ortholog of mammalian Plk1) (Sakchaisri et al., 2004). Compared to Hsl1, two other septin-associated protein kinases, Gin4 and Kcc4 (which are apparent paralogs, but less related to Hsl1) have a supporting, but less significant role, in these events (Ma et al., 1996; Barral et al., 1999; Kusch et al., 2002). It should be appreciated that because Clb2-Cdc28 phosphorylation of Swe1 creates phosphosites that are docking motifs for binding the Polo boxes of Cdc5, thus priming Swe1 for Cdc5-mediated phosphorylation (Asano et al., 2005; Harvey et al., 2005), the Hsl7- and Hsl1-dependent down-regulation of the nuclear pool of Swe1 partially alleviates Clb2-Cdc28 inhibition. This initially modest increase in Clb2-Cdc28 activity then is able to initiate a self-reinforcing burst of autocatalytic activation of Cdk1 by targeting more and more of the population of Swe1 molecules for even more efficient Cdc5-mediated phosphorylation, thereby unleashing more and more Clb2-Cdk1 activity. Thus, once initiated, these processes lead to rapid hyper-phosphorylation and nearly complete destruction of Swe1 (Shulewitz et al., 1999; Sreenivasan and Kellogg, 1999).

Most importantly, Hsl1 is only active in aiding the targeting of Swe1 for destruction when Hsl1 is associated with a correctly

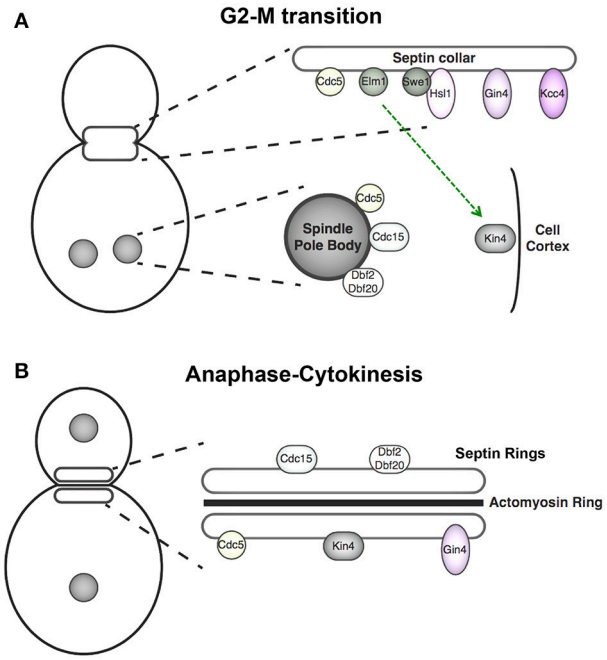

FIGURE 2 | Cell cycle-dependent localization of septin-associated protein kinases. (A) Components of the septin-monitoring morphogenesis checkpoint localize to the septin collar at G2 where they promote Swe1 degradation, and thus full activation of cyclin B-bound Cdk1 and M phase entry. At anaphase of mitosis, the APC protein-ubiquitin ligase terminates this pathway by degrading its pivotal component, the protein kinase Hsl1. At this point in the cell cycle, the protein kinases of the MEN are located at the spindle pole bodies (SPBs) and Kin4, the protein kinase central to the spindle position checkpoint, is localized to the mother cell cortex. Kin4 and the MEN components act in concert to delay mitotic exit until one spindle pole body has been properly segregated into the daughter bud. **(B)** Upon execution of anaphase, spindle elongation, SPB segregation, and initiation of mitotic exit, the MEN components relocalize to the septin rings, where these protein kinases phosphorylate targets that help promote cytokinesis. Gin4 remains septin collar-associated from G1 through cytokinesis.

assembled septin collar at the bud neck (McMillan et al., 1999, 2002; Shulewitz et al., 1999; Finnigan et al., 2016b). Disruption of the septin collar using mutant septin alleles results in failure to recruit Hsl1 and Hsl7 to the bud neck, thereby stabilizing Swe1, which is then able to impose a pronounced cell cycle delay that is manifest by the formation of markedly elongated buds (Shulewitz et al., 1999; Longtine et al., 2000), a phenotype that is not observed if cells lack Swe1 (Barral et al., 1999; McMillan et al., 1999; McMurray et al., 2011).

Directly fusing Swe1 to a septin subunit does not fully bypass the requirement for Hsl1 and Hsl7 in Swe1 degradation (King et al., 2012), indicating that simple tethering of Swe1 at the septin collar for subsequent phosphorylation by Cla4 and Cdc5 is not the sole function of the Hsl1 and Hsl7 enzymes. Binding of Hsl1 to septins occurs via uniquely evolved septin-binding sequences (residues 611–950) downstream of its N-terminal kinase domain, which, to operate efficiently, must act in concert with a C-terminal phosphatidylserine-binding element (KA-1 domain) (Finnigan et al., 2016a,b). Thus, Hsl1 recruitment to the bud neck likely serves as a dual "sensor," reporting that both septin collar assembly and the plasma membrane lipid composition have achieved the proper state to initiate the destruction of Swe1 and thereby license passage from G2 to M phase. Hsl1 catalytic activity is dispensable for its recruitment to the septins (Finnigan et al., 2016b), but may be required, directly or indirectly, in recruiting Hsl7 to the bud neck (Kang et al., 2016). Hsl7 is a substrate of Hsl1 (Cid et al., 2001), and Hsl1 is also extensively autophosphorylated (Barral et al., 1999), but the functional consequences of these modifications remain to be determined.

Hsl1 (and the checkpoint) may have additional roles besides sensing the state of septin organization and plasma membrane composition. A recent study has suggested that some of these same components, in conjunction with an additional bud neck-localized protein kinase, Elm1, have a role in monitoring bud size/shape prior to licensing entry into mitosis (Kang et al., 2016). Elm1 is able to phosphorylate the activation loop in Kcc4 and Gin4 *in vitro* (Asano et al., 2006), and presumably serves as their upstream activating kinase *in vivo* (Koehler and Myers, 1997; Bouquin et al., 2000). In contrast, under the same conditions, Elm1 does not directly phosphorylate Hsl1 *in vitro* (Asano et al., 2006), but there are claims that Elm1-mediated phosphorylation is required for full Hsl1 activity *in vivo* (Szkotnicki et al., 2008). It has been suggested that Elm1 interacts with Hsl7, thereby impeding engagement of Hsl7 with Hsl1; in this way, Swe1 recruitment to the bud neck and its degradation are delayed to allow time for sufficient bud growth (Kang et al., 2016). It has been reported that localization of septins, Hsl7 and Elm1 all depend upon local membrane curvature (Bridges et al., 2016; Kang et al., 2016); it is possible, therefore, that these factors serve as sensors of cell geometry, thereby integrating this input into the timing of the decision of when to allow passage through G2-M. Although Gin4 and Kcc4 have been implicated in septin collar assembly (Longtine et al., 1998) and in the Swe1 degradation pathway (Barral et al., 1999), more recent evidence indicates that these enzymes likely exert their effects more indirectly by modulating the plasma membrane lipid distribution (Roelants et al., 2015) (see further below). Indeed, like Hsl1, Gin4 and Kcc4 also possess phosphatidylserine-binding C-terminal KA-1 domains that are required for their function *in vivo* (Moravcevic et al., 2010).

MITOTIC EXIT

In addition to the role of the septin collar in integrating signals that determine when to initiate entry into mitosis, growing evidence implicates septin structures at the bud neck in coordinating certain aspects of the signaling network that regulates exit from mitosis. Mitotic exit is facilitated, in large part, by a protein kinase signaling cascade, dubbed the Mitotic Exit Network (MEN) (Bardin and Amon, 2001; McCollum and Gould, 2001), that gauges successful spindle elongation by monitoring the location and status of the spindle pole bodies (SPBs) (Smeets and Segal, 2002; Hotz and Barral, 2014; Falk et al., 2016). Once anaphase has been achieved, signals emanating from

the SPB in the daughter cell initiates a pathway that serves to reverse the actions of Cdk1 by unleashing the phosphoprotein phosphatase Cdc14 (Mocciaro and Schiebel, 2010; Bremmer et al., 2012). Components of the MEN regulatory circuitry in yeast have features that suggest that it may be the antecedant of the Hippo tumor-suppressor pathway in animal cells (Avruch et al., 2012; Harvey and Hariharan, 2012; Rock et al., 2013).

The core components of MEN (**Figure 2A**, lower) are the small Ras-related GTPase Tem1, which, in its GTP-bound state, binds to and activates the protein kinase Cdc15, which, in turn, is the upstream activator of two paralogous protein kinases Dbf2 and Dbf20 that, to be activated by Cdc15 and functional, need to associate with an essential regulatory subunit Mob1. The primary role of activated Dbf2/Dbf20-Mob1 complexes is to phosphorylate Cdc14, which both releases it from its inhibitory anchor in the nucleolus (Azzam et al., 2004) and prevents its nuclear import (Mohl et al., 2009), allowing its activity to spread throughout the cell to inactivate Clb-bound Cdk1 and reverse the Cdk1-dependent phosphorylation of many Cdk1 substrates (Visintin and Amon, 2000; Meitinger et al., 2012).

Whether Tem1 is in its inactive GDP-bound state or in its activated GTP-bound state is controlled by events at the SPB, completely independent of the septin collar. In brief, when no SPB has been segregated into the daughter bud, the Tem1 GTPase-activating protein (GAP) complex, Bfa1-Bub2, is activated by the protein kinase Kin4 located at the cortex of the mother cell and, hence, Tem1 remains in its inactive state (D'Aquino et al., 2005; Pereira and Schiebel, 2005). This regulatory scheme is referred to as the spindle positioning/orientation checkpoint (SPOC) (Caydasi and Pereira, 2012; Ibrahim, 2015). However, there is a clear connection to events at the septin collar. First, it was observed that septin-defective mutants exhibited aberrations in MEN signaling outputs (Castillon et al., 2003). Second, Kin4 (and its paralog Frk1) are, like Gin4, Kcc4, and Hsl1, members of the branch of yeast AMPK-like protein kinases (Rubenstein and Schmidt, 2007); and, indeed, it has been shown that, as for Gin4 and Kcc4, Elm1 is the upstream protein kinase responsible for phosphorylation of T209 in the activation loop of Kin4 and, further, that this modification is essential for Kin4 catalytic activity (Caydasi et al., 2010; Moore et al., 2010). Moreover, localization of Elm1 to the bud neck is critical for its phosphorylation and activation of Kin4 (Caydasi et al., 2010; Moore et al., 2010).

The action of phosphoprotein phosphatase PP2A bound to one of its two, yeast, B-type regulatory subunits, Rts1, was also implicated as a positive factor required for maintaining Kin4 at the mother cell cortex and at mother-cell SPBs (Chan and Amon, 2009). However, it was previously shown that Rts1-bound PP2A is required for maintenance of septin ring organization during cytokinesis (Dobbelaere et al., 2003). Thus, the role of Rts1-PP2A is likely indirect and simply to preserve the structure of the septin collar and, thus, the localization of active Elm1 there, supporting maintenance of adequate levels of activated Kin4 in the mother cell.

The daughter cell-specific protein Lte1, despite its sequence resemblance to other guanine nucleotide exchange factors (GEFs), does not appear to function as the GEF for Tem1. Rather, it appears to promote formation of GTP-bound Tem1 by binding to and inactivating Kin4 and excluding the kinase from the daughter SPB, thereby preventing activation of the Bfa1-Bub2 GAP (Bertazzi et al., 2011; Falk et al., 2011).

All of the MEN components, including the protein kinases Cdc5 (Sakchaisri et al., 2004), Cdc15 (Lee et al., 2001), and Dbf2/Dbf20 (Frenz et al., 2000), localize first to the SPB and then to the bud neck (**Figure 2**). At the SPB, Cdc5 functions in MEN by acting in concert with Tem1 to promote SPB recruitment and activation of Cdc15 (Rock et al., 2013). Cdc5 function depends on its cellular location, as revealed by experiments in which Cdc5 recruitment was artificially directed primarily to the SPB or primarily to the bud neck; at the SPB, Cdc5 was necessary for efficient mitotic exit and, at the bud neck, Cdc5 clearly promoted Swe1 degradation (Sakchaisri et al., 2004). The two C-terminal polo boxes of Cdc5 are required for stable association of this protein kinase at each of these two subcellular locations (Song et al., 2000; Lowery et al., 2005), but the protein target (s) at each location that carry the phospho-epitopes to which the polo boxes bind have not been well defined. Given the role that members of the polo family of protein kinases, including Cdc5, play in driving multiple cell cycle events subsequent to initial substrate phosphorylation by cyclin B-bound Cdk1 (Barr et al., 2004; Archambault and Glover, 2009), and the role that the septin collar plays in regulating the sub-cellular distribution of Cdc5, the coordination between septin dynamics and Cdc5 localization, as well as between septin dynamics and Elm1 localization (Thomas et al., 2003), provide regulatory inputs that contribute to ensuring that Swe1 degradation (and mitotic entry) always precedes mitotic exit. Indeed, the septin collar is the passageway through which any and all components segregated between a mother and daughter cell must pass and, hence, is a cellular structure ideally situated to monitor such cell cycle events.

CYTOKINESIS

In addition to its role in tethering of factors involved in controlling the spatial and temporal aspects of the cell cycle, the septin collar also has functions in regulating membrane dynamics. At the cortex of a mother cell or its bud, where the ER is in close apposition to the PM, there are periodic intimate protein-and lipid-mediated connections between the two, referred to as PM-ER junctions (Manford et al., 2012; Gatta et al., 2015). However, at the bud neck, the septin filaments tightly coat the cytosolic surface of the PM at this location (Byers and Goetsch, 1976; Bertin and Nogales, 2012), preventing sterically the formation of such ER-PM contact sites. Although extensions of the ER can be seen to pass through the bud neck, at the location of the septin collar *per se*, the ER surface appears denuded of ribosomes and, further, that this band of smooth ER acts as a barrier to diffusion of ER membrane proteins (but not ER lumenal proteins) (Luedeke et al., 2005). On the one hand, it has been reported that establishment of this specialized ER domain and its function in restricting diffusion depends on localized recruitment of the actin—and

formin-associated protein Bud6 by the septin Shs1 (Luedeke et al., 2005), whereas another more recent study claims that a bridged interaction between Shs1 and the integral ER membrane protein Scs2 is responsible for erecting this ER diffusion barrier (Chao et al., 2014). The evidence in support of both claims is not compelling for several reasons. First, more recent analysis indicates that very little of the total cellular pool of Bni6 is located at the bud neck [see entry for Bud6/Aip3/Ylr319c at the Yeast Protein Localization Plus Database (YPL+) at the Univ. of Graz, Austria; http://yplp.uni-graz.at/index.php], and Scs2 is a demonstrated component of a major class of the ER-PM junctions. Second, because *shs1*Δ cells are viable and do not display severe growth and morphology defects under most conditions (Iwase et al., 2007; Finnigan et al., 2015b), this specialized, purportedly Shs1-mediated, domain in the trans-bud neck ER doesn't seem to be very important for viability or efficient cell cycle progression. Finally, affixing the ER to the septin collar at the bud neck, and thereby risking impeding the passage of chromosomes and other cellular organelles, would not seem to make much sense physiologically for efficient cell division.

In any event, to complete each cell cycle, the spindle midbody remnant must be removed, the plasma membranes resealed, and the CW septa deposited between them, to create two, separate and independent yeast cells. In *S. cerevisiae*, cytokinesis and cell separation proceed via the concerted action of actomyosin ring (AMR) formation and contraction (Bi et al., 1998) with concomitant synthesis of the chitinaceous primary septum (Nishihama et al., 2009). To permit these events to happen following anaphase, the septin collar is split into two gasket-like bands via a mechanism that is still completely unknown (McMurray and Thorner, 2009; Bi and Park, 2012; Wloka and Bi, 2012; Juanes and Piatti, 2016). However, after septin collar assembly and release from the morphogenesis checkpoint, the elevation of Clb2-Cdc28 activity commences the process of promoting AMR formation early in M phase and, once localized to the septin collar, Cdc5 generates a local pool of activated (GTP-bound) Rho1 at the bud neck via phosphorylation and activation of one of the primary Rho1 GEFs (Tus1) (Yoshida et al., 2006). Activated Rho1, in turn, is known to stimulate formin-dependent assembly of the cytokinetic AMR and type II myosin contractility (Yoshida et al., 2006; Ramkumar and Baum, 2016). In addition, activated Rho1 also binds directly to and stimulates protein kinase Pkc1 (Kamada et al., 1996), which controls the transcription of genes needed for CW synthesis (Jung and Levin, 1999) and other events needed for cell cycle completion (Darieva et al., 2012), as well as two enzymes required for CW production (the 1,3-β-D-glucan synthase Fks1 and its paralog Gsc2/Fks2) (Mazur and Baginsky, 1996). In this way, the septins provide a platform by which the cell cycle machinery is linked to both the cytoskeletal machinery and the CW synthesis machinery that each contribute to the successful execution of yeast cytokinesis. Difficult to reconcile with these findings (and the evidence discussed earlier for its role in septin ring *disassembly*), however, is the claim that F-BAR and MHD protein Syp1 is phosphorylated in a Rho1- and Pkc1-dependent manner to promote septin collar *assembly* (Merlini et al., 2015).

The "split" septin collar appears to have two main roles. For certain proteins needed for AMR assembly, e.g., Bni5 (Lee et al., 2002), the septins continue to serve their scaffold function by mediating direct physical association with these factors, thereby anchoring and concentrating them at the bud neck (Patasi et al., 2015; Finnigan et al., 2015a, 2016a). For other proteins, e.g., Sec3 involved in localized deposition of secretory vesicles (Dobbelaere and Barral, 2004) and Chs2 involved in septum construction (Foltman et al., 2016), the two septin bands of the split collar seems to act like corrals and barriers to diffusion, thereby physically trapping these factors between them and thus confining them at this location indirectly (Dobbelaere and Barral, 2004; McMurray et al., 2011; Finnigan et al., 2016a).

In addition to Cdc5, the other two protein kinases of the MEN cascade, Cdc15 and Dbf2/Dbf20-Mob1 complexes, are recruited to the split septin rings during late anaphase, shortly after Cdk1 inactivation (Frenz et al., 2000; Song et al., 2000; Xu et al., 2000; Luca et al., 2001; **Figure 2B**). At this location, these enzymes recruit and phosphorylate many factors directly involved in the coordination of AMR contraction and primary septum formation. In particular, at the bud neck, Dbf2-Mob1 phosphorylates another F-BAR protein, Hof1 (Meitinger et al., 2011), which also contains a C-terminal SH3 domain important for its function (Oh et al., 2013). Hof1 associates with septin structures throughout G1-S phase and while the AMR is assembled between the rings of the split septin collar primarily via direct interaction with Cdc10 (and Cdc12) (Vallen et al., 2000; Oh et al., 2013; Finnigan et al., 2016a). Association of Hof1 with the septins is mediated by a coiled-coil element in Hof1, and phosphorylation at a single Ser within this region by bud neck-localized Dbf2-Mob1 (an event that requires priming by prior phosphorylation of Hof1 by Cdk1 and Cdc5) promotes disassociation of Hof1 from the septin rings (Meitinger et al., 2013). This displacement then allows Hof1 to associate with the AMR and initiate contraction, but the mechanism by which it does so has not been elucidated yet.

In addition to its binding to the AMR, Hof1 also forms a complex with Inn1 and Cyk3 at the septin ring in late anaphase (Sanchez-Diaz et al., 2008; Nishihama et al., 2009). Recruitment of these three components to the bud neck requires MEN signaling activity (Meitinger et al., 2010), and thus presumably their phosphorylation, and all are required for efficient cytokinesis because they contribute to coordinating AMR contraction with primary septum formation (Meitinger et al., 2012; Wloka and Bi, 2012; Juanes and Piatti, 2016). As mentioned above, formation of the primary septum requires the localized activity of chitin synthase Chs2 between the bands of the split septin collar. After Chs2 has been sequestered at this position, Dbf2-Mob1 translocates to the split rings and directly phosphorylates and activates Chs2 (Oh et al., 2012). The dynamic relocation of the protein kinases of the MEN cascade to the split septin collar provides an elegant solution to help ensure that cell division only occurs after successful chromosome segregation. However, the mechanisms that promote recruitment of these kinases to the septins are unknown. Moreover, the SPOC protein kinase Kin4 also localizes to the septin rings late in anaphase, yet its function at the bud neck is not understood.

Finally, cytokinesis cannot be completed unless and until two intact and separable PMs have been generated. In this regard, there is evidence that two types of septin-associated protein kinases spatially and temporally modulate the PM lipid bilayer assymmetry at the bud neck. At sites of polarized growth, the protein kinase Fpk1 (and its paralog Fpk2) phosphorylate and stimulate two PM-localized flippases (Dnf1 and Dnf2), which translocate phosphatidylethanolamine (PtdEth) and phosphatidylserine from the outer to the inner leaflet of the PM (Nakano et al., 2008; Roelants et al., 2010). However, at the bud neck, septin-anchored protein kinase Gin4 phosphorylates and inhibits Fpk1 (and Fpk2) function (Roelants et al., 2015). Thus, flipping of aminoglycerophospholipids is prevented in a highly localized manner. Preventing flipping of aminoglycerophospholipids in this way contributes to enhancing the efficiency of cell division because alterations that compromise flippase function suppress the inviabiity phenotype of a variety of mutations (*hof1*, *inn1*, *cyk3*, and *myo1*, the type II myosin of the AMR) known to cause defects in cytokinesis (Roelants et al., 2015). Moreover, Gin4 is released from the septin collar just before it splits and the AMR contracts, whereas the bulk of Fpk1 remains (Roelants et al., 2015), presumably fine-tuning the timing of transbilayer lipid flipping necessary for the completion of PM scission and cell separation. The mechanisms responsible for the cell cycle-coupled ejection of Gin4 and the recruitment of Fpk1 are not known, although Gin4 is phosphorylated and activated in a cell cycle-dependent manner at the G2-M transition, apparently by Clb2-Cdc28 (Altman and Kellogg, 1997) and, conversely, the septins are required for the mitosis-specific activation of Gin4 (Carroll et al., 1998). In any event, by the spatio-temporal control that Gin4 exerts on Fpk1 activity, it is clear that the septins at the bud neck are critical for protein kinase-mediated regulation of localized PM remodeling.

REGULATION OF SEPTIN ORGANIZATION

The actions of several septin-associated protein kinases also seem to regulate septin organization. The Cdc42-stimulated protein kinase Cla4 (yeast ortholog of mammalian PAKs) directly phosphorylates septins Cdc3 and Cdc10 both *in vitro* and *in vivo*, and Cla4 is necessary for both the formation of the septin collar and the regulation of septin dynamics at specific points in the cell cycle (Dobbelaere et al., 2003; Schmidt et al., 2003; Versele and Thorner, 2004).

Septin subunit Shs1 is the most extensively phosphorylated septin and is a substrate of Gin4 (Mortensen et al., 2002), as well as of the G1 cyclin-dependent protein kinases Cdc28/Cdk1 and Pho85 (yeast ortholog of mammalian Cdk5) (Egelhofer et al., 2008). The latter marks are removed at the end of mitosis via bud neck recruitment of Rts1-bound PP2A, an event thought to aid in initiating splitting of the septin collar (Dobbelaere et al., 2003), and which is coincident with Gin4 phosphorylation of Shs1 on a distinct subset of residues (Mortensen et al., 2002; Asano et al., 2006). These data suggest that cell cycle-dependent phosphorylation states of Shs1 are key in regulating septin dynamics. However, arguing against this hypothesis, strains expressing Shs1 alleles with most of the residues phosphorylated by Cdks mutated to either to Ala or phosphomimetic Asp displayed no discernible septin-defective phenotype (Finnigan et al., 2015b). Furthermore, most of these phosphorylation sites are within a poorly conserved segment of Shs1, and deletion of this entire region does not lead to any loss of Shs1 function *in vivo* (Finnigan et al., 2015b). Perhaps phosphorylation of Shs1 by Cdks and Gin4 is redundant with additional mechanisms that regulate septin assembly, but the precise consequence of these phosphorylation events on septin structure and/or function remains unclear. In marked contrast, Shs1 is phosphorylated at a single site by the protein kinase Rad53 in response to DNA damage and replication stress (Smolka et al., 2006, 2007) and a phosphomimetic mutation of this single residue displays a prominent growth defect (Finnigan et al., 2015b), suggesting that this direct phosphorylation might represent a checkpoint whereby cell cycle progression can be delayed (via perturbation of the septin collar) to permit time for repair of the DNA damage.

The purported role that Gin4 has in proper septin collar assembly at the bud neck has very low penetrance (Longtine et al., 1998) and appears to represent only a kinetic delay (McMurray and Thorner, 2009). Given the close apposition of septin structures with the PM in the cell (Byers and Goetsch, 1976; Bertin et al., 2012) and the effect of lipids on the state of septin assembly *in vitro* (Bertin et al., 2010; Bridges et al., 2014), the phenotype exhibited by *gin4*Δ mutants might be explained by the lack of proper control of Fpk1 function and the ensuing effects on local PM lipid composition. A consequence of Gin4-mediated inhibition of flippase function (via inhibition of the flippase-activating protein kinase Fpk1) is a pronounced reduction in the inner leaflet concentration of PtdEth in the PM (Roelants et al., 2015). A low inner leaflet PtdEth level leads to activation of Cdc42 by suppressing the function of Cdc42-specific GAPs (Saito et al., 2007; Das et al., 2012), and activated Cdc42 plays a significant role in localized tethering of the factors needed for initial recruitment of septins to the site of incipient bud emergence (Iwase et al., 2006; Okada et al., 2013).

OUTLOOK AND PROSPECTUS

Depending on their assembly state, septin-based structures provide dynamic platforms from which the action of a significant number of protein kinases can be directed both spatially and temporally. Moreover, as observed for other protein kinase-scaffold interactions (Ferrell, 2000; Good et al., 2011; Langeberg and Scott, 2015), signaling emanating from septin-associated kinases can be channeled to particular co-localized targets conferring specificity and can be insulated from improper substrates to ensure fidelity. Moreover, where necessary, co-recruitment of multiple protein kinases permits signal propagation in the appropriate sequence and enables cross-talk to elicit coincident and combinatorial outputs.

Moreover, these septin structures serve as sensors that transmit upstream cues, such as cell cycle timing and membrane curvature, to their associated kinases. Certain of these kinases also regulate septin structure and organization, establishing an extremely complex feedback system which is yet to be fully understood. As highlighted through the course of the above discussion, there are still many mechanistic aspects of the control of septin-associated protein kinases that remain to be delineated. Hence, this area of cell biology and biochemistry remains a fertile area for exploring the role of cellular structures in regulating signaling enzymes, and vice versa.

AUTHOR CONTRIBUTIONS

AP, GF, and FR prepared the draft and JT edited and submitted the final version of the manuscript.

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Analysis of Septin Reorganization at Cytokinesis Using Polarized Fluorescence Microscopy

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Septins are conserved filament-forming proteins that act in diverse cellular processes. They closely associate with membranes and, in some systems, components of the cytoskeleton. It is not well understood how filaments assemble into higher-order structures *in vivo* or how they are remodeled throughout the cell cycle. In the budding yeast *S. cerevisiae*, septins are found through most of the cell cycle in an hourglass organization at the mother-bud neck until cytokinesis when the collar splits into two rings that disassemble prior to the next cell cycle. Experiments using polarized fluorescence microscopy have suggested that septins are arranged in ordered, paired filaments in the hourglass and undergo a coordinated 90° reorientation during splitting at cytokinesis. This apparent reorganization could be due to two orthogonal populations of filaments disassembling and reassembling or being preferentially retained at cytokinesis. In support of this idea, we report a decrease in septin concentration at the mother-bud neck during cytokinesis consistent with other reports and the timing of the decrease depends on known septin regulators including the Gin4 kinase. We took a candidate-based approach to examine what factors control reorientation during splitting and used polarized fluorescence microscopy to screen mutant yeast strains deficient in septin interacting proteins. Using this method, we have linked known septin regulators to different aspects of the assembly, stability, and reorganization of septin assemblies. The data support that ring splitting requires Gin4 activity and an anillin-like protein Bud4, and normal accumulation of septins at the ring requires phosphorylation of Shs1. We found distinct regulatory requirements for septin organization in the hourglass compared to split rings. We propose that septin subpopulations can vary in their localization and assembly/disassembly behavior in a cell-cycle dependent manner at cytokinesis.

Keywords: septins, cytokinesis, polarized light microscopy, Shs1

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INTRODUCTION

Septins are a conserved family of cytoskeletal GTP-binding proteins that function in many cellular processes including cytokinesis, cell polarity, and membrane remodeling in many eukaryotic cell types (Gilden and Krummel, 2010; Mostowy and Cossart, 2012; Khan et al., 2015). To contribute to these diverse processes, septins polymerize into filaments and then assemble into higher-order structures associated with the cell cortex (Frazier et al., 1998; Bertin et al., 2008; Spiliotis and Gladfelter, 2012; Bridges et al., 2014). In the budding yeast *Saccharomyces*

cerevisiae, septins assemble into a ring at the new bud site and this ring transitions into an hourglass which splits into two rings at cytokinesis (Haarer and Pringle, 1987; Ford and Pringle, 1991; Kim et al., 1991). The formation of higher-order septin structures is required for proper septin function, and for decades the underlying architecture of these higher-order structures remained uncertain (Byers and Goetsch, 1976; Rodal et al., 2005; Bertin et al., 2012). Recent platinum-replica transmission electron microscopy studies provided clear ultrastructural details of septin higher-order structures in yeast over the course of the cell cycle (Ong et al., 2014). In this approach, predominantly paired filaments parallel to the mother-bud axis were detectable in the hourglass early in the cell cycle, whereas at cytokinesis two orthogonal arrays (parallel and circumferential to axis) of paired septin filaments could be seen and finally after splitting all filaments appeared to be circumferential. The exact ultrastructure in a mature hourglass prior to cytokinesis is not clear and it is possible that the circumferential filaments assemble prior to the onset of cytokinesis as the intensity of septins continues to increase through time in G2/M (Chen et al., 2011). It is not yet clear what the function is of the process of splitting the hourglass into two rings. There is evidence that the split structure forms a corral for the cytokinetic apparatus to trap it locally however it is not clear that the corral is needed for efficient cytokinesis (Dobbelaere and Barral, 2004; Wloka et al., 2011).

The budding yeast *S. cerevisiae*, where septins were first discovered, contains 5 mitotic septins: three septins that assembly into the core of the heteromeric rod (Cdc12, Cdc10, Cdc3) while either Cdc11 or Shs1 occupies the terminal position (Hartwell, 1971; Bertin et al., 2008; Garcia et al., 2011). *In vivo* it is not yet definitive the proportion of rods that have Shs1 or Cdc11 at the terminal positions or if there are rods that are polar and have Cdc11 on one end and Shs1 on the other end. The molecular role of Shs1 is unclear, since it is clearly necessary for higher order structure organization but it is not required for cells to undergo cytokinesis except in sensitized backgrounds (Hartwell, 1971; Mino et al., 1998; Garcia et al., 2011; Ong et al., 2014; Booth et al., 2015). Previous studies have assessed the role of C-terminal extension (CTE) in Shs1 localization and function in both *S. cerevisiae* and *Ashbya gossypii* (Versele and Thorner, 2004; Meseroll et al., 2012, 2013; Finnigan et al., 2015). In *A. gossypii*, the CTE is required to build structures of a specific size and the CTE is phosphorylated on multiple sites *in vivo*. The phosphorylation is not essential for ring scaling but it does contribute to protein turnover in higher-order assemblies and phosphomimetic alleles are lethal (Meseroll et al., 2012). Similarly, the CTE of ScShs1 is highly phosphorylated and the sites are not essential for viability in a sensitized background (Egelhofer et al., 2008; Garcia et al., 2011). However, whether or not the sites contribute to reorganization or splitting of the ring at cytokinesis has not been investigated. A hypothesis we test in this paper is if regulation of Shs1 is required for septin reorganization at cytokinesis.

Insights into septin organization through the cell cycle in live cells have come from using polarized fluorescence microscopy. This microscopy technique provides organizational information in addition to localization of cellular structures through fluorescence imaging. The approach takes advantage of the existence of the dipole moment of GFP, which allows GFP to be preferentially excited by and emit linearly polarized light parallel to the dipole moment (Inoué et al., 2002). If the movement of GFP is restricted relative to its septin fusion partner, then the dipole moment of GFP will act as a readout of underlying septin organization (Vrabioiu and Mitchison, 2006, 2007). Previously, polarized fluorescence microscopy was used by our group and others to assess the organization of septin higher order structures over the course of the cell cycle and found septins to be highly anisotropic (ordered). It was shown that septins rapidly and dramatically reorganize their ensemble organization by 90° over the course of septin ring splitting (Vrabioiu and Mitchison, 2006, 2007; DeMay et al., 2011a,b).

We set out to understand the root of this striking rearrangement of the highly-ordered septin cortex during cytokinesis. The molecular basis for a large-scale rearrangement of a network of cytoskeletal filaments between two, highly anisotropic yet orthogonal states is difficult to envision. Several models for this transition have been proposed previously: (1) a concerted rotation of the entire higher-order assembly; (2) the selective departure of a majority population of filaments that then "reveals" a second, orthogonal population; and (3) a triggered disassembly and reassembly of filaments into a new ensemble that is perpendicular to the starting state (Vrabioiu and Mitchison, 2006; DeMay et al., 2011a). Work from the Barral lab showed that the collar becomes more "fluid" at cytokinesis based on recovery from photobleaching which could be consistent with either a rotation or disassembly/reassembly model (Dobbelaere and Barral, 2004). Analysis of septin filaments *in vitro* have shown them to be highly flexible, that they associate with the membrane through avidity and that longer filaments are more stably bound (Bridges et al., 2014, 2016). These properties could enable a regulated fragmentation and departure of a population of filaments, key components of models 2 and 3. Strong support for the third model came from EM ultrastructural and photoactivation studies from Bi and Svitkina, making it the most well substantiated at this point, although it is still possible that circumferential rings assemble in mature hourglasses and these could be retained at cytokinesis (Ong et al., 2014). The key question is: what promotes such a swift rearrangement of molecular order within such a small window of time and space?

Our goal in this study was to begin to identify the molecular requirements of septin reorganization at cytokinesis. We assessed the organization of septins in a panel of mutant yeast strains using polarized fluorescence microscopy. Our results suggest phosphorylation-based modifications of septins, Gin4 kinase activity and the anillin-like protein Bud4 are important for maintaining a subpopulation of septins at the bud neck before, during and after cytokinesis.

MATERIALS AND METHODS

Yeast Strains and Strain Construction

We generated *S. cerevisiae* Cdc12-conGFP (AGY169; DHD5 yeast with AGB467: pRS416-Sc*CDC12*-conGFP3) based on rationale from previous work (Vrabioiu and Mitchison, 2006,

2007; DeMay et al., 2011a,b). pRS416-Sc*CDC12*-conGFP3 was generated by removing nucleotides encoding 3 amino acids of the N-terminal alpha-helix of GFP and 4 amino acids of the C-terminal region of Cdc12. AGB455 (pSc*CDC12*) was generated by amplifying Sc*CDC12* from *S. cerevisiae* genomic DNA using the oligonucleotides AGO1181 and AGO1182. The PCR product was ligated into the pRS416 plasmid (AGB441) and verified by test digestions using PspXI and SlaI and sequencing (Dartmouth College Core Facility, Hanover, NH). Next, the plasmid AGB459 (pScCdc12-GFP) was generated by first amplifying the GFP insert from the plasmid AGB005 using the oligonucleotides AGO1187 and AGO1188 and ligating the product into the digested AGB455 plasmid. Again this plasmid was verified with test digestions and sequencing (Dartmouth College Core Facility, Hanover, NH). Constraining of GFP to Cdc12 was completed using PCR to amplify the entire AGB459 using the oligonucleotides AGO1203 and AGO1204 which contain homology over the *CDC12*-GFP linker and this amplification removed nucleotides encoding 4 amino acids from the N-terminus of GFP and 3 amino acids from the C-terminus of Cdc12. AGB467 (pRS416-Sc*CDC12*-conGFP) was verified by sequencing (Dartmouth College Core Facility, Hanover, NH). Once verified, AGB467 was transformed into wild-type lab strain (AGY000), and the following yeast deletion strains: *bnr1Δ, bni1Δ, bud4Δ, cla4Δ, gin4Δ, rts1Δ,* and *shs1Δ* (kindly provided by Erfei Bi, AGY028-AGY035 to generate AGY066-AGY073). Doug Kellogg generously supplied us with *shs1* phosphomutant strains (Egelhofer et al., 2008) and the plasmid AGB467 was transformed into these strains as well to generate AGY317-AGY320. To generate strains to measure septin intensity over the cell cycle the plasmid AGB553 (E1915 YIplac128-GFP-Sc*CDC3:LEU* a gift from Erfei Bi) was integrated into the same strains as the AGB467 plasmid generating the strains AGY131-AGY137, and AGY323-AGY327. All strain, plasmid, and oligonucleotide information is present in **Tables 1–3**.

Yeast Culture and Preparation

For imaging, *S. cerevisiae* cells were grown overnight in appropriate media with proper selection for the specific plasmids, collected by centrifugation, and resuspended in 2x low fluorescence minimal media (LFMM). Cells were mounted on gel pads of 1.4% agarose and LFMM on glass depression slide, covered with a coverslip (no. 1.5), sealed with Valap, and imaged.

Time-Lapse Imaging and Intensity Analysis

Time-lapse recordings for estimating septin intensity of mutant strains through the cell cycle were acquired with a Nikon Eclipse Ti-E inverted wide-field microscope equipped with a 60x (1.4 N.A.) plan-apochromat oil objective and a Andor Zyla 4.2 sCMOS camera. A Chroma DAPI/FITC/TRITC/Cy5 quad filter set was used for fluorescent imaging of GFP. The fluorescent light source was a Spectra LED lamphead and images were acquired with 12% laser power, 100 ms exposure, and 90 s time intervals.

Using FIJI, individual cells were cropped from the timelapse recordings and the mean background-subtracted septin intensity at the mother-bud neck were determined for every time point for a given cell using MATLAB. These septin intensity values were saved and plotted in an Excel file. For every cell, a time zero (***t0***) was manually determined. This ***t0*** represented the maximum septin intensity value close (not more than 4–5 time points) to the visible ring split. In most cases the quantitatively determined ***t0*** was not more than two time points (3 min) away from the visible split. This quantitative approach was taken to account for different visible split times depending on how a given image was automatically contrasted by FIJI. ***t0*** therefore represented the quantitative onset of ring splitting. Since the primary focus of this study was the septin concentration change during ring splitting, all following analyses were limited to 24 min before and after ***t0***. Percent change over the entire 24 min was determined and reported in **Table 4**. Since the majority of the strains analyzed showed biphasic septin disassembly rates over the course of ring splitting, the disassembly rate was determined for both phases of disassembly (phase 1 and phase 2). Phase 1 and phase 2 were distinguished by the different slopes of the biphasic intensity curves. If a strain did not show biphasic disassembly, then it is reported as monophasic and the intensity from $t = 0$ to $t = 24$ min after splitting; Phase 2 is the rate of disassembly from the last time point of Phase 1 to $t = 24$. After disassembly rates were determined, the fold change compared to wild-type was calculated.

Polarization Microscopy

All polarization fluorescence images (except the *shs1*-phosphomutant strains) were acquired using the MF-PolScope (Abrahamsson et al., 2015). The MF-PolScope was set up on an inverted Olympus IX-83 microscope body, equipped with an Olympus 60x (1.3 N.A.) silicon-oil immersion objective, and the LC-compensator for polarization-controlled excitation light, with a green 515/30 nm emission filter (Semrock) placed before an EMCCD camera (Andor iXon-888). The fluorescence light source used was a blue LED module of an X-cite XLED1 (Lumen Dynamics). Images were acquired through Micro-manager (www.micro-manager.org) using the OpenPolScope software (www.openpolscope.org).

Polarized fluorescence measurements of the *shs1*-phosphomutant strains and *bud4Δ* splitting events were acquired with the Zeiss Axio Imager.M1 wide-field microscope with five linear polarized filters (Chroma 21033a) in a filter wheel (Ludl Electronic Products, cat. no. 99A075) and UV blocker 420 nm LP emission filter 32 nm (Chroma, cat. no. E420LPv2-32). The fluorescent light source used was an EXFO X-Cite 120 lamp. Images were acquired through Micro-manager (www.micro-manager.org) using the OpenPolScope software (www.openpolscope.org).

Once acquired, all polarization images were analyzed using the OpenPolScope software. Analysis involved internal background corrections of every cell, performed by selecting a ROI in the cytoplasm, and a ROI in the septin hourglass structure and then processed for anisotropy. The anisotropy formula calculates $\frac{I_{max}-I_{min}}{I_{max}+I_{min}}$ in each pixel, based on the bleach corrected fluorescence intensity values. Circular statistics were used to calculate the ensemble orientation and anisotropy for each

TABLE 1 | Yeast strains used in this study.

Strain	Relevant Genotype	Reference
AGY000	DHD5 (MATa/MATα, ura3-52/ura3-52, leu2-3,112/leu2-3, 112, his3-11, 25/his3-11,15)	
AGY028	YEF3572 Mat a *bud4Δ*:his	Erfei Bi
AGY029	YEF3922 *rts1Δ*:kan	Erfei Bi
AGY030	YEF1342 Mata *cla4Δ*:his	Erfei Bi
AGY031	YEF1238 Mat a *gin4Δ*:trp	Erfei Bi
AGY032	YEF5687 Mat a *shs1Δ*:trp	Erfei Bi
AGY034	YEF6005 Mat α *bni1Δ*:his	Erfei Bi
AGY035	YEF1732 Mat α *bnr1Δ*:his	Erfei Bi
AGY066	pRS416-Sc*CDC12*-conGFP/YEF3572 Mat a *bud4Δ*:his	This study
AGY067	pRS416-Sc*CDC12*-conGFP/YEF3922 *rts1Δ*:kan	This study
AGY068	pRS416-Sc*CDC12*-conGFP/YEF1342 Mata *cla4Δ*:his	This study
AGY069	pRS416-Sc*CDC12*-conGFP/YEF1238 Mat a *gin4Δ*:trp	This study
AGY070	pRS416-Sc*CDC12*-conGFP/YEF5687 Mat a *shs1Δ*:trp	This study
AGY072	pRS416-Sc*CDC12*-conGFP/YEF6005 Mat α *bni1Δ*:his	This study
AGY073	pRS416-Sc*CDC12*-conGFP/YEF1732 Mat α *bnr1Δ*:his	This study
AGY075	Sc*CDC11*-GFP::His, Sc*SHS1*-mCherry::Gen	H. Ewers
AGY131	E1915 Ylplac128-GFP-Sc*CDC3*:LEU/YEF1238 Mat a *gin4Δ*:trp	This study
AGY132	E1915 Ylplac128-GFP-Sc*CDC3*:LEU/YEF5687 Mat a *shs1Δ*:trp	This study
AGY133	E1915 Ylplac128-GFP-Sc*CDC3*:LEU/YEF3572 Mat a *bud4Δ*:his	This study
AGY134	E1915 Ylplac128-GFP-Sc*CDC3*:LEU/YEF6005 Mat α *bni1Δ*:his	This study
AGY135	E1915 Ylplac128-GFP-Sc*CDC3*:LEU/YEF1732 Mat α *bnr1Δ*:his	This study
AGY136	E1915 Ylplac128-GFP-Sc*CDC3*:LEU/YEF3922 *rts1Δ*:kan	This study
AGY137	E1915 Ylplac128-GFP-Sc*CDC3*:LEU/YEF1342 Mata *cla4Δ*:his	This study
AGY169	pRS416-Sc*CDC12*-conGFP/DHD5	This study
AGY311	DK186 (Mat a, his3-11,15, leu2-3, 112, trp1-1, ura3-52, ade2-1, can1-100, GAL+, bar1)	Egelhofer et al., 2008
AGY313	DK912 (Mat a, his3-11,15, leu2-3, 112, trp1-1, ura3-52, ade2-1, can1-100, GAL+, bar1, *shs1Δ*::shs1-ps2)	Egelhofer et al., 2008
AGY314	DK966 (Mat a, his3-11,15, leu2-3, 112, trp1-1, ura3-52, ade2-1, can1-100, GAL+, bar1, *shs1Δ*::shs1-ps1)	Egelhofer et al., 2008
AGY315	DK985 (Mat a, his3-11,15, leu2-3, 112, trp1-1, ura3-52, ade2-1, can1-100, GAL+, bar1, *shs1Δ*::shs1-ps4)	Egelhofer et al., 2008
AGY317	pRS416-Sc*CDC12*-conGFP/DK186	This study
AGY318	pRS416-Sc*CDC12*-conGFP/DK912	This study
AGY319	pRS416-Sc*CDC12*-conGFP/DK966	This study
AGY320	pRS416-Sc*CDC12*-conGFP/DK985	This study
AGY323	E1915 Ylplac128-GFP-Sc*CDC3*:LEU/DK186	This study
AGY325	E1915 Ylplac128-GFP-Sc*CDC3*:LEU/DK912	This study
AGY326	E1915 Ylplac128-GFP-Sc*CDC3*:LEU/DK966	This study
AGY327	E1915 Ylplac128-GFP-Sc*CDC3*:LEU/DK985	This study
DLY5487	*SHS1*-GFP::kan-1, mat a	Danny Lew

TABLE 2 | Plasmids used in this study.

Plasmid #	Name	Vector	Relevant insert	Reference
AGB005	pAGT141	pUC19	GFP	
AGB441	pRS416	pRS416	–	
AGB455	pRS416-Sc*CDC12* locus-stop	pRS416	Sc*CDC12* locus-stop	This study
AGB459	pRS416-Sc*CDC12*-GFP	pRS416	GFP	This Study
AGB467	pRS416-Sc*CDC12*-conGFP	pRS416	conGFP4-GEN3 (4D4)	This study
AGB553	E1915 Ylplac128-GFP-Sc*CDC3*:LEU	Ylplac128	GFP-Sc*CDC3*	Erfei Bi

given septin structure. To do this, a 6 × 6 pixel ROI was selected in the center of the septin structure so as to exclude edges of the septin structure with isotropic measures from GFP β-barrel orientation along the curve of the septin ring. The pixel-by-pixel orientation, anisotropy, and intensity for each septin structure was then exported, and used to calculate the

TABLE 3 | Oligonucleotides used in this study.

Plasmid #	Name	Sequence 5′-3′
AGO1181	ScCdc12 locus PspXI F	GGTGCCTCGAGGGGCTTCAAAACTGCTAGGTCGGATTC
AGO1182	ScCdc12 locus SalI R	GGAGGTCGACTTTTAAATGGGATTTTTTTACTTGCAAGCTTTTGACCTGCTCTTC
AGO1187	Sc GFP tagging SalI F	GGTGGTCGACGGCGCGGGCGCAGGTGCCGGTGCAAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCC
AGO1188	Sc GFP tagging EcoRI R	GGCGGAATTCCTATGCGTCCATCTTTACAGTCC
AGO1203	MVB128 Sc*Cdc12*-conGFP F	GCTTGCAAGTAAAAAAATCCGAACTTTTCACTGGAGTTG
AGO1204	MVB128 Sc*Cdc12*-conGFP R	CAACTCCAGTGAAAAGTTCGGATTTTTTTACTTGCAAGC

TABLE 4 | Average percent disassembly, rate change, rate fold change for each phase of disassembly observed.

Strain	% disassembly (phase1)	Rate (% disassembly/ min) phase 1	Rate (% disassembly/ min) phase 2	Monophasic (disassembly/min)	Rate fold change phase 1	Rate fold change phase 2
CDC3-GFP	56.6	6.3	3.4	–	1.0	1.0
CDC11-GFP	58.9	6.5	3.4	–	1.0	1.0
SHS1-GFP	51.0	5.7	2.5	–	0.9	0.7
SHS1-GFP (DLY)	52.4	7.0	2.3	–	1.1	0.7
bni1Δ	62.5	10.4	1.7	–	1.7	0.5
bnr1Δ	74.7	10.0	3.3	–	1.6	1.0
*bud4Δ**	98.3	–	–	12.0	1.9	3.5
cla4Δ	41.2	6.9	4.7	–	1.1	1.4
gin4Δ	51.3	–	–	2.1	0.3	0.6
rts1Δ	49.2	8.2	4.1	–	1.3	1.2
shs1Δ	56.7	–	–	2.4	0.4	0.7
shs1ΔC	99.5	10.3	5.9	–	1.6	1.8
CDC3-GFP (W303)	99.1	–	–	4.1	1.0	–
shs1Δ-ps1	92.8	–	–	3.9	0.9	–
shs1Δ-ps2	97.7	–	–	4.1	1.0	–
shs1Δ-ps4	100.0	–	–	4.0	1.0	–

Rate fold change is normalized to the relevant wild-type background, Phase 1 was identified visually by the first rate of disassembly in the slopes of the biphasic intensity curves. If a strain did not show biphasic disassembly, then it is reported as monophasic and the intensity from t = 0 to t = 24 min after splitting; Phase 2 is the rate of disassembly from the last time point of Phase 1 to t = 24.

**bud4Δ decreased in intensity faster than wild-type yeast; therefore, disassembly rate was determined only for the first 7.5 min.*

ensemble orientation weighted by anisotropy and intensity of each ring, and of the entire population of cells. In addition we also calculated the population variance ($PopVar = 1 - (\frac{Vector\ Average\ Anisotropy * Intensity}{Scalar\ Anisotropy * Intensity})$). A detailed description of how to use the OpenPolScope software for polarization imaging and analysis is present in *Current Protocols in Cell Biology* (McQuilken et al., 2015).

RESULTS

Septin Concentration at the Bud Neck Decreases at Ring Splitting for Multiple Septin Subunits

Previous data from our lab and others have shown that there is a 90° reorientation of the organization within the septin hourglass structure over the course of septin ring splitting at cytokinesis in *S. cerevisiae* cells (Vrabioiu and Mitchison, 2006; DeMay et al., 2011a,b). We had hypothesized in our work that one explanation of the apparent reorganization is that there are two populations of septins in the hourglass and this could be seen by Ong et al. (2014) at least at the onset of cytokinesis. Upon ring splitting, one of these sub-populations is thought to leave the bud neck and another population assembles and/or is retained perpendicular to the original population. What drives the disassembly and reassembly process of septins within such a small region of the cortex in a short window of the cell cycle?

