Published in final edited form as:
Dev Cell. 2011 April 19; 20(4): 563–571. doi:10.1016/j.devcel.2011.03.007.

Protein Phosphatase 2A-SUR-6/B55 Regulates Centriole Duplication in *C. elegans* by Controlling the Levels of Centriole Assembly Factors

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Summary

Centrioles play a crucial role in mitotic spindle assembly and duplicate precisely once per cell cycle. In worms, flies, and humans, centriole assembly is dependent upon a key regulatory kinase (ZYG-1/Sak/Plk4) and its downstream effectors SAS-5 and SAS-6. Here we report a role for protein phosphatase 2A (PP2A) in centriole duplication. We find that the PP2A catalytic subunit LET-92, the scaffolding subunit PAA-1, and the B55 regulatory subunit SUR-6, function together to positively regulate centriole assembly. In PP2A-SUR-6-depleted embryos, the levels of ZYG-1 and SAS-5 are reduced and the ZYG-1- and SAS-5-dependent recruitment of SAS-6 to the nascent centriole fails. We show that PP2A physically associates with SAS-5 in vivo, and that inhibiting proteolysis can rescue SAS-5 levels and the centriole duplication defect of PP2A-depleted embryos. Together our findings indicate that PP2A-SUR-6 promotes centriole assembly by protecting ZYG-1 and SAS-5 from degradation.

Keywords

centriole duplication; PP2A; ZYG-1; SZY-20; *C. elegans*

Introduction

In animal cells, the size and number of microtubule-organizing centers, or centrosomes, are determined by the centrioles, barrel-shaped organelles having ninefold rotational symmetry (Marshall, 2009). Centrioles organize centrosomes and thus, the number of centrosomes per cell generally correlates with the number of centrioles. A typical centrosome possesses an orthogonally arranged pair of centrioles surrounded by a halo of pericentriolar material (PCM), a substance from which the microtubules are nucleated and anchored.

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Centrioles duplicate precisely once per cell cycle with a new daughter centriole assembling next to each preexisting centriole during S phase (Strnad and Gonczy, 2008). Defects in this process lead to abnormal numbers of centrioles, a state that has been linked to both chromosome missegregation and tumor formation (Basto et al., 2008; Castellanos et al., 2008; Ganem et al., 2009; Silkworth et al., 2009). Although the structural complexity of centrioles varies among species, studies in worms and humans suggest the existence of a common centriole assembly pathway that involves the activities of a small set of conserved duplication factors (Delattre et al., 2006; Kleylein-Sohn et al., 2007; Pelletier et al., 2006).

The centriole assembly pathway has been described most thoroughly in the *C. elegans* embryo where the kinase ZYG-1 plays a key role (O'Connell et al., 2001). Related to vertebrate Plk4 and *Drosophila* Sak, ZYG-1 localizes early to the nascent centriole in a step that requires the coiled-coil protein SPD-2 (Delattre et al., 2006; Pelletier et al., 2006). ZYG-1 then recruits a complex of two conserved coiled-coil proteins SAS-5 and SAS-6, that is required for formation of the central tube, the first known structural intermediate (Dammermann et al., 2004; Delattre et al., 2006; Delattre et al., 2004; Leidel et al., 2005; Pelletier et al., 2006). SAS-5 and SAS-6 are also required for the stable incorporation of SAS-4, a coiled-coil protein that promotes the addition of nine sets of microtubules that surround the central tube (Dammermann et al., 2008; Delattre et al., 2006; Kirkham et al., 2003; Leidel and Gonczy, 2003; Pelletier et al., 2006).

Recently, attention has focused on the role of phosphorylation in centriole assembly. Kitagawa et. al (2009) have found that ZYG-1 promotes stable association of SAS-6 with the nascent centriole through phosphorylation. Also, human Plk4 has been shown to prevent centriole overduplication by negatively regulating its own levels through autophosphorylation (Guderian et al., 2010; Holland et al., 2010). While roles for kinases in centriole assembly have been established, the possible involvement of phosphatases remains an open question. Like kinases, phosphatases regulate a myriad of cellular processes and do so with a high degree of specificity. The most abundant phosphatases are members of the PP1 and PP2A family. These multimeric enzymes contain a catalytic subunit characteristic of a specific phosphatase family and a regulatory subunit that determines substrate specificity (Virshup and Shenolikar, 2009).