We hypothesized that whether a given septin filament is released or stays localized at the neck could be explained by the composition of septin heteromeric rods, specifically the terminal subunit, which is occupied by either Cdc11 and/or Shs1. If Cdc11 or Shs1 specified which complexes were retained or exited from the neck, we predicted that Cdc11 and Shs1 would display different rates of change in concentration at splitting. To assess this, we monitored the abundance of 3 different septins at the mother-bud neck over the course of septin ring splitting. We used widefield fluorescence microscopy to image GFP-Cdc3, Shs1-GFP (in two different strain backgrounds), and Cdc11-GFP every 90 s to ensure sufficient time resolution for assessing intensity differences and kinetics (see *Materials and Methods*). Using two

different Shs1-GFP strains we observed the accumulation of Shs1 in the septin hourglass is only ∼50% of the level of either Cdc3 or Cdc11 (**Figure 1A**). Interestingly, we also observed that the decrease of all three septins over the course of ring splitting occurred in a biphasic manner. The first phase occurred within the first ∼10 min after ring splitting. This first phase of septins leaving the bud neck is consistent with the timing of the isotropic "transition" period observed previously (DeMay et al., 2011a). Time-lapse imaging indicated that all septin proteins decrease in abundance with comparable rates during ring splitting and decrease by ∼50% in concentration over the first phase of disassembly (phase 1), regardless of their starting abundance (**Figure 1A**, **Table 4**, and consistent with what was seen for Cdc3 in Dobbelaere et al., 2003; Wloka et al., 2011). When we measure the difference in disassembly rates over the first phase of disassembly between the different septins there was little difference in the rates (**Table 4**). Additionally, when Cdc11 and Shs1 are tagged in the same cell with different fluorophores, the decrease in abundance at the neck is nearly simultaneous for each protein, further indicating that these two subunits behave similarly with regard to septin dynamics in this part of the cell cycle (discrepancy between times of -0.3 ± 0.3 min, $N = 40$ cells, difference not significant from 0 with $p > 0.41$). These data indicate that the difference between the septin populations

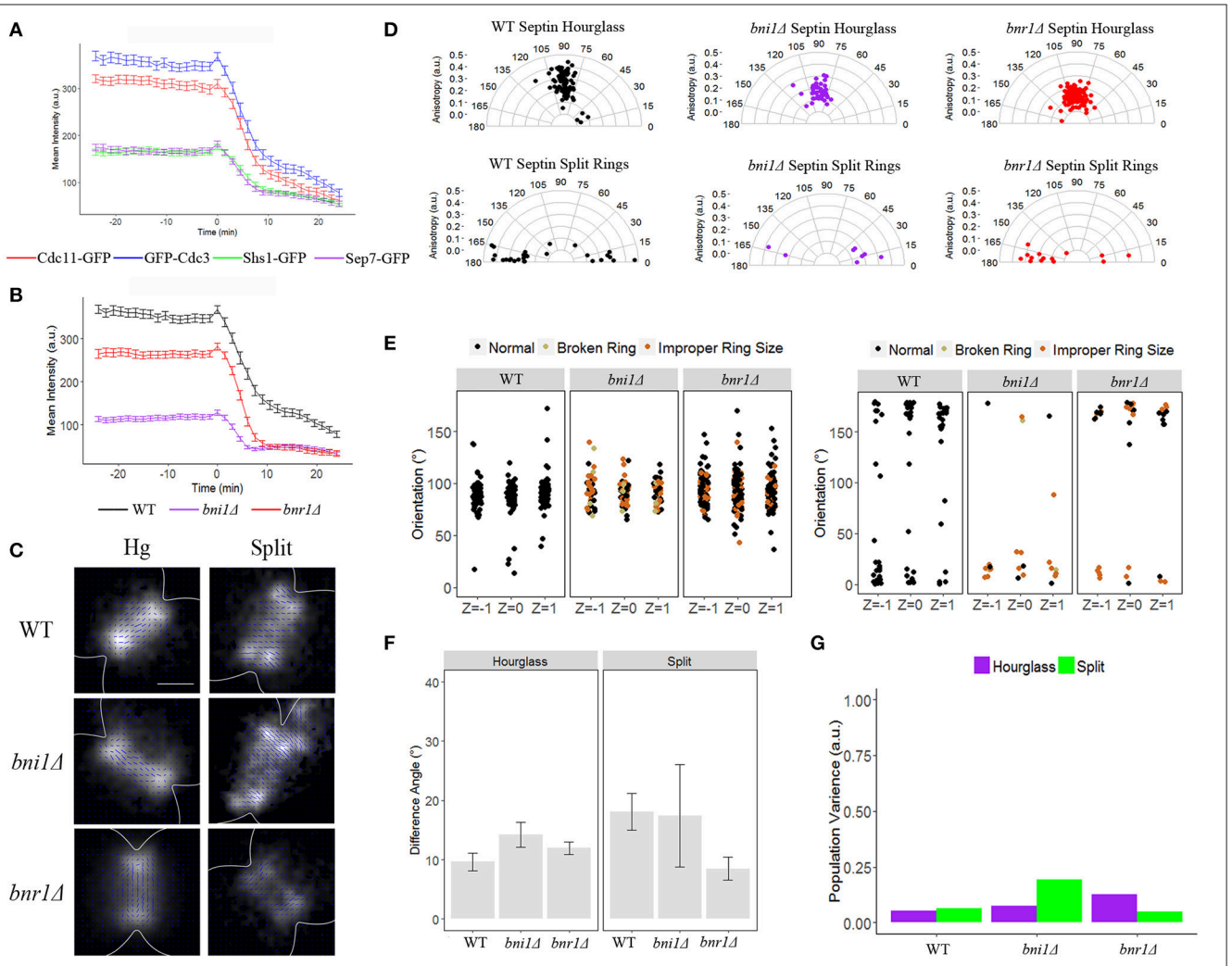

FIGURE 1 | The formins, Bni1 and Bnr1, are not required for the reorganization of the septin ring. (A) Septin intensity of Cdc11-GFP (red), GFP-Cdc3 (blue), Shs1-GFP (green), and Sep7-GFP (purple) over the course of septin ring splitting (time = 0). Error bars represent standard error for each time point ($N = 100$ cells). **(B)** Septin intensity of GFP-Cdc3 in wild-type (black), *bni1Δ* (purple), and *bnr1Δ* (red) over the course of septin ring splitting (time = 0). Error bars represent standard error for each time point ($N = 100$ cells). **(C)** Representative polarization images of wild-type, *bni1Δ* and *bnr1Δ* cells expressing ScCdc12-conGFP in the hourglass and split structures. The blue lines represent the calculated dipole orientation and their length is scaled according to anisotropy**.** Scale bar 0.5 μm. **(D)** Polar plots of net dipole orientations for individual hourglass and split septin rings scaled by anisotropy (hourglass $N > 45$ cells, split rings $N > 8$ cells). **(E)** Scatter plot of net dipole orientations for individual hourglass and split septin rings in three different focal planes. Color scale indicates visible phenotypes of septin rings [broken ring (tan), improper ring size (orange), (hourglass $N > 30$ cells, split rings $N > 8$ cells)]. **(F)** Difference in net orientation measures between top and bottom focal planes for hourglass and split structures. Error bars denote standard error. **(G)** Population variance in hourglass and split ring structures.

that stay associated with the cortex and that are released into the cytoplasm is not simply the presence or absence of Cdc11 or Shs1.

Septin Reorganization Does Not Require the Formins

We next used time-lapse imaging and polarization microscopy to assess the contribution of septin interacting proteins on the organization and behavior of septins in the splitting transition. We first tested the hypothesis that a subset of septins could be specified to disassemble or be anchored via connections with F-actin assemblies. The yeast formin Bnr1 is regulated by Shs1 indicating these two factors are functionally interacting at this window of the cell cycle (Buttery et al., 2012). We assessed how the two formins, Bni1 and Bnr1, influence septin intensity changes and reorganization over septin ring splitting (**Figure 1B**). In strains lacking formins we observed a decrease in the initial intensity of septins in the hourglass, suggesting the actin cytoskeleton contributes partially to the accumulation of septins at the mother bud neck prior to ring splitting. This defect was most severe in *bni1Δ*, where septin intensity at the hourglass was reduced by ∼65% relative to the hourglass in wild-type cells. However, the rate at which the septin sub-population exited the bud-neck during ring splitting was again observed as a biphasic disassembly, was not substantially affected in any of the strains (0.7-fold faster for phase 1 and 0.5-fold slower for phase 2 in *bni1Δ* and 0.6-fold faster in phase 1 and no fold change difference for phase 2 *bnr1Δ*, **Table 4**) consistent with previous reports (Feng et al., 2015).

We next asked if the formins were required for the characteristic 90° reorganization. To address this, we used polarization microscopy to measure the organization of the septin hourglass and split rings in wild-type, *bni1Δ*, and *bnr1Δ* strains containing Cdc12-conGFP (see *Materials and Methods*, **Figures 1C–E**). Specifically, we used multifocus (MF-PolScope) microscopy to simultaneously capture different Z-positions along with polarized fluorescence information so as to determine if orientation was comparable at all positions in the ring at the same time (Abrahamsson et al., 2015). When the orientation is consistent between Z-slices of the top, middle, and bottom of septin assemblies, we have interpreted this to mean that the prevailing population of septins are "paired filaments" (DeMay et al., 2011a,b).

As previously published from our lab, the septin hourglass of wild-type cells expressing Cdc12-conGFP exhibit GFP dipoles with a net orientation perpendicular to the mother-bud axis, while septin rings exhibit a net orientation parallel to the mother-bud axis (DeMay et al., 2011a,b). The net orientation of GFP dipoles in *bni1Δ* and *bnr1Δ* are comparable to wild-type on average however the anisotropy is lower for both mutants suggesting a slightly less well-ordered structure (**Figure 1D**, **Table 5**). We categorized each orientation data point by the state of the ring (broken, aberrant size) but could not see any correlation between grossly misorganized rings and orientations substantially deviating from 90° (**Figure 1D**). There seems to be little difference between the net orientation in the top, middle, and bottom focal planes in the two formin mutants suggesting pairing or symmetric organization relative to the plasma membrane (**Figures 1E,F**). In addition, we also estimated the population variance, which is a measure of the difference in average dipole orientation between cells with the same type of structure, and found little difference in each strain over the entire population compared to wild-type cells (**Figure 1G**). These results suggest that although Bni1 and Bnr1 are required for the proper accumulation and organization of septins in the septin hourglass, they are not required for proper timing of disassembly and reorientation of septins at ring splitting (**Figures 1B–E**).

TABLE 5 | Average anisotropy for all strains.

Strain	HG Anisotropy	S Anisotropy
CDC12-conGFP	0.25	0.22
bni1Δ	0.16	0.18
bnr1Δ	0.12	0.18
bud4Δ	0.15	0.02
cla4Δ	0.13	0.17
gin4Δ	0.05	0.17
rts1Δ	0.11	0.25
shs1Δ	0.03	0.05
shs1ΔC	0.02	0.06
CDC3-GFP (W303)	0.12	0.15
shs1Δ-ps1	0.01	0.09
shs1Δ-ps2	0.06	0.01
shs1Δ-ps4	0.05	0.05

HG = Hourglass and S = Split.

Septins in Cells Lacking *bud4* Have Highly Misorganized Rings after Splitting

We next examined other septin interacting proteins beginning with Bud4, an anillin-like protein. Bud4 has been shown to associate with septins and mutants do not have two split septin rings making them a key candidate in regulation of septin orientation in this process (Wloka et al., 2011). We looked at septin intensity through time over ring splitting in the *bud4Δ* mutant. We observed septins leave the bud neck in a monophasic manner (2-fold faster than wild-type in phase 1, and 3.5-fold faster than wild-type in phase 2). This rapid disassembly from the bud neck is consistent with previous data that Bud4 is required for stability of split septin rings during and after cytokinesis (**Figure 2A**, **Table 4**, Wloka et al., 2011; Eluère et al., 2012; Kang et al., 2013). The ring disassembled asymmetrically with one side much faster than the other but the analysis here was averaged across the whole ring to be consistent with other measurements in the study. Similar to *bnr1Δ* mutants, *bud4Δ* mutants showed a reduced septin accumulation within the hourglass with ∼70% the intensity observed in wild-type hourglasses.

Using polarization microscopy, we observed little difference in terms of septin hourglass orientation, a slight decrease

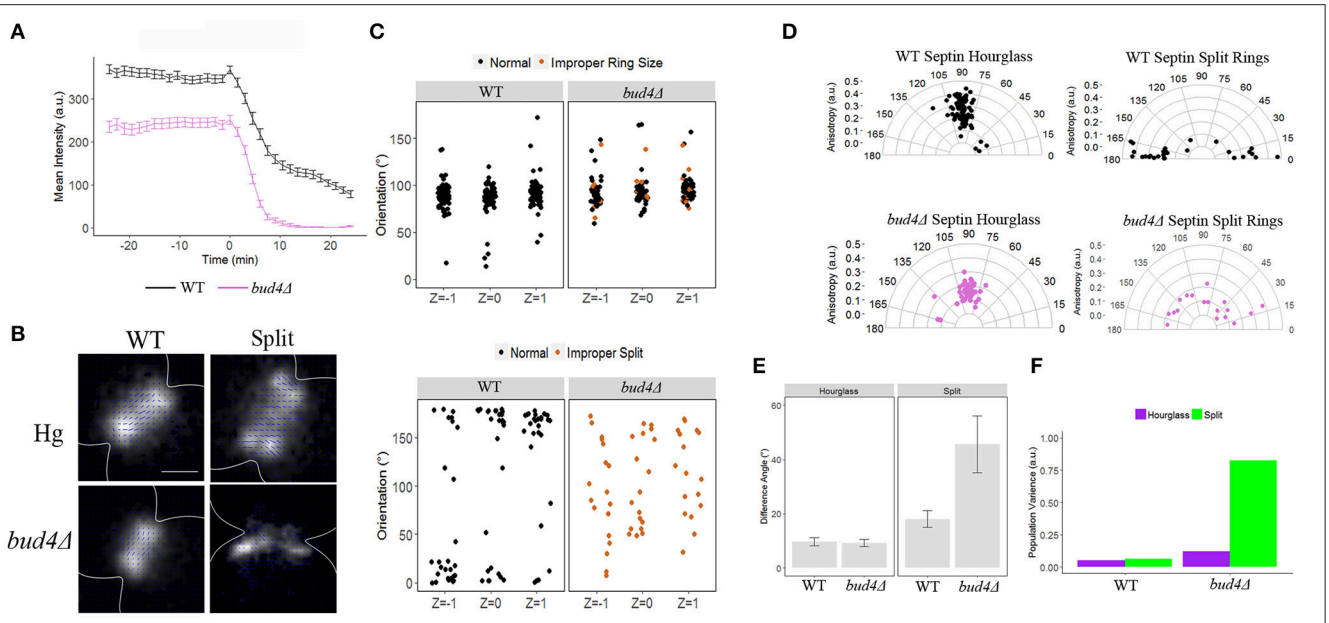

FIGURE 2 | Analysis of cells lacking Bud4. (A) Septin intensity of GFP-Cdc3 in wild-type (black) and *bud4Δ* (pink) over the course of septin ring splitting (time = 0). Error bars represent standard error for each time point ($N = 100$ cells). **(B)** Representative polarization images of wild-type containing pSc*CDC12*-conGFP, and *bud4Δ* containing pSc*CDC12*-conGFP in the hourglass and split structure. The blue lines represent the calculated dipole orientation and their length is scaled according to anisotropy. Scale bar 0.5 μm. **(C)** Scatter plot of net dipole orientations for individual hourglass and split septin rings in three different focal planes. Color scale indicates visible phenotypes of septin rings [improper ring size (orange), Improper split (orange, lower panel), (hourglass $N > 50$ cells, $N = 18$ cells, respectively)]. **(D)** Polar plots of net dipole orientations for individual hourglass and split septin rings scaled by anisotropy (hourglass $N > 50$ cells, $N = 18$ cells, respectively). **(E)** Difference in net orientation measures between top and bottom focal planes for hourglass and split structures. Error bars denote standard error. **(F)** Population variance in hourglass and split ring structures.

in anisotropy, and comparable population variance across all focal planes compared to wild-type (**Figures 2B–F**, **Table 5**). Any variability in GFP dipole orientation observed in the *bud4Δ* mutant could not be explained by visible septin defects (**Figure 2C**). However, when we analyzed the orientation of split rings in the *bud4Δ* we observed a severe decrease in anisotropy, across all focal planes suggesting that Bud4 is also required for proper organization of septin split ring structures (**Figures 2C–F**, **Table 5**). This is again consistent with previous data that Bud4 is required for the stability of the septin split rings (Wloka et al., 2011; Eluère et al., 2012; Kang et al., 2013).

Gin4, but Not Cla4 or Rts1, Contributes to Rate of Disassembly at Splitting

The isotropic transition between the two orthogonal organization states is quite transient, lasting just a few minutes, indicating that the cue to direct disassembly and/or reassembly could be a post-translational modification. Recipients of the modification could be a subpopulation of septins, a septin regulator and/or modifiers of the local membrane composition. We examined two kinases, Cla4 and Gin4, and one phosphatase, Rts1, that have been shown to contribute to septin ring behavior (Longtine et al., 2000; Mortensen et al., 2002; Dobbelaere et al., 2003; Gladfelter et al., 2004; Versele and Thorner, 2004; Roelants et al., 2015). Both *gin4Δ* and *cla4Δ* cells showed a decrease in septin accumulation in the hourglass, both exhibiting ∼50% reduction in intensity relative to the wild-type hourglass (**Figure 3A**, green and gold lines). In addition, we observed that septins do not leave the bud neck in a biphasic manner in the *gin4Δ* mutant (**Figure 3A**). Instead, septins leave the bud neck at a constant rate that is slower in both phases compared to wild-type cells (∼75% the rate of wild-type in phase 1, and ∼30% slower than the rate wild-type in phase 2, **Table 4**). In contrast, in *cla4Δ* cells septins still left the bud neck in a biphasic manner similar to the rate seen in wild-type cells (0.1-fold faster than wild-type in phase 1, and 0.4-fold faster than wild-type in phase 2, **Table 4**). Notably, Rts1 had little effect on septin accumulation in the hourglass, and no effect on disassembly rate relative to wild-type cells (**Figure 3A**, **Table 4**). Thus, Gin4, and to a lesser extent Cla4, are required for the sharp decrease in septin abundance at the neck that accompanies ring splitting.

We next used polarization microscopy to measure the organization of the septin hourglass and split rings in *cla4Δ*, *gin4Δ*, and *rts1Δ* mutant strains (**Figures 3B–D**). In *cla4Δ* and *rts1Δ* cells the spread of orientations for the GFP-dipole in the hourglass increased (thus the population variance increased), but the net orientation remained ∼90° with some decrease in anisotropy (**Figures 3C,D,F**, **Table 5**). In both *cla4Δ* and *rts1Δ* strains the split structure was not appreciably different from wildtype (**Figures 3B–D**). Unlike *cla4Δ* and *rts1Δ*, organization within the septin hourglass in the *gin4Δ* mutant was highly disorganized, while the split structure maintained

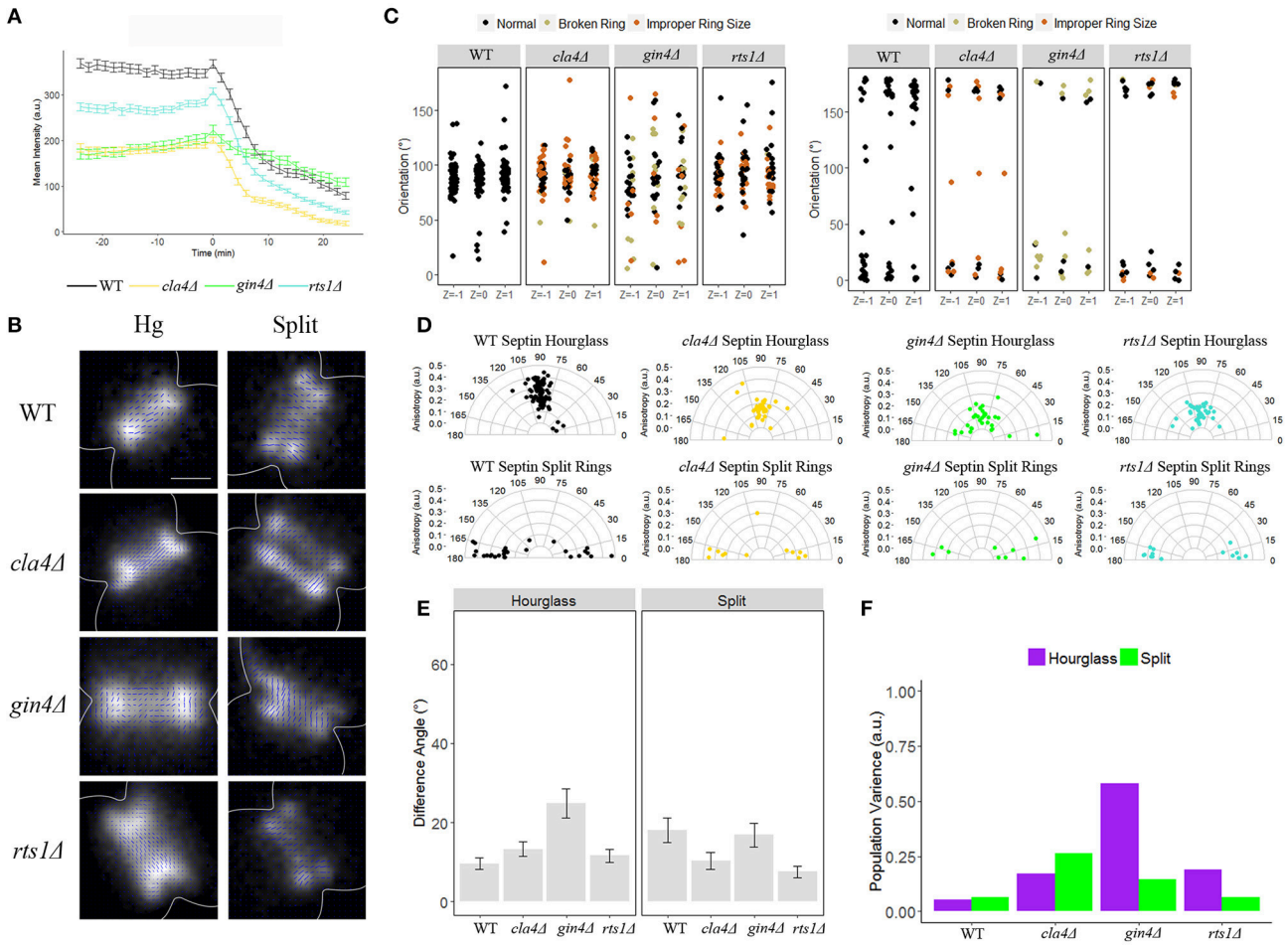

FIGURE 3 | The kinase Gin4 is required for organization of the septin hourglass, but not the septin split rings. (A) Septin intensity of GFP-Cdc3 in wild-type (black), *cla4Δ* (yellow), *gin4Δ* (green), and *rts1Δ* (turquoise) over the course of septin ring splitting (time = 0). Error bars represent standard error for each time point (N = 100 cells). **(B)** Representative polarization images of wild-type containing pSc*CDC12*-conGFP, *cla4Δ* containing pSc*CDC12*-conGFP, *gin4Δ* containing pSc*CDC12*-conGFP, and *rts1Δ* containing Sc*CDC12*-conGFP in the hourglass and split structure. The blue lines represent the calculated dipole orientation and their length is scaled according to anisotropy. Scale bar 0.5 μm. **(C)** Scatter plot of net dipole orientations for individual hourglass and split septin rings in three different focal planes. Color scale indicates visible phenotypes of septin rings [broken ring (tan), improper ring size (orange), (hourglass $N > 30$ cells, split rings $N > 9$ cells)]. **(D)** Polar plots of net dipole orientations for individual hourglass and split septin rings scaled by anisotropy (hourglass $N > 30$ cells, split rings $N > 9$ cells). **(E)** Difference in net orientation measures between top and bottom focal planes for hourglass and split structures. Error bars denote standard error. **(F)** Population variance in hourglass and split ring structures.

its ordered, net orientation of ∼0/180° (**Figures 3B–D**). This data indicates that Gin4 is required for ordered septin hourglass organization and efficient disassembly but not the split ring organization. As with *bni1Δ*, *bnr1Δ*, and *bud4Δ*, little difference between the net orientation in the top, middle, and bottom focal planes was observed, and the increase in variability in GFP-dipole orientations within the population could not be correlated with visible septin higher-order structure defects because morphologically aberrant and normal appearing rings had a similar spread of orientations (**Figures 3C,E**). These data are consistent with a well-appreciated role for Gin4 in establishing the organization of septins but also indicates it is involved in executing the switch-like disassembly process. However, the septins that are recruited/retained after ring splitting do not require Gin4 for normal orientation or order.

Shs1 Contributes to Organization of Hourglass, Split Rings and Timing of Splitting

It is possible that phosphorylation on a subset of septin rod complexes could generate the two distinct pools of septins at cytokinesis that are either in a state of disassembly or stay assembled at the membrane. As Shs1 has the most evidence of phosphorylation, we examined septin organization in cells lacking Shs1 (Mortensen et al., 2002; Egelhofer et al., 2008).

We first looked at septin intensity over ring splitting in a *shs1Δ* mutant strain. We observed a defect in the accumulation of GFP-Cdc3 to the septin hourglass (∼70% reduction compared to wild-type intensity), and similar to what we observed in *gin4Δ* mutant cells, a monophasic disassembly rate of septins from the bud neck slower than either observed phase in wild-type cells (0.6-fold slower than wild-type phase 1, and 0.3-fold slower than wild-type phase 2, **Table 4**, **Figure 4A**) When observed by polarization microscopy, there was a dramatic increase in the disorganization of both the hourglass and split ring structures in the *shs1Δ* mutant strain, consistent with what has been seen for *shs1Δ* mutants by cryo-EM tomography and platinum replica EM (**Figures 4B–D**, Bertin et al., 2012; Ong et al., 2014). Septin disorganization was reflected in a wide spread of GFP-dipole orientations and thus an increased variance within the population, and a decrease in anisotropy (**Figures 4D,F**, **Table 5**). As with the previous mutant strains we analyzed, the difference in spread of angles was not associated with visible defects in septin structures, and no obvious difference was observed in GFP-dipole net orientation between the top, middle, and bottom focal planes (**Figures 4C,E**).

It is clear that complete loss of Shs1 leads to gross abnormalities in the construction and organization of septin structures throughout the cell cycle making it difficult to disentangle assembly defects from a role of Shs1 in the splitting and reorganization process. We hypothesized the highly phosphorylated C-terminus of Shs1 could be contributing to septin organization. We therefore, examined cells carrying *shs1ΔC*, where the C-terminus of Shs1 is eliminated. *shs1ΔC* cells showed a similar decrease in septin accumulation in the hourglass as the *shs1Δ* null strains, but interestingly still showed biphasic disassembly of septins over the course of splitting (**Figure 4A**, **Table 4**). When septin organization was assessed using polarization microscopy, both hourglasses and split rings in *shs1ΔC* were disorganized, similar to *shs1Δ* (**Figure 4**, **Table 5**). Taken together these data suggest the C-terminus of Shs1 is not important for the biphasic exit of septins from the mother-but neck but is necessary for proper organization of septin higher order structures throughout the cell cycle. What aspect of the Shs1 C-terminus makes it important for septin organization but not septin disassembly?

We next analyzed the organization of septin higher-order structures in mutants of Shs1 that have altered phosphorylation sites (Egelhofer et al., 2008). Several *shs1* phosphorylation defective mutants were evaluated that had been previously generated based on results from SILAC mass spectrometry experiments in the Kellogg lab. These were ps1 (Pho85 sites), ps2

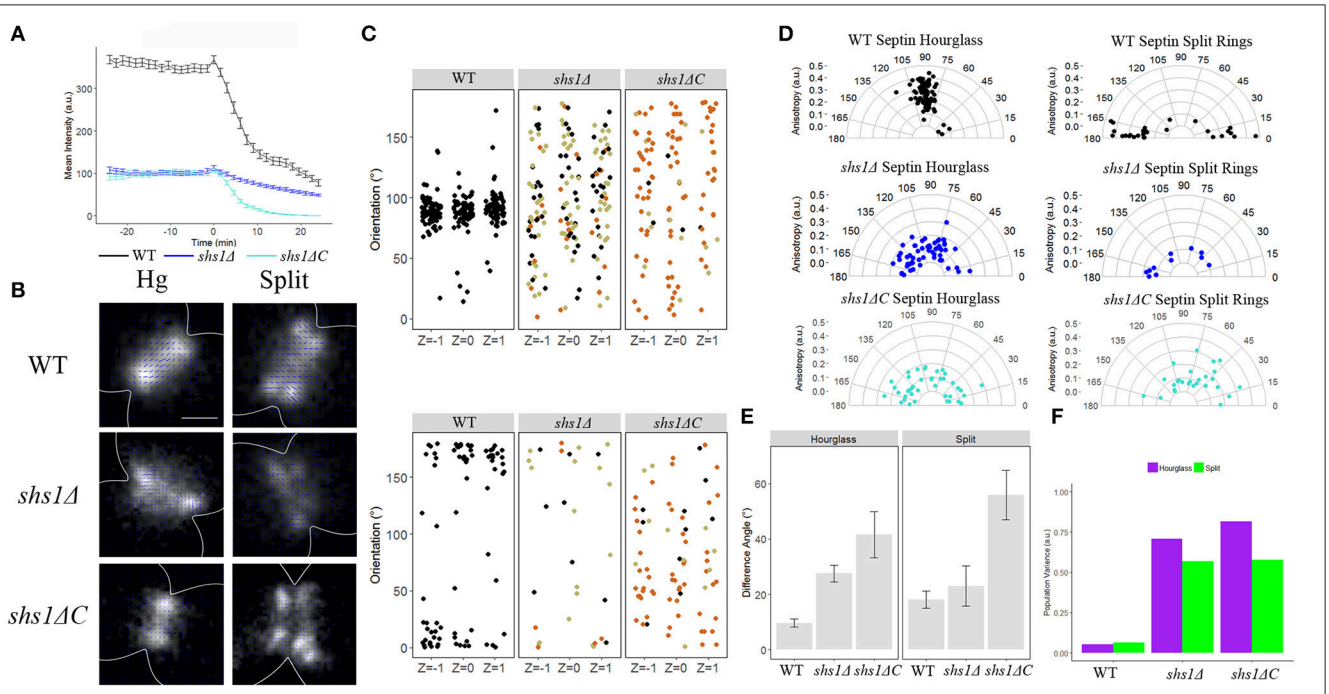

FIGURE 4 | The septin Shs1 is required for the proper organization of all septin higher order structures. (A) Septin intensity of GFP-Cdc3 in wild-type (black) and *shs1Δ* (blue) over the course of septin ring splitting (time = 0). Error bars represent standard error for each time point ($N = 100$ cells). **(B)** Representative polarization images of wild-type containing pSc*CDC12*-conGFP, *shs1Δ* containing pSc*CDC12*-conGFP, and *shs1ΔC* containing pSc*CDC12*-conGFP in the hourglass and split structure. The blue lines represent the calculated dipole orientation and their length is scaled according to anisotropy. Scale bar 0.5 μm. **(C)** Scatter plot of net dipole orientations for individual hourglass and split septin rings in three different focal planes. Color scale indicates visible phenotypes of septin rings [broken ring (tan), improper ring size (orange), (hourglass $N > 55$ cells, split rings $N > 10$ cells)]. **(D)** Polar plots of net dipole orientations for individual hourglass and split septin rings scaled by anisotropy (hourglass $N > 55$ cells, split rings $N > 10$ cells). **(E)** Difference in net orientation measures between top and bottom focal planes for hourglass and split structures. Error bars denote standard error. **(F)** Population variance in hourglass and split ring structures.

(CDK sites), and ps4 (Pho85/CDK sites) (**Figure 5A**; Egelhofer et al., 2008). Septin disassembly rates were notably faster in the wild-type strain background of these mutant alleles, which could be due to background mutations such as in *BUD4*, which is consistent with the observed asymmetric splitting also observed in certain *bud4Δ* strains (Voth et al., 2005). The *shs1* ps mutants showed strikingly distinct assembly and disassembly defects amongst each other. *shs1-ps1* and *shs1-ps4* had substantially diminished levels of septins in assembled hourglasses, ∼50 and 80% reduction in intensity relative to wild-type, respectively (**Figure 5B**). In contrast, *shs1-ps2* cells had similar septin levels at the hourglass compared to wild-type as gauged by GFP-Cdc3 intensity. Interestingly, none of the *shs1* ps mutants had a different rate of disassembly from wild-type (**Table 4**). This is potentially a result of the strain background interfering with any differences in rates relative to one another; however, these data still suggest that phosphorylation may be able to tune the affinity of septins for membrane at the neck or septin-septin interactions for the assembly of the hourglass.

All the *shs1* phosphorylation-deficient mutants were comparably highly disorganized and showed a wide distribution of orientations and cell-to-cell variability in both hourglass and asymmetric split ring structures (**Figures 5C–F**, **Table 5**). The impact of these mutations is comparable to a complete null mutant of *SHS1*. These data attest to the key importance of Shs1 and likely its regulation by phosphorylation in modulating the abundance and organization of septin higher-order structures but do not support a major role of these modifications in the splitting process.

DISCUSSION

What could specify the bifurcation of behavior within a population of septins such that disassembly and reassembly/retention processes basically coexist in time and space? Our goal was to identify features of septins themselves and regulators that might be responsible for this transition in filament organization. One hypothesis we tested was that the septin Shs1 might specify which septins are prone to departure versus reassembly/retention. The difference in abundance of the two terminal septins within the hourglass is consistent with evidence that Shs1 has the ability to cap septin rods, but not polymerize with other Shs1 containing rods (Finnigan et al., 2015). However, we did not see a substantial difference in the rate

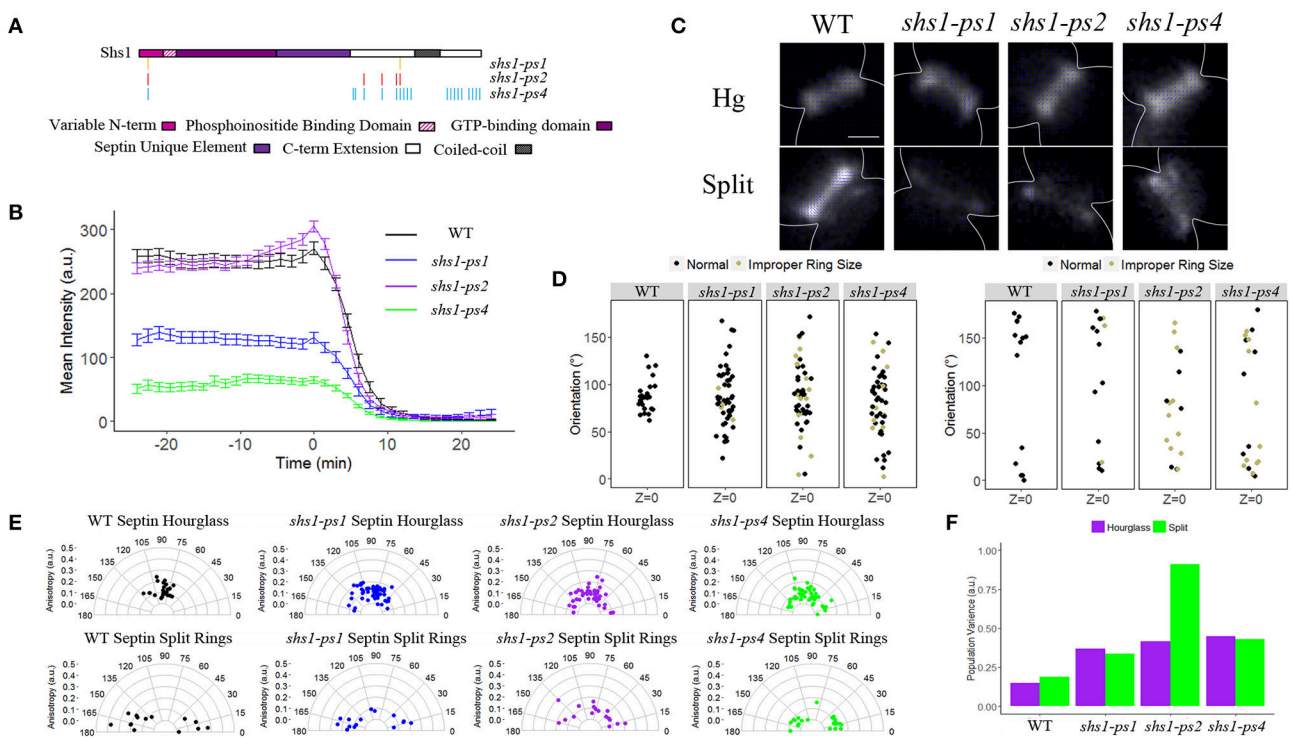

FIGURE 5 | Shs1 phosphorylation modulates septin abundance, splitting dynamics, and organization. (A) Schematic of Shs1 protein domains with mutated phosphorylation sites highlighted for each shs1-phosphomutant (adapted from Egelhofer et al., 2008). **(B)** Septin intensity of GFP-Cdc3 in W303 background (black), *shs1-ps1* (blue), *shs1-ps2* (purple), and *shs1-ps4* (green) over the course of septin ring splitting (time = 0). Error bars represent standard error for each time point (N = 100 cells). **(C)** Representative polarization images of W303 background containing Sc*CDC12*-conGFP, and *shs1*-phosphomutants containing Sc*CDC12*-conGFP in the hourglass and split structure. The blue lines represent the calculated dipole orientation and their length is scaled according to anisotropy. Scale bar 0.5 μm. **(D)** Scatter plot of net dipole orientations for individual hourglass and split septin rings in three different focal planes. Color scale indicates visible phenotypes of septin rings [broken ring (tan), improper ring size (orange), (hourglass N > 26 cells, split rings N > 14 cells)]. **(E)** Polar plots of net dipole orientations for individual hourglass and split septin rings scaled by anisotropy (hourglass N > 26 cells, split rings N > 14 cells]. **(F)** Population variance in hourglass and split ring structures.

at which Cdc11 and Shs1 depart the neck indicating that simply different terminal subunits do not specify the subpopulations at splitting. These results do not lead to a simple explanation for specifying the different fates of the hourglass and split ring septin filaments based on differential behavior of Cdc11 and Shs1 during the transition.

We hoped that through careful analysis of splitting kinetics and organization states that we would be able to dissect the molecular control of the transition. We identified several categories of defects in both the kinetics of splitting and septin organization by combining timelapse imaging with polarized fluorescence analysis (**Table 6**): (1) Decreased abundance in the hourglass (*bni1Δ, bnr1Δ, bud4Δ, gin4Δ, cla4Δ, rts1Δ*, *shs1Δ, shs1ΔC,* and *shs1-ps1* and *-ps4*); (2) Increased rate of disassembly (*bud4Δ*); (3) Monophasic rate of disassembly (*gin4Δ*, *shs1Δ);* (4) Misorganized hourglass but organized split rings (*gin4Δ*); (5) Organized hourglass but misorganized split rings (*bud4Δ*); (6) Misorganized hourglass and split rings (*shs1Δ*, *shs1ΔC shs1-ps1,2 and 4)*; and (7) correctly oriented but less ordered (low anisotropy) assemblies (*bni1Δ, bnr1Δ, cla4Δ, rts1Δ*). Of note, many regulators (Class1), including those influencing F-actin have an appreciable impact of the abundance of septins in the hourglass structure. It is unclear whether this is due to some altered membrane composition in these mutants or direct impacts on septins that may lower their affinity for the membrane.

In this study, we had aimed to identify mutants that would have specific defects in reorientation. However, the only cases where we see definite defects in reorientation is in scenarios where the ring fails to split or the split rings are unstable such as what happens in cells lacking Bud4. A clear challenge that emerged from this study is that perturbing many of the factors that we suspect might contribute to the process leads to defects in the starting higher-order structure of the septin hourglass. In many cases these defects were not detectable until analysis with polarized light. It is difficult to disentangle the degree to which defects arise due to poor construction as opposed to an inability to respond appropriately to cell cycle triggers of the transition. Future work will need to expand this screen to the vast array of septin regulators and interacting proteins that are known to exist with the hope of still identifying a regulator that might be responsible for this transition without contributing to the organization *per se*. It is likely that this next phase of screening will require identifying partial loss-of-function or separation-of-function alleles because it is probable that whatever responds to or executes the signal for reorientation also contributes to overall septin organization.

TABLE 6 | Results summary.

Defect category	Mutant(s)
Decreased abundance in the hourglass	*bni1Δ, bnr1Δ, bud4Δ, gin4Δ, cla4Δ, rts1Δ*, *shs1Δ, shs1ΔC* and *shs1-ps1* and *-ps4*
Increased rate of disassembly	*bud4Δ*
Monophasic rate of disassembly	*gin4Δ, shs1Δ*
Misorganized hourglass but organized split rings	*gin4Δ*
Oraganized hourglass but misorganized split rings	*bud4Δ*
Misorganized hourglass and split rings	*shs1Δ*, *shs1ΔC, shs1-PS*
Correctly oriented but less ordered (low anisotropy) assemblies	*bni1Δ, bnr1Δ, cla4Δ, rts1Δ*

Despite the constellation of phenotypes, there are a few key aspects of the data that point to potential mechanisms relevant to the splitting transition. In particular, consistent with previous observations, Bud4 is required for a proper split ring state (Wloka et al., 2011; Eluère et al., 2012; Kang et al., 2013). We were interested to note that the reorientation did not occur in the absence of splitting. This suggests that the reorientation is concomitant with and potentially requires the splitting process. One possible reason for this is that the reorientation also involves reacting to changes in local membrane curvature and composition that are happening at cytokinesis and also contribute to the reassembly process. On a molecular level, the data indicate that either Bud4 is required for receiving the cell cycle cue of splitting and/or is an essential guide or anchor for the reassembly process that creates the split ring structures.

Another clue emerges from the similar behavior of *gin4Δ* and *shs1Δ* which both lose the biphasic disassembly observed in wild-type cells, and thus are slow in going through the transition. This suggests that Gin4 is targeting either Shs1 or is impacting a feature of the transition that Shs1 is required for as well. In the absence of Gin4 activity or Shs1, septins remain associated with the membrane longer. This could be due to Gin4 phosphorylating a subpopulation of septins or other substrates that influence septin affinity for the membrane. We assessed the role of septin phosphorylation (which may or may not be due to Gin4) and see that non-phosphorylatable mutants altered septin levels in the hourglass and monophasic disassembly. Unfortunately, uncertainty about the background of these mutants with regard to the *BUD4* locus makes it challenging to interpret the rate of disassembly in these strains because the disassembly was more rapid than expected in controls. Analysis of an *shs1* mutant that lacks the entire C-terminus was illuminating in that it also has a major defect in the recruitment of septins to the hourglass however it shows a biphasic disassembly that is not slowed compared to wildtype, unlike the *shs1* null. This mutant still retains a phosphorylation site at T6 that is missing in the PS alleles, pointing to this residue as a potentially important site for regulating contact with the membrane for both assembly of the hourglass and release of septins at splitting. Overall, the data point to Gin4 and Shs1 as modulators of the rate of change in septin dynamics and orientation at late stages of the cell cycle. It is likely that this process involves phosphorylation-dependent changes in membrane composition and/or septin-membrane binding that change the affinity of septins for the membrane and possibly change the preference of septins to bind membranes of specific curvatures.

A real power of using polarized fluorescence microscopy is the ability to detect aspects of macromolecular organization not

readily apparent in standard total fluorescence measurements and to assess the degree of order in an ensemble of molecules. In our previous work we have seen that no matter how we view the hourglass or rings, the average dipole orientation was the same and we interpreted this to mean that a majority of septins are symmetric relative to the membrane supporting paired filaments. Our motivation for using the MF-PolScope was to be able to simultaneously capture multiple focal planes in the panel of mutants in hopes of detecting defects in pairing that might inform the mechanisms of reorientation. To our surprise, we could see no evidence of systematic differences in orientation that were dependent on focal plane suggesting that pairing may not be impacted in these diverse mutant backgrounds. In summary, this work set out to analyze the requirements for septin rearrangement at cytokinesis in budding yeast. The findings point to a function for Shs1, Gin4, and Bud4 in this process but there is clearly much more work to be done to understand how the dramatic cytoskeletal reorientation occurs in this small window of time and space.

AUTHOR CONTRIBUTIONS

MM performed experiments, assembled figures, analyzed data and wrote manuscript, MJ performed experiments and analyzed data, SM and AV assisted in data analysis, RO contributed instrumentation and expertise in analysis, AG designed the study and wrote the paper.

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MINI REVIEW
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Septins Focus Cellular Growth for Host Infection by Pathogenic Fungi

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One of the key challenges faced by microbial pathogens is invasion of host tissue. Fungal pathogens adopt a number of distinct strategies to overcome host cell defenses, including the development of specialized infection structures, the secretion of proteins that manipulate host responses or cellular organization, and the ability to facilitate their own uptake by phagocytic mechanisms. Key to many of these adaptations is the considerable morphogenetic plasticity displayed by pathogenic species. Fungal pathogens can, for example, shift their growth habit between non-polarized spores, or yeast-like cells, and highly polarized hyphal filaments. These polarized filaments can then elaborate differentiated cells, specialized to breach host barriers. Septins play fundamental roles in the ability of diverse fungi to undergo shape changes and organize the F-actin cytoskeleton to facilitate invasive growth. As a consequence, septins are increasingly implicated in fungal pathogenesis, with many septin mutants displaying impairment in their ability to cause diseases of both plants and animals. In this mini-review, we show that a common feature of septin mutants is the emergence of extra polar outgrowths during morphological transitions, such as emergence of germ tubes from conidia or branches from hyphae. We propose that because septins detect and stabilize membrane curvature, they prevent extra polar outgrowths and thereby focus fungal invasive force, allowing substrate invasion.

Keywords: septins, filamentous fungi, branch, germ tube, invasion, pathogenesis

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INTRODUCTION

Fungi are responsible for many of the world's most serious diseases, ranging from crop diseases—which each year severely restrict the global harvest of many of our most important food sources—to human diseases that claim many hundreds of thousands of lives annually (Brown et al., 2012; Fisher et al., 2016). Understanding fungal pathogens is therefore vital from economic, social, and humanitarian standpoints. There is an urgent need for more durable resistance to crop pathogens and more effective drugs to treat human diseases. Fungal pathogens share many conserved features, irrespective of the hosts they have evolved to infect, and these offer insight into the origins of fungal pathogenesis and the most likely targets for intervention and broad spectrum control of fungal disease. One of the most basic features of fungal pathogens is their ability to undergo shape changes, in order to traverse the outer barriers to infection, and gain entry to underlying tissue, which they can invade and colonize with great efficiency.

Fungi can grow as non-polarized yeast-like cells that are well-suited for moving through aqueous environments like blood in an animal host, or water on a leaf surface. Many fungi also resist

environmental stress by forming non-polarized dormant resting bodies, such as sclerotia, and a huge assortment of spores to transmit pathogens long distances in search of new prey (Emmett and Parbery, 1975; Mendgen et al., 1996; Tucker and Talbot, 2001; Wang and Lin, 2012). Non-polarized fungal cells can switch to highly polarized filamentous growth, ideal for invading a heterogeneous environment, such as the tissue of an animal organ or the spaces between plant cells (Nemecek et al., 2006). This basic dimorphic ability has been further developed by pathogenic fungi that differentiate an array of specific infection cell types from fungal hyphae (Mendgen and Deising, 1993). Fungi can, for instance, form appressoria that develop enormous invasive forces—sufficient to break plastic surfaces in the laboratory—which they use to breach the tough outer cuticle of plants, rupturing epidermal cell walls to penetrate plant cells directly. This is exemplified by the rice blast fungus, *Magnaporthe oryzae*, which can generate turgor of up to 8.0 MPa (80 atmospheres) that is applied at the leaf surface to break the strong and resilient rice cuticle that coats rice leaves and stems (Wilson and Talbot, 2009). Appressoria can, however, also be used by insect pathogens to launch a huge proteolytic attack on hosts, to facilitate entry to insect bodies, where pathogens such as *Metarhizium anisopliae*, can rapidly colonize and immobilize their hosts. (Hajek and St. Leger, 1994; Zhang et al., 2010) Root pathogens, meanwhile, differentiate lobed hyphopodia from hyphae to infect root tissue. Fungal pathogens, in fact, can form a plethora of infection structures, such as compound appressoria and infection cushions (Mendgen et al., 1996). This ability to change shape and switch rapidly between non-polarized and polarized cell types is therefore fundamental to fungal pathogenesis and, indeed, the fungal lifestyle more generally.

We recognize that septins play critical roles in cytokinesis, cell cycle control, spatial compartmentalization, and as ER and plasma membrane diffusion barriers. Though these roles are undoubtedly important for fungal growth and pathogenesis, they have been extensively reviewed elsewhere (Joo et al., 2005; Gladfelter, 2006, 2010; Lindsey and Momany, 2006; Lichius et al., 2011; Hernandez-Rodriguez and Momany, 2012; Mostowy and Cossart, 2012; Spiliotis and Gladfelter, 2012; Bridges and Gladfelter, 2014). In this mini-review, we focus instead on the role of septins in the morphogenetic switch from non-polar to polar growth, generally seen in filamentous fungi as the formation of polar outgrowths, and the importance of this switch for tissue invasion and fungal pathogenesis.

SEPTIN-DEPENDENT HOST INFECTION BY FUNGAL PATHOGENS

An increasing body of work suggests that septins play important roles in fungal pathogens (Douglas et al., 2005; Bridges and Gladfelter, 2014; Khan et al., 2015; Vargas-Muniz et al., 2016). An examination of fungal septin mutants suggests that a critical septin role is defining and restricting polar outgrowths that allow the fungus to invade and explore host tissue. In fungal pathogens of plants and insects these polar outgrowths often elaborate specialized structures to penetrate a protective barrier. Strikingly, in most fungal pathogens deletion of septin-encoding genes leads to an increase in polar outgrowths and a decrease in virulence. In the few cases where deletion of septins does not result in increased polar outgrowths, virulence is not reduced (**Table 1** and **Figure 1**). In the rice blast fungus *M. oryzae*, for example, it has been shown that the invasive appressorium requires septins to function (Dagdas et al., 2012). A hetero-oligomeric septin ring is involved at the initial point of appressorium differentiation, forming at the base of the fungal germ tube. Septin deletion mutants often bifurcate at this point forming two germ tubes and appressoria (**Figures 1O–S**). Later, however, during appressorium maturation a hetero-oligomeric septin ring forms at the base of the appressorium, once it reaches a critical turgor threshold. The septin ring scaffolds F-actin, re-modeling this into a toroidal network at the appressorium pore—a specialized zone where the cell re-polarizes to form a rigid penetration hypha to rupture the rice cuticle. The septin ring also acts as a lateral diffusion barrier, holding in place polarity determinants, such as Cdc42, the actin polymerization machinery, such as Las17 (part of the Arp2/3 complex), and BAR proteins involved in membrane curvature generation, as well as endocytosis and exocytosis. In this way, the appressorium pore acts as the specialized frontier; the point at which the host-pathogen interface is first established and a complex signaling hub in the fungal cell (Yan and Talbot, 2016). Many processes are coordinated at this zone, including the generation of protrusive actin-driven force, associated membrane curvature generation, membrane biogenesis, and cell wall biosynthesis, collectively coordinated to focus new anisotropic growth at a single point where the enormous invasive force is deployed. The septin complex forms once the appressorium reaches a threshold of turgor pressure, and requires the regulated synthesis of reactive oxygen species, catalyzed by the Nox2 NADPH oxidase complex, which is necessary for plant infection (Ryder et al., 2013). Regulated ROS may directly play a role in F-actin polymerization, while also being necessary for recruiting other polarization factors, such as Chm1 and Tea1, to the pore (Dagdas et al., 2012).

A further example of the significance of septins in host infection is provided by the wheat head blight pathogen, *Fusarium graminerarum,* where the core septins FgCdc3, FgCdc11, and FgCdc12 (but not FgCdc10), are necessary for fungal development and virulence. Targeted deletion of *FgCdc3*, *FgCdc11*, or *FgCdc12* led to defects in growth, conidiation, and morphology, with foot cells elaborating an extra polar outgrowth in the form of a bifurcated conidium. The *ΔFgcdc3, ΔFgcdc11*, and *ΔFgcdc12* mutants also showed greatly reduced virulence on wheat. In contrast, the *ΔFgcdc10* mutant had wild type growth, morphology, and virulence (Chen et al., 2016). In the corn smut pathogen *Ustilago maydis*, although septins are not necessary for primary plant infection, they are required for full symptom development in which tumors are formed that result in large-scale teliospore production. Septin deletion mutants were affected in these later stages of smut disease development and elaborated bipolar rather than monopolar filaments and extra germ tubes from teliospores (**Figures 1A–C**). These mutants also showed reduced virulence on corn (Alvarez-Tabares and

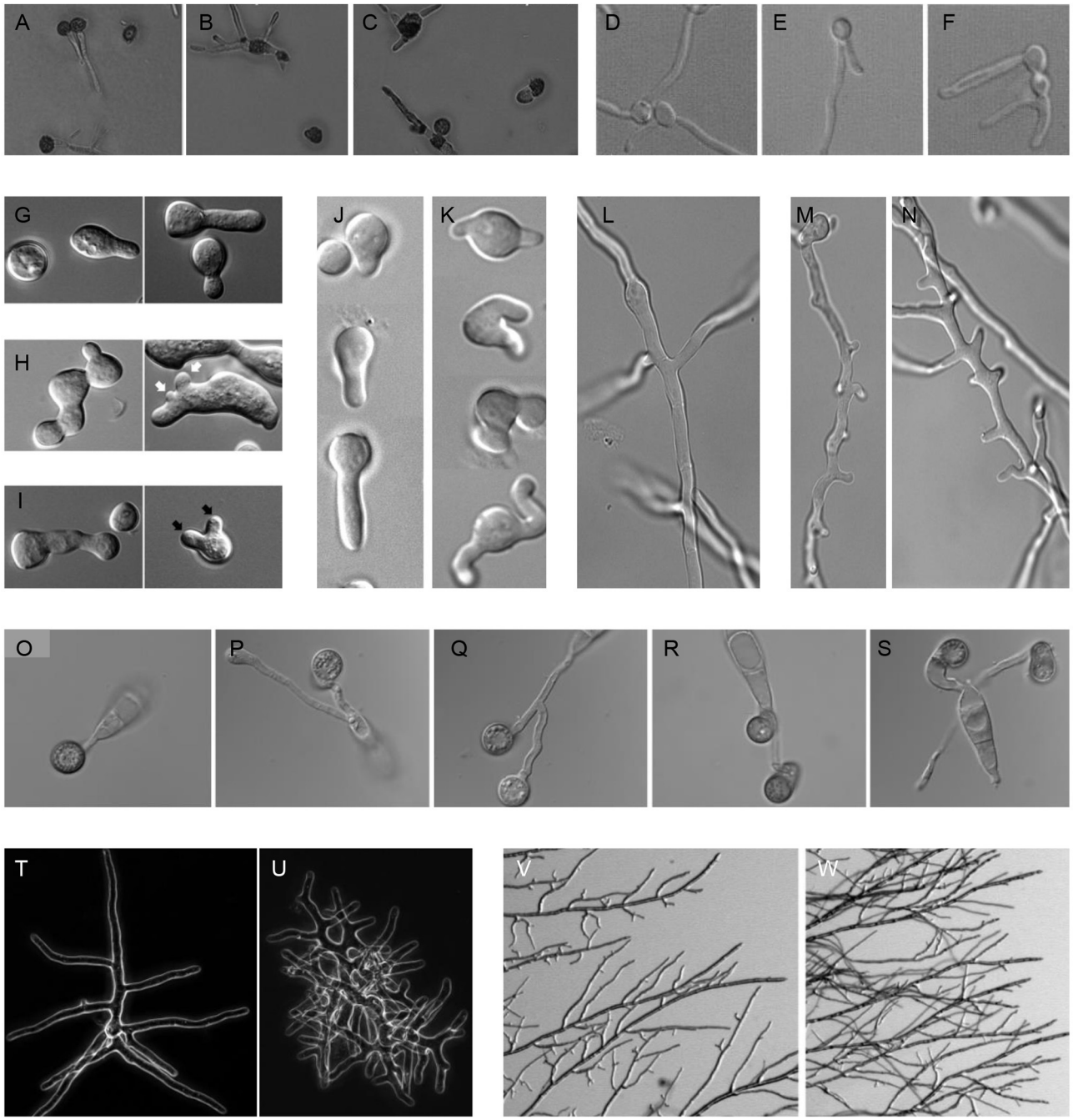

FIGURE 1 | Polar outgrowths of septin mutants. (A–C) Germinating spores of *Ustilago maydis* incubated at 22°C **(A)** WT, **(B)** *sep1*$^{cdc3}\Delta$, **(C)** *sep2*$^{cdc12}\Delta$ (Alvarez-Tabares and Perez-Martin, 2010). **(D–F)** Filaments of *Candida albicans,* **(D)** WT, **(E)** *cdc10Δ*, **(F)** *cdc11Δ* (Warenda and Konopka, 2002). **(G–I)** Germinating spores of *Neurospora crassa*, **(G)** WT, **(H)** *Δcdc-3*, **(I)** *Δcdc-10.* Black arrows denote multiple germ tube emergence. White arrows show multiple conidial anastomosis tube emergence (Berepiki and Read, 2013). **(J–K)** Germinating spores and **(L–N)** emerging branches of *Aspergillus nidulans*, **(J, L)** WT, **(K, M, N)** *ΔaspB*^{cdc3} (Hernandez-Rodriguez et al., 2012). **(O–S)** Germinating spores of *Magnaporthe oryzae*, **(O)** WT, **(P)** *Δsep3*^{cdc3}, **(Q)** *Δsep4*^{cdc10}, **(R)** *Δsep5*^{cdc11}, **(S)** *Δsep6*^{cdc12} (Dagdas et al., 2012). **(T, U)** Branching colonies of *Ashbya gossypii*, **(T)** WT, **(U)** *Agcdc12Δ* (Helfer and Gladfelter, 2006). **(V, W)** Branching colonies of *Neurospora crassa,* **(V)** WT, **(W)** *Δcdc-3* (Berepiki and Read, 2013).

Perez-Martin, 2010). In *Ashbya gossyipii*, a filamentous fungus that can causes stigmatomycosis disease on cotton, *Aghsl1Δ* or *Agcdc12Δ* septin deletion strains showed extra polar outgrowths from hyphae that the authors describe as "kinked hyphae" and which appear to be similar to hyperbranching (Helfer and Gladfelter, 2006; **Figures 1T–U**).

TABLE 1 | Polar outgrowths and virulence phenotypes of fungal septin mutants.

Fungus	Mutant	Extra/altered Polar outgrowths[a]?	Virulence[b]	References
Ashbya gossypii	*Aghsl1Δ* *Agcdc12Δ*	Yes (BR, authors call "kinked hyphae")	NA	Helfer and Gladfelter, 2006
Aspergillus fumigatus	*ΔaspA^{cdc11}* *ΔaspB^{cdc3}* *ΔaspC^{cdc12}*	No	Hypervirulent in *Galleria mellonell*	Vargas-Muniz et al., 2015
	ΔaspB^{cdc3}		Virulent in mice	
	ΔaspD^{cdc10} *ΔaspE*		Virulent in *G. mellonella*	
Aspergillus nidulans	*ΔaspA^{cdc11}* *ΔaspB^{cdc3}* *ΔaspC^{cdc12}*	Yes (GT and BR)	NA	Lindsey et al., 2010; Hernandez-Rodriguez et al., 2012, 2014
	ΔaspD^{cdc10} *ΔaspE*	No		
Candida albicans	*cdc10Δ* *cdc11Δ*	Yes (FIL clusters)	Reduced virulence in mice	Warenda and Konopka, 2002; Warenda et al., 2003
Cryptococcus neoformans	*cdc3Δ* *cdc12Δ*	Yes, Clamp cells emerge, but do not fuse, aberrant protrusions from basidia	Reduced virulence in *Galleria*	Kozubowski and Heitman, 2010
	cdc11Δ *cdc10Δ*		NA	
Fusarium graminearum	*ΔFgcdc3* *ΔFgcdc11* *ΔFgcdc12*	Yes (Extra spore from footcell)	Reduced virulence on wheat	Chen et al., 2016
	ΔFgcdc10	No	Virulent on wheat	
Magnaporthe oryzae	*Δsep3^{cdc3}* *Δsep4^{cdc10}* *Δsep5^{cdc11}* *Δsep6^{cdc12}*	Yes (GT with terminal appressorium)	Avirulent on rice	Dagdas et al., 2012
Neurospora crassa	*Δcdc-3* *Δcdc-11* *Δcdc-12* *Δcdc-10*	Yes (GT and BR)	NA	Berepiki and Read, 2013
Ustilago maydis	*sep1^{cdc3}Δ* *sep2^{cdc12}Δ* *sep3^{cdc11}Δ* *sep4^{cdc10}Δ*	Yes (GT and FIL)	Reduced virulence on corn	Alvarez-Tabares and Perez-Martin, 2010

[a]*Abnormal polar outgrowths observed along hyphae, perpendicular to growth axis, relative to WT. GT, germ tube emergence from spore; BR, branch emergence from hypha; FIL filament emergence from hypha. Septation and nuclear phenotypes not shown.*
[b]*Virulence relative to WT controls.*

In the pleomorphic yeast human pathogen *Candida albicans,* targeted mutation of the *cdc10* and *cdc11* core septins led to clustering of filament emergence and a reduction in virulence and invasive growth capacity in mouse infections (Warenda and Konopka, 2002; Warenda et al., 2003; **Figures 1D–F**). In the basidiomycete human pathogen *Cryptococcus neoformans,* deletion of septin-encoding genes led to loss of fusion in protrusive clamp cells and attenuation of virulence in *Galleria* infections (Kozubowski and Heitman, 2010). By contrast, deletion of septin-encoding genes in the human pathogen *Aspergillus fumigatus* did not result in extra germ tubes or branches, though septation and conidiation were reduced (Vargas-Muniz et al., 2015). Strikingly, *A. fumigatus ΔaspA^{cdc11}*, *ΔaspB^{cdc3}*, or *ΔaspC^{cdc12}* mutants actually showed enhanced virulence in a *Galleria melonella* (waxmoth larva) model of infection, while *ΔaspD^{cdc10}* and *ΔaspE* showed wildtype virulence. The only septin deletion mutant to be tested in a mouse model of disease, however, *ΔaspB^{cdc3}* showed wildtype virulence.

The increased polar outgrowth phenotype is also seen in non-pathogenic filamentous fungi. In the model *Aspergillus nidulans, ΔaspA^{cdc11}*, *ΔaspB^{cdc3}*, and *ΔaspC^{cdc12}* strains showed a dramatic increase in germ tube and branch emergence (Lindsey et al., 2010; Hernandez-Rodriguez et al., 2012; **Figures 1J–N**). Similarly *Neurospora crassa Δcdc-3, Δcdc-11*, *Δcdc-12*, and *Δcdc-10* strains all made extra germ tubes and branches (Berepiki and Read, 2013; **Figures 1G–I, V–W**). Though these fungi are not generally considered pathogens, presumably protrusive growth is important to their ability to explore and invade the heterogeneous substrates they colonize as saprotrophs.

HOW DO SEPTINS FOCUS INVASIVE GROWTH BY FUNGI?

Given the roles identified for septins in fungal invasion of living hosts and non-living substrates, what is their likely function and

can more general conclusions be made? The localization pattern of septin complexes at the periphery of fungal invasive cells is strikingly conserved, even in very diverse cell types. Septin rings, for instance, form at points of hyphal constriction, and at zones of new polarized growth (Berepiki and Read, 2013). Indeed, whenever new polarized outgrowths are formed, they appear to be flanked by septin assemblages that correspond to points of maximal membrane curvature (Gladfelter, 2006). This strong association points to a role for septins in sensing and stabilizing membrane curvature, consistent with recent *in vitro* studies of septins that show their ability to condition micrometer-scale membrane curvature generation. A recent important study showed how septins can act as sensors of micrometer scale plasma membrane curvature in *A. gossyppi*. Septins appear to preferentially localize to regions of positive curvature at the base of polarized outgrowths, branches from hyphae (Bridges et al., 2016). Bridges and co-workers showed that septins localize to the most highly curved regions of branches. When mixed with lipid bilayer-coated silica beads, purified hetero-oligomeric septin complexes of Cdc10, Cdc3, Cdc12, and Cdc11-SNAP adsorbed to beads of 1–3 μm in diameter. The adsorption was therefore dependent on the curvature of the beads, which corresponded with the curvature observed at the base of branch points. The detection of curvature appears to be an intrinsic characteristic of septins that enables them to act as landmarks for stabilizing and amplifying surface topologies.

This remarkable ability of septins to stabilize and amplify new topologies is critical to the capacity of fungi to focus force at a single point—a necessary prerequisite to invasive growth—and their ability to form outgrowths at other points along hyphae. During extension of penetration hyphae it might be necessary, for example, to prevent other branches from forming, thereby focusing growth along a single rigid filament, while at other times, particularly once tissue is invaded, the ability to branch extensively and in a pattern optimized to explore host tissue, is key to the most efficient occupation of a surrounding substrate. We argue that both focused invasion and efficient exploration must involve septins to regulate cortical F-actin interactions, corral proteins associated with endocytosis and exocytosis that collectively regulate membrane homoeostasis, and to specifically deploy polarity determinants that ultimately define new points of growth. This idea is supported by the striking phenotypes observed due to loss of septins in filamentous fungal pathogens described above (**Table 1** and **Figure 1**). These include abnormal polarized outgrowths that form along hyphae perpendicular to the growth axis of the hypha, by aberrant branch patterning, or hyper-branching phenotypes, and with associated mis-regulation of the sites of septation, or spore separation. When considered along with the loss of virulence associated with these septin mutants (Warenda et al., 2003; Alvarez-Tabares and Perez-Martin, 2010; Kozubowski and Heitman, 2010; Dagdas et al., 2012; Chen et al., 2016), it seems likely that septins are critical for cell shape determination which is a fundamental characteristic of infection-associated morphogenesis.

Finally, the role of the aspE septins, the presumed ancestral septins found in filamentous fungi and certain ciliates and algae, but missing from yeasts and animals (Pan et al., 2007; Nishihama et al., 2011; Yamazaki et al., 2013), may offer a clue to how filamentous fungi have maintained a diverse septin repertoire to condition morphogenetic plasticity. Perhaps AspE-type septins facilitate unique hetero-oligomeric associations during different developmental stages. Evidence for such a role comes from the analysis of *ΔaspE* mutants in the multicellular growth stages of *A. nidulans* where higher order structures containing three core septins (AspA^{cdc11}, AspB^{cdc3}, and AspC^{cdc12}) require AspE, but those containing all four core septins do not (Hernandez-Rodriguez et al., 2014). Intriguingly, AspE localizes in cortical patches lining the entire plasma membrane, a position that seems well-suited to organizing membranes for polar outgrowths.

In conclusion, septins appear pivotal to the ability of fungi to regulate their surface topologies to facilitate the invasion of diverse substrates—both non-living and, importantly in this context, living host tissue. Septins may fulfill such a role because they are able to rigidify the cortex to prevent aberrant polar outgrowth, while assembling specifically at points where branching and re-polarization are required. How such septin distribution is determined, however, remains unclear. Does it, for example depend solely on membrane curvature-dependent recruitment of septins? If so, what are the spatial co-ordinates that might determine sites of septin-dependent polarization? In this context, what is the role of BAR domain proteins, implicated in generating and sensing nanometer-scale membrane curvature? Do they initiate the process of invasive growth? And, more broadly, how is spatial organization of the hyphal cortex actually controlled? Is it a consequence of gradients of endocytotic and exocytotic activity that provide longitudinal co-ordinates along a hypha thereby facilitating septin aggregation at correct locations, or is this instead an intrinsic characteristic of septins themselves, as suggested by *in vitro* studies (Bridges and Gladfelter, 2016). It is clear that to answer such questions, the roles of septins in focused invasion and generation of cellular protrusions will need to be explored in much greater detail. To achieve this, there is, for example, a need for specific analysis of septin function by generation of conditional mutants, or by conditional inhibition of septin aggregation during the infection process. The use of gene silencing or conditional alleles of septin genes may offer the means to do this most effectively, so that septin assembly can be prevented directly at the point of primary host infection, or later during invasive growth. The dynamics of septin assembly within living fungal cells during the infection process also requires further analysis. This is, of course, challenging because it depends on the ability to conditionally manipulate septins, while at the same time carrying out live cell imaging. However, this is not beyond the scope of current methodologies, such as super-resolution microscopy, while correlative light and electron microscopy could provide ultrastructural analysis of such assemblies, followed by cryo-electron microscopy to study *in vivo* assembly dynamics in unparalleled detail. Deployment of these approaches in pathogenic fungi undergoing host infection, will allow a comprehensive testing of our hypothesis that septins prevent aberrant polar outgrowths and thereby focus fungal

invasive force at appropriate points for substrate invasion and fungal pathogenesis. An exciting prospect.

AUTHOR CONTRIBUTIONS

MM and NT synthesized source material, generated ideas and conclusions, and co-authored the mini-review.

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MINI REVIEW
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Uncovering the Roles of Septins in Cilia

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Septins are a family of GTP-binding proteins that associate with cellular membranes and the cytoskeleton. Their ability to polymerize into filamentous structures permits them to serve as diffusion barriers for membrane proteins and as multi-molecular scaffolds that recruit components of signaling pathways. At the cellular level, septins contribute to the regulation of numerous processes, including cytokinesis, cell polarity, cell migration, and many others. In this review, we discuss emerging evidence for roles of mammalian septins in the biogenesis and function of flagella and cilia, and how this may impact human diseases such as ciliopathies.

Keywords: septin, cilia, flagella, diffusion barrier, ciliogenesis

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INTRODUCTION

Septins are a family of poorly-characterized filamentous GTP-binding components of the cytoskeleton that orchestrate a variety of cellular processes including cytokinesis, cell migration, cell polarity, and cell-pathogen interactions (Fung et al., 2014). They were initially discovered in yeast in 1971 (Hartwell, 1971), and have since been identified in many eukaryotes (Onishi and Pringle, 2016). The number of septin genes varies widely amongst species, for example *Caenorhabditis elegans* has only two (UNC-59 and UNC-61) while there are thirteen in humans (SEPT1–SEPT12, SEPT14). In mammals, this complexity is further increased by use of alternative promoters and splicing, producing many different isoforms (for example see McIlhatton et al., 2001), making it challenging to fully understand the cell and molecular biology of these proteins.

Structurally, all mammalian septins contain a central GTP-binding domain, have variable N and C-termini, and the C-termini have different numbers of coiled-coil domains. Based on these features and sequence similarities, mammalian septin genes can be categorized into four subgroups (SEPT1, 2, 4, 5; SEPT6, 8, 10, 11, 14; SEPT7; SEPT3, 9, 12). Most cell types express at least one member of each group and these proteins assemble into ordered apolar complexes (Fung et al., 2014).

At the cellular level, septins are often found in structures with micron-scale membrane curvature (Bridges et al., 2016) such as the cytokinetic furrow in dividing cells (Maddox et al., 2007; Estey et al., 2010), the base of dendritic spines in neurons (Tada et al., 2007), the base of phagocytic cups (Huang et al., 2008), the annulus of sperm tails (Kwitny et al., 2010) and the base of primary cilia (Hu et al., 2010). In the following sections, we summarize what is known about septin function in general, discuss the evidence for their presence in flagella and different types of cilia, and speculate about their possible roles.

KNOWN FUNCTIONS OF SEPTINS

The involvement of septins in myriad cellular processes indicates that they may have more than one function. Perhaps the best-studied example of multi-functionality occurs in yeast cytokinesis where septins form a ring from which the bud emerges (Longtine and Bi, 2003). Here, their filamentous nature allows them to function as a macromolecular scaffold to facilitate protein-protein interactions, where they recruit nearly 100 proteins, including bud-specific machinery and regulators of the actin cytoskeleton to the bud site (Gladfelter et al., 2001). This septin ring also acts as a diffusion barrier, limiting the lateral diffusion of membrane proteins between the mother and the bud (Takizawa et al., 2000).

Septins also bind to membranes, particularly to negatively charged lipids such as PIP2 (Zhang et al., 1999). This ability appears to also be influenced by the curvature of the membrane itself (Bridges et al., 2016) suggesting septins preferentially assemble on membrane surfaces of defined shape and charge. The binding of septins to membranes has been shown to promote membrane tubulation *in vitro* (Tanaka-Takiguchi et al., 2009), promote the retraction of blebs from the cell membrane (Gilden et al., 2012) and stabilize the plasma membrane to allow directed cell migration in T cells (Tooley et al., 2009).

Other functions ascribed to septins include their ability to affect the organization and dynamics of other cytoskeletal elements. For example, Drosophila septins were shown to bundle and curve actin filaments (Mavrakis et al., 2014). Septins have also been implicated in altering microtubule dynamics by binding to, and inhibiting the activity of, the microtubule stabilizing protein MAP4 (Kremer et al., 2005). Interaction of microtubules with septins appears to influence microtubule growth by suppressing catastrophe at growing plus ends (Bowen et al., 2011) causing microtubules to track along septin filaments.

FLAGELLA AND CILIA

In vertebrates there are two types of cilia: (a) motile cilia, which are structurally identical to flagella, and (b) non-motile cilia, also called primary cilia. Diseases resulting from defects in cilia are collectively called ciliopathies, although the nature of the disease depends on the type of cilia affected. Flagella and cilia are microtubule-based organelles protruding from the cell surface. The term "flagellum" is used when a single motile cilium is used by cells for locomotion (for example, on mammalian sperm; **Figure 1A**). Motile cilia are more frequently found in large clusters on the cell surface and are involved in moving the extracellular fluid, rather than moving the cell itself. They are found lining the airway tract ependyma in the brain and the oviducts. Defects in motile cilia lead to ciliary dyskinesia and sterility. In contrast is the single motile primary cilia covering the node of the vertebral embryos, where a single cilium per cell moves in a circular manner to create the left-right asymmetry essential for correct positioning of visceral organs in the developing embryo (Baker and Beales, 2009). Defects in nodal cilia result in situs inversus, or loss of the typical asymmetry of the organs.

Rather than motile cilia, most cells have a single "primary" immotile cilium protruding from the membrane. Once thought to be extraneous structures (Webber and Lee, 1975), they are now considered important sensory organelles that act as cellular antennas to transmit extracellular cues into the cell. They are sites for the regulation of several developmental signaling pathways such as non-canonical Wnt and Sonic hedgehog pathways (Sasai and Briscoe, 2012). Diseases associated with loss of primary cilia include Meckel–Gruber Syndrome, Bardet–Biedl syndrome, Joubert syndrome and Polycystic kidney disease. These multisystem disorders frequently include retinal degeneration and cyst formation in liver and kidneys but interestingly may also include situs inversus, suggesting that genetic alterations resulting in the expression of dysfunctional ciliary proteins may affect more than one type of cilium (Baker and Beales, 2009).

In part, this is likely due to the fact that the structure of cilia is conserved across different cell types and species. When viewed in cross-section, cilia can be divided into three regions: basal body, transition zone, and axoneme (**Figure 1B**). The basal body is the region at the base of the cilium, which bears the centriole from which the ciliary machinery arises. Here, the 9 triplet microtubules of the mother centriole are attached to the periciliary membrane by transition fibers. The body of the cilium, or axoneme, follows and contains 9 doublet microtubules. In motile cilia, the microtubules are arranged in a "9+2" arrangement where the 9 doublets surround a central pair of singlet microtubules. In contrast, immotile primary cilia lack the central pair with their "9+0" arrangement.

In both the motile and primary cilia, the proximal region of the axoneme is known as the transition zone, and was shown to contain Y-shaped structures linking the microtubules doublets to the ciliary membrane (Reiter et al., 2012). This region is thought to provide some sort of gating to control the movement of proteins and lipids in and out of the cilia, which would explain the unique profile in both the ciliary membrane and lumen. Most but not all primary cilia, and some motile cilia, also have an endocytic membrane domain called the ciliary pocket near the base of the cilium (Ghossoub et al., 2011).

Intriguingly, several zebrafish studies have indicated the importance of septins for the proper formation and function of cilia, where loss-of-function studies have generated several phenotypes that resemble human ciliopathies (Dash et al., 2014). Hence, an understanding of the role of septins in ciliogenesis at a cellular level is critical to our understanding of ciliopathies.

LOCALIZATION OF SEPTINS IN CILIA

Often the subcellular distribution of a protein can give insights into its function, so understanding septin localization in cilia is critical. Surprisingly, however, even though septins are involved in ciliogenesis in a variety of cell types and organisms, reports vary regarding their location within cilia.

Septins were first seen in a cilium-related structure when they were localized to the annulus of sperm flagella, a structure that has been suggested to separate the membrane compartments of

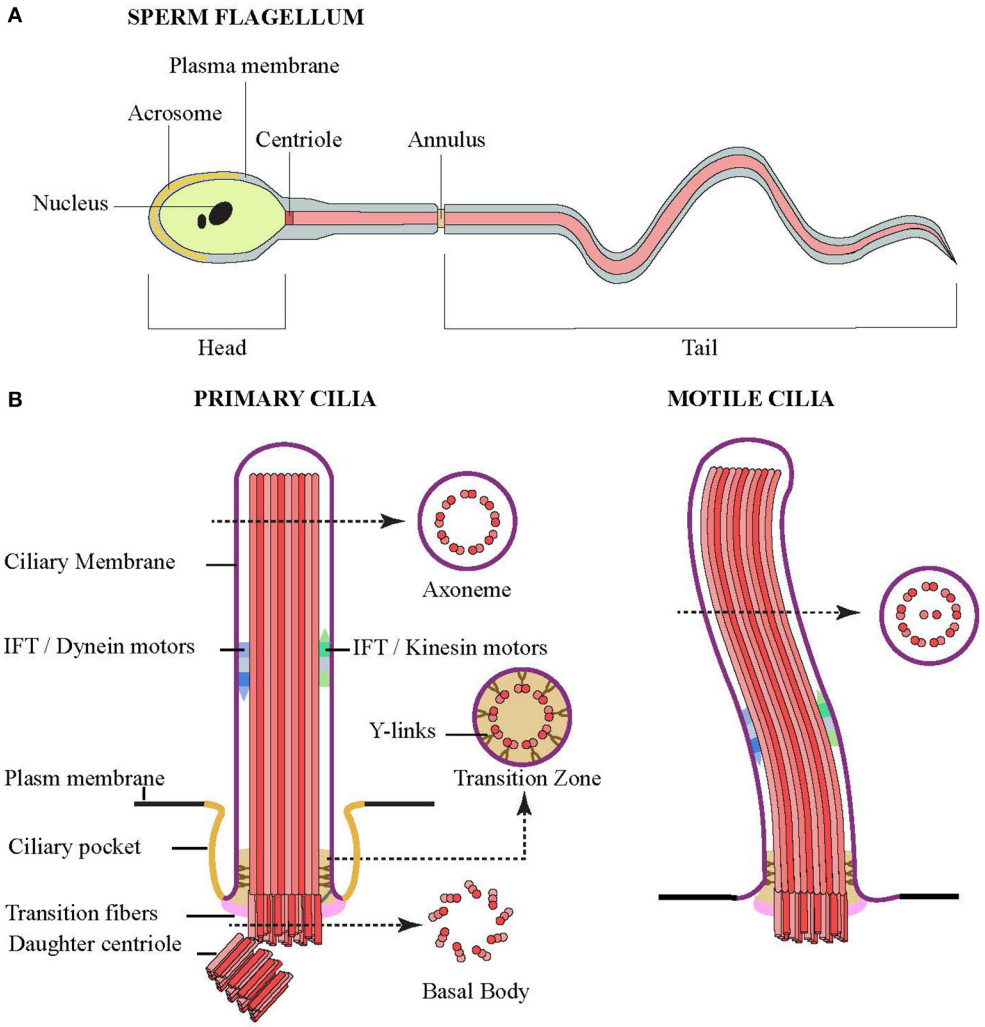

FIGURE 1 | Structures of flagella and cilia. (A) Schematic diagram showing the structure of sperm composed of head (and nucleus), middle piece (centriole and annulus) and tail. The middle piece and tail are the prominent structure of flagellum that compose of a core of microtubules, whose movement is powered by flagellar dynein. **(B)** Schematic side view of primary (left) and motile (right) cilia. Cross sections showing microtubule organization of the axoneme, transition zone and basal body are indicated by arrow.

the posterior and anterior tail regions (Cesario et al., 1995; Ihara et al., 2005; Kissel et al., 2005; Steels et al., 2007). Sperm from SEPT4-null mice exhibit a defective morphology where a clear annulus is missing, resulting in a fragile segment that causes the sperm's tail to have sharp bends, rendering the sperm immotile. A defective sperm annulus and the bent tail phenotype are also seen in sperm from infertile men with SEPT12 mutations and in SEPT12-mutant mice, confirming the importance of septins in sperm structural integrity and motility. In sperm from the SEPT4-null mice, basigin, a membrane protein that moves freely but is usually confined to anterior region of the tail, was found distributed all over the tail's plasma membrane, indicating that the compartmentalizing forces had been lost with the loss of the septin ring (Kwitny et al., 2010). The ring-like distribution is not always the only septin pattern in sperm, as later studies using human sperm showed septins, such as SEPT12, SEPT6 and SEPT2, localized from the centriole to the annulus (Kuo et al., 2015; **Figure 2A**).

The idea that septins might form a ring-like diffusion barrier in cilia was supported by the observation that SEPT2 could be seen as a ring at the base the primary cilia of mammalian cells (Hu et al., 2010) although some signal was reported along the axoneme (**Figure 2B**). In IMCD3 mouse kidney cells, the SEPT2 ring was localized to the transition zone and depletion of SEPT2 resulted in a significant shortening of the cilia. To test the function of SEPT2 at the transition zone, Nelson and colleagues designed GFP fusions of several ciliary transmembrane proteins, including serotonin receptor 6, Smoothened, and fibrocystin, and measured their fluorescence recovery after photobleaching (FRAP). Little exchange of these proteins between ciliary and

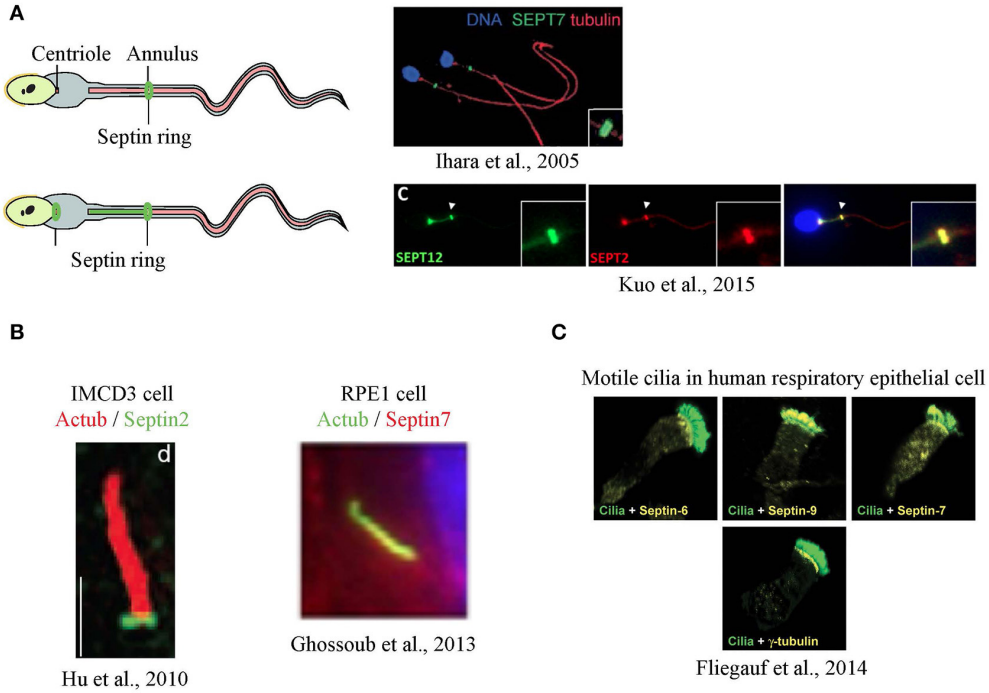

FIGURE 2 | Localization of septins in flagella and cilia. (A) Septins form a ring at the annulus of mouse sperm (Ihara et al., 2005), and a secondary signal was also at the centriole in humans (Kuo et al., 2015) **(B)** In primary cilia, septins were detected as a ring at transition zone (Hu et al., 2010) or along the axoneme (Ghossoub et al., 2013) **(C)** In airway epithelia, different septins are detected at the base, along the axoneme or both in motile cilia (Fliegauf et al., 2014). Images in this figure are reproduced with permission from the relevant publishers.

non-ciliary pools was observed in control cells, despite both pools being mobile (Hu et al., 2010). However, the short cilia that formed upon depleting SEPT2 showed an increased entry of ciliary transmembrane proteins suggesting loss of a diffusion barrier.

Similarly, SEPT2 and SEPT7 had a ring-like appearance at the base of motile cilia in *Xenopus* embryos, and knockdown resulted in fewer and shorter cilia (Kim et al., 2010). Septin localization at the base of motile cilia was controlled by Fritz, a protein involved in organizing polarized cilia growth (Collier et al., 2005). Polymorphisms in Fritz were found in patients of Bardet-Biedl and Meckel-Gruber syndromes, suggesting a possible link between septin regulation and certain ciliopathies (Kim et al., 2010). It should be noted that SEPT7 was also present along the axoneme and at the basal body of these cilia.

If a septin ring at the base of cilia formed the diffusion barrier, and this barrier was needed for proper ciliogenesis, then septins should always be present as rings at the base of cilia. However, septins have more consistently been localized along the axoneme of cilia in many cell types and animal models (Ghossoub et al., 2013; Dash et al., 2014; Fliegauf et al., 2014; Zhai et al., 2014). For example, using various human cells lines such as RPE1 (human retinal pigment epithelial cell line) and hFE (human inner foreskin), Benmerah and colleagues found SEPT2, SEPT7 and SEPT9 to be co-localized along the axoneme in primary cilia and to regulate ciliary length, yet no rings were reported. Transiently expressed fluorescent septin fusion proteins also localized to the axoneme and FRAP revealed that axonemal SEPT2 was not exchanged with cytoplasmic septins, raising the possibility that it might be a structural component of the axoneme. In addition, they observed similar axonemal staining *in vivo* including in human kidney tubular cells (Ghossoub et al., 2013). This axonemal distribution is also supported by evidence from proteomic and proximity ligation experiments (Ishikawa et al., 2012; Mick et al., 2015).

While not always located at sites where diffusion barriers would be predicted to exist, septins could be involved in diffusion barrier formation. Indeed, Peterson and colleagues identified a protein complex containing 9 proteins (including B9D1 and TMEM231) that localized to the transition zone of primary cilia (Chih et al., 2012). Generation of knockout mice lacking either B9D1 or TMEM231 caused increased diffusion into the cilium and impaired hedgehog signaling, suggesting that these proteins comprised the diffusion barrier. Interestingly, depletion of SEPT2 inhibited assembly and recruitment of the complex to the transition zone.

Surprisingly, recent studies have shown that different septins had distinct localizations in motile cilia of human respiratory epithelial cells (Fliegauf et al., 2014). Some septins were found both at the ciliary base and the axoneme (SEPT2, 7, 9), while some were only at the base (SEPT6, 8) or the axoneme (SEPT11) (**Figure 2C**). This raises the possibility that distinct

septin complexes could carry out different functions within cilia in the same cell.

In summary, the differential distribution of septins could arise in several ways. Septins at the base may accumulate there because they sense the micron-scale curvature of the ciliary membrane at this site (Bridges et al., 2016). Alternatively, the formation of ring-like structures of septins at the transition zone could be due to the concentration of specific lipids (Vieira et al., 2006) and/or other proteins at the base of cilia. With respect to septins in the axoneme of motile and primary cilia in various human cells, septins may play a structural role in the axonemal matrix (Ghossoub et al., 2013; Fliegauf et al., 2014). Since FRAP and live imaging of RPE1 cells showed no exchange between axonemal and cytoplasmic pools of septins, nor movement of septins within the axoneme, septins are not components of the intraflagellar transport system that moves proteins within cilia. However, septin accumulation in the axoneme could be due to the fact that septins directly bind microtubules (Bai et al., 2013) and the microtubules within the cilium are very stable. It is also important to consider that the apparently contradictory distributions of septins in cilia could be due to technical factors such cell type, specificity of antibody, fixation conditions, and maturation stage of the cilia. This could particularly be the case for staining at the base since many antibodies falsely stain centrosomes/basal bodies.

WHAT COULD BE THE FUNCTION OF SEPTINS IN PRIMARY CILIA?

The first function proposed for septins in cilia was as a diffusion barrier. Although septins are not always located as a ring at the base of cilia, several studies showed a loss of restricted diffusion into the remnant cilia following septin loss. In addition, loss of Hedgehog signaling following knockdown of septins was observed in Xenopus, Zebrafish and mammalian cells (Hu et al., 2010; Kim et al., 2010; Zhai et al., 2014). However, several cautions are required in considering these results. First, Hedgehog signaling depends on the presence and proper length of cilia, and since these have been impacted by loss of septins, so too may Hedgehog signaling. Second, the base of cilia has been shown to have a unique lipid composition (Vieira et al., 2006) that may itself contribute to restricted diffusion. It would be important to know if the absence of septins alters this lipid domain. Third, the remnant cilia that form in the absence of septins may be abnormal in many contexts including improper targeting of receptors to cilia or altered endocytosis. Changes in trafficking or recycling could affect the distribution of receptors in ways that resemble, but are distinct from, a simple passive restriction of lateral diffusion in the plane of the membrane (Trimble and Grinstein, 2015).

Depletion of septins consistently results in the loss or shortening of cilia and loss of ciliary function. A major consideration for all of these types of studies is whether the effect is due to the function of the septin directly at the cilium, or indirectly through one of its many effects on signaling pathways, membrane traffic or the cytoskeleton. For example, soluble tubulin levels are known to affect cilia length—more free tubulin results in longer cilia while less free tubulin leads to shorter cilia (Sharma et al., 2011). Septins have been shown to alter tubulin dynamics such that depletion of septins increases the numbers of stable (acetylated) microtubules (Kremer et al., 2005). This increased microtubule stabilization presumably reduces free tubulin levels globally in the cell, which could therefore be responsible for reduced cilia length. Similarly, since septins have widely accepted roles as scaffolding complexes, loss of signaling events normally occurring on septin filaments could impact signals required for cilia elongation. For example, the cilia and basal bodies of the SEPT7b morphants were also disoriented and resembled mutants in intraflagellar transport proteins (Jones et al., 2008; Cao et al., 2010). In Xenopus embryos, SEPT7 functions together with the planar cell polarity (PCP) protein Fritz to regulate ciliogenesis (Kim et al., 2010). Therefore, it is possible the role of SEPT7 in ciliogenesis is linked to the PCP pathway via Fritz (Park et al., 2015).

The function of PCP pathway in ciliogenesis is regulated by the exocyst complex, where the PCP protein Disheveled mediates recruitment of a sec8-positive vesicle to the basal body (Park et al., 2008). Septins also interact and regulate the exocyst complex by directing it to the correct location and affect the activity of SNARE proteins important in membrane fusion (Beites et al., 1999, 2005; Amin et al., 2008; Estey et al., 2010), suggesting that changes in membrane traffic caused by septins could also impact cilia growth and function. As well, SEPT7 was shown to interact with Rab8 (Dash et al., 2014), a small GTPase that functions in vesicle trafficking and ciliogenesis (Nachury et al., 2007; Yoshimura et al., 2007). Septins could also affect vesicle traffic via the cytoskeleton since they associate with microtubules and guide the direction of microtubule growth (Bowen et al., 2011; Nölke et al., 2016) and may control vesicular transport along them (Spiliotis et al., 2008).

CONCLUSIONS

In sum, despite ample evidence linking septin function to ciliogenesis, there remain more questions than answers about their roles in this process. Since septin depletion *in vivo* gives phenotypes implicating nodal, motile and primary cilia, the function of septins in ciliogenesis likely involves something conserved among all cilia. Future work will be needed to decipher the contributions of septins to ciliogenesis, and to examine their possible contribution to ciliopathies.

AUTHOR CONTRIBUTIONS

OP, ME, and WT wrote the manuscript. OP and ME contributed equally to this work.

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Functional Redundancy of Septin Homologs in Dendritic Branching

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Septins are cytoskeletal GTPases present in nonpolar heteromeric complexes that assemble in a palindromic fashion from two to eight subunits. Mammalian septins function in several fundamental cellular processes at the membrane-cytoskeleton interface including dendritic branching in neurons. Sequence homology divides the 13 mammalian septin genes into four homology groups. Experimental findings suggest that septin function is redundant among septins from one homology group. This is best understood for the isoforms of the SEPT2 group, which form a homodimer at the center of septin complexes. *In vitro*, all SEPT2-group septins form recombinant hexameric complexes with two copies of SEPT6 and SEPT7. However, it remains unclear to what extent homologs septins can substitute for each other in specific cellular processes. Here, we use the experimental paradigm of dendritic branching in hippocampal rat neurons to ask, to what extent septins of the SEPT2-group are functionally redundant. Dendritic branching is significantly reduced when SEPT5 is downregulated. In neurons expressing SEPT5-shRNA, simultaneously expressed SEPT2-GFP, and SEPT4-GFP colocalize with SEPT7 at dendritic spine necks and rescue dendritic branching. In contrast, SEPT1-GFP is diffusely distributed in the cytoplasm in SEPT5 downregulated neurons and cannot rescue dendritic branching. Our findings provide a basis for the study of septin-specific functions in cells.

Keywords: septin, dendritic spine, neuron

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INTRODUCTION

The septins are a family of conserved GTPases present in all eukaryotes except land plants and have first been described as cell division cycle (cdc) mutants in yeast (Hartwell, 1971). Mammals express at least 13 different septin genes and differential splicing yields even more polypeptides. Sequence homology analysis classifies septins into four groups: the septin2 group (SEPT1, 2, 4, and 5), the septin3 group (SEPT3, 9, and 12), the septin6 group (SEPT6, 8, 10, 11, and 14; Macara et al., 2002; Pan et al., 2007). SEPT7 forms a homology group in itself and is considered to be essential for the assembly of complexes into filaments (Kinoshita, 2003). Human septins are not expressed at the same levels in all tissues suggesting that individual septins are required for distinct tissue-specific functions (Hall et al., 2005; Peterson and Petty, 2010). However, the function of many septins may be redundant to a certain extent, since knockout mice of SEPT3, 4, 5, and 6 show no detectable or very specific defects (Peng et al., 2002; Ono et al., 2005; Fujishima et al., 2007; Tsang et al., 2008). The knockout of certain septin genes induced an upregulation in protein expression levels of others in the same homology group. These findings suggest a possible functional redundancy between septin genes (Peng et al., 2002). SEPT7 and SEPT9 on the other hand seem to be essential since the respective knockouts are embryonic lethal in mice (Füchtbauer et al., 2011; Menon et al., 2014).

The crystal structure of the heterohexameric human septin complex consists of two SEPT2, SEPT6, and SEPT7 molecules, respectively. The septin complex shows a palindromic arrangement of septin molecules with alternating interactions between their GTPase domains and their combined N-and C-termini. In this complex, two SEPT2 subunits (septin2 group) occupy the center of the complex forming a flexible hinge (Sirajuddin et al., 2007). Two SEPT6 subunits (septin6 group) link the SEPT2 dimer to the SEPT7 molecules at both ends of the complex. SEPT3 group septins can bind to SEPT7 and therefore the human septin protofilmanent exists in heterohexamers and heterooctamers (Kim et al., 2011; Sellin et al., 2011, 2014), however no crystal of the heterooctameric complex exists so far.

SEPT7 is essential for the stability of septin complex heterohexamers and heterooctamers (Sellin et al., 2011). The other septins from the same homology group seem to be incorporated into the complex in a modular fashion (Kinoshita, 2003; Sellin et al., 2011, 2014). For instance, *in vitro* co-expression experiments show that all members of the septin2 homology group assemble into complexes with SEPT6 and SEPT7 in SEPTX/6/7 complexes with a stoichiometry of 2:2:2 *in vitro* (with X standing for SEPT1, SEPT2, SEPT4, or SEPT5; Kinoshita, 2003). While for SEPT1 and SEPT2, no knockout animal has been reported, the SEPT4 and SEPT5 knockout mice yielded highly specific phenotypes, such as male sterility for SEPT4 (Ihara et al., 2005) and problems in synapse maturation (Yang et al., 2010) and a complex behavioral phenotype (Suzuki et al., 2009) for SEPT5. Likewise, in the SEPT5 knockout, the expression of SEPT2, another member of the same subgroup, is up-regulated 3-fold at the protein level (Peng et al., 2002). Nevertheless, there is neither direct evidence that individual septins can substitute for other members of the same group in functional complexes in cells nor that septin complex function strictly depends on the exact subunit composition.

Here we asked whether a single septin could be functionally replaced by a different member of the same homology group in dendritic branching of hippocampal neurons. In neurons the SEPT5/SEPT7/SEPT11 complex is required for the development of a complex branched dendritic morphology (Tada et al., 2007; Garcia et al., 2011). This complex localizes to dendritic branch points and contains with SEPT5, SEPT11 and SEPT7 one member of the septin2 homology group, the septin6 group and SEPT7. The other septin2 group homology members, SEPT1, SEPT2 and SEPT4 are absent from this complex (Tsang et al., 2011).

We find that when SEPT5 is downregulated by RNA-interference, simultaneously expressed GFP-tagged SEPT2 or SEPT4 localizes to dendritic branching points and spine necks. Both, SEPT2-GFP and SEPT4-GFP, colocalize with endogenous SEPT7 and exchange at low rates when photobleached, suggesting successful incorporation into septin complexes. Furthermore, reduced dendritic complexity after SEPT5 downregulation is rescued by simultaneous expression of SEPT2-GFP or SEPT4-GFP. In contrast, SEPT1-GFP is unable to rescue dendritic branching in neurons expressing SEPT5 shRNA. In agreement with this finding, SEPT1 does not colocalize with SEPT7 in neuronal septin structures when SEPT5 protein levels are downregulated and both SEPT1-GFP and SEPT7 are absent from dendritic spine necks in such neurons. Instead, SEPT1-GFP is mobile in the cytoplasm.

In conclusion, we show that SEPT2-GFP and SEPT4-GFP localize to and incorporate into neuronal septin complexes and rescue SEPT5 function in neuronal dendritic morphology. In contrast, SEPT1-GFP shows a different phenotype in neurons and cannot rescue the loss of function of SEPT5. Our results provide evidence for a functional difference between individual septin2 group members in hippocampal rat neurons and an experimental model to further investigate isoform-specific functions of septins.

RESULTS

SEPT2-GFP is Recruited to the Dendritic Spine Septin Complex after Downregulation of SEPT5 in Hippocampal Rat Neurons

In cultured rat hippocampal neurons, a complex consisting of SEPT5, SEPT7, and SEPT11 has been reported to co-immunoprecipitate and to localize to dendritic branching points (Tada et al., 2007; Garcia et al., 2011). In a first experiment, we confirmed the colocalization of endogenous SEPT5, SEPT7, and SEPT11 in our primary rat hippocampal neuron cultures by immunofluorescence staining (**Figure 1A**).

We here hypothesized that other members of the septin2 group might replace SEPT5 in its localization and function. The scheme in **Figure 1B** illustrates our experimental setting on the basis of the crystal structure of the septin complex (Sirajuddin et al., 2007). We aim to downregulate SEPT5 from the functional dendritic spine septin complex by RNA interference. We hypothesized that if we then exogenously expressed individual other septin2 homology group members, SEPT1, SEPT2, and SEPT4 might be able to reconstitute a recombinant complex together with the native SEPT11 and SEPT7 *in vivo*.

To realize this experiment, we cotransfected a vector to knock down SEPT5 by RNA interference with a vector to exogenously express SEPT1, SEPT2, or SEPT4 coupled to GFP, respectively, in hippocampal rat neurons cultured for 7 days *in vitro* (DIV 7). In addition to these two constructs, we cotransfected mRFP as a fluorescent marker to visualize neuronal morphology and neurons were grown for 5 more days to ensure proper downregulation of SEPT5 as done before (Tada et al., 2007; Ewers et al., 2014).

First, we verified the downregulation of SEPT5 by immunostaining the cotransfected neurons against SEPT5 and compared the fluorescence signal intensity to non-transfected neighboring neurons. Cells transfected with shRNA against SEPT5 showed significant reduction in fluorescence intensity levels when compared to control cells (**Supplementary Figure 1**). We quantified the downregulation by calculating the fluorescence intensity (FI) ratio between FI values measured in transfected over neighboring non-transfected neurons (**Supplementary Figure 3A**).

As a proof of concept, we assessed the localization of expressed SEPT2-GFP in these cells. SEPT2-GFP localized to the base of dendritic filopodia in an arc-shaped form (**Figure 1C**), similar

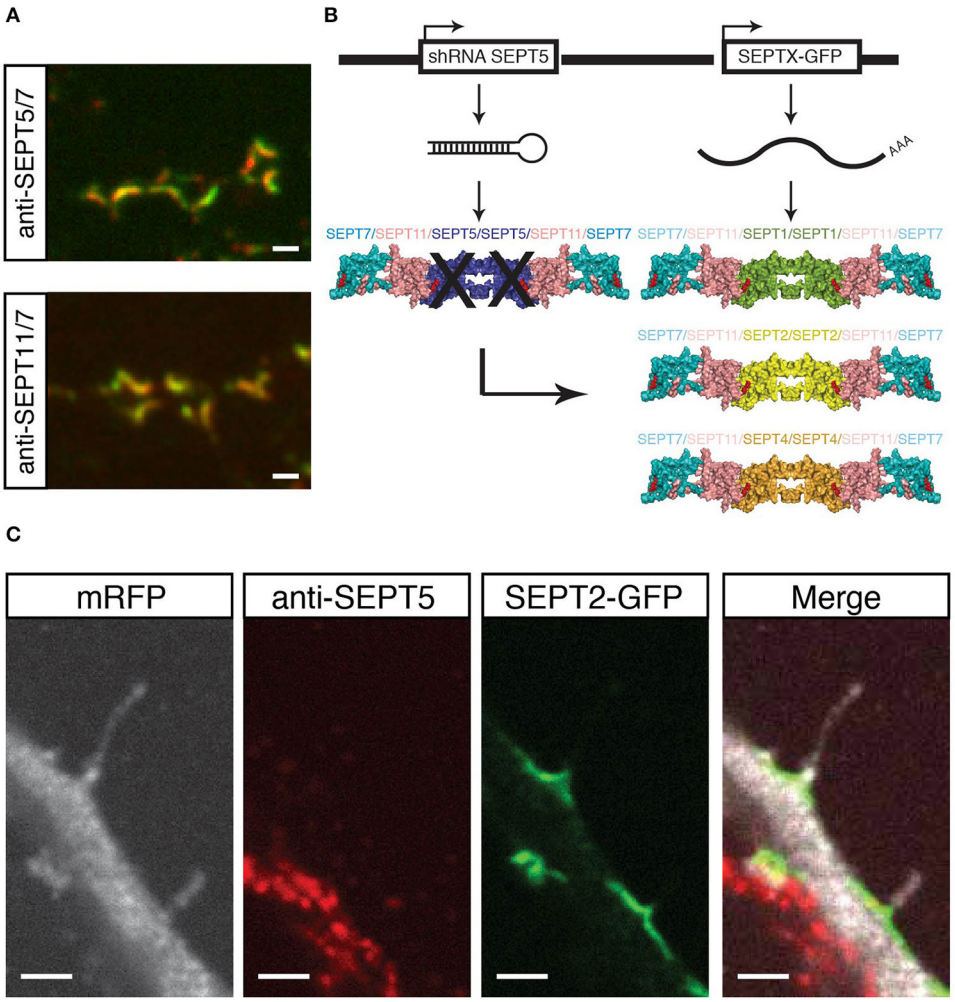

FIGURE 1 | Experimental setup and proof of principle. (A) Coimmunostaining of SEPT5 (green) and SEPT7 (red, top) or SEPT11 (green) and SEPT7 (red, bottom) respectively in hippocampal rat neuron cultured *in vitro* and fixed on DIV 21. Scale bar is 1 μm. **(B)** Experimental setup for septin replacement assay. We cotransfected two vectors into cultured hippocampal neurons at DIV 7. One vector bears a shRNA against SEPT5 to knock-down SEPT5 expression, the other expresses a GFP-tagged version of another SEPT2-group septin. The expected results are different recombinant septin complexes in targeted cells. **(C)** Fluorescence micrograph of a neuron cotransfected with a shRNA against SEPT5, a plasmid encoding mRFP as a marker (white) and a plasmid encoding SEPT2-GFP (green). After 5 days of expression, cells were fixed, and immunostained against endogenous SEPT5 (red). Scale bar is 2 μm.

to SEPT5, SEPT7, and SEPT11 in control cells. At the same time, the same transfected cell does not show a significant SEPT5 fluorescence signal by immunostaining above background level, while the neighboring cell shows a strong SEPT5 fluorescence signal (**Figure 1C**). In combination with the quantification of the FI signal this demonstrates that we were able to downregulate SEPT5 in hippocampal rat neurons and to exogenously expressed SEPT2, another member of the septin2 group, simultaneously.