Here we find that LET-92, the PP2A catalytic subunit (hereafter referred to as $PP2Ac^{LET-92}$), physically associates with SZY-20, a negative regulator of ZYG-1 (Song et al., 2008). We find that $PP2Ac^{LET-92}$ is required for centriole duplication in *C. elegans*, and that it functions in this context as a holoenzyme consisting of the catalytic subunit $PP2Ac^{LET-92}$, the scaffolding subunit $PP2Aa^{PAA-1}$, and the regulatory subunit SUR-6. Further, we show that the most critical role of $PP2Ac^{LET-92}$-SUR6 in centriole assembly is to protect ZYG-1 and SAS-5 from degradation. Our work thus identifies $PP2Ac^{LET-92}$-SUR-6 as a new component of the centriole assembly pathway, and suggests that centriole duplication is controlled by the coordinated action of kinases and phosphatases.

Results

SZY-20 physically interacts with protein phosphatase 2A

Previously, we identified the RNA-binding protein SZY-20 as a factor that negatively regulates ZYG-1 levels at the centrosome (Song et al., 2008). To understand how SZY-20 controls ZYG-1, we sought to identify proteins that physically interact with SZY-20. Using affinity-purified α-SZY-20 antibodies, we immunoprecipitated SZY-20 from whole worm extracts and analyzed the precipitated material by mass spectrometry. Among the proteins that specifically co-precipitated with SZY-20, we identified one, that when depleted by

RNAi, reduces centrosome number in the early embryo. Here we focus on this factor, PP2Ac^{LET-92}, the sole *C. elegans* protein phosphatase 2A catalytic subunit.

To confirm the SZY-20-PP2Ac^{LET-92} interaction, we immunoprecipitated SZY-20 from whole worm extracts, and analyzed the immunoprecipitated material by western blotting. In agreement with our mass spectrometry results, we detected PP2Ac^{LET-92} among the material precipitated with α-SZY-20 antibodies (Figure 1A). Given our data indicating a physical association between SZY-20 and PP2Ac^{LET-92} it seemed plausible that SZY-20 is a substrate of PP2A. Consistent with this idea, our mass spectrometry data indicates that SZY-20 is indeed a phosphoprotein (Figure S1A–C), and depletion of PP2Ac^{LET-92} results in a significant and reproducible reduction in mobility of SZY-20 on SDS-PAGE gels (Figure 1B). The change in mobility appears to be due to phosphorylation, as phosphatase treatment of SZY-20 from *let-92(RNAi)*-treated worms restored its normal mobility (Figure S1D). We conclude that PP2Ac^{LET-92} physically associates with SZY-20 *in vivo* and regulates the phosphorylation level of SZY-20.

Disruption of the PP2A holoenzyme blocks centriole duplication

In *C. elegans* embryos, a maternal block to centrosome duplication is manifest by a distinct phenotype: a bipolar first division followed by formation of monopolar spindles at the two-cell stage (O'Connell et al., 2001). This phenotype arises because the two sperm centrioles can separate and set up the poles of the first bipolar spindle, but can only organize monopolar spindles when partitioned to the daughter cells. Strikingly, we found that depletion of PP2Ac^{LET-92}, although pleiotropic in phenotype, resulted in a nearly identical centrosome defect: a bipolar spindle during first division followed by monopolar spindles during second division (Figure 1E and Movie S1). In addition, PP2Ac^{LET-92}-depleted embryos fail to properly align their chromosomes on a metaphase plate, exhibit a delay in cell cycle progression, and display defects in mitotic exit such as a failure to reform nuclei and to decondense chromatin. A defect in mitotic exit results from depletion of PP2A-B55α in human cells (Schmitz et al., 2010), indicating that this role is conserved between nematodes and humans. To quantify the effect of PP2Ac^{LET-92} depletion on centrosome duplication, we performed live imaging of *let-92(RNAi)* embryos expressing GFP-SPD-2 to mark centrosomes and mCherry-histone to mark chromatin (Movie S2). Reducing PP2Ac^{LET-92} completely blocks centrosome duplication (0/34 successful events, n=18 embryos). We conclude that PP2Ac^{LET-92} is required for centrosome duplication in *C. elegans*.

The B55 regulatory subunit SUR-6 directs the role of PP2A in centriole assembly

PP2A functions as a heterotrimeric complex consisting of a catalytic subunit, a scaffolding subunit, and a regulatory subunit. In *C. elegans*, the scaffolding subunit is encoded by a single gene *paa-1*. Depletion of PP2Aa^{PAA-1} produces defects in chromosome alignment, cell cycle progression, and mitotic exit that are similar to those induced by PP2Ac^{LET-92} depletion (data not shown). In addition, depletion of PAA-1 results in the formation of monopolar spindles during the second embryonic cell division (Figure 1F), confirming the involvement of the PP2A holoenzyme in centrosome duplication. The specificity of the PP2A holoenzyme is determined by association with a specific regulatory subunit. By homology, we identified eight genes in *C. elegans* that encode PP2A regulatory subunits. We used RNAi to knockdown each of these genes and screened for a centrosome duplication defect. Only RNAi of *sur-6*, encoding a B55 family member involved in Ras/Raf/MEK/ERK signaling (Kao et al., 2004), produced an obvious duplication defect (Figure 1G). In these embryos we observed both monopolar spindles and asymmetric spindles in which one spindle pole was smaller than the other (Figure 1G). Asymmetric spindles arise when centriole duplication is partially inhibited (Delattre et al., 2004; Kirkham et al., 2003).