SEPT2-GFP and SEPT4-GFP, but not SEPT1-GFP Localize with Endogenous SEPT7 at Dendritic Spine Necks and Branches

We next asked, whether generally the exogenous expression of septin2 homology group members in neurons downregulated for SEPT5 resulted in interactions only between the exogenous septins or also with other endogenous septins. To address this question we assessed whether the GFP-fusions of SEPT1, SEPT2, and SEPT4 colocalized with the core member SEPT7 in the arc-shaped structures formed at dendritic spine necks and performed immunofluorescence staining of endogenous SEPT7 in cotransfected neurons.

We observed that both SEPT2-GFP and SEPT4-GFP localized to the neck of dendritic spines and branching points (**Figure 2A**). Moreover, a quantitative analysis of the overlap between the septin2 group member's GFP signal and the SEPT7 immunofluorescence signal confirmed that SEPT2-GFP or SEPT4-GFP localize to structures also containing SEPT7 (**Figure 2B**). In contrast, the SEPT1-GFP fluorescence signal intensity was distributed homogenously over the entire neuron downregulated for SEPT5. Accordingly, we conclude that

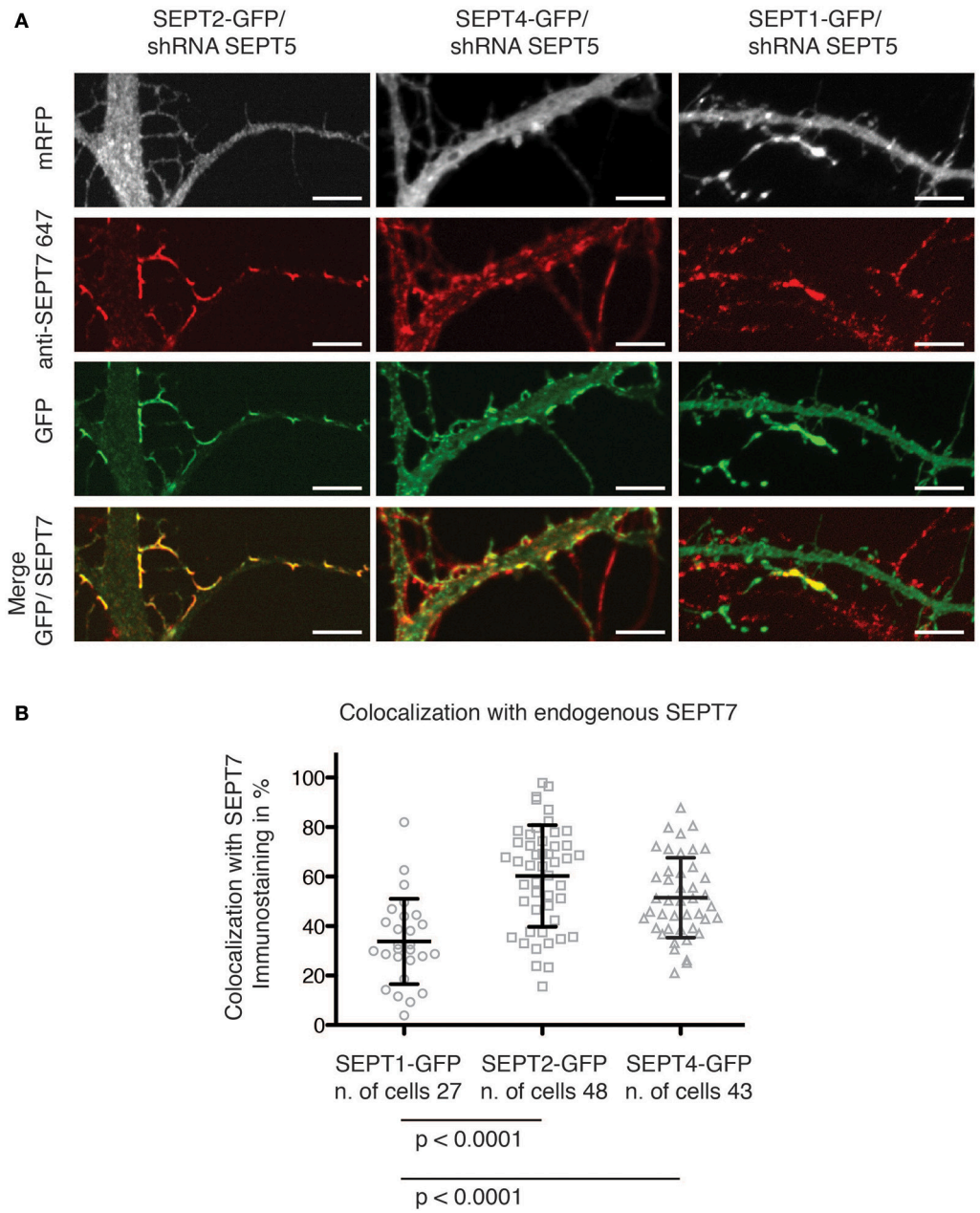

FIGURE 2 | Colocalization of GFP-tagged SEPT1, SEPT2, and SEPT4 with endogenous SEPT7. (A) Fluorescence micrographs of dendrites from neurons in which SEPT5 expression was downregulated by shRNA and that express SEPT1-GFP, SEPT2-GFP, and SEPT4-GFP (green) with immunofluorescence staining against endogenous SEPT7 (red). Note the absence of SEPT7 staining from the SEPT1-GFP expressing cell. Scale bars are 1 μm. **(B)** Quantification of the overlap between the fluorescence signals of SEPT7 with SEPT1-GFP (circles), SEPT2-GFP (squares), or SEPT4-GFP (triangles). The percentage of overlap of SEPT1-GFP/SEPT7-AF 647 ($n = 27$ cells) is significantly smaller than that of SEPT2-GFP/SEPT7-AF 647 ($n = 48$ cells, unpaired Mann-Whitney test $p < 0.0001$) and of SEPT4/SEPT7-AF 647 ($n = 43$ cells, unpaired Mann-Whitney test $p < 0.0001$). A Kruskal-Wallis test on the whole dataset resulted in $p < 0.0001$.

SEPT1-GFP did not concentrate at dendritic spine necks or branches.

Interestingly, when we quantified the immunofluorescence staining against endogenous SEPT7 in SEPT5-shRNA transfected cells, we observed a significant decrease in the FI levels of SEPT7 in cells depleted of SEPT5 (**Supplementary Figure 3B**). This is in accordance with published data on SEPT5 knockout mice that showed a clear reduction of SEPT7 protein levels in the brain of these mice (Peng et al., 2002). Remarkably, on the single cell level, we were able to show that expression of SEPT2-GFP and SEPT4-GFP rescues the FI signal of SEPT7 in SEPR5-shRNA treated cells (**Supplementary Figures 2**, **3B**), while the expression of SEPT1-GFP does not.

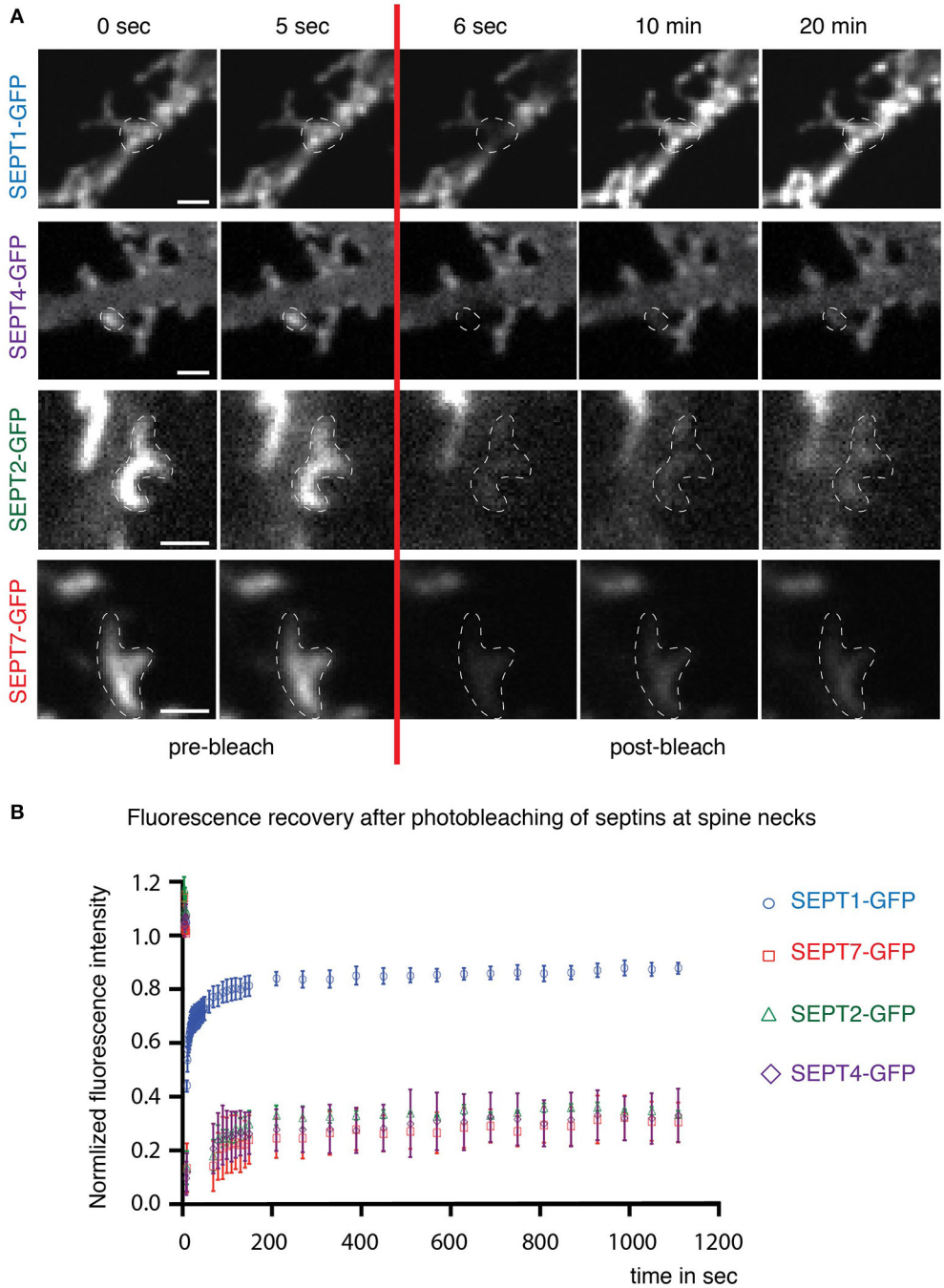

FIGURE 3 | Dynamics of septin2 group homologs and SEPT7 at dendritic spine necks. (A) Fluorescence recovery after photobleaching (FRAP) time course experiment on septin structures at the base of dendritic spines. Two images before (0 s; 5 s) and three after (6 s; 10 min; 20 min) the bleaching pulse are shown. A broken white line emphasizes the photobleached and quantified area. Note the homogenous distribution of SEPT1-GFP and the discrete arc-shaped structures at the base of spines formed by SEPT2-GFP, SEPT4-GFP, and SEPT7-GFP. Scale bars are 1 μm. **(B)** Quantification of FRAP experiments performed in live cells at DIV 7 + 7 to DIV 7 + 9. The recovery of the fluorescence signal is plotted against the time using the mean value and the standard error of the mean from three independent experiments. Total number of septin structures from all three experiments: SEPT2-GFP, green diamonds: $n = 30$, SEPT1-GFP, blue circles: $n = 33$, SEPT4-GFP, purple triangles: $n = 33$, SEPT7-GFP, red squares: $n = 34$.

The septin2 homology group members SEPT1, SEPT2, and SEPT4 are not found in rat neuron septin complexes containing SEPT5 at detectable levels (Tsang et al., 2011). Nevertheless, in our experiments, exogenous expressed SEPT4-GFP as well as SEPT2-GFP localized together with endogenous SEPT7 in higher-order structures at dendritic spine necks and branching

points. In addition, SEPT2-GFP and SEPT4-GFP expression remarkably restored SEPT7 fluorescence intensity levels. Our data demonstrate that some but not all septin2 homology group members can replace SEPT5 in neuronal septin complexes.

SEPT2-GFP and SEPT4-GFP, but not SEPT1-GFP Assembles in a Stable Complex at Dendritic Spine Necks and Filopodia

We have shown before by fluorescence recovery after photobleaching (FRAP) that septins form stable, immobile higher-order structures at spine necks (Ewers et al., 2014). To examine whether the septin2 homologs were incorporated in such stable higher-order structures in hippocampal rat neurons after SEPT5 knockdown, we performed FRAP experiments of overexpressed SEPTX-GFP fusion proteins at dendritic spine necks.

SEPT1-GFP fluorescence (**Figure 3A**, first row) recovered quickly at dendritic spine necks (**Figure 3B**) with a mobile fraction of about 80%, consistent with a cytoplasmic localization. In contrast, only a small fraction of SEPT2-GFP and SEPT4-GFP in SEPT5 downregulated neurons exchanged slowly between the septin higher-order structures located at the base of spines or filopodia in hippocampal rat neurons and the soluble pool in the cytoplasm (**Figure 3A**, second and third row and **Figure 3B**). This slow recovery agrees with the behavior of SEPT7-GFP in neurons with endogenous SEPT5 expression levels (**Figure 3A**, fourth row and **Figure 3B**). Consequently, we conclude that SEPT2-GFP, SEPT4-GFP, and SEPT7-GFP are stably integrated into the septin structure at the base of dendritic spines.

SEPT2-GFP and SEPT4-GFP, but not SEPT1-GFP Rescues Dendritic Branching in Hippocampal Rat Neurons

A functional septin complex is required for proper dendritic branching in hippocampal neurons (Tada et al., 2007) since the knockdown of either SEPT5 or SEPT7 leads to reduced dendritic arbor complexity. Simultaneously, the downregulation of either SEPT5 or SEPT7 also caused a reduction in the expression level of the respective other. We thus hypothesized, that SEPT1-GFP, which is unable to rescue septin complex localization, might not be able to rescue the dendritic branching defect caused by SEPT5 downregulation, while SEPT2-GFP and SEPT4-GFP might. To test this hypothesis, we cotransfected either SEPT1-GFP, SEPT2-GFP, or SEPT4-GFP with shRNA against SEPT5 and mRFP as a tracer at DIV7 and fixed the neurons after 5 days of combined SEPT5 knockdown and SEPTX-GFP expression. Images of the entire neuron including its dendrites were acquired and the complexity of dendrite branching was analyzed by Sholl analysis (Sholl, 1953). Consistent with previous results, dendritic complexity was strongly reduced in neurons downregulated for SEPT7 and SEPT5 (**Figures 4A,B**, *p*-values in **Supplementary Table 2**). Exogenous expression of either SEPT4-GFP or SEPT2-GFP in these cells rescued the dendritic branching morphology (**Figures 4A,C**, *p*-values in **Supplementary Table 2**). In line with the FRAP results and colocalization experiments, SEPT1-GFP on the other hand was not able to restore dendritic branching complexity in cells treated with shRNA against SEPT5. We conclude that the function of SEPT5 in dendritic branching is not redundant between all septin2-group members and can be rescued by SEPT2 and SEPT4 only.

DISCUSSION

Septin complexes are assembled from several different septins from the four groups classified by sequence homology in dependence on their tissue-specific expression. In this study, we investigated the hypothesis that septins belonging to the same homology group can substitute for each other in the septin complex and in cellular function.

Several *in vitro* and *in vivo* findings are supportive of this notion. Recombinant expression of SEPT6 and SEPT7 together with members of the septin2 homology group in insect cells showed that each member of this homology group can assemble into a complex with SEPT6 and SEPT7 (Kinoshita, 2003). Furthermore, the relatively mild phenotypes of the knock-out of septin2 group members SEPT4 and SEPT5 in mice suggest a functional redundancy of homologs within the same septin group. For instance, in knock-out mice of SEPT5, SEPT2 from the same homology group showed increased expression levels (Peng et al., 2002). However, evidence for a functional redundancy of septins from the same homology group on the cellular level is lacking. Hence, we choose to investigate whether other homologs from group 2 can substitute for SEPT5 in its specific function in dendritic branching. In hippocampal neurons, SEPT5 colocalizes at dendritic branching points and at the neck of spines with SEPT11 and SEPT7 (Tada et al., 2007; Garcia et al., 2011). SEPT5 knockdown by shRNA reduces the complexity of the dendritic arbor (Tada et al., 2007; Garcia et al., 2011). SEPT5 is the only member exclusively expressed at high levels in hippocampal rat neurons (Tsang et al., 2011), rendering it an optimal system to study septin-specific function. In our experimental set up, we thus downregulated SEPT5 by shRNA and simultaneously expressed septin2 group members SEPT1-GFP, SEPT2-GFP, or SEPT4-GFP to investigate, whether they would rescue SEPT5 function in dendritic branching.

When we exogenously expressed SEPT2-GFP and SEPT4-GFP in SEPT5 downregulated neurons, both colocalized with endogenous SEPT7 at dendritic branching points and dendritic spine necks. In contrast, SEPT1-GFP showed no significant colocalization with SEPT7. The integration of exogenously expressed septins into native complexes has been demonstrated before. In K562 cells, the expression of exogenous septins close to the endogenous level leads to their incorporation into septin complexes and eventually to replacement of the endogenous version in these (Sellin et al., 2011). We chemically fixed the neurons after 5 days of knockdown of endogenous SEPT5 and overexpression of another group 2 homology member. At this time, exogenous SEPT2-GFP and SEPT4-GFP both were found in discrete localizations where they quantitatively colocalized with endogenous SEPT7 and endogenous SEPT7 colocalized

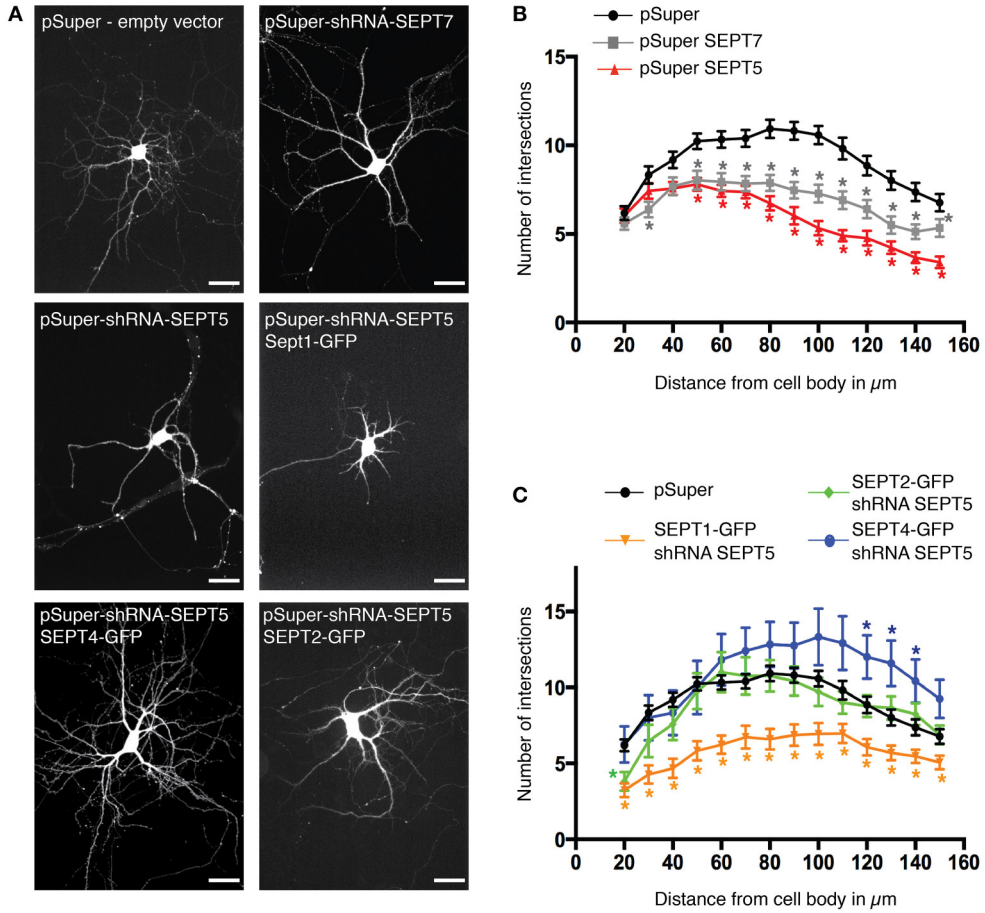

FIGURE 4 | Rescue of SEPT5 function in dendritic branching. (A) Fluorescence micrographs of hippocampal neurons coexpressing mRFP as a marker for cell morphology with shRNA constructs and constructs expressing GFP-tagged septins. Shown is the mRFP channel in grayscale to visualize the cell outline. Scale bars are 50 μm. **(B)** Quantification of dendritic branching by Sholl analysis in control cells and after shRNA-mediated knockdown of SEPT5 or SEPT7 expression. The branching complexity is significantly reduced in neurons transfected with shRNA against SEPT5 ($n = 30$ cells, red triangle) or SEPT7 ($n = 32$ cells, gray square) when compared to control cells ($n = 43$ cells, black circle). Significance difference between the control cells and the knock down of SEPT5 or SEPT7 is highlighted by an asterisk next to the data point in the respective color code when the $p < 0.02$ (p-values from a multiple t-test in **Supplementary Table 2**). **(C)** Quantification of dendritic branching by Sholl analysis in control cells and after shRNA-mediated knockdown of SEPT5 expression with simultaneous expression of SEPT1-GFP ($n = 30$ cells, yellow triangles), Sept2-GFP ($n = 27$ cells, green diamonds), or SEPT4-GFP ($n = 12$ cells, blue circles). Significance difference between the control cells and the three different conditions of SEPT5 knock down and simultaneous expression of another SEPT2 group member is highlighted by an asterisk next to the data point in the respective color code when the $p < 0.02$ (p-values from a multiple t-test are listed in **Supplementary Table 2**).

quantitatively with them. We thus conclude that SEPT2-GFP and SEPT4-GFP are incorporated into endogenous septin complexes.

In the same study, Sellin and coworkers showed when the core unit consisting of SEPT2 and/or SEPT5 was depleted in K562 cells they found a significant decrease in heteromer assembly of SEPT7, SEPT9, and septin6 group members by ultracentrifugation analysis. This finding suggests that the core unit of septin2 group members is important to nucleate septin complex assembly. In our study we did not detect a strong colocalization of SEPT1-GFP with SEPT7 and SEPT1-GFP did not localize with SEPT7 to dendritic spine necks and branching points. In addition, immunolabeling of SEPT7 in neurons in which SEPT1-GFP substitutes SEPT5 showed a decrease in the intensity of the SEPT7 fluorescence signal. This suggests that SEPT1-GFP was not able to nucleate septin complex formation in hippocampal rat neurons, whereas SEPT2-GFP and SEPT4-GFP were able to induce proper complex formation and as a result, proper localization.

SEPT7 stability in cells requires the presence of septin6 and septin2 group homology members and vice versa. SEPT7 by itself is insoluble when expressed recombinantly in bacteria or eukaryotic cells (Kinoshita, 2003). At the same time, SEPT7 binding to SEPT2 and SEPT6 containing heteromers contributes to their stability. Hence, we can conclude that SEPT7 colocalization with SEPT2-GFP and SEPT4-GFP results from productive complex assembly. In addition, the GFP-tag seemingly does not interfere with the function of the septin2 core members to initiate septin complex assembly. Taken together

it seems that the cause of the failure of SEPT1-GFP to rescue dendritic branching lies in its failure to recruit functional septin complexes to dendritic branching points and possibly even failure to incorporate into functional septin complexes. This stands in contrast to biochemical experiments demonstrating assembly of SEPT1/SEPT6/SEPT7 complexes and thus that SEPT1 homodimers can exist at the core of a stable septin complex. In neuronal complexes, SEPT11 is prominent as a SEPT6 group member linking the septin2 group members and SEPT7. A failure of SEPT1-GFP to form functional complexes could thus be due to problems with the interaction of the SEPT1 GTPase interface with the septin6 group member SEPT11 in neuronal septin complexes. A biochemical interactome study of septins found all septin2 group members except SEPT2 to interact with SEPT11 (Nakahira et al., 2010), but detailed biochemical studies involving SEPT1 are lacking.

On the other hand, it is clear that septin2 group members bear different posttranslational modifications. The serine/threonine kinase Aurora-B for example phosphorylates SEPT1 *in vitro* and colocalizes with SEPT1 at the midbody during cytokinesis (Qi et al., 2005). SEPT1 knock-down in mouse oocytes causes spindle defects and impaired chromosome congression as well (Zhu et al., 2011) suggesting a functional connection between SEPT1 and AuroraB. SEPT1 contains three serines (Ser248, Ser307, and Ser315) phosphorylated by AuroraB (Qi et al., 2005) that in other members of the septin2 homology group are missing. While in non-mitotic hippocampal neurons, this very mechanism will likely not play a role, unknown regulatory mechanisms based on posttranslational modifications may allow SEPT2-GFP and SEPT4-GFP to form complexes with SEPT11 and SEPT7 at dendritic spine necks, but not SEPT1-GFP.

In our study, we used the longest available splice isoforms of SEPT1, SEPT2, and SEPT4, respectively (**Supplementary Figure 4**). While in rats only one SEPT2 peptide is expressed, for other SEPT2 group members, especially SEPT4, multiple known and predicted splice isoforms exist (**Supplementary Table 3**). Our conclusions are thus limited to the specific isoforms of each homolog that we expressed in the SEPT5 downregulated neurons. These isoforms contain all canonical septin features, such as the N-terminal domain with the polybasic region, the GTPase domain, the septin unique element and the C-terminus with the coiled-coil domain (**Supplementary Figure 4**) and very little difference in their amino acid sequence length. All the more this suggests that the functional difference between the SEPT1 isoform and SEPT5, SEPT2, and SEPT4 isoforms used here, lies rather not in specific domains, but on the level of the specific amino acid sequence of different isoforms.

Our data suggest that significant basic functions can be redundant between septin2 group homologues. SEPT2 and the longest isoform of SEPT4, respectively, instead of SEPT5, rescued a complex cellular process such as dendritic branching. In this particular function, some septins may rather play a structural than a functional role, which would be executed on the level of the complex or by a different septin within it instead. SEPT1 could not rescue dendritic arborization and could not rescue SEPT7 expression. This may be due to a requirement of a specific SEPT6 group septin as a "bridge" to SEPT7 to form complexes that is not expressed in neurons. It may also point to a function of SEPT1 outside of the septin complex.

Septin biology with 13 genes in mammals and many more splice isoforms that assemble in a modular fashion into functional complexes is very difficult to tackle experimentally. The discovery and investigation of isoform specific functions using established experimental paradigms promises to be an important tool to further our understanding of cellular functions of septins in health and disease.

MATERIALS AND METHODS

Cloning of Septin Constructs

The plasmids pSuper-control (scrambled shRNA), pSuper-Sept5, pSuper-Sept7, pßactin-Sept7-GFP, and pßactin-Sept2-GFP were kind gifts from the laboratory of Morgan Sheng. The cloning as well as the design of the shRNA are described in Tada et al. (2007). The cDNAs encoding for *Rattus norvegicus* SEPT1 and SEPT4 originate from the mammalian gene collection (SEPT1: MGC109124; SEPT4: MGC156532). Further detailed information about the SEPT2 group isoforms used in this study is listed in **Supplementary Table 3**. The plasmids used in this publication were generated by inserting Sept1 or Sept4 into pßactin-GFP using BsrgI and ClaI sites. All plasmids used in this study are listed in **Supplementary Table 1**.

Hippocampal Culture, Transfection, and Immunocytochemistry

Humane killing for preparation of rat primary hippocampal neurons conformed to local King's College London ethical approval under the UK Supplementary Code of Practice, The Humane Killing of Animals under Schedule 1 to the Animals (Scientific Procedures) Act 1986, or in accordance with the guidelines issued by the Swiss Federal Act on Animal Protection, respectively. All efforts were made to minimize animal suffering and to reduce the number of animals used. The preparation and cultivation of primary hippocampal neurons from E18 Sprague-Dawley rats was performed as described previously (Kaech and Banker, 2006): rat embryos removed from a time-mated sacrificed rat were placed in ice-cold HBBS and decapitated. Brains were transferred into a new dish of ice-cold HBSS, cerebral hemispheres were removed and freed from their meninges. The hippocampi were dissected and collected in ice-cold HBSS. Hippocampi were then incubated in trypsin solution (Life Technologies) prewarmed to 37°C for 15 min. Singularization of neurons was performed by carefully pipetting up and down in a 1000 µl pipette tip. Neurons were plated on polylysine pre-coated coverslips with a density of 2–3 $\times$ 10^5 cells/dish and cultured in neurobasal medium supplemented with B-27 and GlutaMax (all from Life-Technologies) at 37°C in 5% CO_2. After 3 days *in vitro* (DIV) Cytarabine was added with a concentration of 5 µM to inhibit cell growth.

Depending on their developmental stage neurons were transfected using Lipofectamin 2000 (Life Technologies, cat. no. 11668-027) between DIV 7 or DIV 8. For the control experiment and septin downregulation the following amounts

of DNA were transfected per 6 cm dish: pSuper-control: 0.5 μg, pSuper-SEPT5: 0.5 μg, pSuper-SEPT7: 0.9 μg; mRFP was transfected as a marker to identify transfected cells in the same respective amounts. The concentration of the simultaneously cotransfected SEPTX-GFP constructs (0.4–0.8 μg plasmid) was adjusted such that no overexpression artifacts were observed and localization profiles looked similar to that of endogenous septins.

Transfected neurons were cultivated further for at least 5 days. Fixation was performed for 10 min at room temperature in 4% paraformaldehyde and 2% sucrose in PBS (pH 7.4). Cells were washed for 15 min in PBS containing 50 mM NH_4Cl and treated for 5 min with 0.25% Triton X-100. Subsequently, cells were blocked with 4% goat serum, 1% bovine serum albumin (BSA) and 0.002% NaN_3 in PBS for 45 min. Primary antibody treatment was done for 2 h at room temperature (RT) or overnight at 4°C using either rabbit anti-SEPT7 (1:1000, kind gift from M. Kinoshita, Nagoya University, Japan), guinea-pig anti SEPT7 (Tada et al., 2007), rabbit anti-SEPT5 (1:200, kind gift from Bill Trimble, University of Toronto, Canada) or rabbit anti-SEPT11 (kind gift from Barbara Zieger, University of Freiburg). Secondary antibody staining was performed with anti-rabbit AlexaFluor647 and anti-guinea-pig AlexaFluor568 (Life Technologies) for 30 min at RT. Cells were washed twice in 1% BSA and 0.002% NaN_3 in PBS and mounted on Vectashield H-1000 (Vector Laboratories, Inc. CA, USA).

Image Acquisition and Quantification

Confocal images for the analysis of fluorescence intensity, colocalization or morphology were acquired on a custom-made spinning disk confocal fluorescence microscope based on an Olympus IX71 (Zeiss) equipped with a CoolSnap HQ2 CCD-camera (Photometrics) and controlled by the MAG Biosystems acquisition software MetaMorph, V.7.6.4.0. Objectives with a 20x (Olympus UPlanSApo NA 0.75 air) or 40x (Olympus PlanSApo N, NA 1.42 oil) magnification were used. Z-stacks were acquired through the entire cell body when cells were imaged with the 40x objective. For further analysis the z-stacks were transformed into maximum intensity projections using NIH ImageJ.

Fluorescence Intensity Measurements

Efficient down-regulation of SEPT5 and SEPT7 in neurons transfected with shRNA was quantified using immunofluorescence images of transfected cells compared to non-transfected cells in the same scan. The mean fluorescence intensities were measured on maximum projections for three different regions of interest (ROI) in branched dendrites of transfected and neighboring non-transfected neurons. Next to the dendrites the background fluorescence was determined and subtracted from the measured dendritic regions. A ratio from these obtained values of transfected cells to non-transfected cells was calculated: a ratio close to 1 means no change in the protein expression level, whereas a ratio <1 means a decreased protein expression level caused by RNA interference. Statistical analysis was performed using Prism Version 6 (Graph Pad software).

Colocalization Analysis of Septins in Fixed Neurons

Colocalization analysis was performed in NIH ImageJ. To do so, the individual fluorescence channels were converted into black (0) and white (1) pixel binary images. The two binary images were then multiplied. To determine the percentage of the overlapping fluorescence signal the number of white pixels in the multiplied image was divided by the number of white pixels in the image of the overexpressed septin of interest. Statistical analysis was performed using Prism Version 6 (Graph Pad software).

Morphological Analysis of Fixed Neurons

Sholl analysis was performed using the Sholl Analysis Plugin v1.0 of NIH ImageJ. To avoid false crossings due to background fluorescence resulting from other neurons the images were first processed with the Neurite Tracer of the NIH ImageJ plugin NeuronGrowth (Fanti et al., 2008). The Sholl Analysis plugin itself was not able to detect all dendrites due to variations in fluorescence intensity along individual dendrites. Therefore, binary images of the hand-traced dendrites were created with equalized gray values in the NeuronGrowth plugin.

The middle of the cell body was selected manually. The other parameters in the Sholl Analysis plugin were set such that a concentric circle starting at a radius of 20 μm increased in steps of 10 μm and ended at a radius of 150 μm (radius span = 0 μm, span type = mean). The algorithm counted how many intersections occurred between the concentric circles and the dendrites to determine the dendrites' morphological complexity. Statistical analysis was performed using Prism Version 6 (Graph Pad software).

Live Cell Imaging and Fluorescence Recovery after Photobleaching (FRAP) Experiments

Live-cell FRAP measurements were performed using the custom-made spinning disk confocal microscope as described under "Image Acquisition And Quantification." The 100x Olympus (PlanSApo N, NA 1.40 oil) objective and the sample holder stage were heated to a temperature of 37°C. Neurons (DIV7 + 7 to DIV7 + 9) were imaged in a buffer containing 145 mM NaCl, 5 mM KCl, 10 mM Glucose, 10 mM Hepes, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.2% BSA. The FRAP laser and time course imaging were controlled using I-Las Version 1 of the Mag Biosystems software and images were captured with an evolve EM-CCD camera (Photometrics). Five prebleaching images were recorded at 1 s intervals. A single 100 ms bleaching pulse was applied to various circular regions along the dendrite. Each region had a size of approx. 0.7 μm diameter. Per measurement 6–8 ROI at dendritic spine necks were bleached. The duration of post bleach time course imaging was adjusted to the observed recovery of the fluorescence signal. SEPT2-GFP, SEPT4-GFP, and SEPT7-GFP expressed in SEPT5 downregulated neurons were imaged for 20 min with intervals of 60 s. To record the faster recovery in SEPT1-GFP expressing cells the first 10 images were recorded at 1 s intervals followed by 20 images at 1.5 s intervals, 10 images at 20 s intervals and 20 images at 60 s intervals.

The time course images were corrected for cellular movement and stage drift during the acquisition. The fluorescence intensity of the bleached ROIs, the whole cell and the background were analyzed using NIH ImageJ. The fluorescence intensity signal was double normalized as described earlier (Phair and Misteli, 2000). All data obtained from the images analysis was plotted and statistical analyzed in Prism Version 6 (Graph Pad software).

AUTHOR CONTRIBUTIONS

HE conceived the project; HE and CK designed experiments; CK, MS, and NZ performed experiments; CK, HE analyzed data; CK prepared the draft of the manuscript and HE. edited it.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcell.2017.00011/full#supplementary-material

Supplementary Figure 1 | Immunofluorescence staining against SEPT5 after treatment of neurons with shRNA against SEPT5. Immunofluorescence images show example cells transfected with shRNA against SEPT5, mRFP as a marker (shown in white, top row), endogenous SEPT5 detected by immunofluorescence (red, second row), and SEPTX-GFP (green, third row), where X stands for SEPT1, SEPT2, or SEPT4. SEPT2-GFP and SEPT4-GFP signals cluster along the dendrite, whereas SEPT1-GFP localizes homogenously within the cytoplasm (all in green). Scale bars are 2 μm.

Supplementary Figure 2 | Immunofluorescence staining against SEPT7 after treatment of neurons with shRNA against SEPT5. Immunofluorescence images show example cells transfected with shRNA against SEPT5, mRFP as a marker (shown in white, top row), endogenous SEPT5 detected by immunofluorescence (red, second row), and SEPTX-GFP (green, third row), where X stands for SEPT1, SEPT2, or SEPT4. Antibody immunostaining against SEPT7 (red) reveals a strong overlap between SEPT2-GFP/SEPT7 or SEPT4-GFP/SEPT7 in the merged images. SEPT2-GFP and SEPT4-GFP localize to higher-order structures at the dendritic spine necks, whereas the SEPT1-GFP signal is spread all over the cell (all in green). Scale bars are 2 μm.

Supplementary Figure 3 | Quantification of immunofluorescence data as shown in Supplementary Figures 1, 2. (A) The boxplot illustrates the fluorescence intensity ratios of neurons immunostained against SEPT5. The transfection of the empty pSuper vector served as a negative control, whereas the same vector containing the shRNA against SEPT5 was employed as a positive control. In all neurons cotransfected with shRNA against SEPT5 and SEPTX-GFP, SEPT5 was efficiently reduced. An unpaired Mann-Whitney test was performed to assess significance (****$p < 0.0001$). "*n*" stands for the number of cells. **(B)** The fluorescence intensity ratios of neurons immunostained against SEPT7 illustrate that transfection of shRNA against SEPT7 efficiently downregulates the protein levels of SEPT7 in neurons (****$p < 0.0001$). In neurons downregulated for SEPT5 the fluorescence intensity ratio of SEPT7 is reduced slightly but significantly (**$p = 0.0029$). A significant increase of the FI SEPT7 signal in SEPT2-GFP/shRNA SEPT5 expressing neurons and SEPT4GFP/shRNA SEPT5 expressing neurons compared to shRNAS5 was detected (****$p < 0.0001$). No statistically significant difference was observed for the FI SEPT7 signal from SEPT1-GFP/ shRNA SEPT5 expressing neurons compared to the FI SEPT7 signal from only shRNAS5 expressing neurons (ns, $p = 0.1221$). An unpaired, two-tailed Mann-Whitney test was performed to assess significance. "*n*" stands for the number of cells.

Supplementary Figure 4 | Scheme showing SEPT2 group isoforms of *Rattus norvegicus*. All isoforms listed are known sequences from cDNA cloning. Shown are the common sequence features that the SEPT2 group isoforms share: a black bar describes the N-terminus that is very variable in length between the different SEPT2 group isoforms. The polybasic region is shown by a black box. The green and red boxes comprise the GTPase domain and septin unique element. All isoforms have a coiled-coil domain in the C-terminus in common. Isoforms that were used in this study are highlighted with a red square.

Supplementary Table 1 | Plasmids used in this study.

Supplementary Table 2 | *P*-values of Sholl analysis. *P*-values were calculated to compare the control morphology with neurons depleted of SEPT7 or SEPT5 and with neurons depleted of SEPT5 and cotransfected with SEPT1-GFP, SEPT2-GFP, or SEPT4-GFP. $P < 0.02$ are highlighted in red.

Supplementary Table 3 | SEPT2 group isoforms of *Rattus norvegicus*. We listed all cloned and predicted isoforms with the Uniprot and NCBI Protein ID reference. We provide the literature reference for the isoforms used in this study in the last column.

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MINI REVIEW
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Septins As Modulators of Endo-Lysosomal Membrane Traffic

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Septins constitute a family of GTP-binding proteins, which assemble into non-polar filaments in a nucleotide-dependent manner. These filaments can be recruited to negatively charged membrane surfaces. When associated with membranes septin filaments can act as diffusion barriers, which confine subdomains of distinct biological functions. In addition, they serve scaffolding roles by recruiting cytosolic proteins and other cytoskeletal elements. Septins have been implicated in a large variety of membrane-dependent processes, including cytokinesis, signaling, cell migration, and membrane traffic, and several family members have been implicated in disease. However, surprisingly little is known about the molecular mechanisms underlying their biological functions. This review summarizes evidence in support of regulatory roles of septins during endo-lysosomal sorting, with a particular focus on phosphoinositides, which serve as spatial landmarks guiding septin recruitment to distinct subcellular localizations.

Keywords: septins, membrane, phosphoinositides, endocytosis, endosome, sorting

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INTRODUCTION

Septins constitute a family of small GTPases that assemble into filaments and higher-order structures in a nucleotide-dependent manner (Kinoshita, 2003). In humans 13 different paralogs are expressed in a cell- and tissue-specific manner, which have been classified into the SEPT2, SEPT3, SEPT6, and SEPT7 subgroups based on sequence similarity. All septins share a central GTPase domain that allows for their oligomerization into filaments (Sirajuddin et al., 2007). The G-domain is flanked by more variable N- and C-terminal extensions, which provide interfaces for the association with non-septin binding partners. Septins have been implicated in a large variety of membrane-dependent processes, including cytokinesis, signaling and membrane traffic. During these events they act as barriers limiting diffusion of membrane-resident factors, but also as molecular scaffolds that orchestrate the recruitment of downstream effectors (Caudron and Barral, 2009). Given the fundamental importance of septin-dependent processes, it is not surprising that several septin family members have been implicated in disease, such as Alzheimer's disease or cancer (Dolat et al., 2014). However, surprisingly little is known about the molecular mechanisms underlying septin-related pathogenesis.

SEPTIN FILAMENTS ASSOCIATE WITH MEMBRANE SURFACES

Septins bind to membrane surfaces enriched in negatively charged phospholipids, in particular phosphoinositides (PIs) (Zhang et al., 1999; Tanaka-Takiguchi et al., 2009). This is mediated by a patch of basic amino acids found in members of the SEPT2, SEPT3, and SEPT7 subgroups, which is located in close proximity to the G-domain. Because septins assemble into hetero-oligomeric filaments the association with negatively charged membrane surfaces might be a cooperative

mechanism facilitated by septin oligomerization. *Vice versa*, it has been noted that membrane association supports the assembly of septins into filaments (Bridges et al., 2014). In the best-characterized septin complex SEPT2/SEPT6/SEPT7 a flexible region at the SEPT2 dimer interface promotes bending of the oligomer (Sirajuddin et al., 2007). This might explain why SEPT2-6-7 filaments can impose positive membrane curvature (Tanaka-Takiguchi et al., 2009). More recently Bridges et al. demonstrated that septins also associate with lipid bilayers devoid of negatively charged lipid species, and in this case sense micron-scale, positive membrane curvature *in vitro* and in living cells (Bridges et al., 2016).

Both, the generation and the recognition of membrane curvature are hallmarks of proteins involved in intracellular membrane traffic (Krauss and Haucke, 2011). Membrane traffic relies on the formation of transport carriers, which depends on the concentration of cargo in confined membrane areas. Furthermore, select machineries need to be assembled to aid membrane deformation into highly curved vesicles, and to promote fission from the donor compartment. Given the proposed scaffolding function of septin filaments, and their well-established roles as diffusion barriers, it is conceivable that septins contribute to carrier formation, for instance by shielding membrane subdomains, by imposing or sensing membrane curvature, or by recruiting appropriate effector proteins assisting vesicle generation.

Septins have been shown to bind to a variety of PI species, which are generated on distinct subcellular membranes (see below for details), and might thus act during carrier formation at different organelles. This is supported by several studies that identified septin binding partners with functions during endo-lysosomal sorting (**Table 1**).

TABLE 1 | Binding partners of mammalian septin family members with implication in endo-lysosomal sorting.

Septin	Binding partner	Process	References
SEPT3	GABA-RAPL2	Autophagy	Nakahira et al., 2010
SEPT3	SNX6	Endosomal sorting	
SEPT3	Myo1b	Endosomal sorting	
SEPT8	RALBP1	Endocytosis, endosomal sorting	
SEPT9	CIN85 (SH3KBP1)	Endosomal sorting	
SEPT8	BLOC-1	Endosomal sorting	Gokhale et al., 2012
SEPT5/ SEPT11	Dynamin	Endocytosis	Maimaitiyiming et al., 2013
SEPT7	AP-3	Endosomal sorting	Traikov et al., 2014
SEPT9	CIN85 (SH3KBP1)	Endosomal sorting	Diesenberg et al., 2015

SEPTINS REGULATE THE FORMATION OF ENDOCYTIC CARRIERS AT THE PLASMA MEMBRANE

Membrane recruitment of septin filaments is supported by $PI(4,5)P_2$ and $PI(3,4,5)P_3$, two PI species found predominantly at the plasma membrane (Zhang et al., 1999). Depletion of plasmalemmal $PI(4,5)P_2$ disrupts the integrity of septin filaments, indicating that this PI orchestrates filament assembly. Moreover, septins have been suggested to support the generation of $PI(4,5)P_2$-enriched microdomains at the plasma membrane, and to thereby promote the formation of junctions transiently formed with the endoplasmic reticulum during store-operated calcium entry (Sharma et al., 2013). This suggests that septin filaments can undergo dynamic re-organization in a spatiotemporally regulated manner, together with $PI(4,5)P_2$ pools at the plasma membrane.

The generation of $PI(4,5)P_2$ initiates many plasma membrane-derived processes, including endocytosis (Krauss and Haucke, 2007), and indeed, several studies implicated septins in endocytic events. Depletion of SEPT2 or SEPT11 in macrophages perturbs phagocytic uptake of opsonized latex beads (Huang et al., 2008), and at sites of phagosome formation both septin family members co-localize with actin-rich structures, at a time when $PI(4,5)P_2$ accumulates at the same spots (**Figure 1A**). Similarly, several septins have been found to assemble in close proximity to actin at the entry site of several pathogens (i.e., *Listeria* and *Candida*) in human non-phagocytic cells, and have been proven important for their internalization (Mostowy and Cossart, 2011; Phan et al., 2013).

Interestingly, entry of some pathogens additionally depends on parts of the clathrin machinery, including clathrin itself, but also the vesicle fission enzyme dynamin, and a number of accessory proteins known to associate with receptor tyrosine kinases during their endocytosis (Veiga and Cossart, 2006). Some of these factors encode SH3-domains, which could potentially interact with proline-rich stretches present in several septins. One of them, CIN85/SH3KBP1, interacts with SEPT9 (Diesenberg et al., 2015) (see below for details) and could thereby link the clathrin machinery to SEPT9-containing filaments. Based on these findings it is tempting to speculate that septins also participate in other actin- and/ or dynamin-dependent endocytic pathways, such as clathrin-mediated and caveolar endocytosis, or macropinocytosis (**Figures 1B,C**). In line with this hypothesis SEPT5 and SEPT11 are found in complexes with dynamin (Maimaitiyiming et al., 2013). Furthermore, yeast septins associate with a subset of endocytic proteins (Renz et al., 2016), including the dynamin-like GTPase Vps1, and the accessory proteins Sla2 (ortholog of the mammalian clathrin- and actin binding protein Hip1R) and Syp1 (ortholog of mammalian FCHo proteins that are believed to nucleate clathrin coat formation at the plasma membrane).

SEPTINS DURING ENDOSOMAL SORTING

Accumulating evidence suggests that septins also associate with membranes of the endo-lysosomal system. A proteomic approach identified several septins together with *bona fide* endosomal proteins on early endosome-like liposomes containing PI(3)P (Baust et al., 2008). Later, it was demonstrated that SEPT6 and SEPT7 on endosomes regulate the biogenesis of multivesicular bodies (MVBs) in a process involving the adaptor complex AP-3

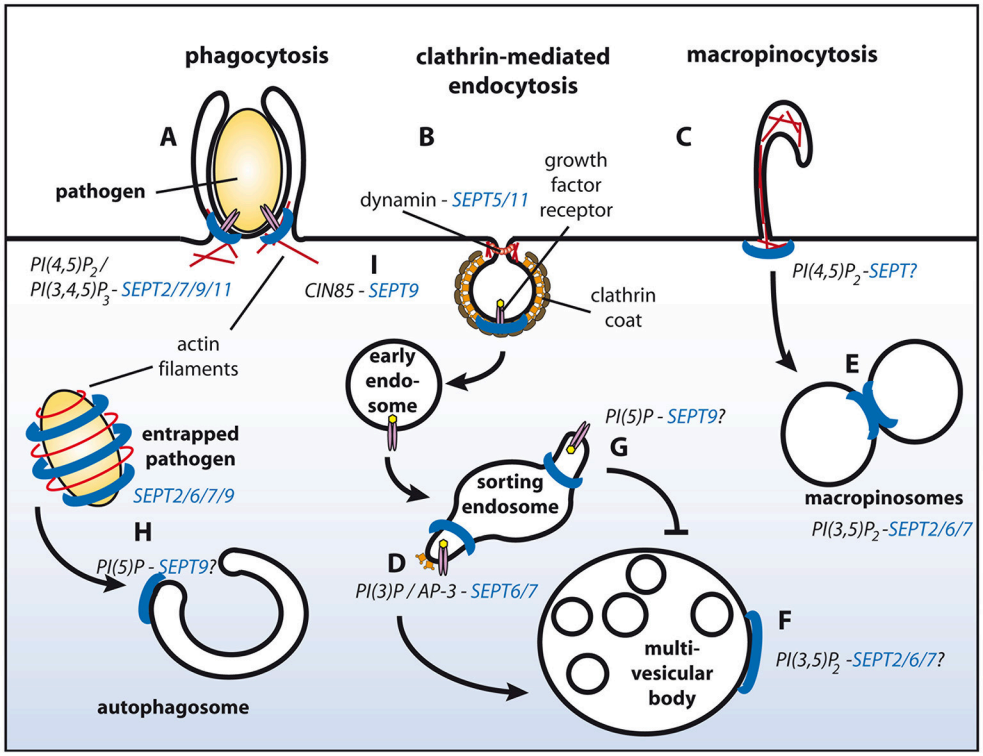

FIGURE 1 | Schematic representation of endosomal sorting events with proven or putative involvement of septins. Septin filaments are indicated in blue, actin filaments in red. SEPT2/7/9/11 promote phagocytosis **(A)**. Through associating with dynamin SEPT5/11 might function during clathrin-mediated endocytosis **(B)**. Interactions of septins with actin filaments might modulate macropinocytosis **(C)**. Complex formation between AP-3 and SEPT6/7 facilitates degradative sorting **(D)**. SEPT2/6/7 promote fusion of macropinosomes **(E)** and potentially of MVBs with lysosomes **(F)**. PI(5)P at endosomes protects cargo from degradative sorting, and might recruit SEPT9 to sorting endosomes **(G)**. SEPT2/6/7/9 form cages around intracellular pathogens and promote their sorting to autophagosomes **(H)**. SEPT9 inhibits ubiquitylation of EGF receptors at the plasma membrane by associating with CIN85, and thereby attenuates degradative sorting at endosomes **(I)**. See text for details.

and ESCRT proteins (Traikov et al., 2014). They thereby facilitate the degradation of ubiquitylated cargo proteins in lysosomes (**Figure 1D**).

During maturation into MVBs PI(3)P-positive endosomes acquire a PI(3,5)P_2-positive identity, which is generated by FYVE finger containing PI kinase (PIKfyve) to promote endo-lysosomal fusion. Interestingly, a PIKfyve-dependent pool of PI(3,5)P_2 recruits SEPT2 to fusion sites on Rab7-positive macropinosomes (Dolat and Spiliotis, 2016; **Figure 1E**). SEPT2 depletion does not impair docking between macropinosomes, but reduces their fusion. It remains elusive if this defect is caused by a direct modulation of the SNARE machinery through SEPT2. Alternatively, SEPT2-containing filaments might directly confer fusogenic properties. Whether SEPT2 is recruited to MVBs as well, is currently unknown (**Figure 1F**).

Several physiological stimuli, but also infection with certain pathogens, can up-regulate PI(5)P on endosomes. In cells infected with *Shigella* this pool of endosomal PI(5)P impedes EGF receptor degradation (Ramel et al., 2011; Boal et al., 2015). Interestingly, exogenous supply of PI(5)P translocates SEPT9 to lipid droplets and possibly other organelles (Akil et al., 2016; **Figure 1G**).

Besides its role at endosomes, PI(5)P can also regulate the biogenesis of autophagosomes through a non-canonical, Vps34-independent pathway (Vicinanza et al., 2015). Interestingly, it has been noted that SEPT9 and SEPT7 are incorporated into septin cages that entrap cytosolic *Shigella* to target them for autophagy (Mostowy et al., 2010; Sirianni et al., 2016; **Figure 1H**). Septin cage formation occurs in concert with, and dependent on proteins involved in autophagy, including p62/SQSTM1, Atg5, Atg6, and Atg7. It is, thus, tempting to speculate that septins have a regulatory role during autophagy.

INDIRECT EFFECTS OF SEPTINS ON DEGRADATIVE SORTING OF RECEPTOR TYROSINE KINASES

Septins can also exert indirect effects on sorting of cargo proteins. We recently noted a profound decrease in surface levels of epidermal growth factor (EGF) receptors upon depletion of SEPT9 (Diesenberg et al., 2015; **Figure 1I**). This effect depends on a proline-rich motif within the SEPT9

N-terminal domain that supports its association with the adaptor protein CIN85/SH3KBP1. CIN85-SEPT9 complexes localize exclusively to the plasma membrane, where SEPT9 is recruited to ligand-engaged receptors in a CIN85-dependent manner. CIN85 promotes down-regulation of EGF receptors through its interaction with the ubiquitin ligase Cbl (Soubeyran et al., 2002). As SEPT9 competes with Cbl for the same binding sites on CIN85 it negatively regulates receptor multi-ubiquitylation and thereby attenuates subsequent degradative sorting of ubiquitylated EGF receptors to lysosomes.

Similar mechanisms might apply for other receptors down-regulated by the CIN85/Cbl module, such as the hepatocyte growth factor Met (Petrelli et al., 2002). In support of this hypothesis, decreased levels of Met have been detected in cells depleted of septins (Mostowy et al., 2011). As Met serves as a docking site for *Listeria*, this might provide an additional explanation for the reduced capability of this pathogen to invade host cells in absence of septins.

Marcus et al. have reported recently that septin oligomerization stabilizes ErbB2 (Marcus et al., 2016), a receptor tyrosine kinase mutated or overexpressed in multiple cancers. SEPT2 and SEPT9 co-localize with Erb2 at the basolateral plasma membrane of gastric cancer cells. Treatment of cells with forchlorfenuron, an inhibitor impairing septin assembly and dynamics, as well as septin depletion aggravate ubiquitin-dependent degradation of ErbB2, similar to what has been seen for EGF receptors. However, as ErbB2 is sorted independently of the CIN85/Cbl-module, alternative effectors apparently act downstream of septins in this case.

CONCLUDING REMARKS

Septins have been found to associate with a variety of PIs at different intracellular membranes, where they regulate a variety of cellular processes. As outlined above individual septin family members can thereby exert distinct effects, depending on their subcellular localizations. This is exemplified by SEPT2, which is recruited to the plasma membrane, to endosomal membranes or to the surface of mitochondria, where it controls unique events (the formation of phagocytic carriers, endosomal membrane fusion or organelle fission, respectively) (Huang et al., 2008; Mostowy and Cossart, 2011; Phan et al., 2013; Dolat and Spiliotis, 2016; Pagliuso et al., 2016). Thus, specificity in SEPT2 membrane recruitment and function must be accomplished through additional factors. This could be other septin family members that assemble with SEPT2 into filaments of distinct compositions, thereby conferring unique PI specificities. The association of filaments with organelle-specific, non-septin binding partners might generate additional flexibility in membrane targeting. Future studies will need to carefully dissect the exact composition of septin scaffolds to allow for a detailed understanding of their functions in endo-lysosomal sorting. Finally, the fact that the application of a septin inhibitor can counteract the stabilization of signaling receptors at the plasma membrane in cancer cells may offer an avenue for the treatment of cancer, and potentially other septin-related diseases.

AUTHOR CONTRIBUTIONS

KS, GR, and MK participated in outlining and writing this Minireview. MK prepared figure and table.

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Regulation of Store-Operated Ca^{2+} Entry by Septins

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The mechanism of store-operated Ca^{2+} entry (SOCE) brings extracellular Ca^{2+} into cells after depletion of intracellular Ca^{2+} stores. Regulation of Ca^{2+} homeostasis by SOCE helps control various intracellular signaling functions in both non-excitable and excitable cells. Whereas essential components of the SOCE pathway are well characterized, molecular mechanisms underlying regulation of this pathway need investigation. A class of proteins recently demonstrated as regulating SOCE is septins. These are filament-forming GTPases that assemble into higher order structures. One of their most studied cellular functions is as a molecular scaffold that creates diffusion barriers in membranes for a variety of cellular processes. Septins regulate SOCE in mammalian non-excitable cells and in *Drosophila* neurons. However, the molecular mechanism of SOCE-regulation by septins and the contribution of different subgroups of septins to SOCE-regulation remain to be understood. The regulation of SOCE is relevant in multiple cellular contexts as well as in diseases, such as the Severe Combined Immunodeficiency (SCID) syndrome and neurodegenerative syndromes like Alzheimer's, Spino-Cerebellar Ataxias and Parkinson's. Moreover, *Drosophila* neurons, where loss of SOCE leads to flight deficits, are a possible cellular template for understanding the molecular basis of neuronal deficits associated with loss of either the Inositol-1,4,5-trisphosphate receptor (IP_3R1), a key activator of neuronal SOCE or the Endoplasmic reticulum resident Ca^{2+} sensor STIM1 (Stromal Interaction Molecule) in mouse. This perspective summarizes our current understanding of septins as regulators of SOCE and discusses the implications for mammalian neuronal function.

Keywords: STIM, Orai, flight, Endoplasmic reticulum, cytoskeletal proteins

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The calcium ion (Ca^{2+}) performs multiple signaling functions to regulate a diverse range of cellular processes including fertilization, cell division, and apoptosis (Berridge et al., 2000; Clapham, 2007; Soboloff et al., 2012). Regulation of calcium signaling is thus of utmost importance in all cell types. Cytosolic Ca^{2+} concentrations are carefully maintained at levels of ~100–200 nM in most cell types either by extrusion of the excess Ca^{2+} into the extracellular space by ATP-driven pumps like the PMCA (Plasma membrane Ca^{2+} ATPase; Brini et al., 2013) or by sequestering excess Ca^{2+} into cellular organelles like the Endoplasmic Reticulum (ER), which functions as an intracellular Ca^{2+} store (Soboloff et al., 2012; Prakriya and Lewis, 2015). Ca^{2+} can enter the cell from the extracellular space in response to a variety of signals. For example, in T cells, activation of the T cell receptor on the cell surface activates intracellular signaling cascades and results in depletion of Ca^{2+} from the intracellular stores. This drop in intracellular store Ca^{2+} activates a mode of extracellular Ca^{2+}

uptake called the store-operated Ca^{2+} entry (SOCE) (Putney et al., 2001; Putney, 2005; Prakriya and Lewis, 2015). The resulting increase in cytosolic Ca^{2+} stimulates translocation of the Nuclear Factor of Activated T-cells (NFAT) from the cytosol to the nucleus, where it turns on transcription of genes important for activated T-cell function (Lewis, 2001). SOCE is the primary mechanism for uptake of extracellular Ca^{2+} in non-excitable cells like T cells. Although discovered in immune cells, Ca^{2+} uptake by SOCE also operates in other non-excitable as well as in excitable cells, such as muscles (Pan et al., 2014) and neurons (Venkiteswaran and Hasan, 2009; Gruszczynska-Biegala et al., 2011; Hartmann et al., 2014).

STORE-OPERATED CA^{2+} ENTRY (SOCE)

A range of extracellular signals can activate membrane-localized G-protein coupled receptors (GPCRs) and/or Receptor Tyrosine Kinases (RTKs) followed by Phospholipase C mediated enzymatic hydrolysis of Phosphatidyl inositol-4,5-bis phosphate (PIP_2) and generation of the intracellular messenger inositol-1,4,5-trisphosphate (IP_3). IP_3 binds to the inositol-1,4,5-trisphosphate receptor (IP_3R), which is primarily localized to the Endoplasmic Reticulum (ER) (Berridge, 2009). The IP_3R is a ligand-gated Ca^{2+} channel and upon ligand binding releases Ca^{2+} from the ER to the cytosol along a favorable concentration gradient. The consequent drop in ER Ca^{2+} is sensed by ER-membrane localized Stromal Interaction Molecules (STIM) (Liou et al., 2005). The drop in ER Ca^{2+} results in release of Ca^{2+} from the luminal EF-hand domain of STIM, followed by oligomerization and translocation of STIM proteins to regions of the ER, which are in close proximity of the plasma membrane (PM), the ER-PM junctions (Zhang et al., 2005; Liou et al., 2007; **Figure 1**). STIM proteins recruited to the ER-PM junctions physically interact with the Ca^{2+}-selective Orai channel located on the plasma membrane (**Figure 1**). In resting cells with replete Ca^{2+} stores, Orai normally remains closed. Upon ER store depletion and STIM translocation, STIM binds to Orai leading to channel opening and the entry of extracellular Ca^{2+}, referred to as store-operated Ca^{2+} entry (Park et al., 2009; **Figure 1**). The resulting increase in Ca^{2+} levels in the cytosol serves as a signal for multiple Ca^{2+}-dependent processes (Feske et al., 2001; Lewis, 2001; Somasundaram et al., 2014; Pathak et al., 2015). Cytosolic Ca^{2+} levels return to resting levels by the action of ATP-driven Ca^{2+} pumps on the ER (Sarco-Endoplasmic Reticulum Ca^{2+} ATPase or SERCA; Periasamy and Kalyanasundaram, 2007) and the PM (Plasma membrane Ca^{2+} ATPase, PMCA; Juška, 2010), which pump Ca^{2+} back from the cytosol to the ER lumen or the extracellular space, respectively (Soboloff et al., 2012; Prakriya and Lewis, 2015). Ca^{2+} from the cytosol can also be taken up along the concentration gradient by the Mitochondrial Ca^{2+} uniporter (MCU), a highly selective Ca^{2+} channel in the inner mitochondrial membrane (Kirichok et al., 2004; Baughman et al., 2011; De Stefani et al., 2011).

Several disease conditions arise as a consequence of either reduced or dysregulated STIM/Orai mediated SOCE. Mutations in genes encoding STIM1 and Orai1 reduce SOCE in T-cells leading to severe combined immunodeficiency (SCID) syndrome (Feske et al., 2006; Maus et al., 2015). STIM1 and Orai1 mediated SOCE also drives tumor metastasis in different kind of cancers (Yang et al., 2009; Chen et al., 2011, 2013). Congenital non-progressive myopathy in humans is associated with a loss of STIM1 and Orai1 (Stiber et al., 2008; McCarl et al., 2009) in agreement with findings *in vivo* and *in vitro* where loss of STIM1 resulted in muscle differentiation defects. The physiological relevance of SOCE in neurons is only just beginning to be understood. Knockdown of single genes encoding *Drosophila* STIM and Orai, *dSTIM* and *dOrai*, respectively, in neurons compromised flight initiation and maintenance (Venkiteswaran and Hasan, 2009). Defects in motor coordination were also observed upon knock out of STIM1 from cerebellar Purkinje neurons in mice (Hartmann et al., 2014). SOCE thus serves important signaling functions in a range of cell types by regulating cellular Ca^{2+} homeostasis.

SEPTINS REGULATE SOCE

Stromal Interaction Molecule (STIM) and Orai are the key molecular components of SOCE, but in addition other proteins like CRACR2A (CRAC regulator 2A; Srikanth et al., 2010) and Junctophilin-4 (Woo et al., 2016) modulate the strength and duration of the Ca^{2+} signal and subsequent downstream events. While CRACR2A facilitates STIM/Orai coupling after ER store-depletion (Srikanth et al., 2010), Junctate and Junctophilin help define ER-PM junctions suitable for STIM/Orai coupling (Srikanth et al., 2012; Woo et al., 2016). A genome wide screen in HeLa cells identified Septins as positive regulators of SOCE (Sharma et al., 2013). Septins are a class of filament forming GTPases that assemble into higher order structures. Primarily they act as diffusion barriers or molecular adaptors (Mostowy and Cossart, 2012). However, septin function can vary depending on the cell type as well as the context of a given cell type. For example, knockout of SEPT7 blocks cell division in fibroblasts but not in lymphocytes (Sellin et al., 2011; Menon et al., 2014). T-lymphocytes in contact with other cells do not require septins for division, whereas single T cells that are not in contact with other cells depend on septins for cell division (Mujal et al., 2016).

Based on their sequence homology, Septins can be classified into four subgroups- SEPT2, SEPT6, SEPT7, and SEPT3 (Kartmann and Roth, 2001; Kinoshita, 2003; Pan et al., 2007). Septins of different subgroups occupy distinct positions in a linear septin filament. Determination of the structure of a hexameric oligomer formed by human SEPT7, SEPT6, and SEPT2 revealed that members of the SEPT2 subgroup occupy the central position whereas SEPT7 occupies the terminal position in such a complex (Sirajuddin et al., 2007). Originally, it was shown that loss of SEPT2, SEPT4, and SEPT5 reduced SOCE significantly in Jurkat T-cells (Sharma et al., 2013). All three belong to the SEPT2 subgroup (Kartmann and Roth, 2001; Kinoshita, 2003; Pan et al., 2007).

In addition to positive regulators of SOCE, given the necessity of tightly regulating cellular Ca^{2+} entry, not surprisingly, negative regulators of SOCE have also been identified (Feng

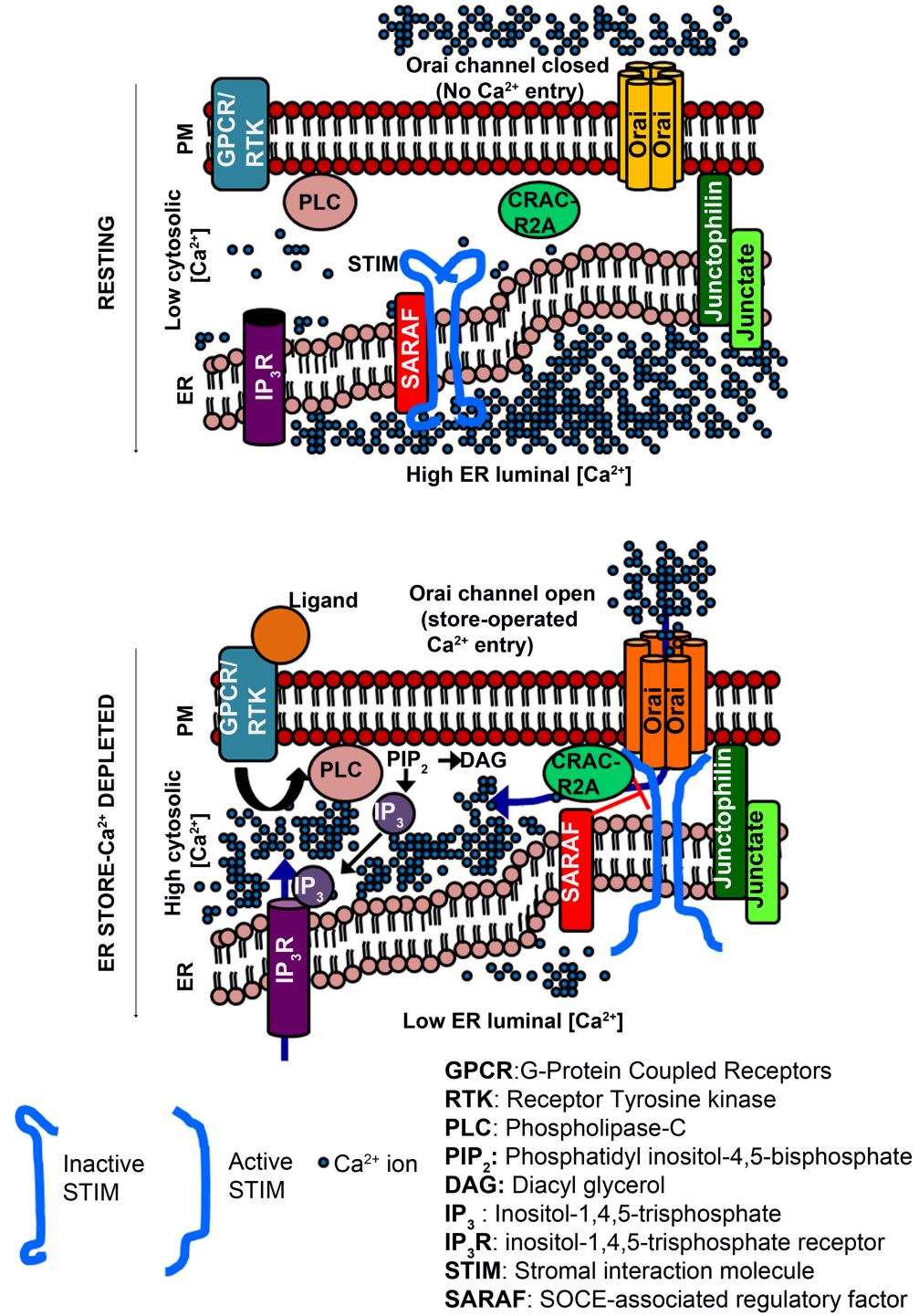

FIGURE 1 | Activation of Orai channels by STIM proteins during store-operated Ca^{2+} entry (Top). Cytosolic [Ca^{2+}] is low compared to the ER luminal [Ca^{2+}]. STIM proteins (inactive form) reside in regions of ER distal from the PM. Orai channels (closed) are distributed in the PM. ER-membrane resident Junctate and Junctophilin proteins (positive regulators of SOCE) help in pre-organizing ER-PM contact sites that facilitate STIM recruitment to the PM-proximal regions of the ER. **(Bottom)** Binding of an extracellular ligand to a PM-resident GPCR/RTK activates PLC and generates IP_3, which binds to ER-resident IP_3R, and is followed by ER store-Ca^{2+} release through the IP_3R. The lowering of ER luminal [Ca^{2+}] activates STIM, which translocates to ER-PM regions where it binds to and opens Orai channels. CRACR2A is a positive regulator of SOCE that helps stabilize STIM/Orai complexes. SARAF is a negative regulator, which destabilizes STIM/Orai clusters to prevent excessive Ca^{2+} refilling. Hexameric Orai channels are depicted in accordance with the crystal structure of *Drosophila* Orai.

et al., 2006; Palty et al., 2012). For example, SARAF (SOCE-associated Regulatory Factor) regulates SOCE by destabilizing STIM1/Orai1 complexes (Palty et al., 2012). Recent genetic and cellular experiments in *Drosophila* neurons demonstrated that reduced levels of dSEPT7 support store-independent Ca^{2+}-entry through dOrai. Thus, SEPT7 functions as a negative regulator of the *Drosophila* Orai channel in neurons (Deb et al., 2016). The *Drosophila* genome contains five genes encoding septins (Neufeld and Rubin, 1994; Field et al., 1996; Adam et al., 2000) that have been classified into three subgroups based on their sequence homology with mammalian septins (Cao et al., 2007). dSEPT1 (or "Sep1") and dSEPT4 (or "Sep4") belong to the SEPT2 subgroup while dSEPT2 (or "Sep2) and dSEPT5 (or "Sep5") belong to the SEPT6 subgroup of septins (**Figure 2A**). Similar to mammals, dSEPT7 is the only member of the SEPT7 subgroup in *Drosophila* (Cao et al., 2007). There are no representative members of the SEPT3 subgroup in *Drosophila*. An important question that arose from these findings is whether Septin subunits regulate SOCE differentially in *Drosophila* neurons as compared to mammalian T-cells.

SEPTIN SUBGROUPS AND REGULATION OF THE ORAI CHANNEL

The role of septin subgroups and how they might independently modulate SOCE in mammalian cells remains to be addressed. Recent work in *Drosophila* neurons suggests a complex picture. Reduction of SOCE in neurons affects flight initiation and maintenance in *Drosophila* (Venkiteswaran and Hasan, 2009). Simultaneous knockdown of dSEPT1 and dSEPT4 in neurons reduced neuronal SOCE and resulted in flight deficits in *Drosophila*, indicating that the SEPT2 subgroup of septins, dSEPT1 and dSEPT4, function as positive regulators of SOCE in *Drosophila* flight circuit neurons (Deb et al., 2016). These observations are in agreement with studies in mammalian cells (Sharma et al., 2013), suggesting that the role of SEPT2 subgroup

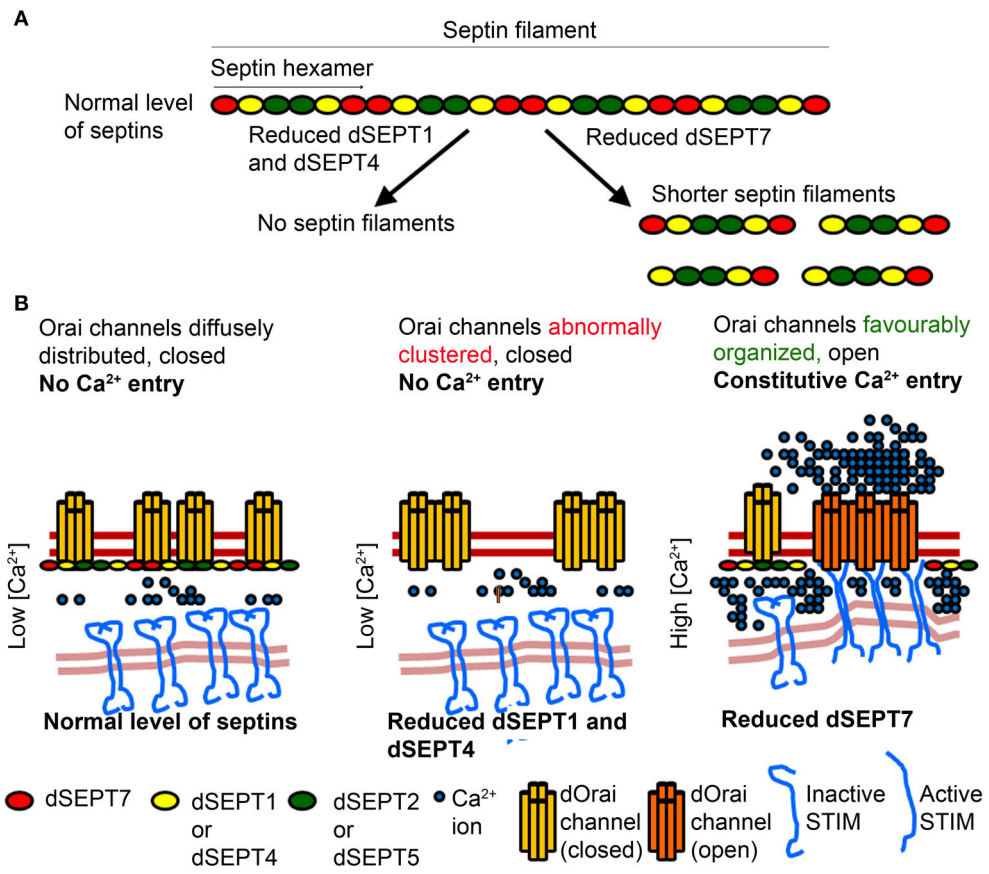

FIGURE 2 | Proposed mechanism by which Septins of the SEPT2 subgroup affect Septin filaments and Orai activation differently from SEPT7 (A) Septin subunits belonging to different subgroups form hexameric complexes that are arranged end to end to form linear non-polar filaments. Reduction of the SEPT2 subgroup, dSEPT1 (or Sep1) and dSEPT4 (or Sep4), results in loss of septin filaments. Reduction of dSEPT7 (or Pnut) results in formation of shorter septin filaments. **(B)** In resting cells with normal level of Septin subunits, septin filaments help organize lipid domains in the PM, with closed Orai channels. Reduction of the SEPT2 subgroup leads to loss of septin filaments and favor's a closed conformation of Orai channels. Reduction of dSEPT7 results in shorter septin filaments, which facilitate STIM/Orai coupling and the distribution of Orai in lipid domains, where a constitutively open conformation is favored. Septin filament and Orai architecture depicted in the model are based on genetic and cellular studies but have not been demonstrated experimentally.

septins in SOCE is evolutionarily conserved across cell types. Septin 7 regulation of SOCE in *Drosophila* neurons appears more nuanced. Reduction of dSEPT7 had no significant effect on SOCE in *Drosophila* neurons. However, reduction of dSEPT7 in *Drosophila* neurons with mutations in the IP_3R or with reduced levels of the Ca^{2+} sensor dSTIM, enhanced uptake of extracellular Ca^{2+} (Deb et al., 2016). This Ca^{2+} entry occurred without depletion of ER store Ca^{2+} and was abrogated by introducing a dominant negative form of dOrai, suggesting that reduction or loss of dSEPT7 activated dOrai constitutively in *Drosophila* neurons. In agreement with this, basal cytosolic Ca^{2+} levels were significantly higher in resting neurons with reduced or no dSEPT7 when compared to wild-type neurons. dSEPT7 also forms a hexameric complex with dSEPT2 and dSEPT1 similar to the mammalian SEPT7-SEPT6-SEPT2 complex (Field et al., 1996). An intriguing question that arises from Septin/SOCE findings in *Drosophila* neurons is how two subgroups of Septin subunits, viz., dSEPT1/4 and dSEPT7, both of which form part of the same complex, perform antagonistic rather than synergistic functions for Orai channel activation. Whereas, dSEPT7 acts as a negative regulator or "molecular brake" of the dOrai channel in *Drosophila* neurons, SEPT2 subgroup septins (dSEPT1/4) exhibit an essential role in SOCE activation. A possible explanation for these contrasting observations is that SEPT7 can function independently of other septin subunits, but existing data from mammalian cells do not support this idea (Sellin et al., 2011). Though an independent function for dSEPT7 cannot be ruled out, an alternate explanation for these apparently contradictory findings might lie in the assembly order of these septins during formation of septin complexes and the subsequent filament structure.

Septins of the SEPT2 subgroup nucleate septin complex formation. Knockdown of the SEPT2 subgroup destabilizes septin complexes in mammalian cells (Sellin et al., 2011). In *Drosophila* mutations in the GTPase domain of dSEPT1, which belongs to the SEPT2 subgroup, prevents stable septin complex formation whereas mutations that attenuate GTPase function of dSEPT7 do not affect it's ability to form a stable complex with wild-type dSEPT1 and dSEPT2 (Akhmetova et al., 2015). Mutant studies of genes encoding *Drosophila* SEPT6 subunits (*dSEPT2* and *dSEPT5*) suggest that similar to mammalian cells a SEPT6 class subunit is necessary for functional Septin complexes (O'Neill and Clark, 2016). In mammalian cells, reduction of SEPT7 affects the formation of complexes with septin hexamers but allows formation of heterodimeric and heterotetrameric complexes containing SEPT2 and SEPT6 (Sellin et al., 2011). We hypothesize that partial reduction of dSEPT7 leaves SEPT2-SEPT6 complexes intact and results in formation of smaller septin filaments, because filament elongation requires dSEPT7 at the two termini (**Figures 2A,B**). Septin filaments are known to help in the formation of lipid domains in the plasma membrane. For example, boundaries created by septin filaments prevent lateral diffusion of proteins between the membrane of mother and daughter cells in yeast (Barral et al., 2000; Faty et al., 2002). Thus, we speculate that in resting cells, septin filaments help in sequestering dOrai in lipid domains that are non-permissive to STIM/Orai interactions and Orai opening. Partial loss of dSEPT7 in resting cells results in shorter septin filaments and helps organize membrane lipids in a conformation that is permissive to STIM/Orai coupling and Orai opening (**Figure 2B**). Indeed, partial reduction of dSEPT7 resulted in constitutive activation of dOrai and increased clustering of dOrai in resting cells (Deb et al., 2016).

In mammalian cells, knockdown of SEPT2/4/5 resulted in abnormal clustering of Orai1 in resting conditions (Sharma et al., 2013). Septins bind phosphoinositides through a polybasic domain (Zhang et al., 1999; Bertin et al., 2010) and the polybasic domain of mammalian SEPT4 binds preferentially to PIP_2 (Zhang et al., 1999). In cells with SEPT2/4/5 knockdown, diffuse and homogeneous distribution of Orai1 was replaced by Orai1 clusters that correlated with an altered arrangement of PIP_2 in the PM (Sharma et al., 2013). These observations suggest that septin filaments help maintain PIP_2 organization in the PM necessary for Orai activation after store-depletion. The abnormal Orai1 clusters are a likely cause for reduced STIM1/Orai1 coupling observed in cells with knockdown of SEPT2/4/5 after ER-store depletion. Because SEPT2/4/5 subunits are required to nucleate septin complex formation, we hypothesize that their reduction destabilizes septin filaments. Very likely, this affects Orai organization in resting cells, and upon SOCE stimulation negatively impacts both STIM1/Orai1 coupling and Orai1 opening (**Figure 2B**). Additional cellular studies and higher resolution analysis of Orai organization by electron microscopy in cells with knockdown of either SEPT7 or SEPT2/4/5 subunits should help elucidate the mechanisms by which different Septin subunits control Orai organization and channel opening.

SEPTINS AND THE ER-PM TRANSLOCATION OF STIM

An essential step in regulation of SOCE is movement of the ER-Ca^{2+} sensor STIM to ER-PM junctions after store depletion, followed by its interaction with Orai, leading to channel opening and Ca^{2+} entry. Interestingly, septin filaments were detected both in the vicinity of the PM as well as at the ER in *Drosophila* neurons and reduction of dSEPT7 increased the intensity of dSTIM near the PM in resting neurons (Deb et al., 2016). A role for Septin regulation of STIM movement is also indicated in mammalian cells where knockdown of the SEPT2 subgroup of septins led to a delay in STIM recruitment to the ER-PM regions after ER-store depletion (Sharma et al., 2013). These data suggest that septins also help in the recruitment of STIM to the ER-PM junctions. Taken together these findings support the idea that septin filaments localized at the ER regulate STIM translocation and consequently its affect on Orai organization. In addition, at the PM, Septin filaments interact with PIP_2 and probably function as diffusion barriers. In the case of dSEPT7 knockdown, partial loss of the diffusion barrier might allow Orai opening, whereas knockdown of the SEPT2 subgroup results in complete or near-complete loss of Septin filaments and consequently a complete loss of the diffusion barrier, which impacts Orai activation negatively.

Several questions that need addressing emerge from these observations. Firstly, the nature of Septin complexes formed in *Drosophila* neurons and their specific organization in the ER and PM regions before and during SOCE need to be understood. Secondly, a better molecular understanding of dOrai channel opening by reduction of dSEPT7 is necessary. The observation that Ca^{2+} entry through Orai upon dSEPT7 reduction remained unaffected by knockdown of dSTIM suggests that the Orai channel in neurons with reduced dSEPT7 may exist in a conformation, that allows Ca^{2+} entry in the absence of SOCE. Importantly, we do not know as yet the effect of complete loss of dSEPT7 on SOCE. If dSEPT7 null neurons lack Septin filaments, it is possible that this too will prevent Orai channel opening. Finally, at this stage it is not known how knockdown of dSEPT1/4 (SEPT2 subgroup in *Drosophila*) alters clustering and activation of dOrai and conversely if knockdown of SEPT7 in mammalian cells has similar effects on Orai mediated Ca^{2+} entry as seen after dSEPT7 reduction in *Drosophila* neurons.

The observation that reduction of dSEPT7, restored normal flight in flies with reduced IP_3R or STIM function suggests that Septin 7 could be a target for alleviating conditions arising from reduced SOCE in neurons (Deb et al., 2016). Dysregulated Ca^{2+} signaling is linked to several neurodegenerative disorders (Egorova et al., 2015). If indeed, SEPT7 functions as a "molecular brake" on the Orai channel in mammalian neurons, it could be an important therapeutic target for diseases resulting from reduced intracellular Ca^{2+} signaling in neurons.

AUTHOR CONTRIBUTIONS

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

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The Mammalian Septin Interactome

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Septins are GTP-binding and membrane-interacting proteins with a highly conserved domain structure involved in various cellular processes, including cytoskeleton organization, cytokinesis, and membrane dynamics. To date, 13 different septin genes have been identified in mammals (*SEPT1* to *SEPT12* and *SEPT14*), which can be classified into four distinct subgroups based on the sequence homology of their domain structure (SEPT2, SEPT3, SEPT6, and SEPT7 subgroup). The family members of these subgroups have a strong affinity for other septins and form apolar tri-, hexa-, or octameric complexes consisting of multiple septin polypeptides. The first characterized core complex is the hetero-trimer SEPT2-6-7. Within these complexes single septins can be exchanged in a subgroup-specific manner. Hexamers contain SEPT2 and SEPT6 subgroup members and SEPT7 in two copies each whereas the octamers additionally comprise two SEPT9 subgroup septins. The various isoforms seem to determine the function and regulation of the septin complex. Septins self-assemble into higher-order structures, including filaments and rings in orders, which are typical for different cell types. Misregulation of septins leads to human diseases such as neurodegenerative and bleeding disorders. In non-dividing cells such as neuronal tissue and platelets septins have been associated with exocytosis. However, many mechanistic details and roles attributed to septins are poorly understood. We describe here some important mammalian septin interactions with a special focus on the clinically relevant septin interactions.

Keywords: septins, septin-multimers, septin-interacting proteins, platelets, human endothelial cells

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INTRODUCTION

Septins (SEPTs) have been originally discovered in *Saccharomyces cerevisiae* as a family of proteins associated with cytokinesis and cell morphology. They received their name due to the involvement in the septum formation during yeast budding and are expressed in all eukaryotes, except in plants (Hartwell et al., 1974; Kinoshita and Noda, 2001; Pan et al., 2007). The first human septin was described in 1994 (Nakatsuru et al., 1994). To date, 13 functional septin genes encoding tissue-specific and ubiquitous expressed septins (*SEPT1* to *SEPT12* and *SEPT14)* have been identified (Kinoshita, 2003a). The number of septins varies between eukaryotic cells, from one in some algae to 13 in humans (Cao et al., 2009; Nishihama et al., 2011). Septins are an evolutionarily highly conserved protein group and belong to the Ras-like GTPase superclass of phosphate-binding loop (P-loop) NTPases (Leipe et al., 2002). All known septins (30–65 kDa) share a common structural domain organization (**Figure 1A**): A highly conserved central GTP-binding region, a variable amino-terminus, and a carboxyl-terminus often predicted to form coiled-coil structures, possibly involved in mediating protein-protein interactions (Trimble, 1999). Between the N-terminus and

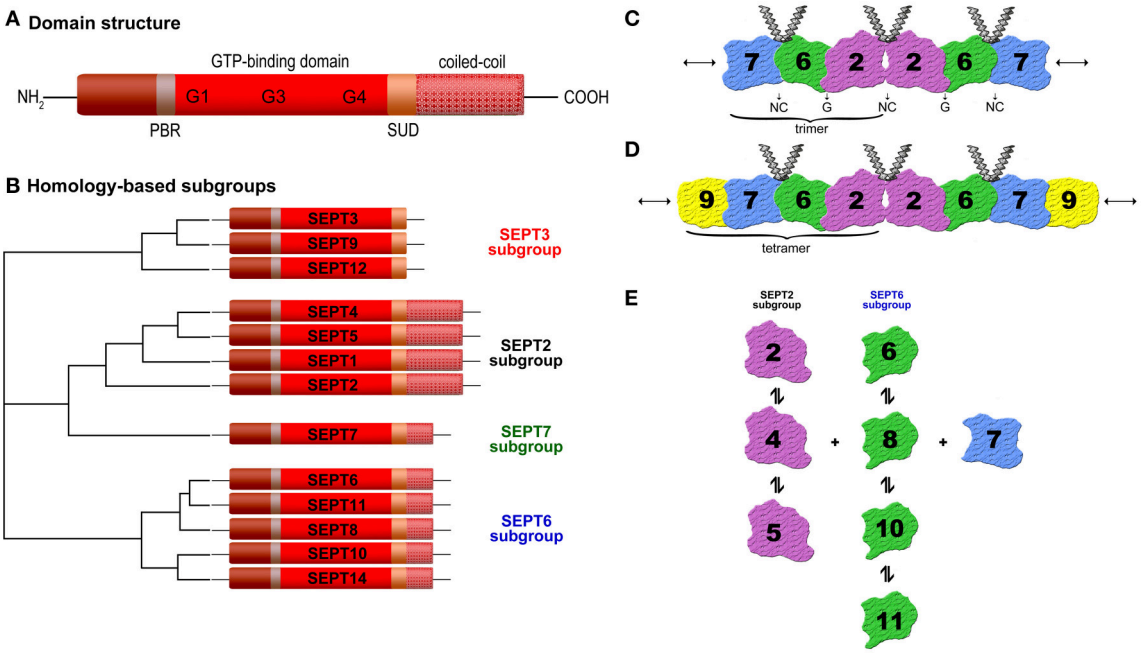

FIGURE 1 | (A) Schematic septin domain structure. Septins share a conserved GTP-binding domain, a phosphoinositide-binding polybasic region (PBR), a septin unique domain (SUD), and most of them one or more coiled-coil domains. The length and amino acid sequence of the N- and C-terminus vary (according to Trimble, 1999). **(B)** Homology-based subgroups. The 13 human septins (SEPT1 to SEPT12 and SEPT14) are classified into four subgroups (SEPT3, SEPT2, SEPT7, and SEPT6) based on sequence homology and coiled-coil domains (Macara et al., 2002; Kinoshita, 2003b). **(C)** Structure of the SEPT2-6-7 complex. Two copies of each septin are symmetrically arranged (SEPT7-6-2-2-6-7) to generate a hexamer by alternating N- and C-termini (NC) and G-interface (GTP-binding domain) (Sirajuddin et al., 2007). **(D)** Structure of the SEPT2-6-7-9 complex (Sandrock et al., 2011; Kim et al., 2011). **(E)** Binding preferences of individual septins to other septins (Sandrock et al., 2011).

the GTPase domain, a short polybasic region (PBR) is found in most septin sequences, which binds directly to phosphatidylinositol bisphosphate (PIP_2), and may be responsible for mediating interactions with plasma membranes (Casamayor and Snyder, 2003). A septin-unique domain (SUD) of unknown function exists C-terminally (Pan et al., 2007). The GTPase domain (G-domain) itself has a mixed α-helix/β-sheet secondary-structure and contains three motifs: G1 (GxxxxGK[s/T]) is characterized by the presence of the P-loop and is capable of interacting with nucleotide phosphate groups. Both G3 (DxxG) and G4 (xKxD) are directly associated with GTP binding (Sirajuddin et al., 2009). Based on their sequence homology and the number of coiled-coil domains, mammalian septins can be subdivided into four different groups termed according to their founding member SEPT2, SEPT3, SEPT6, or SEPT7 (**Figure 1B**). The SEPT2 subgroup (SEPT1, 2, 4, 5) contains two coiled-coil domains. The SEPT3 subgroup (SEPT3, 9, 12) has no coiled-coil domain. The SEPT6 (SEPT6, 8, 10, 11, 14) and the SEPT7 subgroup (SEPT7), respectively comprise one coiled-coil domain (Macara et al., 2002; Kinoshita, 2003b).

SPECIFICITY OF SEPTIN INTERACTIONS

Unlike RAS-like GTP-binding proteins the septins can assemble into multimeric complexes including two or more subunits depending on the organism (Field et al., 1996; John et al., 2007; Sellin et al., 2011a). The classification of mammalian septins into four subgroups has relevance for the formation of these septin complexes because individual septins from each group can polymerize into a variety of higher-order structures, such as filaments, bundles, or rings (Mostowy and Cossart, 2012). Several studies have shown subgroup-restricted binding preferences of mammalian septins (**Table 1**) and that the typical filamentous form involves a hetero-trimer as its core module (Sirajuddin et al., 2007; Nakahira et al., 2010; Sandrock et al., 2011; Sellin et al., 2011a). Sirajuddin et al. first solved the crystal structure of the mammalian septin complex containing SEPT2, 6, and 7 and demonstrated its hexameric composition with mirroring symmetry arranged as a head-to-head trimer in the order 7-6-2-2-6-7 (Sirajuddin et al., 2007) (**Figure 1C**). In humans septins self-assemble predominantly into hetero-hexamers or hetero-octamers, whereas the hetero-octamers additionally contain SEPT3 subgroup members (Sellin et al., 2014). Sandrock et al. and Kim et al. showed that SEPT9 caps the ends of octameric complexes with SEPT7 (9-7-6-2-2-6-7-9) (Kim et al., 2011; Sandrock et al., 2011) (**Figure 1D**).

The mammalian six subunit core heteromers are apparently stable protein complexes (Sellin et al, 2011b) but several studies showed that within one septin subgroup the individual members can substitute for one of the others at the same position of the

TABLE 1 | Subgroup-specific septin-septin binding preferences, septin-interacting proteins, and their physiological relevance.

Septin subgroup	Preferred partner		References
SEPT6 group	SEPT2 group, SEPT3 group, SEPT7 group		Nakahira et al., 2010; Sandrock et al., 2011
SEPT2 group	SEPT6 group		Nakahira et al., 2010; Sandrock et al., 2011
SEPT3 group	SEPT7 group		Nakahira et al., 2010; Sandrock et al., 2011
Protein	**Interacting septin**	**Physiological relevance**	**References**
CENP	Septin 1, 2, 4, 5, 7, 9	Exocytose, intracellular trafficking	Nakahira et al., 2010
SNX6	Septin 2, 5, 6, 8, 11	Exocytose, intracellular trafficking	Nakahira et al., 2010
Sec6/8	Septin 2, 4, 6, 7	Vesicle transport	Ihara et al., 2005
Syntaxin1A	Sepin 2, 5	Vesicle transport	Beites et al., 2001
VAMP1	Septin 4, 11	Vesicle transport	Zhang et al., 2000
Transferrin receptor	Septin 4, 11	Vesicle transport	Zhang et al., 2000
FLNA	Septin 9	Cytoskeleton organization, vesicle transport	Nakahira et al., 2010
SH3KBP1	Septin 9	Cytoskeleton organization, vesicle transport	Nakahira et al., 2010
IFT27	Septin 3, 7	Vesicle transport, endocytose	Nakahira et al., 2010
Ra1ABP1	Septin 3, 7	Vesicle transport, endocytose	Nakahira et al., 2010
Actin cytoskeleton	Septins	Multiple functions	Kinoshita et al., 2002; Spiliotis et al., 2005
Microtuble cytokeleton	Septins	Multiple functions	Surka et al., 2002; Nagata et al., 2003; Kremer et al., 2005; Joo et al., 2007; Bowen et al., 2011; Mostowy and Cossart, 2012
Pospholipid membrane	Septins	Multiple functions	Spiliotis et al., 2008; Tanaka-Takiguchi et al., 2009
Anillin	Septin 2	Filament organization	Spiliotis et al., 2005
α-Tubulin	Septin complexes	Regulation of actin and tubulin polymerization	Nagata et al., 2003
Cytochalasin D	Septin 2, 6	Regulation of actin polymerization	Spiliotis et al., 2005
Latrunculin	Septin 2, 6	Regulation of actin polymerization	Spiliotis et al., 2005
MAP4	Septin 2-6-7 complex	Modulation of microtuble dynamics	Bowen et al., 2011
HDAC6	Septin 7	Dendritic development	Ghossoub et al., 2013
ERK3	Septin 7	Dendritic morphology	Ageta-Ishihara et al., 2013
Aurora-B	Septin 1	Mitose, cytokinesis	Hu et al., 2012
Cdk1/Pin1	Septin 9	Cytokinesis	Qi et al., 2005
Drp1	Septin 2	Mitochondrial fission	Estey et al., 2013
Tau, NFTs	Septin 1, 2, 4	Neuronal differentation and growth	Pagliuso et al., 2016
Parkin	Septin 5	Regulation of neuronal differentation and growth	Takehashi et al., 2004
α-Synuclein	Septin 4	Regulation of neuronal differentation and growth	Dong et al., 2003; Ihara et al., 2003
MLL	Septin 5, 6, 9, 11	Myeloid-lymphoid leukemia	Megonigal et al., 1998; Zieger et al., 2000
BORG3-cdc42	Septin 6, 7	Cell polarity, cytokinesis, vesicle transport	Engidawork et al., 2003; Kuo et al., 2015
BORG4-AP-3	Septin 6, 7	Regulation of endocytose	Joberty et al., 2001; Baust et al., 2008
KIF17	Septin 9	Intracellular protein transport	Nakahira et al., 2010; Traikov et al., 2014
UBE21, SUMO, PIAS	Near all septins	Protein degradation	Nakahira et al., 2010

complex *in vivo* and *in vitro* (Nakahira et al., 2010; Sandrock et al., 2011). For example, SEPT2 in the SEPT2-6-7 complex is replaceable by SEPT4 or 5 (**Figure 1E**), SEPT6 by SEPT8, 10, or 11. Several different trimers have been described, including SEPT3-5-7 (Fujishima et al., 2007), SEPT4-5-8 (Martinez et al., 2004), SEPT5-7-11 (Xie et al., 2007), SEPT7-11-9b (Nagata et al., 2004). The subunit heteromers depend on SEPT7 for stability (Sellin et al., 2011a). Mutations of a potential phosphorylation site within SEPT7 regulates the binding to all other septins (Sandrock et al., 2011). The higher-order septin structures are assembled from a mixture of hexamers and octamers, which all include the SEPT7 and variable SEPT2, 3, and 6 subgroup members. The composition of the septin complex is cell-type specific and essential for certain functions. At the mammalian sperm annulus SEPT12 as well as SEPT9 can flank the SEPT2-6-7 hexamers to form octamers (e.g., 12-7-6-2-2-6-7-12 or 12-7-6-4-4-6-7-12), suggesting a critical role in sperm motility (Kuo et al., 2015). These interaction studies in yeast and immunoprecipitation approaches have hinted toward the existence of diverse non-canonical septin complexes, but future studies are needed to determine the factors determining heteropolymer assembly.

Septins polymerize and interact with other septins via two interaction interfaces (G- and NC-interface) (Sirajuddin et al.,

2007, 2009). The G-interfaces comprise the GTP-binding domain whereas the NC-interfaces involve the N- and C-terminal regions, which are brought into close proximity upon folding (Sheffield et al., 2003). This means, each septin subunit assembles and extends apolar filaments arranged in a palindromic order by alternating NC- and G-interface associations (**Figure 1C**). Upon GTP-binding a conformational change in the switch regions is provoked, which affects the G- and the NC-interface. The GTP-binding capacity of septins is essential for septin-septin interactions and fundamental to ensure structural integrity, in a way that GTP-binding and its hydrolysis control assembly and disassembly of filaments and also the stability of the interface within the septin polymer (Zeraik et al., 2014). In the SEPT2-6-7 complex SEPT2 and 7 bind to GDP, while SEPT6 binds to GTP (de Almeida Marques et al., 2012). A Sept11 mutant, which showed reduced GTPase activity, was unable to form filaments (Hanai et al., 2004). Moreover, the GTPase domain seems to form homo-dimers and homo-filaments *in vitro* (Garcia et al., 2006; Huang et al., 2006; Nakahira et al., 2010). Studies showed that single septins may be unstable and assemble into homo-dimers or a fibrillary aggregated form called amyloid in the absence of GTP and by unbalanced stoichiometries. Depending on the temperature SEPT2 can exist as a dimer and contains regions within its G-domain sequence with a tendency to aggregate and/or form amyloids (Pissuti Damalio et al., 2012). Thereby, a decrease of α-helical content and a gain in β-sheet structure has been observed. Also homo-multimers have been reported for human SEPT2 in both the GTP- and GDP-bound states (Huang et al., 2006). Furthermore, Garcia et al. reported that SEPT4-G (an intermediate structure of the GTPase domain of human SEPT4) can form homo-filaments and amyloid-like aggregates (Garcia et al., 2007, 2008).

Due to alternative mRNA splicing many human septin genes present several variants. Cell culture models showed that the human SEPT9 exists as multiple isoforms, which have a common G-domain but differ in both length and sequence of the N-terminus. The SEPT9 isoform and expression level determine the higher-order arrangement of septin filaments (Sellin et al., 2012). In cells lacking SEPT7 mutational analysis of interaction surfaces reveals that SEPT9 exists as monomer (Kim et al., 2011; Sellin et al., 2011a). Besides the G-domain, the C-terminal domains and their coiled-coil regions are important determinants for filament assembly and stability and are important for recognition and binding of partner molecules (de Almeida Marques et al., 2012). For example, some septins interact with proteins (CENP-E/F, SNX6), which are associated with intracellular trafficking and exocytosis or are part of the kinetochore via coiled-coil domains (Nakahira et al., 2010) (**Table 1**). Filaments containing septins are implicated in exocytosis and are closely involved in membrane transport and fusion (Blaser et al., 2004; Ihara et al., 2005). Septins may associate to provide a targeting system to recruit secretory vesicles to appropriate docking/fusion sites leading to the correct organization of proteins along the plasma membrane. In brain lysates of rats the septins SEPT2, 4, 6, and 7 are associated with the exocyst complex sec6/8 that is essential for neuronal vesicle transport (Hsu et al., 1998). In neurons SEPT2 and 5 have been shown to interact with syntaxin 1A, a t-SNARE protein predominantly present on the plasma membrane, and they copurify with synaptic vesicles (Beites et al., 1999, 2001; Zhang et al., 2000). Bartsch et al. demonstrated the colocalization of SEPT4 and 11 with the vesicle-associated protein synaptobrevin 1 (VAMP1) and the endocytotic transferrin receptor (Bartsch et al., 2010). Moreover, SEPT9 interacts with filamin A (FLNA) and SH3-domain kinase binding protein 1 (SH3KBP1) via N-terminus (Nakahira et al., 2010), two proteins involved in cytoskeleton organization and vesicle transport (van der Flier and Sonnenberg, 2001; Spiliotis et al., 2005). Further septin partners associated with vesicle transport are other Ras-like GTPases (IFT27, Ra1ABP1) (Nakahira et al., 2010).

Mammalian septins interact with actin (Kinoshita et al., 2002; Joo et al., 2007) and microtubule cytoskeletons (Surka et al., 2002; Nagata et al., 2003; Kremer et al., 2005; Spiliotis et al., 2008; Bowen et al., 2011; Sellin et al, 2011b), as well as with phospholipid membranes (Tanaka-Takiguchi et al., 2009; Bertin et al., 2010). Thus, they assemble at specific locations in the cell to coordinate changes in membrane and cytoskeletal organization by acting as cell scaffolds for protein recruitment to specific sites in a cell and/or as lateral diffusion barriers in the plasma membrane to compartmentalize discrete cellular domains. Unlike actin and microtubules, septin complexes are apolar along the longitudinal axis in recombinant systems. One important actin-binding protein, which interacts with septins at the cell surface is anillin (Kinoshita et al., 2002). Anillin mediates the septin filament organization along actin bundles by recruiting and stabilizing myosin, actin, and regulatory kinases (Joo et al., 2007; Maddox et al., 2007). Septin complexes interact with α-tubulin and control actin and tubulin polymers (Surka et al., 2002). In fibroblasts cytochalasin D or latrunculin inhibit the actin polymerization and the septin filaments disappear while septin rings occur, which are not associated with actin (Kinoshita et al., 2002). The microtubule-associated protein MAP4, which is required for modulation of microtubule dynamics during mitosis and cytokinesis, is another septin binding partner. MAP4 is recruited to the SEPT2-6-7 complex via the direct interaction of its C-terminal proline-rich domain with SEPT2 (Kremer et al., 2005). SEPT2 together with MAP4 is involved in the organization of primary cilia (Ghossoub et al., 2013). In addition, SEPT7 is associated with transport of organelles and regulates dendritic morphology (Xie et al., 2007) by interaction with α-tubulin deacetylase HDAC6 (Ageta-Ishihara et al., 2013) or extracellular signal-regulated kinase 3 (ERK3) (Brand et al., 2012). In developing axon collateral branches, SEPT7 influences the remodeling of microtubules into filopodia, a process required for successful formation of branches (Hu et al., 2012). Tubulin-associated SEPT2 facilitates vesicle transport from the Golgi to the plasma membrane (Spiliotis et al., 2008). Furthermore, septins are involved in cell division. SEPT1 is a target of aurora-B, which is an important serine/threonine kinase required for chromosome segregation and cytokinesis (Qi et al., 2005). Phosphorylation of SEPT9 by cyclin-dependent kinase 1 (Cdk1) regulates association with the proline isomerase (Pin1), which is crucial for the disjunction of daughter cells (Estey et al., 2013).