In contrast to *let-92(RNAi)*, the effect of *sur-6(RNAi)* was relatively mild; embryos did not possess obvious defects in cell cycle progression or mitotic exit but did occasionally exhibit anaphase bridging of chromatin. Also unlike *let-92(RNAi)*, *sur-6(RNAi)* did not completely block centrosome duplication: 24% of spindles were monopolar and 47.5% were asymmetric (Table S1). The lack of a completely penetrant centriole duplication defect could reflect incomplete depletion of SUR-6 protein by RNAi (Figure 1J), or alternatively functional redundancy between SUR-6 and other PP2A regulatory subunits. To address this, we re-screened these genes using a sensitized genetic background. At the semipermissive temperature of 20°C, centrosomes in the temperature-sensitive *zyg-1(it25)* mutant fail to duplicate just 2.9% of the time. (Figure 1I and Table S1). When subjected to RNAi of each regulatory gene, only *sur-6(RNAi)* had a significant effect, increasing the rate of monopolar spindle formation 22-fold to 64% (Figure 1H and Table S1). These results suggest that the role of PP2A in centrosome duplication is predominantly, if not entirely, directed by SUR-6, and that the lack of a more robust centriole duplication defect upon *sur-6(RNAi)* is due to incomplete depletion.

To determine where in the cell SUR-6 acts, we produced affinity-purified anti-SUR-6 polyclonal sera. The sera identified a band of the correct size by immunoblotting, and the intensity of this band was significantly reduced by *sur-6(RNAi)*(Figure 1J). Further, the SUR-6 antibody also coprecipitated $PP2Ac^{LET-92}$ from worm extracts (Figure 1C). Immunostaining of embryos produced diffuse cytoplasmic staining that was significantly reduced by *sur-6(RNAi)* (Figure 1K, L). This result suggests that the function of $PP2Ac^{LET-92}$-SUR-6 is not limited to the centrosome and may function broadly in the cell to regulate centriole duplication.

The SZY-20-$PP2Ac^{LET-92}$ interaction plays a minor role in regulating centriole duplication

We next wanted to determine where in the centriole duplication pathway PP2A functions. SZY-20 negatively regulates centriole duplication (Song et al., 2008) and appears to be a substrate of $PP2Ac^{LET-92}$-SUR-6 (Figures 1B, S1D and S1E). Thus, PP2A might promote duplication by repressing SZY-20. To address this, we depleted $PP2Ac^{LET-92}$ in strain OC470, a *szy-20(bs52)* mutant that expresses GFP-SPD-2 and mCherry-histone. Strong knockdown of $PP2Ac^{LET-92}$ in this strain still produced a complete block to duplication (n=38 events), indicating that the centriole duplication defect of *let-92(RNAi)* embryos is not due to an overactive SZY-20 protein. However, the *szy-20(bs52)* mutation could partially suppress a weaker knockdown of $PP2Ac^{LET-92}$ (7 of 26 centrosomes duplicated in *szy-20(bs52)* mutants versus 0/18 in controls), suggesting that one minor role for PP2A is to repress SZY-20. Overall, failure of the *szy-20(bs52)* mutation to strongly affect the *let-92(RNAi)* duplication defect indicates that the critical role of PP2A in centriole duplication lies elsewhere in the pathway.

PP2A functions closely with ZYG-1 and SAS-5

We have shown that genes encoding core centriole assembly factors exhibit robust genetic interactions with each other (Kemp et al., 2004). Mutations in two different centriole assembly genes often fail to complement one another, with the double heterozygotes exhibiting high levels of embryonic lethality and centriole duplication errors. This interaction, termed non-allelic non-complementation, occurs when two genes function closely together in a common process; in a double heterozygote, the process is rendered inefficient enough to yield an observable phenotype. To determine if *let-92* also behaves in this manner, we asked if the *let-92(s504)* mutation would fail to complement the *zyg-1(b1)* allele. Animals heterozygous for either *zyg-1(b1)* or *let-92(s504)* had low levels of embryonic lethality, yet strikingly the *zyg-1(b1)/+; let-92(s504)/+* double heterozygotes exhibited 100% embryonic lethality (Figure 2A). We also tested the *zyg-1(b1)* mutation

against the *let-92(s677)* allele and again detected a very strong interaction: 61% (n=1197) of the embryos produced by *zyg-1(b1)/+; let-92(s677)/+* hermaphrodites were inviable compared to 2.8% (n=611) for *let-92(s677)/+* progeny. We then examined embryos from *zyg-1(b1)/+; let-92(s504)/+* hermaphrodites and observed a prominent centrosome duplication defect (Figure 2B–D). Live DIC imaging of these embryos revealed that the block to centriole duplication was complete (0 of 48 centrioles duplicated; movie S3). These results show that PP2Ac^{LET-92} behaves genetically in the same manner as other core centriole duplication factors and indicate that PP2Ac^{LET-92} functions closely with ZYG-1.