Recently SEPT2 has been shown to participate in dynamin-like protein Drp1-dependent mitochondrial fission (Pagliuso et al., 2016). Misregulation of human septins is associated with numerous diseases, like neurodegenerative disorders. For example, septins are involved in Alzheimer's disease (AD) because they interact with neurofibrillary tangles (NFTs). SEPT1, 2 and 4 bind the microtubule-associated protein tau, a major component of the neurofibrillary tangles, which is important for neuronal differentiation and growth (Kinoshita et al., 1998). Furthermore, genotype studies of polymorphic SEPT3 alleles in human neuronal cells indicated a significant difference between AD patients and controls (Takehashi et al., 2004). Another binding partner of septins is parkin, which is mutated in autosomal-recessive juvenile parkinsonism (ARJP) (Kitada et al., 1998). Parkin, an ubiquitin ligase, interacts with SEPT5 and mediates its degeneration (Choi et al., 2003; Son et al., 2005). A loss of parkin causes accumulation of SEPT5 in neurons of patients with ARJP and induces selective dopamine-dependent neurodegeneration. SEPT5 inhibits the release of dopamine (Zhang et al., 2000; Choi et al., 2003; Dong et al., 2003; Son et al., 2005). In Parkinson's disease or dementia SEPT4 is accumulated in cytoplasmic aggregates colocalizing with the dopamine receptor α-synuclein. Sept4 deficient mice exhibit diminished dopaminergic neurotransmission due to the lack of SEPT4 (Ihara et al., 2003, 2005, 2007). SEPT4 is a distinct gene product with a >90% identity to SEPT5 (Zieger et al., 2000). Some human septin proteins (SEPT5, 6, 9, and 11) have been cloned as fusion partner of myeloid-lymphoid leukemia *MLL* (also referred to as *ALL1* or *HRX*) genes. These fusion proteins consist of almost the entire open reading frame of the involved septin and the N-terminus of MLL (Megonigal et al., 1998; Kojima et al., 2004). Thus, misregulation of human septins plays a role in cancer. SEPT9 supports HIF-1α-mediated transcription in tumor cells (Amir et al., 2009), suggesting that SEPT9 is a player in posttranslational modification. Down-syndrome patients showed an increased expression of mixed lineage leukemia septin like fusion protein (MSF)-B, suggesting a hint why Down-syndrome children show a stringent incidence of acute leukemia (Engidawork et al., 2003).

Septins have been shown to interact with BORGs (binding partners of RHO-GTPases; CDC42 effector proteins). For example, BORG3 binds directly to SEPT6 or 7 (Joberty et al., 2001; Sheffield et al., 2003). BORGs (BORG1 to BORG3) interact generally with two types of GTPases, namely septins and CDC42. Overexpression of constitutively active CDC42-GTPase markedly affects the association of BORG3 with septins and disrupts normal septin complex organization leading to a pathological localization of the septins within the cell. Thus, BORGs are important regulators of mammalian septin organization and provide a link between the septins and CDC42-GTPases, which regulate cell polarity, cytokinesis, cytoskeletal remodeling and vesicle transport (Joberty et al., 2001). Baust et al. identified a septin-/BORG-protein network und hypothesized that BORG4 and septins are important regulators for the AP-3 adaptor complex-dependent sorting of the lysosome membrane protein 1 (LAMP-1) to lysosomes (Baust et al., 2008). AP-3 is a member of an adaptor complex, which is involved in the targeting of cargo destined to remain in outer membranes of maturing endosomal compartments. In this complex, SEPT6 and 7 function as regulators of endosome transport by modulating the timely coordinated interaction of AP-3 (Traikov et al., 2014). In addition, SEPT9 interacts with the kinesin 2 family motor KIF17, which is a cargo/scaffold protein, suggesting the importance of SEPT9 in transport mechanisms in neurons (Bai et al., 2016). SEPT9 also regulates growth and accumulation of lipid droplets, which are frequently observed in hepatitis C virus infection (HCV), by a phosphatidylinositol-5-phosphate and microtubule-dependent binding mechanism in HCV-infected cells (Akil et al., 2016).

Nakahira et al. described septin interactions with several proteins that play a role in protein degeneration and functionally associated with the ubiquitin and sumoylation cycles (e.g., UBE21, SUMO, or PIAS) (Nakahira et al., 2010). Sumoylation generally controls assembly, localization, stability and other functions of protein complexes (Schmidt and Muller, 2003; Johnson, 2004).

SEPTIN INTERACTIONS IN PLATELETS AND HUMAN ENDOTHELIAL CELLS

In platelets several septins (SEPT2, 4, 5, 7, 8, 9, and 11) are expressed, which seem to be important for regulating platelet function (Yagi et al., 1998; Zieger et al., 2000; Blaser et al., 2003; Bartsch et al., 2010; Sandrock et al., 2011). Transmission electron microscopy revealed that SEPT4 and 8 surround the α-granules, as it had been shown for SEPT5, suggesting that they may be components of the same complex in platelets and play in such a way a general regulatory role in platelet biology. Activation of platelets by agonists resulted in the translocation of SEPT4 and 8 to the platelet surface indicating a possible functional role of these proteins in platelet granular transport and secretion (Dent et al., 2002; Blaser et al., 2004; Martinez et al., 2006). Platelets from Sept5 deficient mice showed altered granule release (serotonin) and revealed a decreased threshold for agonist-mediated platelet aggregation, suggesting that SEPT5 is involved in platelet physiology (Dent et al., 2002). Sept5 deficient mice presented a marked increase in bleeding symptoms compared to wild-type mice. The regulatory factors promoting vesicle docking and fusion after platelet stimulation are unknown so far, but it might be a large multimeric complex of various proteins involved in platelet biology. The interaction in a macromolecular complex in platelets between SEPT5 and syntaxin-4, which is involved in vesicle transport, suggests a cooperation of these proteins supporting the critical role of SEPT5 in granule secretion. Interestingly, in platelets SEPT5 has a strong affinity for other septins, such as SEPT4 and 8, which are expressed together in various tissues (Blaser et al., 2002; Martinez et al., 2004). In addition, SEPT5 has been shown to be colocalized with SEPT6 in the periphery of the platelet and in the cytoplasma and was associated with platelet microtubules in the SEPT5-6-7 complex promoting the important role of septins in granule trafficking through their association with the microtubule network. Martinez et al. revealed furthermore

that SEPT9 is part of the SEPT5-6-7-9 complex in platelets (Martinez et al., 2006). In platelets and HUVECs, SEPT5 has been identified as an interaction partner of SEPT11, which may also be involved in regulated secretion (Blaser et al., 2006). Many septins are expressed ubiquitously, for example SEPT6 (Ono et al., 2002), SEPT7, 9, and 11 (Hanai et al., 2004) while a subset appears tissue restricted (Hall et al., 2005; Cao et al., 2007), like SEPT5 (previous: hCDCrel-1; human cell division cycle gene, PNUTL1) (Macara et al., 2002), which is expressed predominantly in platelets, brain, and heart (McKie et al., 1997; Yagi et al., 1998). In addition, SEPT5 deficiency exerts pleiotropic effects on affective behaviors and cognitive functions as shown in Sept5 knock-out mice, which feature delayed acquisition of rewarded goal approach (Suzuki et al., 2009). In HIT-T15 cells mutations in SEPT5 inside the GTP-binding domain lead to an increased granule secretion (Beites et al., 1999). As shown before, in human endothelial cells SEPT4 and 11 are involved in endo- and exocytotic processes by interacting with vesicle-associated proteins (Bartsch et al., 2010). These studies confirm the binding preferences of the SEPT2 subgroup (SEPT4 and 5) with members of the SEPT6 subgroup (SEPT8 and 11) (**Figure 1E**).

Interestingly, mice with Bernard-Soulier-syndrome (BBS) caused by genetic deletion of the platelet glycoprotein (GP) Ibβ (GP1BB) demonstrated increased levels of SEPT5 in the megakaryocytic linage. Overexpression of SEPT5 is associated with fewer and larger platelet α-granules, suggesting that SEPT5 supports normal α-granule size in wild-type littermates (Kato et al., 2004). *SEPT5* was identified as a 5′gene located in close 5′proximity to the *GPIbβ* gene (Zieger et al., 1997). GPIbβ is besides GPIbα a subunit of GPIb, which is a major component of the platelet membrane receptor (GPIb/IX) for von Willebrand factor (Ruggeri, 1991). Both genes, *SEPT5* and *GPIbβ*, are located within the chromosomal locus 22q11.2, a region associated with the DiGeorge syndrome (DGS) and other cardio-facial abnormalities (McDermid and Morrow, 2002). The close relationship of DGS and GPIbβ has been confirmed by chromosomal deletion of 22q11.2 in a patient with both DGS and BSS (congenital absence of the platelet GPIb/V/IX receptor complex) (Budarf et al, 1995).

In a boy with a unique homozygous deletion of the two contiguous genes *SEPT5* and *GPIbβ* resulted in a BSS phenotype (reduced expression of the GPIb/V/IX receptor) combined with a platelet secretion defect (Bartsch et al., 2011). The patient suffered from life-threatening bleedings, which could be hardly stopped. Therefore, he received hematopoietic stem cell transplantation. Because of the deletion of SEPT5 (which is also important for vesicle transport and exocytosis in neurons) the patient showed an additional secretion defect of platelet α-granules. Most probably, the platelet secretion defect resulted in an additive effect regarding his bleeding symptoms besides the BSS defect. Furthermore, the patient is retarded in development and shows autistic streaks. The cortical dysplasia (polymicrogyry) and neurologic dysfunction is likely caused by the SEPT5 deletion. His parents both exhibit a heterozygous deletion on this area.

CONCLUSION

Diverse studies showed cell-specific formation of mammalian septin-multimers and their association with a variety of the cellular processes, such as actin dynamics, microtubule regulation, membrane trafficking, vesicle transport, exocytosis, the assembly of scaffolding platforms, protein degradation, and mechanical stability. Many septins have been associated with diverse human diseases, such as neurodegenerative and bleeding disorders. Homozygous deletion of ubiquitary septins results in embryonic lethality (Roseler et al., 2011). Exact structural properties and many of the molecular details and modes of action remain unclear. The complex relationship between polymerization, bundling, GTPase activity and membrane association needs to be elucidated in future studies.

AUTHOR CONTRIBUTIONS

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

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MINI REVIEW
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Cancer-Related Functions and Subcellular Localizations of Septins

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Since the initial discovery of septin family GTPases, the understanding of their molecular organization and cellular roles keeps being refined. Septins have been involved in many physiological processes and the misregulation of specific septin gene expression has been implicated in diverse human pathologies, including neurological disorders and cancer. In this minireview, we focus on the importance of the subunit composition and subcellular localization of septins relevant to tumor initiation, progression, and metastasis. We especially underline the importance of septin polymer composition and of their association with the plasma membrane, actin, or microtubules in cell functions involved in cancer and in resistance to cancer therapies. Through their scaffolding role, their function in membrane compartmentalization or through their protective function against protein degradation, septins also emerge as critical organizers of membrane-associated proteins and of signaling pathways implicated in cancer-associated angiogenesis, apoptosis, polarity, migration, proliferation, and in metastasis. Also, the question as to which of the free monomers, hetero-oligomers, or filaments is the functional form of mammalian septins is raised and the control over their spatial and temporal localization is discussed. The increasing amount of crosstalks identified between septins and cellular signaling mediators reinforces the exciting possibility that septins could be new targets in anti-cancer therapies or in therapeutic strategies to limit drug resistance.

Keywords: septin, cancer, plasma membrane, actin cytoskeleton, microtubules

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INTRODUCTION

In humans, 13 septin proteins have been identified, which can be clustered into four groups according to their gene structure homology (Kinoshita, 2003; Hall et al., 2005). A plethora of isoforms has been described, and for SEPT4, 8 and 9, long and short N-terminal extensions have been identified (see Hall and Russell, 2012). Such diversity allows a large repertoire of septin assemblies, which could explain the multiplicity of septin subcellular localizations and functions. Septins can oligomerize into repeated and ordered hexamers or octamers, which can organize into higher-order structures, including rings or filaments. Ring structures have been well described in the context of mitotic completion or in differentiated cell structures like at the base of primary cilia or in the spermatozoid annulus (for reviews, Caudron and Barral, 2009; Saarikangas and Barral, 2011; Spiliotis and Gladfelter, 2012). Septins are also observed as rods and filaments, which associate with actin, microtubules (MTs) or membranes and are thus considered as a fourth

component of the cytoskeleton (Mostowy and Cossart, 2012). Septins function as scaffolds for protein-protein interactions, or as diffusion barriers for protein compartmentalization, not only during cell division, but also in an increasing number of processes in interphase cells (for reviews, Mostowy and Cossart, 2012; Fung et al., 2014; Montagna et al., 2015).

Septins have initially been identified as fusion partners with MLL in leukemia (Osaka et al., 1999; Cerveira et al., 2011). Their gene expression is also deregulated in tumors. *SEPT2*, *8*, *9*, and *11* genes are consistently up-regulated, while *SEPT4* and *10* are down-regulated in many cancer cells (Liu et al., 2010; Montagna et al., 2015). A colon cancer diagnosis method based on the quantification of circulating methylated *SEPT9* DNA has even been proposed (for review, Song and Li, 2015). Septin isoform expression (mainly focused on SEPT9 isoforms) has also been studied in a broad range of solid tumors (Scott et al., 2006; Connolly et al., 2011, 2014; Shen et al., 2012; Gilad et al., 2015). Here, we focus on the links between the modulation of septin polymer composition, their differential subcellular localization, and the molecular and cellular pathophysiological mechanisms they affect in cancer, both in mitotic and interphase cells.

ROLES OF SEPTINS IN MITOSIS

By forming highly organized rings at cell division sites, septins have been found to play a crucial role in the spatio-temporal control of yeast budding, and the mechanisms that control septin assembly, remodeling and functions in this context are still thoroughly explored in the yeast model. In mammalian cells, septins have also been identified as one of the contributors of mitosis, and could potentially be implicated in a variety of cancers. Indeed, after Cdk1-mediated phosphorylation, long SEPT9 isoforms become a substrate of the prolyl-isomerase Pin1, and their isomerization is required for cytokinesis completion (Estey et al., 2013). Like other oncogenes and tumor suppressors controlled by Pin1 (for review, Zhou and Lu, 2016), specific SEPT9 isoforms may thus participate in oncogenesis. Also, septins contribute to fulfill proper chromosome congression and correct segregation during the anaphase. In this context, the SEPT2/6/7 complexes seem to be important for the recruitment of the kinesin family protein CENP-E (Spiliotis et al., 2005), which participates in the mitotic checkpoint, and for chromosome movement along MTs during the anaphase. At the onset of telophase, septins concentrate at the central spindle region where they interact with the actomyosin contractile ring via the partner protein anillin (for reviews, Fung et al., 2014; Menon and Gaestel, 2015). Recent data indicate that the anillin-septin ring promotes the intercellular bridge ingression, elongation and narrowing. These steps occur prior to septin and anillin relocalization to the central stem body and to sites of MT constriction. There, the septin ring facilitates the recruitment of Chmp4B, allowing the assembly of the ESCRT III complex, which actually mediates the abcission step (Renshaw et al., 2014). A recent study on the effects of chrysotile fibers (responsible of mesothelioma, lung cancer, and asbestos) points out the role of cytokinesis failure mediated by an overexpression of SEPT2 and by anillin and SEPT9 mislocalizations, in causing aneuploidy, centrosome amplification, and multipolar mitoses (Cortez et al., 2016), which are frequent in cancer cells.

LOCALIZATION-DEPENDENT ROLES OF SEPTINS IN INTERPHASE CELLS

Out of the cell division context, septin contribution to cancer may also involve interphase cells, in a way that is linked to their subcellular location, as described hereafter and summarized in **Figure 1**.

Membrane-Associated Septins

Septin self-assembly into filaments and higher-order structures occur by diffusion-driven annealing on the plasma membrane (Bridges et al., 2014). In return, large septin filament arrays stably interacting with the plasma membrane may modify cortical morphogenesis by imposing membrane curvature (Tanaka-Takiguchi et al., 2009), and affect the cortical rigidity of migrating cells (Tooley et al., 2009), thus contributing to tumor metastasis.

Besides their role in membrane compartmentalization, septins have been implicated in the misregulation of growth factor receptors involved in cancer progression. They can cluster and stabilize plasma membrane proteins (Caudron and Barral, 2009; Hagiwara et al., 2011) including receptor tyrosine kinases. Indeed, membrane-associated SEPT9 prevents CIN85 binding to the ubiquitin ligase Cbl, resulting in reduced ubiquitin-dependent EGFR degradation (Diesenberg et al., 2015). Also, septins are involved in the abnormal persistence of ErbB2 at the plasma membrane of cancer cells via decreased ubiquitylation and degradation (Marcus et al., 2016). In addition, the surface distribution of the c-Met protooncogene is regulated in opposite ways by SEPT2 and 11, but both participate in its interaction with the ligand and anchorage to the actin cytoskeleton (Mostowy et al., 2011), illustrating the importance of the subunit composition of septin filaments in controlling their biological functions.

Septins may also associate with other membrane-bound organelles. SEPT2 and 7, by interacting with DRP1 would concentrate it at the sites of mitochondrial constriction and facilitate their fission (Pagliuso et al., 2016; Sirianni et al., 2016). Mitochondria-associated septins also comprise the isoform 2 of SEPT4 (SEPT4_i2) also called ARTS, which upon pro-apoptotic stimuli, is released in the cytosol where it binds to the XIAP proteins to release the inhibition of caspases and thus promote apoptosis (Edison et al., 2012). ARTS expression has been shown to drop in acute lymphoid leukemias and in lymphomas, and would be implicated in controlling the number of normal stem cells (García-Fernández et al., 2010). This has led to propose ARTS as a valuable therapeutic target against cancer stem cells (Elhasid and Larisch, 2011). Also, septins are implicated in autophagosome formation upon nutrient deprivation in yeast (Barve et al., 2016). These studies provide new clues to understand the role of septins in cancerogenesis and/or adaptation to tumoral environment.

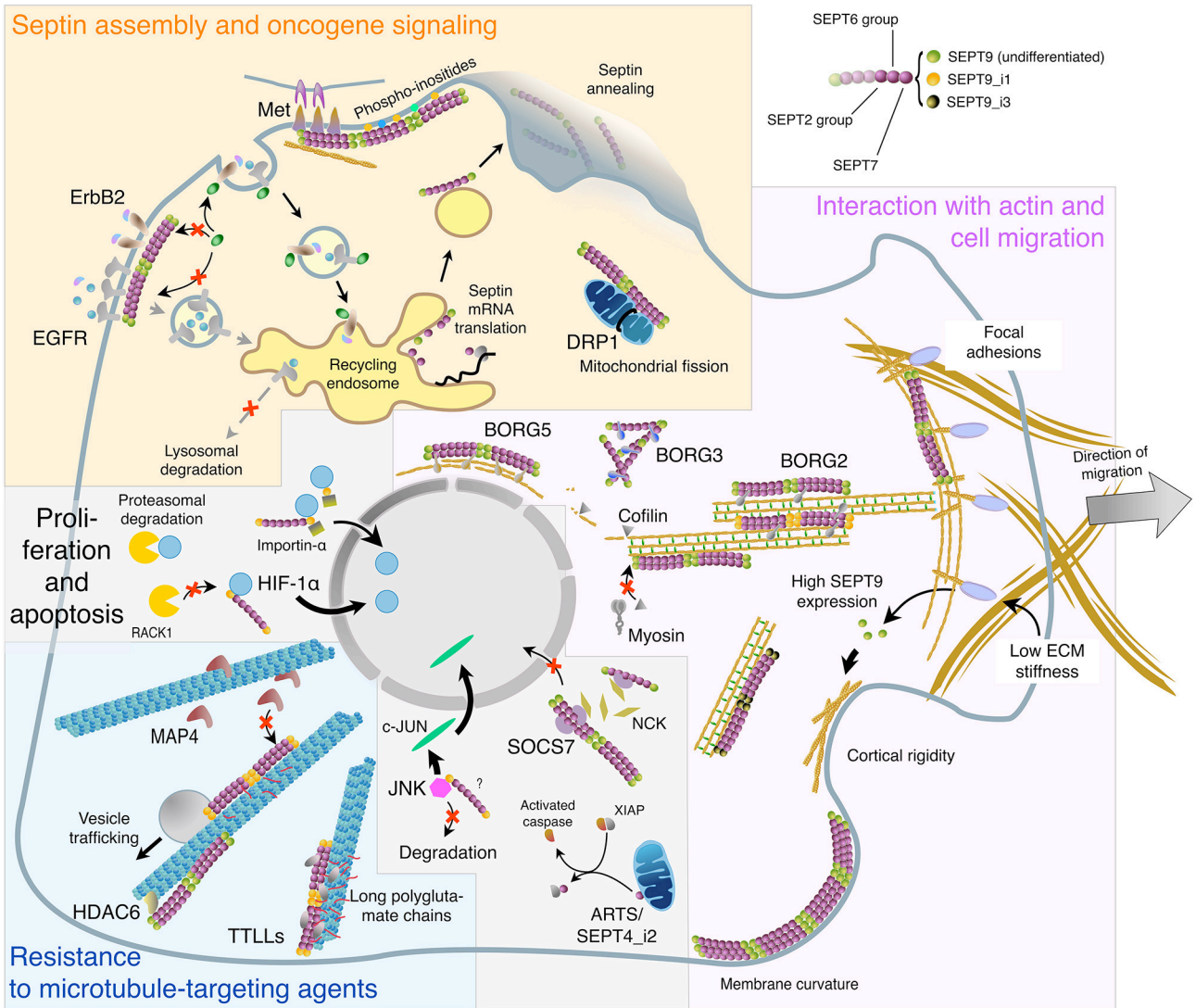

FIGURE 1 | Subcellular localizations and functions of septins in interphase cells in relation with oligomer composition and binding partners. This virtual cell summarizes the main topics described in the text regarding: Septin biosynthesis, annealing, and association with the plasma membrane. Role of septin filaments in oncogene receptor signaling and dynamics, and in mitochondria fission (Yellow panel). Septin localization to the actin cytoskeleton: direct binding or involvement of SEPT9 isoforms and BORG proteins. Implication of septin filaments in actin filament bundling and organization, cell migration, cortical rigidity, and membrane curvature sensing (Magenta panel). Septin binding to microtubules and the links with tubulin post-translational modifications and MAPs in vesicular trafficking and in cell resistance to microtubule-targeting agents (Cyan panel). Septin roles in the nuclear import of signaling factors involved in cell proliferation and in apoptosis. It is not known whether SEPT9 interacts alone or as part of septin oligomers with HIF-1α, importin-α and JNK (Light gray panel). The color code of septin monomers is indicated on the top right of the figure.

Actin-Associated Septins

In many cell types, septin filaments coalign with actin in subcortical regions or along stress fibers (Kinoshita et al., 1997). Actin loss causes septins to form free cytoplasmic rings (Kinoshita et al., 2002). Conversely, SEPT2 depletion attenuates actin bundling (Kinoshita et al., 2002) and disrupts stress fibers (Schmidt and Nichols, 2004). Knocking down SEPT6 and 7 (Kremer et al., 2007) produce a similar loss of actin bundling along with the disruption of cell polarity. These effects may involve septin binding partners that crosslink septins to actin like BORG2 and BORG5, which are two of the five Cdc42-effector proteins of the BORG family (Liu et al., 2014; Calvo et al., 2015). Stress fiber disruption may also involve septin partners that directly regulate actin like the protein Wdpcp (Cui et al., 2013).

Septins control actin remodeling during cell migration and may thus contribute to metastatic cancer cell dissemination and invasion. Septin filaments bind to and stabilize the transverse arc and radial stress fibers in the lamellipodia of migrating cells (Dolat et al., 2014). Such stabilization could

involve SEPT9-mediated prevention of actin depolymerisation by myosin and cofilin (Smith et al., 2015). Septins also contribute to the stabilization of nascent focal adhesions (Dolat et al., 2014), which is necessary for their turnover and thus for effective migration. *Sept9*-knockout mouse embryonic fibroblasts indeed migrate more slowly than wild-type cells (Füchtbauer et al., 2011). Also, SEPT9_i4 is involved in the control of migration directionality in MCF7 breast cancer cells (Chacko et al., 2005). Persistent directional migration is further dependent on SEPT7 and on BORG5, which maintain proper actin filament organization (Liu et al., 2014).

Cell migration and invasion requires epithelial-mesenchymal transition (EMT), which involves the formation of cell protrusions and changes in the way cells interact with the extracellular matrix (ECM). Depletion of SEPT9 in various metastatic cancer cells allows the reversion of EMT and reduces cell spreading, migration and invasion (Shankar et al., 2010). SEPT1 also participates in the spreading of squamous cell carcinoma DJM-1 cells (Mizutani et al., 2013).

Tumor progression also requires neoangiogenesis, which implies the migration of leader cells of the tumor microenvironment. Interestingly, septins are involved in both processes. Indeed, Yeh et al. (2012) proposed that the ECM stiffness controls SEPT9 expression of endothelial cells via integrin signaling, and regulates cell proliferation and peripheral distribution of actin assembly. Regarding tumor-associated fibroblast migration, Calvo et al. (2015) showed that the cohesion between actin fibers and septins is increased due to elevated expression of the crosslinking protein BORG2. This in turn leads to matrix remodeling, favors the activation of highly contractile cancer-associated fibroblasts and promotes cancer cell invasion, angiogenesis, and tumor growth.

Microtubule-Associated Septins

In a few cell types, septin filaments co-align with MTs (Surka et al., 2002; Nagata et al., 2003; for review, Silverman-Gavrila and Silverman-Gavrila, 2008). They compete with the MT-stabilizing protein MAP4 to associate with the MT lattice and reduce MT stability (Kremer et al., 2005; Spiliotis et al., 2008). By contrast, septin-decorated MTs also exhibit lower MT dynamics in MDCK cells (Bowen et al., 2011) although septins do not colocalize with stabilized acetylated or detyrosinated MTs (Spiliotis et al., 2008). However, the relationship between septins and MT acetylation is still unclear as, in dendrites, SEPT7 was found to interact with the tubulin deacetylase HDAC6 (Ageta-Ishihara et al., 2013). In addition, septin filaments interact with polyglutamylated MTs and favor vesicle trafficking along these tracks to maintain the polarity of MDCK cells (Spiliotis et al., 2008).

Cancer chemotherapy often makes use of MT-targeting agents (MTA), which do not only act during mitosis but also interfere with MT dynamics during the interphase. MT-associated septins by modulating the MT environment may therefore modulate MT-based activities. Several septins have been proposed to participate in cancer cell resistance to MTA. Low SEPT10 expression level would promote paclitaxel resistance (Xu et al., 2012), while resistance to paclitaxel and to 2-methyl-estradiol involves SEPT9_i1 overexpression in several cancer cell lines (Amir and Mabjeesh, 2007). Also, misregulations of SEPT9_i1 and i4 have been linked to bad prognosis and resistance to MTA in prostate (Gilad et al., 2015) and breast cancers (Chacko et al., 2012). Consistently, paclitaxel-resistant MDA-MB 231 breast cancer cells display increased SEPT2, 8, 9, 11 levels (Froidevaux-Klipfel et al., 2011). In these cells, septins are displaced from actin fibers to MTs, where they restore higher level of MT dynamics by acting as scaffolding proteins to recruit tubulin polyglutamylation enzymes. Septin recruitment to polyglutamylated MTs result in increased binding of MT modulators that play key roles in controlling catastrophe and rescue events (Froidevaux-Klipfel et al., 2015).

Septins not only form a diffusion barrier at the base of the primary cilium (Hu and Nelson, 2011), but also associate with acetylated MTs in the axonema of RPE-1 cells in which the SEPT2/7/9 complex controls ciliary length (Ghossoub et al., 2013). The loss of TTLL3 activity, a polyglycylase required for robust primary cilium formation has been involved in colon cancer development (Rocha et al., 2014). By analogy with the finding that septin filaments recruit tubulin polyglutamylases on MTs (Froidevaux-Klipfel et al., 2015), the axonema-associated septins could perhaps function to scaffold TTLL3 on ciliary MTs, as it belongs to the same family of enzymes.

Other Roles of Septins in Ill-Defined Locations

As observed for membrane receptors, septins, and more precisely SEPT9_i1, stabilize other signaling proteins like JNK (Gonzalez et al., 2009) or HIF-1α (Amir et al., 2009) by preventing their degradation. However, these studies used total cell extracts and gave no indication of where these events take place in the cell. In both cases, it is not known whether septins function as filaments. By stabilizing JNK, SEPT9_i1 promotes longer JNK signaling, c-Jun phosphorylation, and cyclin D1 expression, leading to enhanced proliferation (Gonzalez et al., 2009). Septins are also involved in the nucleo-cytoplasmic distribution of proteins. By binding to SOCS7, which contains the nuclear import/export signals, the SEPT2/6/7 complex maintains SOCS7 together with the adapter protein NCK in the cytoplasm, thus perturbing cell cycle arrest induced by DNA damage (Kremer et al., 2007). Also, SEPT9_i1 was evidenced to physically bind to importin-α and HIF-1α to promote HIF-1α nuclear translocation and subsequent transcriptional activation (Golan and Mabjeesh, 2013).

MOLECULAR DETERMINANTS OF SEPTIN FILAMENT LOCALIZATION

The high diversity of septin isoform expression and the variety of their assembly into oligomers and higher-order structures suggest a molecular basis for their multiple localizations and functions. The intimate determinants of subcellular septin fate are most often enigmatic but some targeting and/or interaction mechanisms have been described (see **Figure 1**).

One intriguing mechanism has been revealed in fungi and could potentially be generalized to other organisms. In polarized fungal hyphae, septins are locally translated and assembled into

heteromeric complexes on the surface of shuttling endosomes for an efficient long-distance transport and deposition at growth poles (Baumann et al., 2013; Zander et al., 2016). Consistent with such a model, the majority of septin molecules found in the cytosol are already assembled into octamers, while the septin complexes recovered at the plasma membrane are composed of multiple octamers that can anneal (Bridges et al., 2014). Septin rod association into filaments would be driven by interactions with the plasma membrane (Bridges et al., 2014). Such interactions involve the binding to membrane-specific phosphoinositides like PIP2 (Zhang et al., 1999), PI4,5bis-phosphate (Bertin et al., 2010) or PI5P (Akil et al., 2016) and the local curvature of the membrane, which can be efficiently discriminated by septin rods (Bridges et al., 2016).

Septins have been found to bind actin directly (Mavrakis et al., 2014; Smith et al., 2015). The N-terminal tail of SEPT9 was recently evidenced to cross-link actin filaments by binding to three different sites on F-actin (Smith et al., 2015). While anillin has been evidenced to bridge septin filaments with actin during mitosis, BORG2 was recently shown to play a similar role in non-dividing cells (Calvo et al., 2015). BORG1, 2 and 3 were evidenced to directly bind to septin oligomers that comprise SEPT6, and this interaction is negatively regulated by Cdc42 (Joberty et al., 2001; Sadian et al., 2013). Gic1, one of the yeast homologs of human BORG, has been shown to stabilize long septin filaments by binding to the Cdc10 (a septin of the group 3) subunit (Sadian et al., 2013). In contrast, activated BORG3, by strongly binding to the SEPT6/7 interface (Sheffield et al., 2003), causes uncontrolled septin bundling and thus filament loss (Joberty et al., 2001; Kinoshita et al., 2002). More recently, BORG5 was shown to control the localization of septin filaments along the perinuclear actin fibers (Liu et al., 2014). The spatiotemporal control of septin targeting to the actin cytoskeleton may thus depend on the presence of binding partners in the cytoplasm, excluding the participation of anillin, which is sequestered in the nucleus during the interphase (Field and Alberts, 1995). Regulation of BORG availability may also exist, as BORG2 expression increased in response to cancer cell-derived soluble factors and is consistently increased in the stromal compartment of breast cancers (Calvo et al., 2015).

SEPT9, in particular SEPT9_i1, has been linked to many cancers of bad prognosis, to cell resistance to MTA and was found to be overexpressed during the interphase (G1 and S) in breast cancer (Gonzalez et al., 2009). Long SEPT9 isoforms (SEPT9_i1, i2, and i3), which are preferentially incorporated into higher-order structures than short ones (_i4-5; Sellin et al., 2011), contribute to localize septin filaments along interphase MTs (Sellin et al., 2012; Bai et al., 2013; Mizutani et al., 2013). Thus, the localization of SEPT9-containing filaments to actin in the cytoplasm and to MTs in the cilium of RPE-1 cells (Ghossoub et al., 2013), may also rely on the expression of specific *SEPT9* splice variants. By directly binding *in vitro* to MTs through its GTP-binding domain (Nagata et al., 2003), SEPT9_i1 was proposed to target septin filaments to MTs in interphase cells (Surka et al., 2002). The repeated basic motifs in the N-terminal regions of the long SEPT9 isoforms (SEPT9_i1-3) were shown to interact with the acidic regions of tubulin (Bai et al., 2013). However, a more recent study demonstrated that the whole N-terminal domain of mammalian SEPT9_i1 also contains an interaction domain with F-actin (Smith et al., 2015). These findings indicate that other molecular determinants on septins or on their target organelles may play a role in septin association with either cytoskeleton element. Tubulin posttranslational modifications are one of these determinants as highlighted by the differential septin filament locations between sensitive and Taxol®-resistant breast cancer cells, which both express long SEPT9 isoforms (Froidevaux-Klipfel et al., 2015). As already observed in MDCK cells, septin filaments coalign with polyglutamylated MT tracks (Spiliotis et al., 2008). Long lateral polyglutamate chains on tubulin even enhance this association in Taxol®-resistant breast cancer cells, while septins remain associated with actin in their Taxol®-sensitive counterpart (Froidevaux-Klipfel et al., 2015). This differential septin filament localization between MTs and actin filaments correlates with a high level of SEPT9_i1 in resistant cells, while SEPT9_i3 is the main SEPT9 isoform found in the septin filaments of sensitive cells. By bearing 5 more positive charges than SEPT9_i3, SEPT9_i1 would have more affinity for the acidic tail of tubulin, and even more when it bears long polyglutamate chains.

CONCLUDING REMARKS

Although, septin roles in cancer have been largely documented, the understanding of how the spatial and temporal dynamics of septin subcellular localization is regulated remains to be fully addressed. As described above, some clues are emerging, as the incorporation of specific SEPT9 isoforms into oligomers was found to orient the final destination of septin filaments inside the cell. Other septins are also involved, as the SEPT5-containing complexes are enriched in the lamellipodia of squamous cell carcinoma DJM-1 cells, while complexes recovered along MTs exclude this septin (Mizutani et al., 2013).

Besides filament composition, much remains to be learned about the potential roles of septin monomers vs. oligomers and vs. filaments. Indeed, while SEPT2, 7, and 11 are required at the early stages of cytokinesis, only the SEPT9 deletion impairs the final separation of daughter cells (Estey et al., 2010), suggesting a role for SEPT9 even when it is not incorporated into a filament. Also, not only filaments but septin hexamers can mediate actin bending and bundling during actin remodeling at the furrow canal in *Drosophila* embryos (Mavrakis et al., 2014). Therefore, caution must be taken when overexpressing a single septin because it might perturb the original septin filament location and/or function, as observed for some SEPT9 isoforms. Also, a strong expression of individual septins may form homo-oligomers (see Fung et al., 2014; Sellin et al., 2014) that have nothing to do with physiological septin filaments. Furthermore, posttranslational modifications (phosphorylation, acetylation, and sumoylation) may impact the way septins assemble into higher-order structures as described in fungi and will deserve further investigation in the context of cancer (Hernández-Rodriguez and Momany, 2012).

Regarding cancer therapy or cell adaptation and resistance to chemotherapies, the direct targeting of septins could have many side effects. Nevertheless, septins have been proposed as molecular targets in solid tumors, where they are required for cytokinesis completion as opposed to hematopoietic cells (Menon et al., 2014). Alternatively, research of novel therapeutic targets might focus on the perturbation of the subcellular localization of septin filaments. As such, by dampening the lateral interactions between parallel septin filaments at the anaphase spindle midline, alternating electric fields used for the treatment of recurrent glioblastoma induce mitotic catastrophe and subsequent apoptosis (Gera et al., 2015). Septin relocalization in interphase cells might as well be achieved by the targeting of their membrane- or cytoskeletal-binding partners. Understanding the underlying mechanisms of septin subcellular localization therefore deserves more interest, and will be important to focus on in the years to come.

AUTHOR CONTRIBUTIONS

CP, LK, and AB designed and wrote the manuscript.

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REVIEW
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Septin Mutations in Human Cancers

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Septins are GTP-binding proteins that are evolutionarily and structurally related to the *RAS* oncogenes. Septin expression levels are altered in many cancers and new advances point to how abnormal septin expression may contribute to the progression of cancer. In contrast to the *RAS* GTPases, which are frequently mutated and actively promote tumorigenesis, little is known about the occurrence and role of septin mutations in human cancers. Here, we review septin missense mutations that are currently in the Catalog of Somatic Mutations in Cancer (COSMIC) database. The majority of septin mutations occur in tumors of the large intestine, skin, endometrium and stomach. Over 25% of the annotated mutations in SEPT2, SEPT4, and SEPT9 belong to large intestine tumors. From all septins, SEPT9 and SEPT14 exhibit the highest mutation frequencies in skin, stomach and large intestine cancers. While septin mutations occur with frequencies lower than 3%, recurring mutations in several invariant and highly conserved amino acids are found across different septin paralogs and tumor types. Interestingly, a significant number of these mutations occur in the GTP-binding pocket and septin dimerization interfaces. Future studies may determine how these somatic mutations affect septin structure and function, whether they contribute to the progression of specific cancers and if they could serve as tumor-specific biomarkers.

Keywords: septins, cancer, neoplasia, missense mutations, tumorigenesis, oncogenes, tumor suppressors, Ras GTPases

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Septins are GTP-binding proteins that form higher order filamentous structures, which function primarily in the spatial organization and compartmentalization of many cellular processes (Caudron and Barral, 2009; Mostowy and Cossart, 2012; Spiliotis and Gladfelter, 2012). In human cells, septins comprise a family of 13 paralogous genes, which encode 13 different septins (SEPT1-SEPT12, SEPT14) with multiple isoform variants (Kinoshita, 2003b; Pan et al., 2007; Russell and Hall, 2011). Evolutionarily, septins belong to the same class of GTPases as the *RAS* oncogenes (Leipe et al., 2002). Similar to the Ras proteins, the core GTP-binding structure of septins consists of alternating α-helices, β-sheets and loops (P-loops) that come in contact with the phosphate groups of GTP (Leipe et al., 2002). Septins, however, contain additional helices (α0, α5′, and α6) and β-strands (e.g., β7, β8), which in part support tandem dimerization into oligomers and polymers (Sirajuddin et al., 2007, 2009; Macedo et al., 2013). Assembly of these septin heteromers depends on GTP-binding and hydrolysis, which further stabilizes the dimerization interfaces through allosteric effects (McMurray, 2014; Zent and Wittinghofer, 2014; Zeraik et al., 2014). In contrast to the monomeric Ras GTPases, whose function relies on the hydrolysis and exchange of GTP by GTPase activating proteins (GAPs) and guanine exchange factors (GEFs), septins hydrolyze and exchange GTP on their own, albeit at very slow rates (Sheffield et al., 2003; Vrabioiu et al., 2004; Huang et al., 2006; Abbey et al., 2016).

Based on sequence similarity, mammalian septins are categorized in four groups: SEPT2 (septins 1, 2, 4, and 5), SEPT3 (septins 3, 9, and 12), SEPT6 (septins 6, 8, 10, 11, 14) and SEPT7 (Kinoshita, 2003a; Cao et al., 2007; Pan et al., 2007). Due to lack of a critical threonine residue, which coordinates the hydrolysis of GTP, septins of the SEPT6 group are thought to bind GTP constitutively (Sirajuddin et al., 2009; Zent and Wittinghofer, 2014). Septins vary mainly in the N- and C-terminal sequences that flank the GTP-binding domain. SEPT7 and septins of the SEPT6 group contain α-helical coiled-coil domains in their C-terminal tails, while SEPT9 contains an elongated N-terminal tail, which is enriched with prolines and interacts with microtubules and actin microfilaments (Bai et al., 2013; Smith et al., 2015). With the exception of the SEPT6 group, all septins contain a polybasic domain, which has been shown to interact with membrane phosphoinositides (Zhang et al., 1999; Casamayor and Snyder, 2003).

Septins assemble into oligomers and polymers in a combinatorial fashion, forming complexes that include a septin from each one of the four groups (Kinoshita, 2003a; Nakahira et al., 2010; Sandrock et al., 2011). Through their GTP-binding domains, septins dimerize in tandem to form a non-polar hetero-octamer of a 2:2:2:2 stoichiometry (Kim et al., 2011; Sellin et al., 2011). This palindromic dimer of two SEPT2/6/7/9 complexes is posited to be the basic unit of most mammalian septin heteromers; septins of the same group substitute one another within the SEP2/6/7/9 complex. The expression of certain septins, however, varies significantly between different tissues and organs, and the exact composition of septin complexes is not well known (Hall et al., 2005; Connolly et al., 2011a; Sellin et al., 2014). Moreover, septin heteromers of alternative compositions or stoichiometries have been reported, suggesting that the SEPT2/6/7/9 mode of assembly might not be a panacea (Dolat et al., 2014a).

Septins interface functionally with molecular mechanisms that underlie the membrane- and cytoskeleton-based processes of the cell. Named after their roles in partitioning the membranes of the two emerging daughter cells in late mitosis, septins initially became known for their functions in cytokinesis (Longtine et al., 1996; Kinoshita and Noda, 2001; Joo et al., 2005; McMurray and Thorner, 2009). Over time, membrane-associated septins were discovered to maintain diffusion barriers, controlling protein localization, and to modulate exocytic membrane fusion (Kartmann and Roth, 2001; Caudron and Barral, 2009; Bridges and Gladfelter, 2015). Recently, septins were found to affect mitochondrial division (Pagliuso et al., 2016; Sirianni et al., 2016), pointing to as-yet-unknown roles in the biogenesis of membranous organelles. Of note, septins have been implicated in the biogenesis of multi-vesicular bodies and autophagosomes, as well as in lysosomal homeostasis (Mostowy et al., 2010; Traikov et al., 2014; Dolat and Spiliotis, 2016).

In the cytoplasm, septins associate with the actomyosin and microtubule cytoskeletons. The organization and contractile properties of actin-myosin filaments are modulated by septins, which cross-link and bend actin filaments into functional structures such as the cytokinetic contractile ring, the actin stress fibers that power cell migration, and cellular protrusions such as filopodia, pseudopodia and lamellipodia (Kinoshita et al., 1997, 2002; Joo et al., 2007; Kremer et al., 2007; Shankar et al., 2010; Hu et al., 2012; Mizutani et al., 2013; Dolat et al., 2014b; Mavrakis et al., 2014). Similarly, septins affect the organization, dynamics and post-translational modifications of the microtubule cytoskeleton, impacting the morphogenesis of epithelia and neurons (Spiliotis et al., 2008; Bowen et al., 2011; Ageta-Ishihara et al., 2013; Bai et al., 2013; Froidevaux-Klipfel et al., 2015). Moreover, microtubule-associated septins are essential for proper chromosome alignment and segregation during mitosis, and the cytoskeleton-dependent transport of membrane vesicles in interphase cells (Spiliotis et al., 2005, 2008; Bai et al., 2016).

As our knowledge of septin functions continues to expand, it is becoming increasingly evident that abnormalities in septin expression have a major impact on cellular homeostasis and human health. To date, septins are linked to various disease states including neurodegenerative, neuromuscular and blood disorders as well as infertility and developmental disabilities (Dolat et al., 2014a; Marttinen et al., 2015). Notably, septin expression levels are widely altered in almost every cancer type from leukemias and epithelial carcinomas to melanomas and gliomas (Cerveira et al., 2011; Connolly et al., 2011a; Dolat et al., 2014a).

ALTERATIONS OF SEPTIN EXPRESSION IN CANCER

Historically, SEPT9 was the first septin implicated in cancer. In the late 1990s/early 2000s, three independent lines of evidence linked SEPT9 to cancer. First, SEPT9 was identified as a fusion partner of the mixed lineage leukemia (MLL) gene (Osaka et al., 1999), which translocates to various chromosomal loci, giving rise to chimeras that promote the oncogenic potential of MLL; subsequently more septins were identified as MLL fusion partners (Cerveira et al., 2011). Second, the murine SL-3 retrovirus that causes T-cell lymphomas was found to preferentially integrate into the *SEPT9* gene locus (Sorensen et al., 2000). This observation was reminiscent of the seminal discovery of proto-oncogenes, which trigger cancerous growth after insertion of retroviral DNA into the host genome (Hayward et al., 1981; Payne et al., 1982). Third, the human SEPT9 gene was mapped to the chromosomal locus 17q25.3, which is frequently deleted in sporadic ovarian and breast cancers (Kalikin et al., 2000; Russell et al., 2000). Multiple copies of the SEPT9 gene, however, were also found in a variety of human tumors and similar amplifications of the SEPT9 gene were observed in mouse models of breast cancer (Montagna et al., 2003).

Following these early findings, an increasing number of studies began to report the over-expression and down-regulation of specific septins in a variety of hematological malignancies and solid tumors. In addition to the MLL-septin chimeras, the SEPT4/ARTS isoform was found to be down-regulated in acute lymphoblastic leukemia (ALL) and genetic ablation of SEPT4 increased the levels of hematopoietic stem cells, which became resistant to apoptosis (Larisch et al., 2000; Elhasid

et al., 2004; Garcia-Fernandez et al., 2010). Alterations in septin expression have been reported in brain tumors (glioblastomas), skin (squamous cell carcinomas, melanomas), kidney (renal cell carcinomas), colorectal, lung and hormonally regulated cancers such as prostate, breast, ovarian and endometrial cancers (Hall and Russell, 2004; Dolat et al., 2014a; Montagna et al., 2015). In the vast majority of these cancers, septins are over-expressed (**Figure 1**), but occasional down-regulation, ectopic expression and epigenetic alterations have also been reported (Tanaka et al., 2001; Liu et al., 2010; Payne, 2010; Connolly et al., 2011b; Shen et al., 2012; Montagna et al., 2015). Based on these abnormalities, diagnostic tests were developed for urothelial and colorectal cancers, which screen respectively for the expression of the Bradeion isoform of SEPT4 in the urine and the methylation of the SEPT9 gene in the blood (Tanaka et al., 2001, 2003; Grutzmann et al., 2008; Warren et al., 2011; Bongiovanni et al., 2012).

EMERGING ROLES OF SEPTINS IN CANCER

In contrast to classical tumor suppressors and oncogenes, which induce cancer by loss and gain of function mutations, septins are thought to belong to a broader class of cancer genes that affect tumorigenesis as a consequence of altered levels of expression (Sager, 1997; Hall and Russell, 2004). Given that septins function as hetero-oligomers, it is unclear how up- or down-regulation of a septin isoform could contribute to cancer progression. However, a growing number of studies show that abnormalities in the expression levels of a single septin bestow cellular properties akin to tumorigenic phenotypes (Gonzalez et al., 2009; Garcia-Fernandez et al., 2010; Connolly et al., 2011b; Dolat et al., 2014b). Moreover, decreasing the expression of a single septin has been shown to suppress tumor growth *in vivo* (Tanaka et al., 2002; Yu et al., 2016).

Taken together with advances in the cell biological functions of septins, it is now evident that septins are linked to the molecular mechanisms that underlie hallmarks of cancer such as resistance to cell death, proliferation, angiogenesis and invasion and metastasis. The ARTS isoform of SEPT4 (SEPT4_i1) is regarded as a pro-apoptotic tumor suppressor, whose down-regulation in leukemia could render resistance to pro-apoptotic stimuli (Gottfried et al., 2004; Garcia-Fernandez et al., 2010). SEPT9_i1 binds and prevents the degradation of the c-Jun-N-terminal kinase (JNK), which promotes tumor cell proliferation (Gonzalez et al., 2009). Similarly, septins have also been reported to suppress the degradation of the epidermal growth factor receptor (EGFR) and the receptor-protein tyrosine kinase Erb2/HER2, which are linked to signaling pathways that trigger cell proliferation and migration (Diesenberg et al., 2015; Marcus et al., 2016). Notably, SEPT9_i3 is phosphorylated by the cell cyclin-dependent kinase 1 (Cdk1), which controls entry into mitosis and promotes cell proliferation and survival (Liu et al., 2008; Estey et al., 2013).

Sustained proliferation requires a metabolic reprogramming, which includes the internalization of carbon sources and amino acids from the interstitial fluid by macropinocytosis (Bloomfield and Kay, 2016). Ras mutants have been known to upregulate macropinocytosis and septins were recently found to affect the intracellular transport of macropinosomes in a dose dependent manner (Bryant et al., 2014; Dolat and Spiliotis, 2016). Oxygen supply is equally important for the metabolic demands of tumors, which induce angiogenesis under hypoxic conditions (Hoff and Machado, 2012). SEPT9_i1 binds and facilitates the nuclear import of the hypoxia-inducible factor-1α (HIF1α), whose transcriptional activity promotes angiogenesis (Amir et al., 2009; Golan and Mabjeesh, 2013).

Multiple studies have shown that over-expression of SEPT9 isoforms enhances cell motility and invasion, and suggest that SEPT9 upregulation is programmed by the epithelial-to-mesenchymal transition (EMT) that drives the development of carcinomas (Chacko et al., 2005; Shankar et al., 2010; Connolly et al., 2011b; Fuchtbauer et al., 2011; Dolat et al., 2014b). SEPT2 and SEPT7 have also been implicated in the migration and invasion of breast cancer and glioblastoma cells (Jiang et al., 2014; Zhang et al., 2016). While SEPT2/7 promote the cell migration and invasion of breast cancer cells, SEPT7 appears to have the opposite role in gliomas (Jiang et al., 2014; Zhang et al., 2016). Over-expression of other septins such as SEPT1 has been suggested to promote the migration of skin cancer cells (Mizutani et al., 2013).

In addition to these roles, which are intimately associated with the hallmarks of cancer, septins may contribute to the genomic instability of cancer, resistance to anti-cancer drugs and the promotion of cell growth by the tumor microenvironment. Alterations in septin expression affect chromosome alignment and segregation as well as cytokinesis, which may result in the loss or gain of whole chromosomes (Spiliotis et al., 2005; Estey et al., 2010; Thompson et al., 2010; Menon et al., 2014). Indeed, over-expression of SEPT9_i1 in human mammary epithelial cells increases aneuploidy, which correlates with defects in centrosome duplication, chromosome segregation and cytokinesis (Peterson et al., 2011). In cancer cell lines, elevated expression of SEPT9 isoforms (e.g., SEPT9_i1/i4) and down-regulation of SEPT10 have been reported to confer resistance to the anti-cancer microtubule-targeting drug paclitaxel (Amir and Mabjeesh, 2007; Froidevaux-Klipfel et al., 2011; Chacko et al., 2012; Xu et al., 2012). Future studies will determine whether dysregulation of septin expression affects cancer resistance *in vivo*. Interestingly, a recent study showed that *in vivo* septins are required for the remodeling of the extracellular matrix by cancer-associated fibroblasts (CAFs). In addition, septins partly promote CAF-induced tumor growth and pro-angiogenic activity (Calvo et al., 2015). This is the first evidence of septins playing a role in the organization and properties of the tumor microenvironment, which is important for the progression of cancer.