We next asked if we could use this type of genetic interaction to map the role of PP2A, as well as other factors, in the centriole assembly pathway. We constructed and quantified embryonic lethality in all possible double heterozygotes from a set of *spd-2*, *zyg-1*, *sas-5*, *sas-6*, *sas-4*, *let-92* and *paa-1* mutations (Figure 2A). Most pertinent to the current study is the behavior of *let-92*, which shows strong interactions with *zyg-1* and *sas-5* but weak or no interactions with *spd-2*, *sas-6*, and *sas-4*. Again, these double heterozygotes exhibit centriole duplication defects (Figure 2E). These results suggest that the role of PP2Ac^{LET-92} is most closely tied to *zyg-1*- and *sas-5*-dependent steps. Also of interest, *spd-2* only shows a strong interaction with *zyg-1*, consistent with the only known role of SPD-2 in localizing ZYG-1 to sites of centriole assembly (Delattre et al., 2006; Pelletier et al., 2006). In contrast, *zyg-1* shows strong genetic interactions with multiple components including *spd-2*, *sas-5*, *sas-4*, and *let-92*, suggesting that ZYG-1 functions at multiple steps in this pathway.

PP2A is required for recruitment of SAS-5 and SAS-6

A molecular hierarchy describing the order and dependencies with which duplication factors localize to assembling centrioles has been described (Delattre et al., 2006; Pelletier et al., 2006). To determine where in this pathway PP2Ac^{LET-92}-SUR-6 functions, we analyzed the effect of PP2Ac^{LET-92} depletion on the recruitment of duplication factors. We found that *let-92(RNAi)* does not block localization of ZYG-1 to nascent centrioles (Figure 3A and B). We then examined SAS-5 and SAS-6, two downstream effectors of ZYG-1 that form a complex *in vivo* and are mutually dependent for localization to assembling centrioles (Dammermann et al., 2004; Delattre et al., 2004; Leidel et al., 2005). To examine the localization of the SAS-5/6 complex, we followed GFP-SAS-6 utilizing a previously established recruitment assay (Pelletier et al., 2006). In this assay, matings between wild-type males and transgenic females carrying a germ line-expressed *gfp-sas-6 gene* allows one to examine the accumulation of GFP-SAS-6 protein around the unlabeled sperm-derived centrioles. Consistent with previous observations, we found that in control embryos, GFP-SAS-6 was initially detected at centrioles during the first S phase (Figure 3C). In contrast, we often failed to detect centriole-associated GFP-SAS-6 in *let-92(RNAi)* embryos (Figure 3D). Of ten one-cell *let-92(RNAi)* embryos that ranged from S phase to mid prophase, only two showed even a faint GFP-SAS-6 signal around one of the two centrioles. We conclude that PP2Ac^{LET-92}-SUR-6 is required for proper localization of the SAS-5/6 complex to sites of centriole assembly.

PP2Ac^{LET-92}-SUR-6 regulates ZYG-1 and SAS-5 levels

We next tested for direct effects of PP2Ac^{LET}-SUR-6 depletion on centriole assembly factors. Using quantitative immunoblotting, we measured the levels of ZYG-1, SAS-5, and SAS-6 proteins in control and PP2Ac^{LET-92}-depleted embryos. Depleting PP2Ac^{LET-92}, SUR-6, or PAA-1 led to reproducible declines in the levels of ZYG-1 and SAS-5 (Figure 4A and B). We also noted that SAS-5 mobility decreased slightly upon depletion of PP2A subunits (Figure 4B). Quantitatively, PP2Ac^{LET-92}-depleted embryos possess 48% ± 25 (n=6) as much ZYG-1, and 40% ± 15 (n=7) as much SAS-5 as control embryos. As evident in Figure 3B, the reduction in ZYG-1 protein level does not completely block the

localization of ZYG-1 to centrosomes. However, we are currently unsure if the centrosome levels of ZYG-1 are slightly reduced upon PP2Ac^{LET-92} depletion. In contrast to ZYG-1 and SAS-5, the level of SAS-6 appears unaffected by depletion of PP2Ac^{LET-92} or SUR-6 (Figure 4C). Thus, a striking parallel exists between our genetic and biochemical analyses; genetically *let-92* interacts with *zyg-1* and *sas-5* but not with *sas-6*, and biochemically, loss of PP2Ac^{LET-92} affects the levels of ZYG-1 and SAS-5 but not of SAS-6.

To test if PP2Ac^{LET-92}-SUR-6 controls the levels of SAS-5 directly, we immunoprecipitated SAS-5 from wild-type embryonic extracts and probed for PP2Ac^{LET-92}. Consistent with a direct interaction between SAS-5 and PP2Ac^{LET-92}, we detected PP2Ac^{LET-92} in the precipitated material (Figure 4D). We also immunoprecipitated SAS-6 from wild-type embryonic extracts but in contrast to the SAS-5 pull-down experiment, we did not detect PP2Ac^{LET-92} among the coprecipitating proteins (Figure 4D). It should be noted that a significant fraction of SAS-5 exists in a cytoplasmic complex with SAS-6 (Leidel et al., 2005). Thus, it was unexpected that we failed to detect an interaction between SAS-6 and PP2Ac^{LET-92}. This may indicate that PP2Ac^{LET-92} interacts predominantly with the pool of SAS-5 not bound to SAS-6.

Our findings that PP2Ac^{LET-92} physically interacts with SAS-5 and controls SAS-5 levels suggests that PP2A promotes the stability of SAS-5, and that in the absence of PP2A, the reduced amount of SAS-5 (as well as ZYG-1) is unable to support centriole duplication. To investigate this, we determined what effect blocking SAS-5 degradation would have on PP2Ac^{LET-92}-depleted embryos. We knocked down both PP2Ac^{LET-92} and RPT-4, an essential component of the 26S proteosome, and assayed centriole behavior. Remarkably, we found 58% of centrioles (n=26) duplicated in such embryos while centrioles never duplicated (n=24) in paired *let-92(RNAi)* controls (Movie S4). Further, knockdown of RPT-4 could also suppress the genetic interaction between *zyg-1* and *let-92*. Specifically, we found (using slightly less stringent conditions than used for the genetic interaction experiments) that 70% of centrosomes (n=10) duplicated in *zyg-1(b1)/+; let-92(s504)/+* double heterozygotes subject to RPT-4 knockdown compared to 10% (n=10) in controls. Further, despite the fact that RPT-4 knockdown reduces the viability wild-type embryos (27% viability), we found that it actually increases the viability of embryos produced by *zyg-1(b1)/+; let-92(s504)/+* double heterozygotes 10-fold from 0.3% (n=292) in controls to 3.0% (n=448) in *rpt-4*-treated animals. Finally, we investigated the effect of RPT-4 depletion on SAS-5 levels and found that partial knockdown of RPT-4 could suppress the reduction of SAS-5 observed in PP2Ac^{LET-92}-depleted embryos (Figure 4E). Therefore in PP2Ac^{LET-92}-depleted embryos, the level of SAS-5 positively correlates with the ability of centrioles to duplicate.

Discussion

While the involvement of kinases in centriole duplication has been established, the potential involvement of phosphatases has not been well explored. Our identification of PP2Ac^{LET-92}-SUR-6 as a positive regulator of duplication indicates that centriole duplication is governed by both activating and inhibitory phosphorylation events. A recent proteomic study in *Drosophila* found the fly PP2A catalytic and scaffolding subunits associated with embryonic centrosomes (Muller et al., 2010). Consistent with our results, depletion of either PP2A subunit in fly or human tissue culture cells reduced centrosome number suggesting a defect in centriole duplication or segregation (Muller et al., 2010). Thus it seems likely that the role of PP2A in centriole duplication is conserved.

We have identified ZYG-1 and SAS-5 as factors that are targeted by PP2Ac^{LET-92}-SUR-6: the levels of these proteins are diminished upon PP2Ac^{LET-92} or SUR-6 depletion. ZYG-1 is

still detectable at centrosomes in PP2Ac^{LET-92}-depleted embryos (Figure 3A, B), although it remains unclear if ZYG-1 levels at the centrosome are reduced relative to the wild type. However, since ZYG-1 and SAS-5 are both required for SAS-6 localization (Delattre et al., 2006; Delattre et al., 2004; Leidel et al., 2005; Pelletier et al., 2006), the reduced levels of ZYG-1 and especially SAS-5 likely contribute to the failure of SAS-6 to localize to nascent centrioles in the absence of PP2Ac^{LET-92}-SUR-6. This is supported by our finding that inhibiting proteolysis in PP2Ac^{LET-92}-depleted embryos restores both the level of SAS-5 and centriole duplication. Despite this, it still remains possible that PP2Ac^{LET-92}-SUR-6 also functions directly (and independent of its role in regulating ZYG-1 and SAS-5 levels) to promote the recruitment of the SAS-5/6 complex.