A GLOBAL VIEW OF SEPTIN MISSENSE MUTATIONS IN HUMAN CANCERS

In contrast to the alterations in septin expression, which have been extensively documented in a variety of cancers, septin mutations remain virtually undocumented. The Catalog of

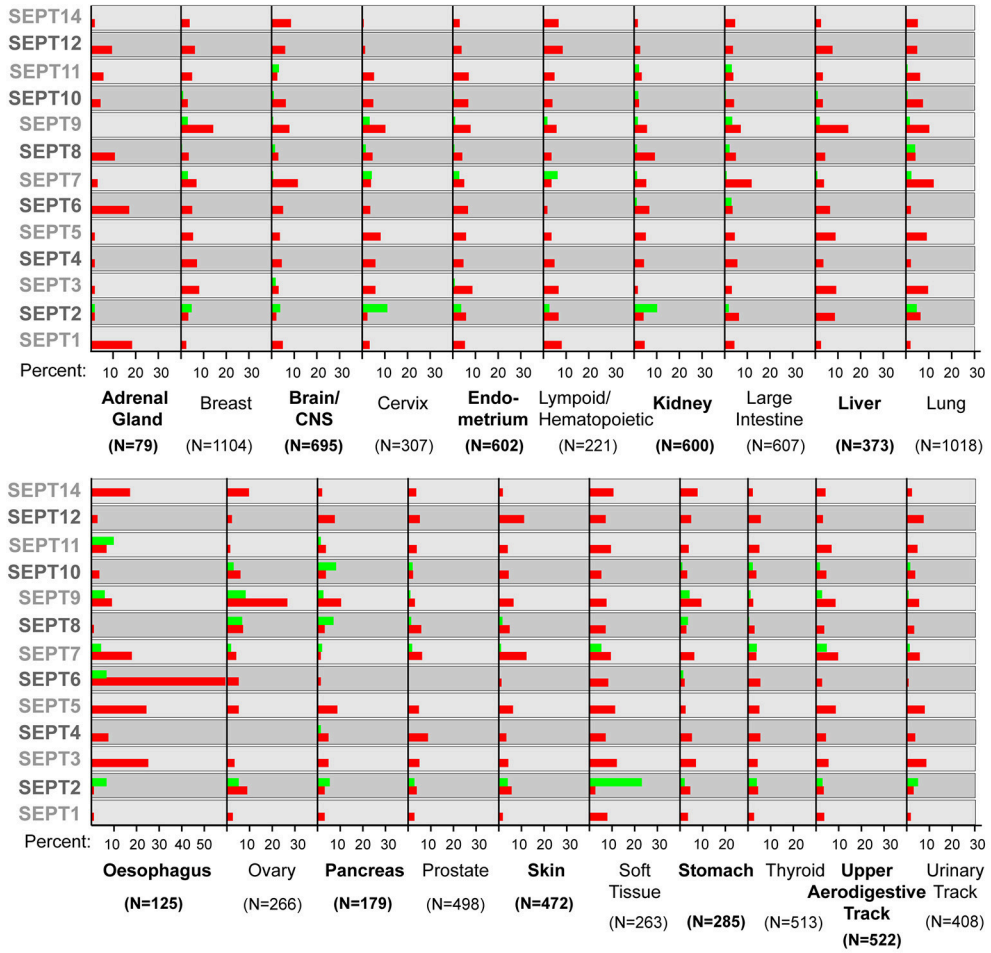

FIGURE 1 | Septin over- and under-expression in human cancers. Bar graphs show the percentage of tumor samples, in which a specific septin is over-expressed (red) or under-expressed (green). In the COSMIC database, the thresh-hold value for over- or under-expression is set at two times the standard deviation from the mean values of expression in tumor samples that were diploid for a septin gene. The number (N) of samples analyzed is shown in parenthesis under each tumor type. Note that several septins are both over- and under-expressed in samples of the same tumor type.

Somatic Mutations In Cancer (COSMIC; http://cancer.sanger.ac.uk/cosmic) is an on-line database that compiles cancer genomic information from scientific publications, The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC) databases. Currently, COSMIC contains data for 42 different primary tumor types (e.g., intestinal, skin, kidney), which were derived from approximately 1.23 million patients. The majority of these samples are from patients with haemotopoietic and lymphoid cancers (∼32%). Large intestine, lung and thyroid cancer samples account for ∼14%, ∼13%, and ∼5% of patients, respectively. All other tumor types are each under 5% of the total pool of patients. Presently, COSMIC contains data from screening a cumulative of 28,611 genes, including isoforms from identical genes, and 301,848 unique mutations are catalogd.

In COSMIC, 693 septin missense mutations are reported; for this review's purpose, we focused on single amino acid substitutions and not on gene deletions, duplications, translocations or epigenetic alterations. A number of these septin mutations are found in different tumor types or different patients with the same type of tumor. Over 20% of the septin mutations correspond to large intestine tumors (**Figure 2A**). Approximately 15% of septin mutations belong to skin cancers, >10% to lung tumors and >5% to endometrial and stomach tumors. The percentage of total septin mutations for any other tumor type is lower than 5%. Given that intestine, skin, endometrial and stomach cancers represent 14.4%, 3.3%, 1.3%, and 2% of the total tumor samples, respectively, the percentage of septin mutations in these tumor types is above the relative abundance of samples. Similarly, the percentage (4–5%) of septin mutations in liver and kidney tumors is more than double the percentage (1.3–1.4%) of these tumors in the total samples screened.

Septins of the SEPT6 group account for 44.6% of total septin mutations, while the SEPT2 and SEPT3 groups each harbor ∼25% of septin mutations (**Figure 2B**). From all 13 septin paralogs, the top two most mutated septins are SEPT14 and SEPT9, which contain ∼15% and ∼10% of total

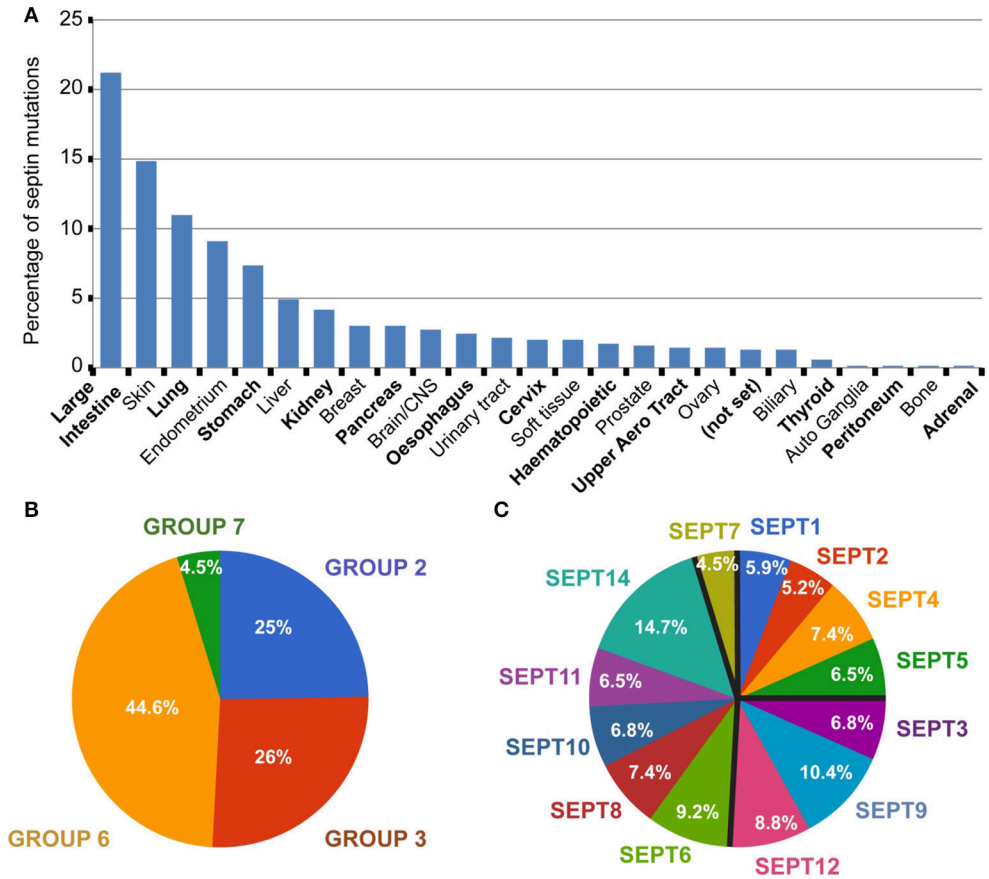

FIGURE 2 | Global view of septin missense mutations in human cancers. (A) All 693 septin missense mutations were grouped per tumor type. Bar graph shows the percentage of total septin mutations that occur in each tumor type. Tumors of uncertain type are designated as "not set." **(B,C)** Pie charts show the percentage of septin mutations per septin group **(B)** and individual septin paralog **(C)**.

mutations, respectively (**Figure 2C**). Each of the remaining septin paralogs account for 5–9% of total mutations. Interestingly, the ubiquitously expressed SEPT7 is the least mutated, accounting for less than 5% of total mutations (**Figures 2B,C**).

Grouping the mutations of each septin paralog under their corresponding tumor types shows that 25–35% of the SEPT2, SEPT4, and SEPT9 mutations are found in large intestine tumors (**Figures 3A,B**). SEPT2 and SEPT9 had notably more mutations (∼14%) in liver and stomach cancers, respectively, relative to all other septins (**Figures 3A,B**). The majority of SEPT12 and SEPT14 mutations occur in skin (20–26%), large intestine (20%) and lung (13–15%) cancers (**Figures 3B,C**). Similar trends are observed for SEPT3, SEPT5, SEPT6, SEPT10, and SEPT11. Compared to all other septins, SEPT7 has a higher percentage of mutations in cancers of the central nervous system (∼13%) and endometrium (∼16%) (**Figure 3D**).

In contrast to the high mutation frequencies of Ras GTPases and other classical oncogenes (Fernandez-Medarde and Santos, 2011; Pylayeva-Gupta et al., 2011; Chang et al., 2016), the incidence of septin mutations appear to be below 3% (**Table 1**). Among the top ten most frequent septin mutations, SEPT14 and SEPT9 top the list with frequencies of 2.56 and 1.86% in skin and stomach cancers, respectively (**Table 1**). The same septins also have the highest frequencies (1.3–1.5%) in tumors of the large intestine. The mutation frequencies (1.25%) of SEPT9 and SEPT6 in endometrial tumors, and of SEPT3 in cervical cancer are also among the highest of all septins. Overall, septin mutations occur with the highest frequency

TABLE 1 | Septins with the highest mutation frequencies *(f)*.

Rank	SEPT	Primary tumor	*f* (%)	Samples screened	Total samples
1*	SEPT14	Skin	2.56	1094	16,706
2	SEPT9	Stomach	1.86	592	24,308
3	SEPT9	Large intestine	1.55	1482	179,020
4	SEPT14	Large intestine	1.35	1482	179,020
5	SEPT9	Endometrium	1.25	640	16,706
–	SEPT6	Endometrium	1.25	640	16,706
7	SEPT3	Cervix	1.24	322	5828
8	SEPT4	Large Intestine	1.21	1482	179,020
9	SEPT12	Skin	1.10	1094	40,749
10	SEPT14	Soft tissue	1.06	567	40,032

**SEPT6 frequency in peritoneal tumors is 10%, but only 10 out of 400 samples have been screened.*

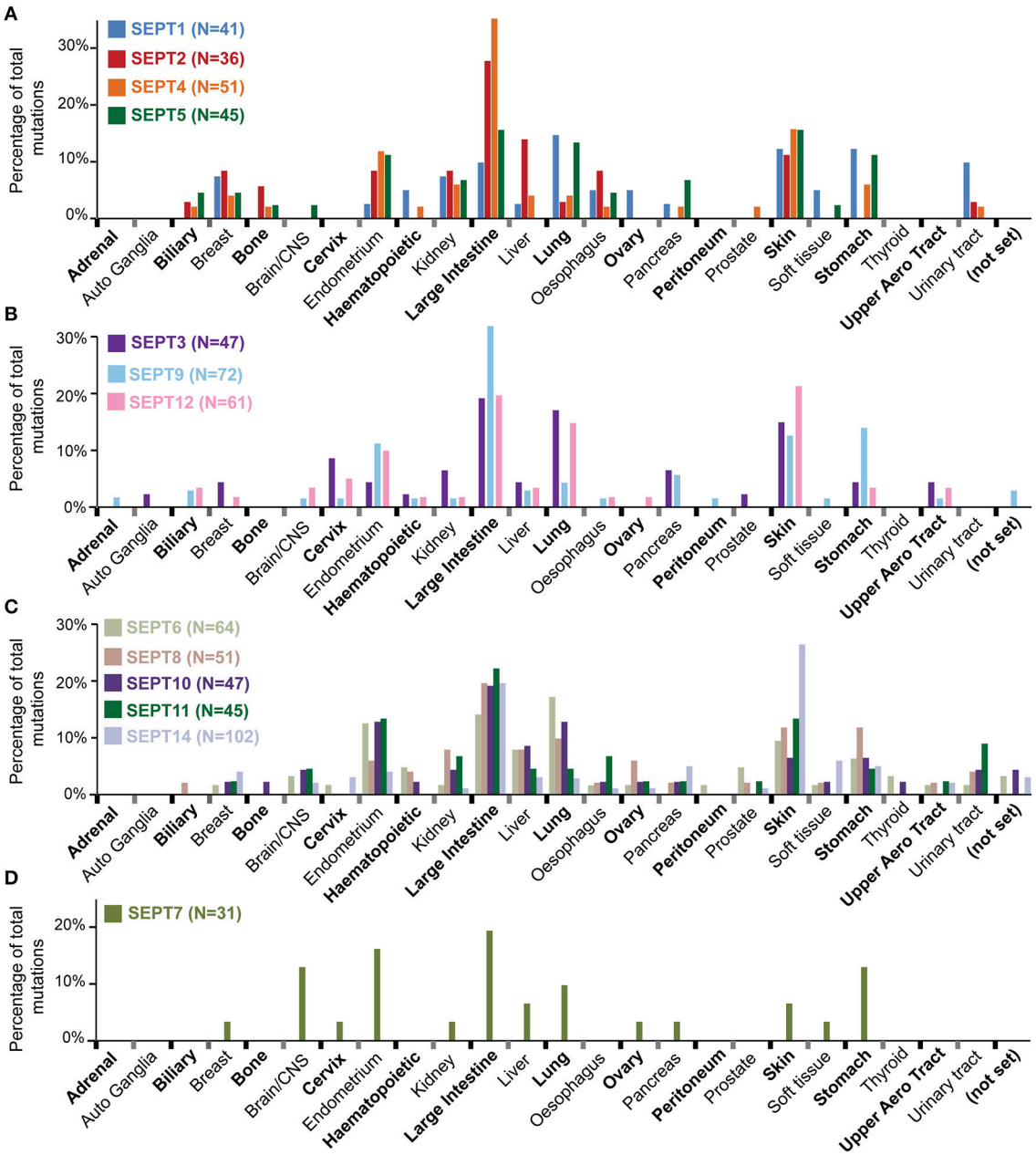

FIGURE 3 | Distribution of septin mutations by tumor type. Bar graphs show the distribution of missense mutations across tumor types for the individual septin paralogs of the SEPT2 **(A)**, SEPT3 **(B)**, SEPT6 **(C)**, and SEPT7 **(D)** groups. Septin paralog-specific mutations were binned under each tumor type and the percentages of total mutations per each tumor type were derived and graphed. The *N* values shown in parenthesis correspond to the number missense mutations identified in COSMIC for the corresponding septin paralogs. Tumors of uncertain identity are designated as "not set."

in cancers of the skin, large intestine, endometrium and stomach.

RECURRING MUTATIONS IN CONSERVED SEPTIN RESIDUES AND DOMAINS

Ras proteins are known for containing highly conserved amino acids that are mutated with high frequencies in a variety of cancers (Fernandez-Medarde and Santos, 2011; Pylayeva-Gupta et al., 2011). These mutational hotspots comprise amino acids, which are key for the hydrolysis of GTP, and have been developed as prognostic markers and targets for anti-cancer therapies (Pylayeva-Gupta et al., 2011; Chang et al., 2016; Lu et al., 2016).

Aligning the sequences of the 13 septin paralogs and scoring all amino acids with missense mutations reveals that there are fully conserved residues with multiple mutations (**Figure 4A**, **Table 2**). In **Figure 4A**, the positions of the most frequently

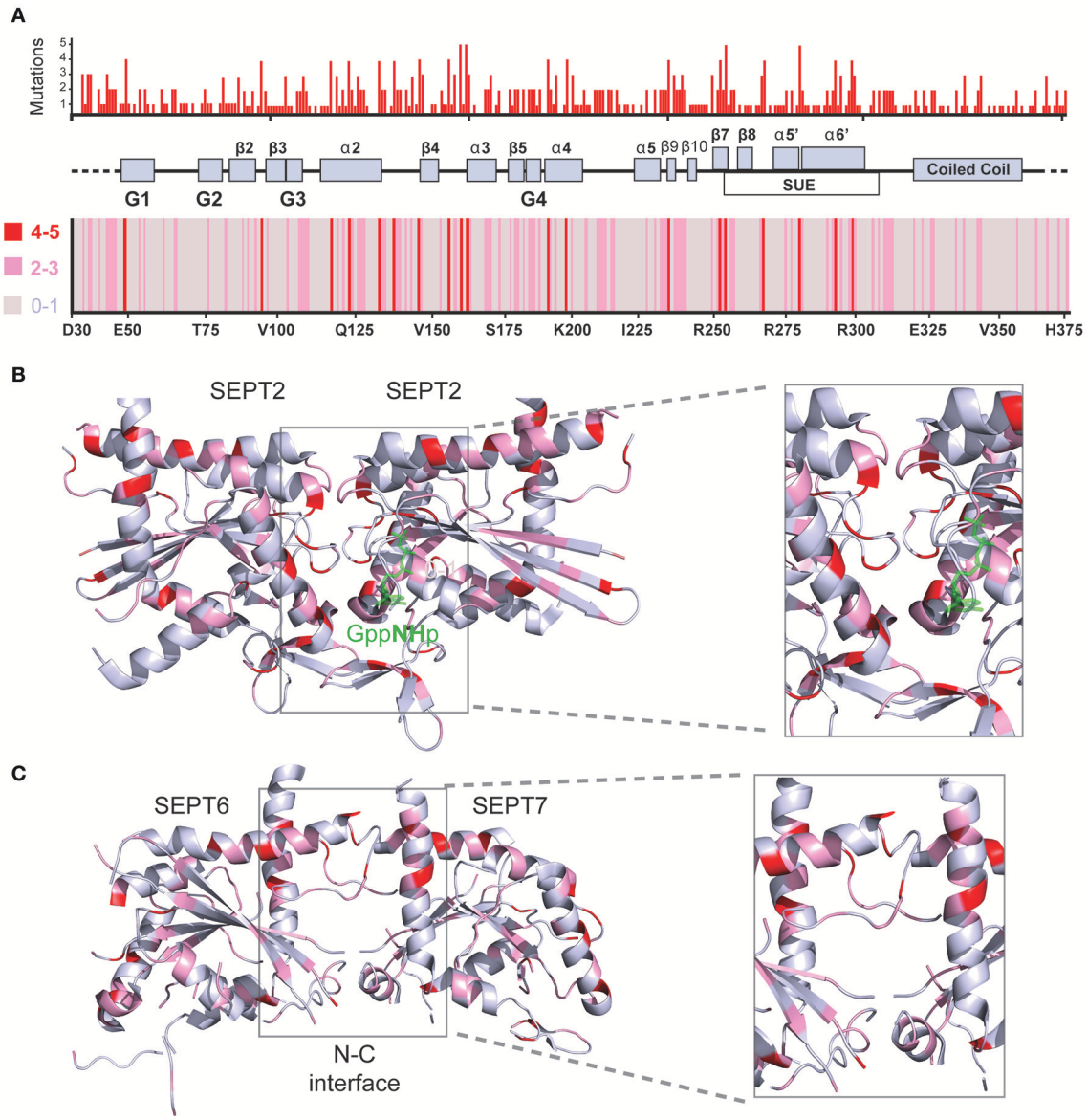

FIGURE 4 | Structural mapping of septin missense mutations. (A) The amino acid sequences of all 13 human septins were aligned with the Clustal Omega program (EMBL-EBI) and amino acid positions were numbered starting with amino acid 30 of SEPT6. Histogram (top) shows the cumulative number of missense mutations that occur for each amino acid position across all septin paralogs. A heat-map (bottom) representation indicates the number of mutations for each amino acid position by color-coding 0-1 mutations in gray, 2-3 mutations in pink and 4-5 mutations in red. A schematic diagram depicts the secondary structure elements and domains that correspond to the amino acid positions on the x-axis of the histogram and heat-map. On the x-axis of the heatmap, the amino acid identities and numbers of SEPT6 are shown for reference purposes. **(B,C)** Ribbon cartoons of the 3D crystal structure of SEPT2 (**B**; PDB code: 3FTQ) and the SEPT6/7 dimer **(C)** from the crystal structure of SEPT2/6/7 (PDB code: 2QAG) depict the location and cumulative number of mutations shown in the histogram and heatmap above. Gray areas contain none or one mutation, while pink and red areas harbor 2-3 and 4-5 mutations, respectively. Insets show magnified views of the GTP-binding pocket and G-G interface **(B)** as well as the N-C dimerization interface of SEPT6/7 **(C)**. Ribbon cartoons were generated with the PyMOL software and amino acid locations were also color-coded in PyMOL.

mutated amino acids are identified by a color-coded heatmap, which indicates the cumulative number of mutations for each amino acid position. Interestingly, several amino acids that contain multiple mutations are positioned in the GTP-binding pocket, the septin unique element and the G-G as well as the N-C dimerization interfaces of septins (**Figures 4B,C**).

MUTATIONS IN THE GTP-BINDING POCKET OF SEPTINS

The GTP-binding pocket of septins comprises the G1 (Walker A) motif GxxxxGKS/T, which forms the P-loop that interacts with the phosphate residues of the GTP, the G4 motif AKAD,

TABLE 2 | Missense mutations in fully conserved amino acids across all septins.

	Polybasic		β1 sheet		.....		α1 helix				.....		β3 sheet		Switch II			α2 helix		.....	
SEPT1				G32E			S39C								G92S				E121K	R126Q	
SEPT2			F38L	G44S															E133K		
SEPT3																					R170H
SEPT4						G156D							L204F						E240K		
SEPT5																G113W					
SEPT6									L62R			V93M		T98M							
SEPT7			F52L								E103D										
SEPT8					G54D														E135V		R149M
SEPT9									L318I			V354I								R399C R399H	
SEPT10																		E151K			R171H
SEPT11		F40L										V92A					D111N			R137C R137H	
SEPT12				G56V				T64M		F70L F70C	E101D						D118E				
SEPT14	G50R			G59R		G64R G64E	S66L					V103I				G116D		E137Q		R148C	R157H R157C R157L

	β4 sheet		.....		α3 helix	β5 sheet	G4 (AKAD)		.....	.....			β7 sheet	β8 sheet	.....		α5′ helix			α6 helix	
SEPT1										P224R											
SEPT2								D185A		P236S		G241A									R300C
SEPT3								D210N D210E		P260S	V263L		R280Q								
SEPT4			G264S									G348D									R408L
SEPT5		F159V													E273K						
SEPT6	C151F	F154L				P181S			Y213C	P234L										T290I	R299H
SEPT7					D177H		A197S														
SEPT8						P183H							R256Q								R301W
SEPT9			G419D						Y473F										R537W		
SEPT10			G183D	L186V									R278H	G283V							
SEPT11																N264Y	F270V	L273P			
SEPT12											V249M		R266L R266W R266Q								
SEPT14							A196V					G249E	R264H						R285Q	T300A	

which interacts with the guanine base of GTP, and the G3 motif DxxG, which binds Mg^{2+} and is essential for the coordination of GTP hydrolysis (**Figure 5A**). The GTP-binding pocket also contains a threonine, which is analogous to the Thr-35 of the G2 sequence of the Ras GTPases (Sirajuddin et al., 2009). In conjunction with the glycine of the G3 motif, this threonine (T78 in SEPT2) is critical for the hydrolysis of GTP and is present in all septins with the exception of those of the SEPT6 group, which are thought to bind GTP constitutively (Sirajuddin et al., 2009; Zent et al., 2011; Macedo et al., 2013; Zent and Wittinghofer, 2014). In addition, an invariant arginine residue, which is positioned in the beginning of the septin unique element, and a highly conserved glutamate, which is located in the α4 helix following the G4 motif, make contact with the guanine base of GTP (**Figures 5B,C**).

In Ras GTPases, the conserved residues G12 and G13 are heavily mutated in human cancers (Pylayeva-Gupta et al., 2011). These residues correspond to the third and fourth amino acids of the G1 Walker A motif GxxxxGKS/T (**Figure 5A**). In septins, only G13 is conserved as an invariant glycine and is mutated only once in SEPT8 (**Figure 5A**). Mutation of this residue in SEPT7 has been previously shown to disrupt GTP-binding and septin dimerization (Abbey et al., 2016). The first invariant glycine of the GxxxxGKS/T motif is mutated in four different septins (SEPT1, SEPT2, SEPT12, and SEPT14) and the invariant serine, which has been shown to affect SEPT7 oligomerization (Abbey et al., 2016), is mutated in SEPT1 and SEPT14.

Mutations in the highly conserved threonine (T78 in SEPT2; G2 sequence), which is responsible for GTP hydrolysis in septins, occur only in SEPT5 and SEPT12. Interestingly, the threonine of SEPT12 (T89) is mutated into methionine, which is the same mutation identified in infertile male patients with asthenoteratozoospermia (Kuo et al., 2012). This mutation is found in a cancer of the central nervous system. Of note, an arginine residue (R198 in SEPT2) that is highly conserved among all septins with the catalytic threonine (T78 in SEPT2) is mutated

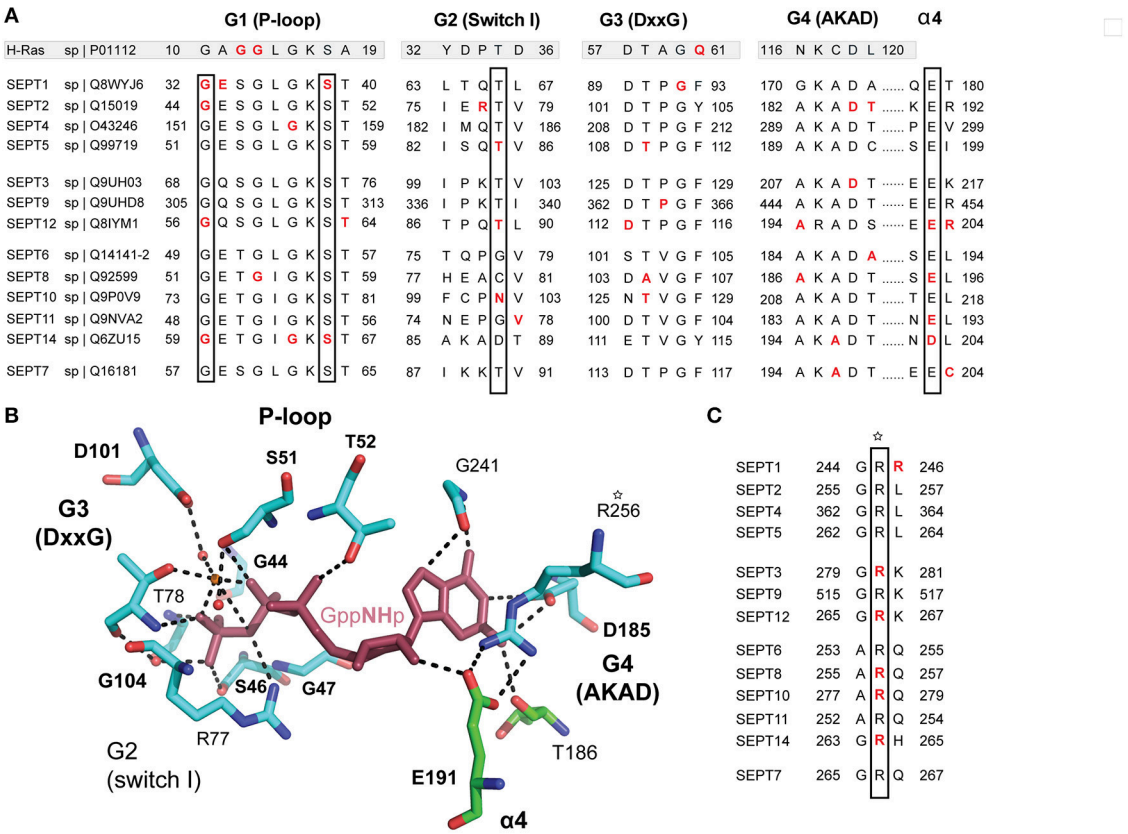

FIGURE 5 | Cancer mutations in conserved residues of the septin GTP-binding pocket. (A) Sequence alignment of the G1 (P-loop), G2, G3, and G4 motifs of all 13 human septins. The corresponding sequences of H-Ras are shown in shaded boxes. The H-Ras amino acids in codons 12, 13, and 61 (highlighted in red font) are sites of highly frequent activating mutations among all *RAS* oncogenes. Septin residues with missense mutations are shown in red font. The positions of highly conserved amino acids with three or more missense mutations across all septins are outlined with rectangle boxes. **(B)** Stick cartoon of the atomic structure of the GTP-binding pocket of SEPT2 (PDB code: 3FTQ) shows the position of highly conserved amino acids, which make contact with the GTP analog GppNHp (maroon) and are mutated in several human cancers. Dashed lines outline interactions between the side chains of amino acids and GppNHp. The atomic representation and bonds (dashed lines) were generated in PyMOL using the "sticks mode" and "measure distance" functions of the software. **(C)** Sequence alignment shows an invariant arginine (asterisk), which is mutated in five different septins and makes contact with the ribose moiety of GTP. This amino acid is also involved in the polar interactions of the G-G interface (**Figure 6**) and is positioned at the beginning of the septin unique element.

in four septins. This arginine is located in the α4 helix, which underlies the GTP-binding pocket.

In the guanine-binding AKAD motif (G4), six mutations are identified in three different amino acids. The invariant alanine in the first position of the AKAD motif is mutated in SEPT2 and SEPT8. The aspartate residue is mutated once in SEPT2 and twice in SEPT3. Remarkably, a mutation in the same amino acid of SEPT12 (D197N) is linked to male infertility and has been shown to disrupt SEPT12 assembly into functional filaments (Kuo et al., 2012). Moreover, the aspartate of the AKAD motif is also the site of a temperature-sensitive mutation (D182N) in the Cdc10 septin of the budding yeast *S. cerevisiae* (Weems et al., 2014). Interestingly, this missense mutation has been shown to switch the nucleotide specificity of p21Ras from guanosine to xanthosine, and is likely to result in a nucleotide-free septin (Schmidt et al., 1996).

The G3 motif DxxG harbors six mutations (**Figure 5A**), but the conserved D and G residues are only mutated once in SEPT12 and SEPT1, respectively. The remaining four mutations occur in the second and third positions of the DxxG motif; three mutations occur in a highly conserved threonine. Among all residues of the GTP-binding pocket, the invariant guanine-interacting arginine of the SUE (R256 in SEPT2) and glutamate of α4 helix (E191 in SEPT2) are the most heavily mutated (**Figures 5A,C**). Interestingly, an invariant glycine (G241 in SEPT2), which is positioned between the β9 and β11 sheets and interacts with guanine, is also mutated in three different septins (**Figure 5B**). Notably, this glycine marks a dominant negative temperature-sensitive mutation (G247E) in the yeast septin Cdc12 (Weems et al., 2014).

Overall, the mutational pattern of the septin G domain does not exhibit the high frequency and amino acid selectivity of Ras GTPases. Given that Ras mutational hotspots such as the G12 and G13 residues of the P-loop are involved in the mechanism of GTP hydrolysis by GTPase-activating proteins (GAPs), this difference may be due to the slow and mechanistically unique GTPase activity of septins, which do not involve GAPs and depend functionally more on hetero-oligomerization than GTP turnover. Thus, activating mutations in the G domain of Ras GTPases could be more self-selective in the evolution of cancer. It is nevertheless evident that a significant number of mutations are clustered in the GTP-binding pocket of septins and involve several invariant residues that come in contact with both the guanine base and phosphates of GTP.

MUTATIONS IN THE G-G AND N-C DIMERIZATION INTERFACES OF SEPTINS

Septins assemble into functional higher order structures by homo- and hetero-dimerization, which is structurally mediated by their GTP-binding domains (Sirajuddin et al., 2007; Kim et al., 2012). The X-ray crystallographic structures of several septin homodimers (e.g., SEPT2, SEPT7, SEPT3) and of the heterotrimeric SEPT2/6/7 have revealed two dimerization interfaces, which are termed G-G and N-C interfaces (Sirajuddin et al., 2007, 2009; Zent et al., 2011; Macedo et al., 2013). The G-G interface involves elements of the GTP-binding pocket including the loop regions between the antiparallel β7 and β8 strands, which are a unique features of the septin G-domain compared to Ras proteins, and the loop regions between the β4 strand and α3 helix (Sirajuddin et al., 2007, 2009; Zent et al., 2011; Macedo et al., 2013). The N-C interface is mediated by interactions between the N- and C-terminal regions of the GTP-binding domains, and is characterized by an upper and lower half. The upper N-C interface involves the N-terminal helix α2 and the C-terminal helix α6 of the SUE domain (Sirajuddin et al., 2007, 2009; Zent and Wittinghofer, 2014). The lower N-C interface is supported by interactions between residues of the N-terminal α0 helices and between amino acids of the C-terminal β2/β3 sheets and N-terminal α0 helix (Sirajuddin et al., 2009; Zent and Wittinghofer, 2014).

In the G-G interface, over 25 mutations are clustered on a 12 amino acid patch of the β4/α3 region (**Figures 6A,B**), and involve amino acids that flank a highly conserved histidine (H158 in SEPT2), which contributes to the tight interactions of the G interface (Weirich et al., 2008; Sirajuddin et al., 2009). Notably, this region includes a phenylanine residue (F156), whose mutation has been shown to disrupt the dimerization of SEPT2 (Sirajuddin et al., 2007). The invariant glycine that precedes H158 is mutated in three different septins (SEPT4, SEPT9, SEPT10) and a highly conserved basic amino acid (arginine or lysine; K161 in SEPT2) is mutated in SEPT6 and all members of the SEPT2 group (**Figures 6B,C**). A highly invariant proline (P155 in SEPT2) is also mutated in three different septins (**Figures 6B,C**). In the region outlined by the antiparallel β7 and β8 sheets, the majority of mutations occur in a glutamate (E191 in SEPT2) and an arginine (R256 in SEPT2), which form salt bridges between opposing septin monomers (**Figures 6A,D**). Mutations in the glutamate residue, which is nearly invariant (aspartate in SEPT14 only), occur in four different septins. R256 is the same invariant arginine of the SUE domain that stacks over the ribose moiety of GTP (see above) and is mutated in five different septins (**Figures 5C**, **6D**). In addition, the preceding arginine (R254) is mutated in three septins of the SEPT2 group. No mutations are identified in the interacting histidine (H270 in SEPT2) and tryptophan (W260 in SEPT2) residues of the β7/β8 sheet region (**Figure 6D**).

In the N-C interface (**Figure 7A**), the majority of septin mutations are clustered on the upper half targeting a major electrostatic interaction between two invariant residues, a glutamate at the end of the α2 helix (E133 in SEPT2) and an arginine in the loop between helix α2 and strand β4 (R138 in SEPT2). Both of these residues are mutated in four different septins (**Figures 7A,B**). Interestingly, several other mutations are clustered in this region targeting highly conserved arginine residues in the α6 helix of the SUE domain (R280/R293 in SEPT6 and R300 in SEPT2), which may contribute to interactions between opposing monomers (**Figure 7B**). Each of these arginines is mutated in four different septins. In the lower N-C interface, an invariant valine (V93 in SEPT2) is the most mutated residue; mutations occur in SEPT6, SEPT9, SEPT11 and SEPT14 (**Figures 7A,C**). No mutations were identified in the valine of the α0 helix (V27 in SEPT2), which interacts hydrophobically with the same valine of the opposing septin monomer; valine is conserved as hydrophobic amino acid

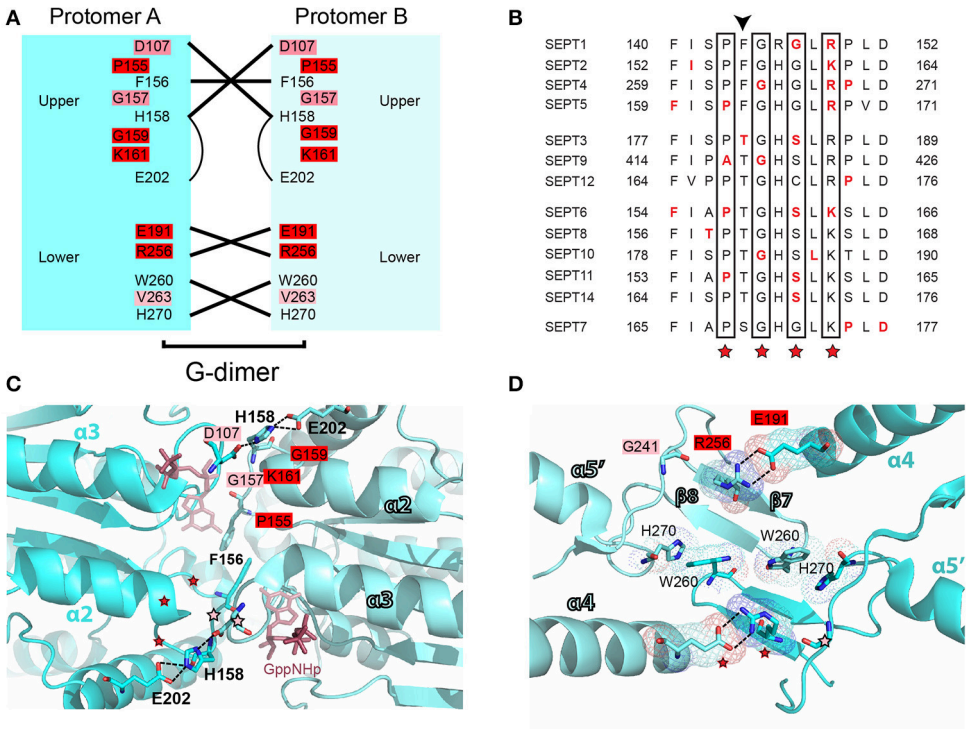

FIGURE 6 | Mutations in the G-G interface of septin oligomerization. (A) Open book diagram of the G-G interface of SEPT2 (PDB code: 3FTQ) shows critical residues and interactions (lines) of this interface. Amino acids with 2-3 and 4-5 mutations are highlighted in pink and red, respectively. Open book diagram is a hand-made representation of the approximate position of each amino acid and of their interactions along the z-axis of the crystal structure of SEPT2. **(B)** Sequence alignment of a region that underlies the interactions of the upper G-G interface. Amino acids with missense mutations are shown in red font. The positions of highly conserved amino acids with three or more missense mutations across all septins are outlined with rectangle boxes and red asterisks. Arrowhead points to a phenylalanine residue, whose mutation has been shown to abrogate the dimerization of SEPT2. **(C,D)** Ribbon cartoons depict the upper **(C)** and lower **(D)** G-G interface of SEPT2 (PDB code: 3FTQ). Dotted lines outline interactions between select residues. Amino acids with two or more mutations are highlighted in pink (2-3 mutations) or red (4-5 mutations), and their positions on the opposing protomer are indicated with asterisks of the same color code. Opposing protomers are shown in cyan and aqua marine colors. The non-hydrolyzable GTP analog GppNHp **(C)** is depicted in ruby red. Cartoons were generated in PyMOL. Select bonds (**C**; dashed lines) and electron densities **(D)** were highlighted using the "measure distance," "mesh," and "dots" functions of PyMOL.

(methionine or isoleucine) in all septins. Similarly, no mutations were observed in the highly conserved phenylalanine (F20 in SEPT2; isoleucine in group 3 septins), which forms a hydrophobic pocket with several residues including an invariant phenylalanine (F58 in SEPT2), which is mutated twice in SEPT12, and an isoleucine (I88 in SEPT2) and lysine (L95 in SEPT2), which are mutated in SEPT14 (**Figure 7C**). Of note, SEPT14 is also mutated in L289 (I281 in SEPT2), which belongs to the SUE domain and projects into the lower N-C interface (**Figure 7C**).

In summary, the polar interactions between E191 and R256 in the G-G interface and between E133 and R138 in the upper N-C interface emerge as mutational hotspots. Every septin contains at least one mutation that is likely to affect the electrostatic interactions of G-G or N-C interfaces. In addition, the loop region between strand β4 and helix α3, and the SUE are domains that harbor many mutations in conserved amino acids. Four arginine residues of the SUE domain are repeatedly mutated in multiple septins, and one these arginine residues (R256 in SEPT2) is involved in both GTP-binding and septin dimerization. Therefore, mutations in this residue could be rather detrimental for the assembly and function of septins.

MUTATIONS IN THE POLYBASIC AND COILED-COIL DOMAINS OF SEPTINS

The polybasic and coiled coil domains of septins, which are respectively positioned on the N- and C-terminal ends of the GTP-binding domain, are known for their roles in membrane-binding, septin assembly and interaction with binding partners (Zhang et al., 1999; Low and Macara, 2006; de Almeida Marques et al., 2012; Kim et al., 2012). Mutations in these domains could impact the intracellular localization and functions of septins. The phosphoinositide-binding polybasic domain is sparsely mutated, containing only a few substitutions in arginine or lysine residues of SEPT1, SEPT5, SEPT3, and SEPT9. Septins of the SEPT6 group are characterized by a paucity of basic amino acids in their respective regions of the polybasic domain and mutations are minimal. By contrast, the coiled-coil-containing C-terminal tails of the SEPT6 group harbor a significant number of mutations. The C-terminal tails of SEPT14 and SEPT6 contain the most mutations, but very few of these mutations are in amino acids of conserved identity or type. The C-terminal tails of the SEPT2 group of septins, which are shorter,

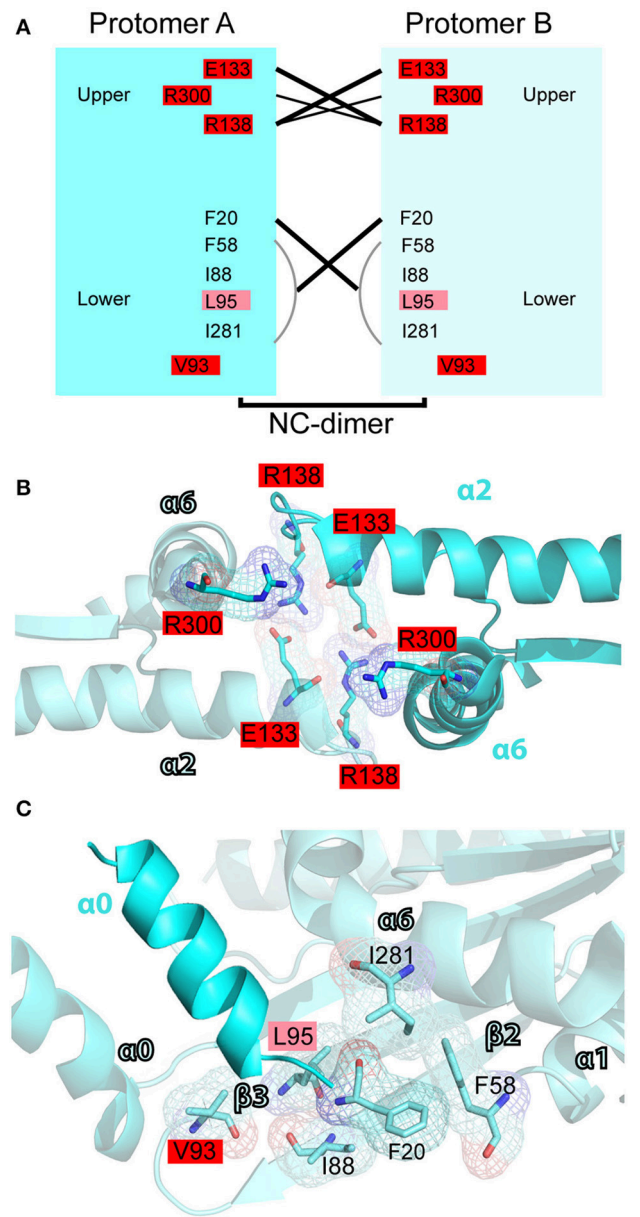

FIGURE 7 | Mutations in the N-C interface of septin oligomerization. (A) Open book diagram of the N-C interface of SEPT2 (PDB code: 3FTQ) shows critical residues and interactions (lines) of this interface. Amino acids with 2-3 and 4-5 mutations are highlighted in pink and red, respectively. Open book diagram is a hand-made representation of the approximate position of each amino acid and of their interactions along the z-axis of the crystal structure of SEPT2. **(B,C)** Ribbon cartoons depict the upper **(B)** and lower (D) N-C interface of SEPT2 (PDB code: 3FTQ). Amino acids with two or more mutations are highlighted in pink (2-3 mutations) or red (4-5 mutations). Cartoons were generated in PyMOL and electron densities were highlighted using the "mesh" function of PyMOL.

exhibit a similar mutational pattern and SEPT7 has the least number of C-terminal mutations per length of amino acid sequence.

SEPT9-SPECIFIC MUTATIONS

Among all septins, SEPT9 has been extensively implicated in human cancer. As reviewed above, SEPT9 is linked to the molecular mechanisms of proliferation, angiogenesis, cell invasion and resistance to anti-cancer drugs. SEPT9 contains a unique N-terminal extension with a basic domain, which binds microtubules, actin and the angiogenic HIF1α, as well as an acidic proline-rich domain, which may interact with proteins that contain Src homology 3 (SH3) domains (Golan and Mabjeesh, 2013; Diesenberg et al., 2015; Smith et al., 2015; Bai et al., 2016). In addition, the N-terminal domain of SEPT9 is phosphorylated by the cyclin-dependent kinase 1 (Cdk1) at the onset of mitosis (Estey et al., 2013). Multiple start codons and alternative splice sites give rise to isoforms of variable N-terminal length and sequence (McIlhatton et al., 2001; McDade et al., 2007). These SEPT9 isoforms are likely to possess different binding partners, functions and properties, and their relative levels of expression could affect cell behavior as demonstrated in studies of cell migration (Robertson et al., 2004; Connolly et al., 2011b; Chacko et al., 2012; Dolat et al., 2014b).

The majority (62.5%) of all SEPT9 mutations map to the N-terminal domain and nearly 70% of these N-terminal mutations occur in the basic domain. Similar to the full-length SEPT9, nearly a third (∼35%) of all N-terminal mutations are found in intestinal cancer samples and approximately 10% are cataloged under skin, stomach or endometrial cancers (**Figure 8A**). Interestingly, mutations in the N-terminal basic domain of SEPT9 are even more prevalent (∼45% of total) in intestinal cancers and mutations in the acidic proline-rich domain of SEPT9 are notably elevated (>20% of total) in stomach cancers (**Figures 8B,C**).

Previous work has shown that the N-terminal 25 amino acids of the longest SEPT9 isoform (SEPT9_i1) are critical for the activation of HIF1α (Golan and Mabjeesh, 2013). In addition, the basic domain of the SEPT9_i1 (amino acids 1-142) contains the microtubule-binding K/R-R/x-x-E/D motifs (Bai et al., 2013). Missense mutations in five of the N-terminal 25 amino acids of SEPT9_i1 were identified in a variety of cancers (intestine, cervix, lung, skin, liver) and mutations in the basic residues (R91, R107) of two R-R-x-E motifs occur in stomach (R91) and intestinal (R91, R107) cancers (**Figure 8D**). Interestingly, R107 is mutated in four different patients with intestinal cancer and corresponds to the second arginine of the R-R-x-E motif of SEPT9_i3, which harbors the R88W mutation of hereditary neuralgic amyotrophy (Hannibal et al., 2009). In the acidic proline-rich domain, missense mutations were identified in four proline residues (**Figure 8D**). Notably, mutations in two amino acids (R45 and P236 of SEPT9_i1) correspond to two of the three PR motifs, which are implicated in the interaction of SEPT9 with the Cbl ubiquitin ligase-interacting protein 85 kD (CIN85), an adaptor protein that promotes the degradation of the epidermal growth factor receptor (EGF-R); SEPT9 prevents binding of CIN85 to ubiquitin ligase (Diesenberg et al., 2015).

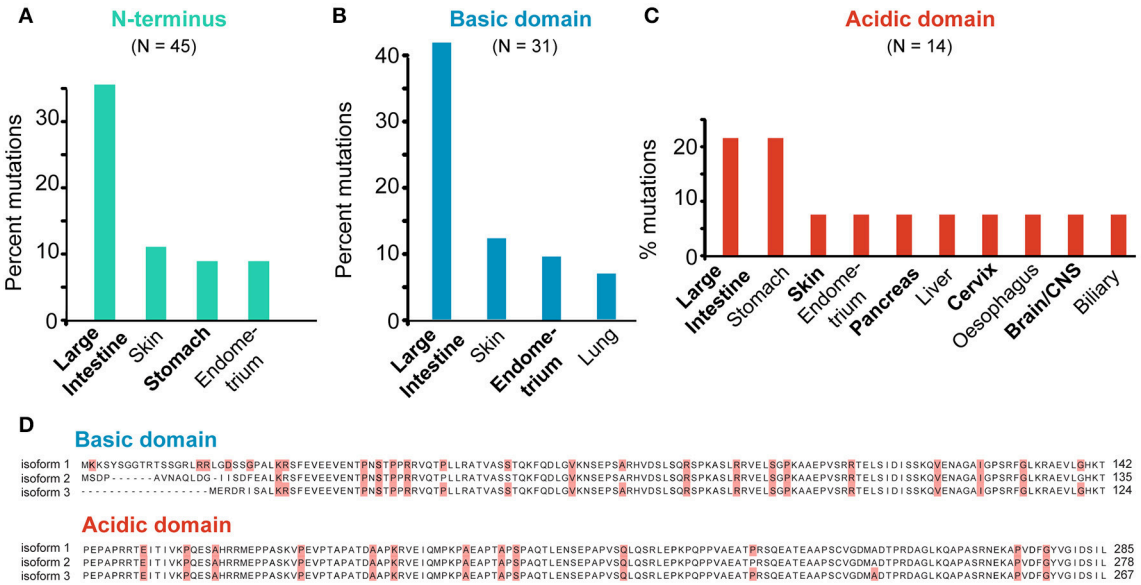

FIGURE 8 | SEPT9-specific mutations. (A–C) All missense mutations identified in the N-terminus **(A)** and N-terminal basic **(B)** and acidic **(C)** domains of SEPT9 were grouped per tumor type. Bar graphs show the tumor types which harbor mutations that account for over 5% of the total mutations identified in each domain of SEPT9. **(D)** Sequence alignments of the N-terminal basic and acidic domains of the SEPT9_i1, SEPT9_i2 and SEPT9_i3 isoforms. Missense mutations are highlighted in red.

CONCLUSIONS AND FUTURE DIRECTIONS

Since the early 2000s, when mammalian septins began to emerge as a new field of research, there have been major advances in our knowledge of the cellular functions of septins. In parallel, mounting evidence has indicated that septin levels of expression are altered in a variety of cancers. A cause-and-effect relationship between these alterations and tumorigenesis is yet to be established. Septins, however, are involved in mechanisms that promote hallmarks of cancer such as sustained proliferation, resistance to cell death, angiogenesis, cell migration and invasion. Additionally, septins have been implicated in chromosomal instability, resistance to anti-cancer drugs and the induction of tumor growth by the cancer-associated microenvironment. Understanding how abnormalities in septin expression contribute to cancer pathology will benefit from studies that directly test how the up- or down-regulation of certain septins impact tumorigenicity and cancer progression. In these efforts, the use of *in vivo* tumor models will be critical for substantiating septin roles in cancer development.

To date, septin mutations in cancer have not been studied. From the missense mutations that are currently cataloged in COSMIC, the mutational profile of septins appears to be different from the mutations of the evolutionarily related *RAS* oncogenes. Unlike *RAS*, whose mutations exhibit high frequencies and involve select amino acids, septin mutations occur with low frequencies and encompass a variety of residues. While more data are needed to ascertain the lack of "hotspot" amino acids in septins, several missense mutations take place on highly conserved amino acids with key roles in GTP-binding and the G-G and N-C dimerization interfaces. Interestingly, a few mutations occur in amino acids that are linked to male infertily and temperature-sensitive phenotypes in budding yeast.

Future studies are necessary to explore the potential roles and utility of these mutations. Do these mutations result in gain or loss of septin function? Do they have dominant negative or recessive effects? Can they be utilized toward manipulating septin functions *in vitro* and *in vivo*? Are they of diagnostic and/or prognostic value for specific cancers and patients? More importantly, are these mutations a mere consequence of the increased genomic instability that characterizes many cancers or do they play an active role in cancer progression? Answers to these questions will provide more clarity on the role of septins in cancer and may lead to new diagnostic and therapeutic strategies.