How might the PP2Ac^{LET-92}-SUR-6 complex regulate ZYG-1 and SAS-5 levels? Our finding that SAS-5 physically associates with PP2Ac^{LET-92} *in vivo* suggests that PP2Ac^{LET-92}-SUR-6 acts to stabilize SAS-5 by dephosphorylation. Regulating protein stability via phosphorylation is common; in vertebrates, Plk4 negatively regulates its own levels by targeting itself for destruction via autophosphorylation (Guderian et al., 2010; Holland et al., 2010; Sillibourne et al., 2010). Autophosphorylation promotes association of Plk4 with βTr-CP, an adapter for the SCF E3 ubiquitin ligase. In flies, inhibition of SCF$^{\beta Tr\text{-}CP}$ leads to elevated levels of Plk4 and centriole amplification (Cunha-Ferreira et al., 2009; Rogers et al., 2009), suggesting that autophosphorylation may function to maintain proper levels of Plk4.

In summary, PP2Ac^{LET-92}-SUR-6 is a core centriole duplication factor and regulates the levels of ZYG-1 and SAS-5. Unlike the other core duplication factors, PP2Ac^{LET-92}-SUR-6 does not appear to function exclusively at centrosomes, as we find that depletion of RSA-1, a regulatory chain that targets PP2Ac^{LET-92} to centrosomes (Schlaitz et al., 2007), does not inhibit centrosome duplication (Figure 2A) and SUR-6 is not enriched at centrosomes (Figure 1K). Thus, PP2Ac^{LET-92}-SUR-6 likely acts within the cytoplasm. Future studies aimed at identifying the specific residues within SAS-5, and possibly ZYG-1, targeted by PP2Ac^{LET-92}-SUR-6 will reveal how inhibitory phosphorylation events contribute to the fidelity of centriole duplication.

Experimental Procedures

Immunoprecipitation and Quantitative Immunoblotting

For whole worm lysates, N2 worms were grown in liquid culture until gravid, harvested by centrifugation, washed several times in lysis buffer (50 mM HEPES, pH7.4, 1 mM EDTA, 1 mM $MgCl_2$, 200 mM KCl, 10% glycerol: (Cheeseman et al., 2004), and gently pelleted. Worm slurries (7ml) were frozen on dry ice and lysed using a One Shot Cell Disrupter (Constant Systems Limited, Northants, UK) set at 40 psi. Crude lysates were centrifuged three times at 382K rpm for 45 min; the resulting high-speed supernatants were used for immunoprecipitation.

For pull-down assays, antibodies to SUR-6, SAS-5, SAS-6 and SZY-20 (Song et al., 2008) were bound to Dynabeads coupled to protein A (Invitrogen) at a ratio of 1μg antibodies to1μl beads. Anti-rabbit-IgG (Invitrogen) served as a negative control. Antibodies were incubated with beads in PBST (1×PBS, 0.1% Triton X-100), rotated overnight at 4°C, and cross-linked to beads according to manufacturer's instructions. The beads were then mixed with 1 ml of the whole worm extract and incubated with rotation overnight at 4°C. The lysate was removed and the beads washed with PBST ten times at room temperature.

For quantitative immunoblotting, embryonic proteins were fractionated on a NuPAGE Bis-Tris Gel (Invitrogen) and transferred to nitrocellulose. α-SZY-20 (Song et al., 2008), α-

SPD-2 (Kemp et al., 2004), α-ZYG-1 (Kemp et al., 2007), α-PP2Ac (Clone 1D6, Millipore), α-actin (Thermo Scientific), and DM1A (Sigma) were used at a 1:500–1500 dilution. IRDye secondary antibodies (LI-COR Biosciences) were used at 1:15,000. Blots were imaged using the Odyssey Infrared Imaging System (LI-COR Biosciences) and analyzed using the same software or ImageJ v1.40G. For quantifying ZYG-1 and SAS-5 levels, values were normalized against tubulin or actin.