AUTHOR CONTRIBUTIONS

ES conceived the review topic, directed the review of septin mutations and wrote the manuscript. DA performed the meta-analyses of COSMIC data and prepared all figures.

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Septins and Bacterial Infection

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Septins, a unique cytoskeletal component associated with cellular membranes, are increasingly recognized as having important roles in host defense against bacterial infection. A role for septins during invasion of *Listeria monocytogenes* into host cells was first proposed in 2002. Since then, work has shown that septins assemble in response to a wide variety of invasive bacterial pathogens, and septin assemblies can have different roles during the bacterial infection process. Here we review the interplay between septins and bacterial pathogens, highlighting septins as a structural determinant of host defense. We also discuss how investigation of septin assembly in response to bacterial infection can yield insight into basic cellular processes including phagocytosis, autophagy, and mitochondrial dynamics.

Keywords: actin, autophagy, cell-autonomous immunity, cytoskeleton, mitochondria, *Listeria*, *Shigella*, septins

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INTRODUCTION

Work has shown that components of the cytoskeleton occupy a central role in innate immunity by promoting bacterial sensing and executing antibacterial functions (Mostowy, 2014; Mostowy and Shenoy, 2015). On the other hand, several intracellular pathogens can exploit the host cytoskeleton for their own advantage to promote invasion, establish a replicative niche, and/or enable dissemination. During infection, some bacteria can invade non-phagocytic cells including epithelial and endothelial cells (Cossart and Sansonetti, 2004; Haglund and Welch, 2011). After entry these pathogens remain enclosed within a membrane-bound compartment or escape to the host cell cytosol (Fredlund and Enninga, 2014). The compartmentalized or cytosolic lifestyle of intracellular pathogens can trigger rearrangements of the cytoskeleton and determine host response to infection. For example, some bacteria that rupture the phagocytic vacuole and escape to the cytosol directly interact with components of the host cytoskeleton to initiate actin-based motility for cell-to-cell spread (Welch and Way, 2013). To counteract bacterial pathogenesis, the cytoskeleton can mediate a variety of cell-autonomous immune defenses, such as activation of the inflammasome (a molecular platform processing inflammatory cytokines) or targeting pathogens to autophagy (a cytosolic degradation process) (Mostowy and Shenoy, 2015).

In comparison to actin, relatively little is known about the role of septins during bacterial infection. Septins are GTP-binding proteins that associate with cellular membrane to form filaments and ring-like structures (Mostowy and Cossart, 2012). They also interact with actin filaments and microtubules, and are therefore considered a cytoskeletal component. Septins were discovered in *Saccharomyces cerevisiae* as crucial for cell division (Hartwell, 1971). Though septins are highly conserved in fungi and animals, their number across eukaryotic species is variable. There are 7 septins in *S. cerevisiae*, 2 in *Caenorhabditis elegans*, 5 in *Drosophila melanogaster*, at least 17 in zebrafish (*Danio rerio*), and 13 in both mice and humans (SEPT1-12 and SEPT14). Vertebrate septins can be classified into 4 subgroups, namely the SEPT2, SEPT3, SEPT6, and SEPT7 subgroups, based on homology of sequence and protein domains (Mostowy and Cossart, 2012) (**Figure 1A**). Septin subunits from the different subgroups interact through their G (consisting

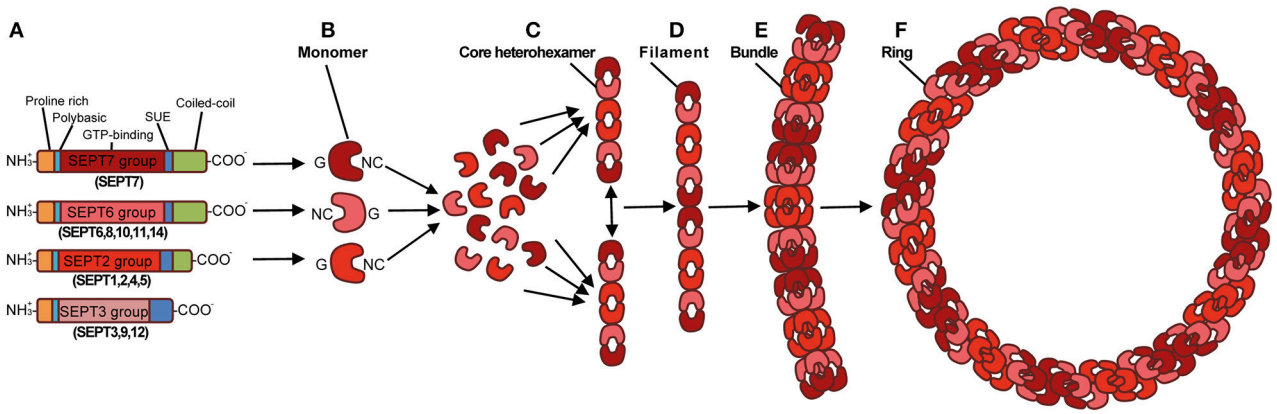

FIGURE 1 | Structure and assembly of the septin cytoskeleton. (A) Prototypical structure of mammalian septins. Humans septins (SEPT1–SEPT12, SEPT14) are classified into four subgroups (SEPT2, SEPT3, SEPT6, SEPT7), and consist of three conserved domains: a phosphoinositide-binding polybasic region, a GTP-binding domain, and a septin unique element (SUE). Septins typically have a proline-rich motif at the amino-terminal and a coiled-coil domain at the carboxy-terminal, although the length of these regions varies. By interacting via their G and NC interfaces, septin monomers **(B)** from different subgroups (shown as different shades of red), form complexes (rods of 32–40 nm in length) that contain six **(C)** or eight (not shown) symmetrically arranged septins (2 monomers from each septin subgroup). When complexes are assembled end-to-end they form non-polar filaments **(D)**. Septin filaments can associate laterally and form bundles **(E)**, bundles of septin filaments can form higher-order structures, such as rings which are ~0.6 μm in diameter **(F)**. Adapted from Mostowy and Cossart (2012).

of the GTP-binding domain) and NC (consisting of the amino- and carboxy-terminal regions) interfaces, forming complexes that can join end-to-end to form filaments. Septin filaments can associate, bundle, and go on to form higher-order structures such as rings (Mostowy and Cossart, 2012) (**Figures 1B–F**).

Septins are involved in numerous biological processes and have been implicated in a wide variety of pathological conditions including cancer, neurodegenerative disorders, and infection. This protein family was first studied in the context of bacterial infection 15 years ago (Pizarro-Cerdá et al., 2002). Identified by mass spectrometry, SEPT9 was associated with the invasion of *Listeria monocytogenes* into epithelial cells. Since then, septins have been associated with a variety of bacterial pathogens and different stages of the host cell infection process (Mostowy and Cossart, 2012; Krokowski and Mostowy, 2016). Here, we review the literature implicating septins in bacterial pathogenesis (Supplementary Table S1), and discuss how investigation of septin-bacteria interplay can provide novel insights into fundamental processes underlying bacterial infection and also host cell physiology.

SEPTIN FUNCTION IN BACTERIAL ADHESION AND ENTRY INTO HOST CELLS

Bacterial adhesion to the host cell is fundamental for some pathogens to establish infection. Adhesion prevents the mechanical clearance of extracellular bacteria (e.g., enteropathogenic *Escherichia coli* and *Clostridium difficile)* and facilitates the host cell entry of invasive bacteria (e.g., *L. monocytogenes, Yersinia* spp., *Shigella flexneri*, and *Salmonella* spp.). In this section, we summarize how several bacterial pathogens manipulate septins at the plasma membrane to support bacterial adherence to the host cell and enable bacterial internalization into both immune and non-immune cells.

Formation of Pedestal-Like Structures during Enteropathogenic *Escherichia coli* Infection

Enteropathogenic *E. coli* (EPEC) is a Gram-negative bacterium responsible for diarrheal disease in humans. By using a type III secretion system (T3SS), a molecular syringe that secretes effector proteins into the host cell, EPEC injects Translocated Intimin receptor (Tir) into the plasma membrane so that it functions as an anchor for Intimin, an effector protein located on the EPEC outer membrane (Croxen et al., 2013). Tir-Intimin interactions mediate the recruitment of Neural Wiskott-Aldrich Syndrome Protein (N-WASP) and the Actin-Related Protein 2/3 (ARP2/3) complex, remodeling of cortical actin filaments, flattening of intestinal microvilli, and ultimately the formation of pedestal-like structures raising the bacteria above the plasma membrane (Croxen et al., 2013; Lai et al., 2013).

A recent study discovered that EPEC infection phosphorylates SEPT9 in a T3SS-dependent manner (Scholz et al., 2015). The depletion or impaired phosphorylation of SEPT9 reduced adherence of EPEC to the host cells and also EPEC-mediated cytotoxicity. How septins and their phosphorylation contribute to EPEC adhesion remains to be established. Work has shown an important role for septin phosphorylation in higher-order assembly into rings (Dobbelaere et al., 2003; Kinoshita, 2003; Hernández-Rodríguez and Momany, 2012; Meseroll et al., 2013). Given that septins are involved in actin dynamics, septin assembly may promote actin rearrangements and membrane dynamics which mediate pedestal biogenesis (Scholz et al., 2015) (**Figure 2A**). The T3SS effector that mediates SEPT9

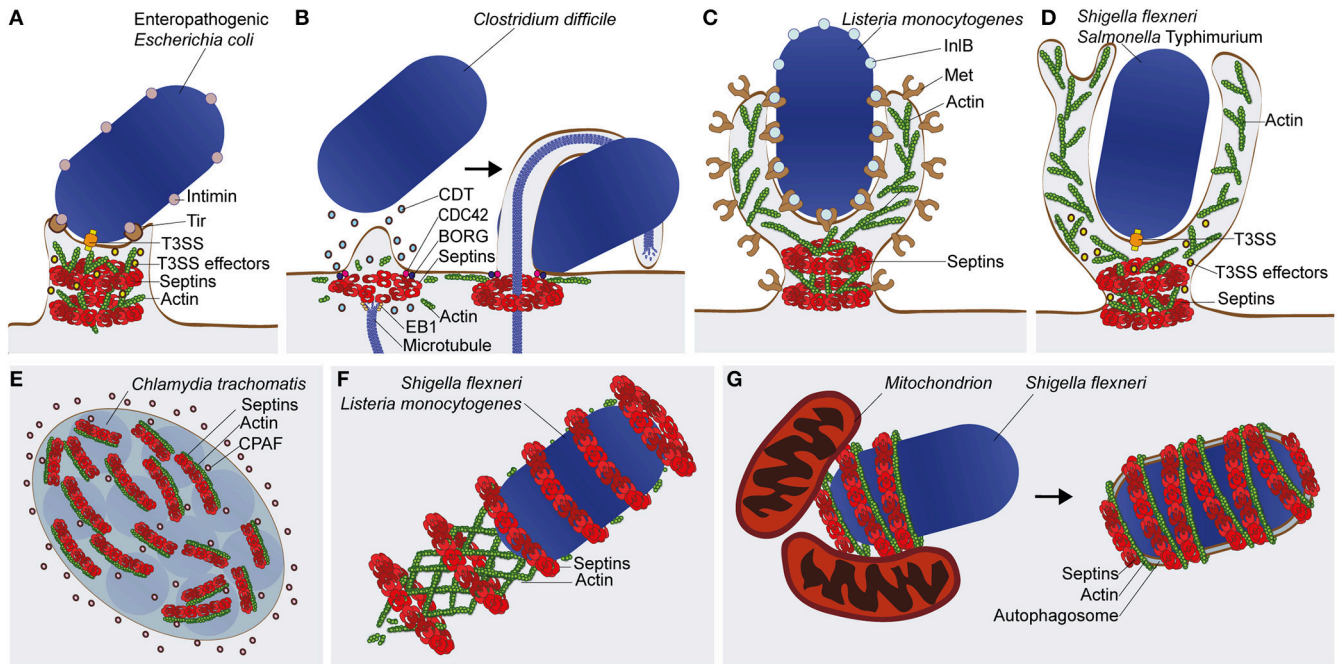

FIGURE 2 | Septin recruitment and function in bacterial infections. (A) The enteropathogenic *E. coli* (EPEC) pedestal. Type 3 secretion system (T3SS) effectors of EPEC are implicated in septin phosphorylation and assembly into filaments and rings. It is proposed that septins assemblies (depicted here as rings) remodel cortical actin for pedestal biogenesis **(B)** The *C. difficile* microtubule-based protrusion. Intoxication with the *C. difficile* toxin (CDT) leads to patches of cortical actin/septin depolymerization followed by rearrangement of septins into ring-like structures, facilitated by the factors Cell Division Control Protein 42 (CDC42) and Binder of Rho GTPase (BORG). By interacting with End Binding 1 (EB1) protein, septins redirect microtubule polymerization and initiate protrusion formation. Protrusions prevent the mechanical clearance of *C. difficile* by wrapping around the bacterium. **(C)** Zipper-mediated entry by *L. monocytogenes*. During invasion of *Listeria* into host cells, interaction of the virulence factor Internalin B (InlB) with its membrane receptor Met, initiates septin recruitment to the entry site where it forms ring-like structures at the base of the phagocytic cup. **(D)** Trigger-mediated entry by *S. flexneri* or *S.* Typhimurium. Septin ring-like structures associate with the bacteria upon injection of T3SS effectors into the host cell to induce membrane ruffles and macropinocytosis at the bacterial entry site. In the case of **(C,D)**, septins at the plasma membrane may facilitate actin remodeling, membrane protrusion, and bacterial internalization by acting as diffusion barriers for lipids and signaling molecules. **(E)** The *C. trachomatis* inclusion vacuole. Septin and actin filaments form a coat that associate with the cytosolic surface of the inclusion vacuole, and contributes to its remodeling to accommodate bacterial growth. The chlamydial enzyme CPAF is able to cleave septins for control of inclusion remodeling. **(F)** The *L. monocytogenes* or *S. flexneri* actin tail. Although dispensable for actin-based motility, septins assemble into rings around a subset of cytosolic bacteria polymerizing actin tails. The precise role of septins in the functionality of the actin tail remains unknown. **(G)** The *S. flexneri* septin cage. Septins assemble into cage-like structures around a subset of cytosolic bacteria polymerizing actin. In the case of *Shigella,* septin cage assembly is promoted by mitochondria and leads to the restriction of bacterial replication by autophagy.

phosphorylation remains unknown, however a previous study performed in *S. cerevisiae* showed that overexpression of the T3SS effector proteins Mitochondrial associated protein (Map) and *E. coli* secretion protein F (EspF) cause abnormal and mislocalized septin assemblies that affect morphogenesis and cell division (Rodríguez-Escudero et al., 2005). The same study implicated Map and EspF in the activation of a phosphorylation cascade via the Mitogen-Activated Protein Kinase (MAPK) pathway. Taken together, it is tempting to speculate that EPEC virulence factors activate a phosphorylation cascade to mediate the recruitment and assembly of septins at the plasma membrane for actin-mediated pedestal formation. Further experiments will be required to address this hypothesis. Notably, increased phosphorylation of SEPT9 has also been identified from infections with other T3SS-positive enterobacteria, including *Shigella* and *Salmonella* spp. (Rogers et al., 2011; Schmutz et al., 2013), raising the possibility that septin phosphorylation is commonly exploited by bacterial pathogens to coordinate rearrangements of the actin cytoskeleton and plasma membrane.

Microtubule-Based Protrusions from *Clostridium difficile* Infection

Hypervirulent strains of *C. difficile* infect humans provoking colitis, antibiotic-associated diarrhea, and occasionally death. The increased virulence of some *C. difficile* isolates is associated with the expression of the *C. difficile* transferase (CDT), a toxin that mediates formation of microtubule-based protrusions, which wrap around bacteria and increase their adherence to the host cell (Schwan et al., 2014). When exposing cells to CDT, the cellular cortex undergoes extensive remodeling, resulting in patches of actin depolymerization. Complexes of SEPT2-SEPT6-SEPT7-SEPT9 are recruited to the plasma membrane shortly after initial depolymerization events and form collar-like structures that initiate CDT-dependent protrusions (Nölke et al., 2016) (**Figure 2B**). Depletion of septins by siRNA or their

inhibition with forchlorfenuron reduced protrusion formation, while septin overexpression increased protrusion emergence. At the protrusion base, septins interact with the GTPase Cell Division Control Protein 42 (CDC42) and its effector Binder of Rho GTPase (BORG) (Nölke et al., 2016). These results are consistent with previous work using non-infected HeLa cells showing the regulation of septin assembly by BORGs (Joberty et al., 2001).

Septins have high affinity to End Binding 1 (EB1), a protein which associates to the plus end of microtubules, suggesting that septins guide microtubule polymerization in the extending protrusion (Nölke et al., 2016). The discovery that septins mediate microtubule apical guidance can have broad implications in cell biology. Septins are well known to localize at the base of the primary cilium, a membrane-delimited eukaryotic appendage also sustained by a microtubule-based scaffold that senses and transduces a variety of extracellular stimuli (Hu et al., 2010). Work has shown that depletion of SEPT2 results in mislocalization of ciliary membrane proteins and impairs ciliogenesis (Hu et al., 2010). Together, these results indicate that septins play a key role in maintenance of membrane compartmentalization and work as a membrane diffusion barrier, a function also attributed to septin rings at the mother-bud neck in *S. cerevisiae* (Barral et al., 2000; Takizawa et al., 2000; Caudron and Barral, 2009).

"Zipper" and "Trigger"-Mediated Entry of Invasive Bacteria

To enter non-phagocytic cells, invasive bacteria have different mechanisms to manipulate host signaling pathways leading to their uptake. Some bacteria, such as *L. monocytogenes* and *Yersinia* spp., can enter host cells via a mechanism called "zippering" that is activated via the direct interaction between bacterial surface components and host cell receptors at the plasma membrane. Other bacteria, such as *S. flexneri* and *Salmonella enterica* serovar Typhimurium, enter non-phagocytic cells by a mechanism called "triggering" that is dependent on the injection of T3SS effector proteins to stimulate host cell membrane ruffling and engulfment via a macropinocytosis-like process.

L. monocytogenes, a Gram-positive bacterium and foodborne pathogen, can stimulate its internalization into host cells via interactions between its surface proteins Internalin (InlA) and Internalin B (InlB) with plasma membrane proteins E-cadherin and Met, respectively (Pizarro-Cerdá et al., 2012). To discover other proteins required for *Listeria* entry, proteomic analysis was performed and identified enrichment of SEPT9 at the entry site during InlB-mediated invasion (Pizarro-Cerdá et al., 2002). Follow-up work revealed that septin collar-like structures assembled in response to actin polymerization at sites of *Listeria* entry (Mostowy et al., 2009b) (**Figure 2C**). Interestingly, SEPT2 promotes *Listeria* invasion while SEPT11 restricts it, suggesting different roles for different septins in bacterial entry (Mostowy et al., 2009a,b, 2011a; Kühbacher et al., 2015). Septins also form collar-like structures at sites of entry of "triggering" bacteria, such as *S. flexneri* and *S.* Typhimurium (Mostowy et al., 2009b) (**Figure 2D**). Here, septins may additionally facilitate membrane fusion events during the bacteria-induced macropinosome trafficking (Dolat and Spiliotis, 2016; Weiner et al., 2016). Together, this highlights septin assembly as a general response to actin-mediated bacterial invasion. In addition to the above studies performed using infection of non-phagocytic host cells, work has shown that SEPT2 and SEPT11 assemble into collar-like structures at the base of the phagocytic cup in macrophages and neutrophils to enable phagocytosis (Huang et al., 2008).

The precise role of septins in mediating bacterial entry remains to be established. Considering their function in compartmentalizing plasma membrane (Kusumi et al., 2012; Bridges and Gladfelter, 2015), septins may recruit host cell receptors, phospholipids, and/or signaling molecules to orchestrate the cytoskeletal rearrangements and membrane extension which underpin formation of the phagocytic cup (Barral et al., 2000; Caudron and Barral, 2009; Mostowy and Cossart, 2011; Ostrowski et al., 2016).

SEPTIN INTERACTIONS WITH INTRACELLULAR BACTERIAL PATHOGENS

After phagocytosis and entry into host cells, bacteria can adopt different intracellular lifestyles. Some invasive pathogens, such as *Chlamydia trachomatis* and *Legionella pneumophila*, can remodel their phagocytic compartment to establish an intracellular niche for persistence and replication. In contrast, other bacterial pathogens such as *L. monocytogenes, S. flexneri*, *Mycobacterium marinum*, and *Rickettsia* spp., can escape from the phagocytic vacuole to invade the host cell cytosol (Ray et al., 2009). In the following section, we discuss the interactions between septins and intracellular bacteria. We focus on septin roles in maintenance of the *C. trachomatis* intracellular niche, and also in the compartmentalization of cytosolic *S. flexneri* for host defense.

Septin Interactions with *Chlamydia trachomatis*

C. trachomatis is an obligate intracellular bacterium that replicates inside an inclusion vacuole delimited by host membranes. The parasitic lifecycle of *Chlamydia* relies on the recruitment of actin and vimentin intermediate filaments to the inclusion (Kumar and Valdivia, 2008). To remodel the cytoskeleton, *C. trachomatis* secretes the protease Chlamydial proteasome-like activity factor (CPAF) into the host cell cytosol and increases the size/flexibility of the inclusion to enable bacterial replication (Kumar and Valdivia, 2008). A recent study revealed that complexes of SEPT2-SEPT11-SEPT7-SEPT9 are also recruited to the cytosolic surface of the *Chlamydia* inclusion, where septins associate with actin filaments (Volceanov et al., 2014) (**Figure 2E**). Inhibition of septin assembly using forchlorfenuron reduced the expansion of inclusion vacuoles. Additionally, depletion of septins by siRNA abolished the formation of septin/actin coats around the *Chlamydia* inclusion and restricted the extrusion of intact

inclusions from host cells. The same study revealed that SEPT2 is a host cell substrate for CPAF, like vimentin. While the exact role of SEPT2-CPAF interactions awaits investigation, it has been suggested that cleavage of SEPT2 by CPAF contributes to dynamic remodeling of the cytoskeleton around the inclusion.

A Role for Septins in Actin Tail Polymerization?

To evade cytosolic immunity, some bacteria that escape from the phagocytic vacuole can polymerize actin for actin-based motility and cell-to-cell dissemination (Welch and Way, 2013). Interestingly, bacteria (including *Listeria* spp., *Shigella* spp., *M. marinum, Burkholderia* spp., and *Rickettsia* spp.) have evolved different mechanisms to polymerize actin tails (Gouin et al., 2005; Welch and Way, 2013). Just as septins are recruited to actin polymerization at the plasma membrane, septins are also recruited to some actin-polymerizing bacteria in the cytosol as ring-like structures (Mostowy et al., 2010) (**Figure 2F**). Although work using septin-depleted cells failed to reveal any role for septins in the functionality of actin tails (Mostowy et al., 2010), it is possible that septins impact actin tails in aspects difficult to detect using transient depletion techniques in tissue culture cells. Given that both actin-based motility and septin assembly can be reconstituted using purified proteins in cell-free conditions (Loisel et al., 1999; Bridges and Gladfelter, 2016), it seems likely that biochemical reconstitution systems will be of great value to elucidate whether septins have a role in the functionality of actin tails.

Septin Interplay with Bacterial Autophagy

Septins can recognize some bacterial pathogens that escape to the host cell cytosol and polymerize actin, forming cage-like structures to entrap bacteria and prevent actin tail formation (Mostowy et al., 2010) (**Figure 2G**). Indeed, the frequency of actin tails is significantly enhanced in septin-depleted cells infected with bacteria normally recognized by septin cages. Recent work has also shown that septin cages assemble to restrict bacterial replication (Sirianni et al., 2016). Septin cages have been observed for both *S. flexneri* and *M. marinum* during infection of tissue culture cells *in vitro* and during infection of zebrafish *in vivo* (Mostowy et al., 2010, 2013; Sirianni et al., 2016). Collectively, these observations highlight septin caging of cytosolic bacteria as an evolutionarily-conserved assembly valuable for host defense (Mostowy, 2014; Mostowy and Shenoy, 2015).

Shigella entrapped in septin cages are also targeted to autophagy (Mostowy et al., 2010; Sirianni et al., 2016) (**Figure 2G**). Autophagy is a membrane trafficking process that can redirect cytosolic material, including bacteria, to lytic compartments (Levine et al., 2011; Mostowy, 2014). A group of autophagy-related (ATG) proteins constitute the core machinery responsible for the formation, elongation, and maturation of the autophagosome. In the case of bacterial autophagy, ubiquitination and its recognition by a set of ubiquitin-binding autophagy receptors (e.g., p62/SQSTM1, NDP52/CALCOCO2), can direct the ATG machinery around bacterial cargo (Khaminets et al., 2016; Maculins et al., 2016). Strikingly, the recruitment of septins and autophagy markers are tightly connected. The depletion of septins (SEPT2, SEPT7, or SEPT9) or autophagy components (p62, NDP52, ATG5, ATG6, or ATG7) abrogates both septin cage assembly and bacterial autophagy (Mostowy et al., 2010, 2011b; Sirianni et al., 2016). These data suggest that, at least during autophagy of *Shigella*, septin cage assembly and autophagosome formation are interdependent (Mostowy and Cossart, 2012). It is interesting to consider that septin cages have been observed during autophagy of *S. flexneri* and *M. marinum*, distinct bacterial pathogens that both polymerize actin via the recruitment of N-WASP (Mostowy et al., 2010, 2013; van der Vaart et al., 2014). In contrast, septin cages have not been observed for *Listeria* spp. (Mostowy et al., 2010), partly because cytosolic *Listeria* polymerize actin tails via ActA, a bacterial effector that mimics host cell N-WASP and prevents the ubiquitination and recognition of bacteria by autophagy (Yoshikawa et al., 2009; Mostowy et al., 2011b). How exactly septins and autophagy are recruited to bacteria remains to be established. Recent work in *S. cerevisiae* has shown that septins are involved in the early stages of autophagosome formation during starvation (Barve et al., 2016). Collectively, these data suggest that septins have a fundamental role in multiple pathways of autophagy, including starvation-induced autophagy and bacterial autophagy.

To better understand the molecules and events required for septin cage assembly, proteomic analysis of the *Shigella*-septin cage was performed (Sirianni et al., 2016). From this approach, 56 host proteins were identified to interact with septins in *Shigella*-infected conditions, including p62 and the ATG8 family member Light Chain 3 B (LC3B). Surprisingly, proteomics also revealed that 21.4% of septin cage-associated proteins were annotated as exclusively mitochondrial. Indeed, high-resolution microscopy showed that mitochondria support septin assembly into the cages that entrap *Shigella* for autophagy. A role for mitochondria in septin cage assembly was confirmed by depletion of factors implicated in mitochondria fission and fusion (Sirianni et al., 2016). The depletion of Dynamin Related Protein 1 (DRP1), which elongates mitochondria and increases their availability as a membrane source for septin assembly, significantly increased septin caging. Conversely, the depletion of Mitofusin 1 (MFN1), which fragments mitochondria and limits their availability as a membrane source for septin assembly, significantly reduced septin caging. *Shigella* invasion is well known to induce mitochondria fragmentation (Carneiro et al., 2009; Lum and Morona, 2014). Remarkably, fragmentation of mitochondria enables *Shigella* to escape from septin cages and autophagy recognition (Sirianni et al., 2016). *L. monocytogenes,* which avoids septin caging, can induce fragmentation of mitochondria via Listeriolysin O (LLO), a pore-forming toxin (Stavru et al., 2011). Together, these observations suggest that fragmentation of mitochondria may be a general mechanism for bacterial pathogens to escape from cell-autonomous immunity (Sirianni et al., 2016).

Important studies have shown that septins recognize micron scale curvature at the plasma membrane, and that septin assembly is membrane-facilitated (Tanaka-Takiguchi et al., 2009; Bridges and Gladfelter, 2015; Bridges et al., 2016). In

agreement with this, septin assemblies are closely associated with invaginations and protrusions of the plasma membrane including the phagocytic cup, the cleavage furrow, and the base of dendritic spines and cilia (Bridges et al., 2016; Lobato-Márquez and Mostowy, 2016). In contrast, septin association with sources of membrane in the cytosol remains to be established. New studies have shown that septins interact with mitochondria and enable mitochondrial fission, in a process called mitokinesis (Pagliuso et al., 2016; Sirianni et al., 2016). Strikingly, septin function at the plasma membrane (e.g., phagocytosis, cytokinesis) and in the cytosol (e.g., septin caging, mitokinesis) all involve actin and non-muscle myosin II (Mostowy and Cossart, 2012; Sirianni et al., 2016). Next, it will be important to investigate different sources of cytosolic membrane (e.g., mitochondria, ER), and their precise role in septin assembly.

CONCLUSIONS

Investigation into the cell biology of bacterial infection has significantly improved our understanding of both infection and cytoskeleton biology (Haglund and Welch, 2011). For example, the study of actin-tail formation by cytosolic bacteria has enabled major discoveries underlying bacterial pathogenesis and actin biology (Welch and Way, 2013). In this review, we have discussed several examples of how investigation of septin assembly in response to bacterial invasion is discovering novel aspects of septin biology and cell-autonomous immunity. Our current knowledge of septin function during infection mostly derives from work performed *in vitro* using non-phagocytic cells (e.g., HeLa). Considering the emerging roles for the host cytoskeleton in cell-autonomous immunity (Mostowy and Shenoy, 2015), future work should address septin function in immune cells. More insight into septin roles in host defense can also come from the use of animal models. Indeed, septin-bacteria interactions can be studied using zebrafish infection models innovated to study the cell biology of infection *in vivo* (Mostowy et al., 2013; Willis et al., 2016). We propose that use of *in vivo* systems, including the zebrafish and mouse, will be essential to illuminate a more complete understanding of septin biology during host defense against bacterial infection.

AUTHOR CONTRIBUTIONS

VT and SM jointly wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcell.2016.00127/full#supplementary-material

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Septins: Regulators of Protein Stability

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Septins are small GTPases that play a role in several important cellular processes. In this review, we focus on the roles of septins in protein stabilization. Septins may regulate protein stability by: (1) interacting with proteins involved in degradation pathways, (2) regulating the interaction between transmembrane proteins and cytoskeletal proteins, (3) affecting the mobility of transmembrane proteins in lipid bilayers, and (4) modulating the interaction of proteins with their adaptor or signaling proteins. In this context, we discuss the role of septins in protecting four different proteins from degradation. First we consider botulinum neurotoxin serotype A (BoNT/A) and the contribution of septins to its extraordinarily long intracellular persistence. Next, we discuss the role of septins in stabilizing the receptor tyrosine kinases EGFR and ErbB2. Finally, we consider the contribution of septins in protecting hypoxia-inducible factor 1α (HIF-1α) from degradation.

Keywords: septins, protein stability, botulinum toxins, receptor tyrosine kinases, hypoxia-inducible factor 1α

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INTRODUCTION

Protein turnover is regulated by intracellular degradation pathways, including the ubiquitin-proteasome and lysosome-autophagy systems. Protein ubiquitylation plays a key role in the regulation of protein degradation. It is well-established that polyubiquitin chains, predominantly but not exclusively with Lys48 linkages, target proteins to proteasomal degradation (Peng et al., 2003; Xu et al., 2009; Kaiser et al., 2011; Kim et al., 2011). Ubiquitylation also acts as a signal to endocytosis (Terrell et al., 1998; Mukhopadhyay and Riezman, 2007) or protein sorting of internalized proteins to multi-vesicular bodies followed by lysosomal degradation (Raiborg and Stenmark, 2009; Ren and Hurley, 2010; Stringer and Piper, 2011). In addition, ubiquitylation regulates signaling by modulating protein-protein interactions (Mukhopadhyay and Riezman, 2007; Yau and Rape, 2016), which may affect localization of the protein and hence its susceptibility to degradation.

The half-life of most cellular proteins is typically a few days (~40–69 h) (Varshavsky, 1996). Soluble proteins usually have a relatively short half-life (several hours), while transmembrane proteins tend to survive longer (2–3 days). At the extremes, some proteins, like hypoxia-inducible factor 1α (HIF-1α), last only seconds (Yu et al., 1998), while others, like the light chain of serotype A botulinum neurotoxin, remain intact for several months (Dolly and Aoki, 2006).

Septins are small GTPases that form hetero-oligomeric structures and act as linkers between the plasma membrane and the intracellular cytoskeleton. Through interactions with transmembrane and cytosolic proteins, septins organize segregated membrane microdomains, and membrane-associated protein complexes. The contribution of septins to the regulation of numerous cellular processes, including cell division, protein trafficking, exocytosis, cell migration, and cell

proliferation, has been described in several excellent reviews (Hall and Russell, 2012; Mostowy and Cossart, 2012; Dolat et al., 2014; Fung et al., 2014). In this review, we will focus on the roles of septins in the regulation of protein stability.

Septins regulate protein stability by affecting several intracellular processes. First, they interact with components of endocytosis and exocytosis machinery (Beites et al., 1999, 2005; Amin et al., 2008; Maimaitiyiming et al., 2013; Phan et al., 2013; Tokhtaeva et al., 2015; Song et al., 2016). Second, they may regulate the interaction between transmembrane proteins and cytoskeletal proteins (Gilden and Krummel, 2010; Hagiwara et al., 2011; Hall and Russell, 2012; Mostowy and Cossart, 2012; Bridges et al., 2016). Third, by forming diffusion barriers, septins can modulate the mobility of transmembrane proteins in lipid bilayers (Hagiwara et al., 2011; Saarikangas and Barral, 2011; Hall and Russell, 2012; Mostowy and Cossart, 2012; Fung et al., 2014; Bridges et al., 2016). Fourth, septins form scaffolds and thus can modulate the interaction of proteins with their adaptor or signaling proteins (Ihara et al., 2007; Spiliotis and Gladfelter, 2011; Ageta-Ishihara et al., 2013; Ghossoub et al., 2013). All these processes can affect trafficking and sorting of proteins to degradative pathways. Finally, septins interact with proteins involved in degradation pathways, including ubiquitin ligases, and de-ubiquitylating enzymes, thus modulating protein turnover rate (Nakahira et al., 2010; Diesenberg et al., 2015; Marcus et al., 2016).

SEPTINS CONTRIBUTE TO THE REMARKABLE STABILITY OF BOTULINUM NEUROTOXIN A LIGHT CHAIN

Botulinum Neurotoxins and Their Stability

Botulism is a life-threatening illness caused by neurotoxins produced by *Clostridium botulinum*. Botulinum neurotoxins (BoNTs) are synthesized as a single-chain 150 kDa polypeptide that is later cleaved by proteases for biological activity into heavy (100 kDa) and light (50 kDa) chains. BoNTs bind through the heavy chain to specific receptors on motor neurons followed by receptor-mediated endocytosis and translocation of their light chains into the cytoplasm (Montal, 2010). Once inside the neuron, the light chain acts as a zinc-dependent endoprotease to cleave one of the SNARE (Soluble *N*-ethylmaleimide-sensitive factor Attachment Protein REceptor) proteins involved in vesicle-membrane fusion. This prevents release of acetylcholine into the synaptic cleft, resulting in neuromuscular paralysis (Dolly and Aoki, 2006; Popoff and Bouvet, 2009; Montal, 2010). As a result, patients with botulism develop severe muscle weakness. The illness may progress to total loss of muscle function, inability to breathe, and death unless supportive care is provided (Arnon et al., 2001).

Of the seven serotypes of BoNT (A–G), human botulism is caused by serotypes A, B, E and rarely by F (Arnon et al., 2001). BoNT/A intoxication lasts surprisingly long: even after 57 weeks following exposure to BoNT/A, humans may still demonstrate 22% muscle paralysis (Eleopra et al., 1998). Current treatment for adult botulism consists of supportive care and passive immunization with heptavalent equine antitoxin (Centers for Disease and Prevention, 2010). Antitoxin lowers death rates and shortens duration of symptoms only if administered within 24 h of disease onset (Arnon et al., 2001; Sobel, 2005). However, antitoxin does not enter neurons and does not reverse paralysis; hence, there is no specific treatment that targets BoNT once it is inside motor neurons.

While they cleave the same SNARE protein (SNAP-25), BoNT/A causes much longer duration of paralysis than BoNT/E (Keller et al., 1999; Adler et al., 2001; Bajohrs et al., 2004). In cultured spinal cord neurons, the proteolytic activity of BoNT/A, and BoNT/E persists for more than 80 days and <1 day, respectively (Keller et al., 1999). When expressed in cultured neuronal cells, the light chain of BoNT/A (LCA) survives significantly longer than the light chain of BoNT/E (LCE) due to less efficient degradation (Fernández-Salas et al., 2004; Tsai et al., 2010; Vagin et al., 2014). However, LCA is cleaved by proteases *in vitro* as efficiently as LCE (Gimenez and DasGupta, 1993; Beecher and DasGupta, 1997; Prabakaran et al., 2001). Thus, intrinsic resistance to intracellular proteases does not explain LCA persistence.

Septins Protect Botulinum Toxin Light Chain a from Intracellular Degradation

Even though LCA does not possess a transmembrane domain, it is localized in clusters almost exclusively at the plasma membrane (Vagin et al., 2014). In contrast, LCE remains in the cytoplasm, where its half-life corresponds to that of typical cytoplasmic proteins (Foran et al., 2003; Fernández-Salas et al., 2004). A double mutation, L428A/L429A, in a dileucine-containing motif (EFYKLL), which is present in LCA but not in LCE, decreases association of LCA with the plasma membrane (Fernández-Salas et al., 2004), prevents its clustered distribution, and shortens the half-life of LCA in cultured neuronal cells (Vagin et al., 2014) and shortens neuroparalytic effects of BoNT/A in mice (Wang et al., 2011). Furthermore, fusion of LCE with BoNT/A stabilizes LCE, while L428A/L429A mutation in the LCA portion of this chimera reverts LCE back into a short-lived protein (Wang et al., 2011). Mass spectrometry analysis identified septins as proteins that interact with LCA but not with the L428A/L429A LCA mutant (**Table 1**) (Vagin et al., 2014). Septins co-localize with LCA in plasma membrane-associated clusters, and the L428A/L429A mutation decreases this co-localization and accelerates ubiquitylation-dependent degradation of LCA (Vagin et al., 2014). Similarly, impairment of septin oligomerization with forchlorfenuron (FCF), decreases LCA clustering, and increases LCA degradation (Vagin et al., 2014). Therefore, the dileucine-mediated formation of membrane-attached LCA- and septin-containing complexes is crucial for the long-lasting stabilization of LCA and LCA-related neuroparalytic activity. The involvement of septins in LCA stabilization is consistent with the reports on enrichment of septins in the presynaptic sites in neurons (Kinoshita et al., 2000; Xue et al., 2004; Yang et al., 2010; Tsang et al., 2011) and interaction of septins with SNARE proteins (Beites et al., 1999; Dent et al., 2002; Ihara et al., 2007; Ito et al., 2009; Wasik et al., 2012; Tokhtaeva et al., 2015).

TABLE 1 | Septin-mediated regulation of protein stability.

Septin- regulated protein	Septin(s) involved in regulation	Protein-septin interaction	Effect(s) of septins on protein stability	Molecular mechanism of septin-mediated effect	References
LCA	Septin 2 Septin 3 Septin 5 Septin 6 Septin 7 Septin 9 Septin 11	Dileucine motif (428L/429L) required for binding	Protect from ubiquitylation-dependent degradation	Unknown	Vagin et al., 2014
EGFR	Septin 9 Septin 2 Septin 7	Not demonstrated	Protects from ubiquitylation and degradation	Septin 9 competes with CBL for binding to CIN85	Diesenberg et al., 2015
ErbB2	Septin 2 Septin 9 Septin 7	Multiprotein complex with several septins	Protect from ubiquitylation and lysosomal degradation	Unknown	Marcus et al., 2016
HIF-1α	Septin 9	GTPase domain of septin 9 required for interaction	Protects from ubiquitylation and degradation	Septin 9 competes for RACK1 binding to HIF-1α	Amir et al., 2006, 2009; Golan and Mabjeesh, 2013; Vardi-Oknin et al., 2013
MET	Septin 2 Septin 11	Unknown	Differently modulate surface expression and association with the cytoskeleton	Unknown	Mostowy et al., 2011
JNK	Septin 9	GTPase domain of septin 9 required for interaction	Protects from degradation	Unknown	Gonzalez et al., 2009

SEPTINS STABILIZE RECEPTOR TYROSINE KINASES

Persistent Signaling of Receptor Tyrosine Kinases

EGFR family receptor tyrosine kinases (ErbB1 or EGFR, ErbB2, ErbB3, ErbB4) trigger signaling cascades that regulate critical cellular processes, such as growth, differentiation, proliferation, adhesion, survival, and migration (Blume-Jensen and Hunter, 2001). One of the primary mechanisms that regulates the duration of downstream signaling after activation is the removal of these receptors from the membrane by endocytosis followed by trafficking back to the cell surface or to lysosomes for degradation (Wiley and Burke, 2001; Sorkin and Goh, 2009). Over-expression and aberrant degradation of ErbB receptors lead to enhanced or continuous signaling, promoting malignant transformation (Sangwan and Park, 2006; Lemmon and Schlessinger, 2010; Tebbutt et al., 2013; Arteaga and Engelman, 2014).

Endocytosis and degradative sorting of EGFR are regulated by the ubiquitin ligase CBL (Levkowitz et al., 1996; Schmidt and Dikic, 2005). EGFR stimulation by ligand binding triggers the recruitment of CBL, which induces multi-mono- and K63-polyubiquitylation of the receptor (Mosesson et al., 2003; Huang et al., 2006, 2013). Whether ubiquitylation is required for endocytosis is still a point of controversy. However, it is generally agreed that CBL-mediated receptor ubiquitylation targets internalized receptors for lysosomal degradation, while non-ubiquitylated receptors are recycled back to the membrane. CBL-interacting protein of 85 kDa (CIN85) mediates the interaction between CBL and EGFR and hence is involved in the downregulation of EGFR (Soubeyran et al., 2002; Haglund et al., 2003).

In contrast to EGFR, ErbB2 does not have a known ligand (Garrett et al., 2003) and is thought to be resistant to endocytic down-regulation (Wang et al., 1999; Haslekås et al., 2005; Roepstorff et al., 2008; Sorkin and Goh, 2008). Ubiquitylation is important for ErbB2 degradation (Vuong et al., 2013), but the involvement of CBL is not clear (Levkowitz et al., 1996; Klapper et al., 2000). Heat shock protein 90 (HSP90) is required for stable expression of ErbB2 at the plasma membrane, and disruption of ErbB2 association with HSP90 allows for recruitment of HSP70 and ubiquitin ligases CHIP and/or Cullin-5, leading to ErbB2 ubiquitylation (Xu et al., 2002; Ehrlich et al., 2009), internalization and lysosomal degradation (Tikhomirov and Carpenter, 2000; Austin et al., 2004; Lerdrup et al., 2006). Proteasomal activity is required for endocytosis and lysosomal targeting of ErbB2 but is not directly involved in ErbB2 proteolysis (Lerdrup et al., 2006, 2007; Roepstorff et al., 2008).

Septins Protect Receptor Tyrosine Kinases from Ubiquitylation, Endocytosis and Degradation

CIN85 is involved in down-regulation of EGFR by tethering CBL to the endocytic machinery in an EGF-dependent manner (Dikic, 2002). A number of studies have suggested that CIN85

participates in both the initial step of EGFR internalization (Soubeyran et al., 2002) and also in receptor trafficking and degradation (de Melker et al., 2001; Kowanetz et al., 2004; Schroeder et al., 2012). Knockdown of CIN85 results in a decrease in EGFR ubiquitylation (Rønning et al., 2011), while prevention of CIN85 phosphorylation affects efficient sorting and degradation of EGFR but has no effect on receptor endocytosis (Schroeder et al., 2012). A recent study has identified CIN85 as an interacting partner of septin 9 (**Table 1**) (Diesenberg et al., 2015). This interaction depends on the presence of a conserved ProArg motif in the N-terminus of septin 9 and mediates the formation of a multiprotein complex of CIN85 with septin 9 and other septins. *In vitro* binding studies suggest that septin 9 competes with CBL for binding to CIN85 (Diesenberg et al., 2015). Stimulation of EGFR with EGF in Hela cells results in the recruitment of the CIN85-septin complex to the plasma membrane. Depletion of septin 9 increases the degree of EGFR ubiquitylation and accelerates its degradation. Taken together, these data suggest that septin 9 negatively regulates EGFR degradation by preventing the association of the ubiquitin ligase CBL with CIN85, resulting in reduced EGFR ubiquitylation, and degradation (Diesenberg et al., 2015).

Another recent study has identified septins as interacting partners and regulators of the persistent expression of ErbB2 (Marcus et al., 2016). Several septins, including septin 2, septin 7, and septin 9, co-localize and interact with ErbB2 at the plasma membrane in gastric cancer cells. Inhibition of septin filament assembly-disassembly with FCF: (1) decreases association of septins with ErbB2, (2) reduces plasma-membrane localization of septins, (3) increases the amount of septin-free ErbB2, (4) induces ubiquitylation of ErbB2, and (5) accelerates its lysosomal degradation. A similar increase in ErbB2 degradation is observed in septin 2-depleted cells. These results imply that normally organized septin filaments protect ErbB2 from ubiquitylation, endocytosis, and lysosomal degradation.

This protective effect of septins is not related to the regulation of ErbB2 interaction with its chaperone HSP90 because the FCF-induced ubiquitylation and degradation of ErbB2 is not altered by geldanamycin (Marcus et al., 2016). This inhibitor down-regulates ErbB2 by disrupting the ErbB2-HSP90 interaction (Tikhomirov and Carpenter, 2000; Lerdrup et al., 2006, 2007). Therefore, distinct and complementary effects of FCF, and geldanamycin present a potential for augmented targeting of ErbB2 persistence in cancer.

FCF-induced ubiquitylation of ErbB2 is unlikely to be due to septin 9-mediated regulation of CBL as has been reported for EGFR (Diesenberg et al., 2015), because ErbB2 does not interact with CIN85 and FCF does not affect levels of EGFR (Marcus et al., 2016). The involvement of other ubiquitin ligases identified as septin interacting proteins (Nakahira et al., 2010; Tokhtaeva et al., 2015) in septin-regulated ErbB2 ubiquitylation has not been evaluated. Alternatively, septins may recruit de-ubiquitylating enzymes to ErbB2, resulting in a lower steady state level of ErbB2 ubiquitylation. In particular, ErbB2 interacts with a de-ubiquitylating enzyme USP9x (Marx et al., 2010; Marcus et al., 2016) that has been shown to protect ErbB2 from bortezomib-induced lysosomal degradation (Marx et al., 2010). In addition, septins play critical roles in endocytosis and exocytosis (Beites et al., 1999, 2005; Maimaitiyiming et al., 2013; Phan et al., 2013; Tokhtaeva et al., 2015; Song et al., 2016), and disruption of septin dynamics with FCF may induce ErbB2 internalization or delay its recycling to the plasma membrane.

SEPTINS STABILIZE HYPOXIA-INDUCIBLE FACTOR 1α

Role of HIF-1α in Cell Responses to Hypoxia

HIF-1 is a transcription factor and key regulator of cellular responses to changes in oxygen concentration that allow cell adaptation and survival under hypoxic conditions (Semenza, 2014). HIF-1 is composed of the oxygen-regulated subunit, HIF-1α, and the constitutively expressed HIF-1β subunit. HIF-1α is constantly produced and degraded under normoxic conditions due to oxygen-dependent hydroxylation, which promotes binding of von Hippel-Lindau protein (VHL), leading to ubiquitylation, and proteasomal degradation of HIF-1α. Under hypoxic conditions, HIF-1α is not hydroxylated, does not interact with VHL, translocates to the nucleus, and binds to hypoxia-response elements in target genes. The expression of over 70 genes is known to be activated at the transcriptional level by HIF-1 (Semenza, 2014). HIF-1α rapidly accumulates under hypoxic conditions and is degraded upon reoxygenation with a half-life of under 1 min (Yu et al., 1998). Another level of post-translational regulation does not depend on oxygen, hydroxylation, or VHL but requires the interaction between HIF-1α and RACK1 (receptor of activated protein C kinase). RACK1 competes with HSP90 for binding to HIF-1α and promotes the proteasome-dependent degradation of HIF-1α (Liu et al., 2007; Liu and Semenza, 2007). Activation of the HIF system has been observed in carcinogenesis and numerous cancers (Semenza, 2012, 2016a,b; Hubbi and Semenza, 2015). Increased levels of HIF-1 activity are often associated with increased tumor aggressiveness, therapeutic resistance, and mortality.

Septin 9 Protects HIF-1α from Degradation

A search for HIF-1α-interacting proteins in human prostate cancer cells identified septin 9 (Sept9_v1 isoform) (Amir et al., 2006). Over-expression of septin 9 decreases ubiquitylation and degradation of HIF-1α and activates HIF-1α-dependent transcriptome (Amir et al., 2006). Co-immunoprecipitation experiments show that septin 9 competes with RACK1 for binding to HIF-1α (Amir et al., 2009). Inhibition of HSP90 induces RACK1-dependent HIF-1α degradation, and the rate of this degradation is significantly lower in cells over-expressing septin 9 (Amir et al., 2009). Taken together, these results implicate septin 9 in oxygen-independent stabilization of HIF-1α. As long as HIF-1α is bound to HSP90 or septin 9, it is protected from RACK1-mediated degradation via the proteasome. In the presence of HSP90 inhibitors, the HIF-1α-HSP90 association is disrupted, leading to a competition between RACK1, which promotes HIF-1α degradation, and septin 9, which confers HIF-1α stabilization (Amir et al., 2009). In support of this

HIF-1α-stabilizing role of septin 9, disruption of normal assembly-disassembly of septin oligomers with FCF induces degradation of HIF-1α, and inhibition in HIF-1α transcriptional activity in various cancer cell types (Vardi-Oknin et al., 2013).

CONCLUDING REMARKS

Septins stabilize integral membrane proteins (EGFR and ErbB2), membrane-associated proteins (LCA), and cytosolic/nuclear proteins (HIF-1α) by attenuating their ubiquitylation and degradation. Septin 9 also interacts with and stabilizes another cytosolic/nuclear protein, c-Jun-N-terminal kinase (JNK) (Gonzalez et al., 2009), but whether septin 9 affects the degree of JNK ubiquitylation has not been investigated. Septin 2 and septin 11 regulate surface expression of MET receptor tyrosine kinase by modulating its mobility in the lipid bilayer and its linkage to the underlying cytoskeleton (Mostowy et al., 2011). However, it is not clear if septins affect MET degradation. Only some of the molecular events underlying the protective effects of septins against protein ubiquitylation and degradation are understood (**Table 1**), and further studies are required to determine the detailed mechanisms of septin dependent protein stabilization.

Understanding the mechanism by which septins contribute to intracellular stability of LCA would provide valuable insights for treating BoNT intoxication. On the other hand, the remarkable stability of LCA is fundamental in the success of BoNT/A for long-term treatment of several disorders as well as cosmetic therapies (Bhidayasiri and Truong, 2005; Chancellor et al., 2013; Esquenazi et al., 2013; Hallett et al., 2013; Naumann et al., 2013; Jost et al., 2015; Choi et al., 2016), and a super-stable LCA would be useful therapeutically.

A better understanding of septin contribution to the abnormal persistence of EGFR and ErbB2 in cancer cells will provide a potential treatment target for aggressive malignancies. Receptor-targeted therapies, such as monoclonal antibodies (e.g., trastuzumab), and tyrosine kinase inhibitors (e.g., gefitinib), are effective against several types of malignancies, but tumors may develop resistance to these agents due to compensatory mechanisms (Hynes and Lane, 2005; Takeuchi and Ito, 2011; Arteaga and Engelman, 2014), emphasizing the evolving need to develop new synergistic treatment strategies.

Knowledge of the pathways of septin contribution in oxygen-independent stabilization of HIF-1α would provide a better understanding of the reasons for HIF-1α over-expression in various cancers even in aerobic conditions, which correlates with poor prognosis, making HIF-1α an important target for cancer therapy. A better understanding of septin-mediated stabilization of HIF-1α opens the way to new therapeutic approaches to target "normoxic" tumor cells (Semenza, 2012; Burroughs et al., 2013; Warfel and El-Deiry, 2014).

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