RNAi

To feed dsRNA, L4 larvae were placed on a lawn of bacteria that carried a plasmid for inducible expression of *let-92*, *paa-1*, *sur-6*, *sas-5*, or *rpt-4* dsRNA (Source Bioscience plc, Nottingham, UK), and allowed to feed for one day at 25°C. Bacteria carrying the dsRNA-feeding vector L4440 served as a negative control. For dual RNAi, plates were seeded with mixtures of bacteria in specified ratios based on the optical densities of the cultures; the total amount of bacteria added to the plate was kept constant by adding the appropriate amount of L4440 bacteria. To test for suppression of *let-92* by *rpt-4*, we compared the effect of a 1:1 mixture of L4440 and *let-92* bacteria (0% duplication, n=24 events) to a 3:4:1 ratio of L4440, *let-92*, and *rpt-4* bacteria (58% duplication, n=26 events) as they both contained the same fraction of *let-92* bacteria. To test for suppression of *let-92(RNAi)* by *szy-20(bs52)*, we compared N2 and *szy-20(bs52)* animals grown on a 1:1 mixture of L4440 and *let-92* bacteria (strong $PP2Ac^{LET-92}$ knockdown) or on a 3:1 mixture of L4440 and *let-92* bacteria (weak knockdown). To test *zyg-1(b1)/+; let-92(s504)/+* double heterozygotes for suppression by *rpt-4*, we compared double heterozygotes grown on L4440 to those grown on a 7:1 mixture of L4440 and *rpt-4* bacteria.

Cytology

The following antibodies were used at a 1:500–2000 dilution: DM1A (Sigma), α-GFP (Roche), α-SPD-2 (Kemp et al., 2004), α-SPD-5 (Hamill et al., 2002), α-ZYG-1 (O'Connell et al., 2001), α-SAS-4 (Song et al., 2008), and Alexa Fluor 488 and 568 secondary antibodies (Invitrogen). Methods and imaging systems for indirect immunofluorescence and confocal and 4D-DIC microscopy have been described (Peters et al.; Song et al., 2008). Image processing was performed with ImageJ v1.40G and Adobe Photoshop CS5.

The SAS-6 recruitment assay was performed essentially as described (Pelletier et al., 2006). Briefly, wild-type males were mated overnight to OD103 females on RNAi plates at 25°C. The next day embryos were immunostained using the α-SAS-4 antibody to mark centrioles and the anti-GFP antibody to detect GFP-SAS-6.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank members of the O'Connell and Song labs, David Weisblat, Alexander Dammerman, Tony Hyman, Markus Decker, Karen Oegema, Martin Srayko, and Orna Cohen-Fix, for sharing strains and reagents and/or providing advice. Some strains were provided by The *Caenorhabditis* Genetics Center and The National Bioresource Project- Japan. This work was supported by the Intramural Research Program of the National Institutes Health (NIH) and by the National Institute of Diabetes and Digestive and Kidney Diseases.

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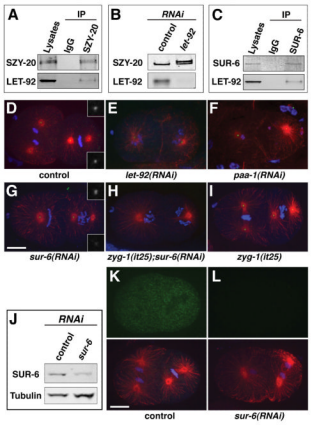

Figure 1. PP2Ac^{LET-92} -SUR-6 regulates centriole duplication

(A) PP2Ac^{LET-92} coprecipitates with SZY-20. (B) Depletion of PP2Ac^{LET-92} results in a decrease in SZY-20 mobility consistent with hyperphosphorylation. (C) PP2Ac^{LET-92} coprecipitates with SUR-6. (D–I) Embryos of indicated genotype stained for microtubules (red), centrioles (SAS-4, green), and DNA (blue). Depletion of SUR-6 in wild-type embryos (G) results in centriole duplication defects such as monopolar spindles (left blastomere) or asymmetric spindles (right blastomere). Insets show symmetrical staining of wild-type spindle poles (D) and asymmetrical staining of *sur-6(RNAi)* spindle poles (G). (H, I) RNAi of *sur-6* enhances the centriole duplication defect of *zyg-1(it25)* embryos raised at permissive temperature. (J) A SUR-6 immunoblot demonstrating the specificity of the α-SUR-6 antibody. RNAi of *sur-6* results in a reduction in the intensity of the SUR-6 band. (K, L) Immunostaining reveals a broad distribution of SUR-6 (green) in the control embryo (K) and a loss of this staining in the *sur-6(RNAi)* embryo (L). Lower panels show the same embryos stained for microtubules (red) and DNA (blue). Bars, 10μm. For supporting data see Figure S1, Table S1, and movies S1 and S2.

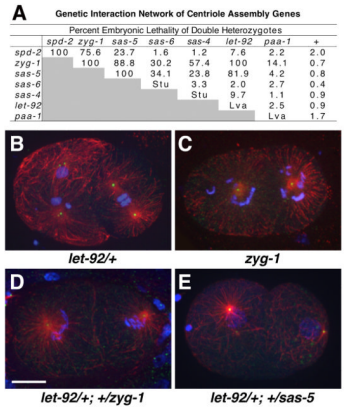

Figure 2. PP2Ac^{LET-92} genetically interacts with other centriole duplication factors

(A) A genetic interaction network of centriole duplication genes. Shown is the percent embryonic lethality of indicated double heterozygotes. Single heterozygotes are listed in last column (+). Some single homozygotes exhibit a sterile uncoordinated (Stu) phenotype and others a larval lethal (Lva) phenotype. Between 345 and 1,521 embryos were counted for each strain. (B–E) Embryos of indicated genotype stained for microtubules (red), centrioles (SAS-4, green), and DNA (blue). Note that *let-92* (B) and *zyg-1* heterozygotes (not shown) assemble bipolar spindles, but *zyg-1* homozygotes (C), and *let-92/+; zyg-1/+* (D) and *let-92/+; +/sas-5* double heterozygotes assemble monopolar spindles. Bar, 10 μm. For supporting data see Movie S3.

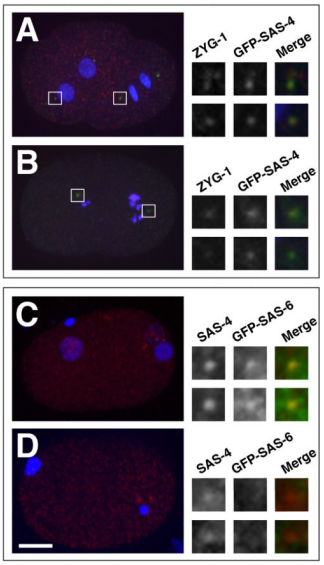

Figure 3. PP2Ac^{LET-92} is required for recruitment of the SAS-5/6 complex

(A) Control and (B) *let-92(RNAi)* embryos stained for ZYG-1 (red), GFP-SAS-4 (green) and DNA (blue). ZYG-1 localizes to centrosomes in PP2Ac^{LET-92} -depleted embryos (insets). (C) Control and (D) *let-92(RNAi)* embryos stained for endogenous SAS-4 (red) GFP-SAS-6 (green-insets only) and DNA (blue). (C) In control embryos, GFP-SAS-6 is recruited to centrioles as evidence by the overlap between the GFP-SAS-6 and SAS-4 signals (insets). (D) In *let-92(RNAi)* embryos, GFP-SAS-6 does not accumulate around the centrioles. Note that control and *let-92(RNAi)* embryos are approximately the same age based on position of DNA and separation of centrioles. The DNA of *let-92(RNAi)* embryos however stays highly condensed.

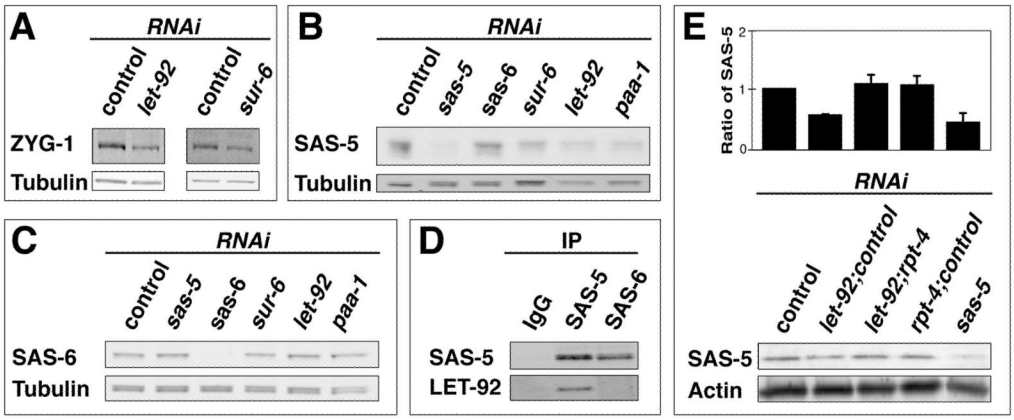

Figure 4. The PP2Ac^{LET-92} -SUR-6 complex regulates the levels of ZYG-1 and SAS-5

(A–C) Quantitative immunoblots of embryonic extracts showing that RNAi of *let-92*, *sur-6*, or *paa-1* reduces the levels of ZYG1 and SAS-5 but not the levels of SAS-6. (D) PP2Ac^{LET-92} coprecipitates with SAS-5, but not with SAS-6. (E) Weak *rpt-4(RNAi)* restores SAS-5 levels in PP2Ac^{LET-92}-depleted embryos. Shown is a graph depicting the average relative levels of SAS-5 with standard deviations from three experiments, and a representative blot. The reduction of SAS-5 levels upon *let-92(RNAi)* is less pronounced in these experiments due to dilution of *let-92* bacteria with control bacteria. Ratios of RNAi bacteria used: *let-92*:control, 1:1; *let-92:rpt-4*:control: 4:1:3; *rpt-4*:control, 1:7. For supporting data see movie S4.