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Regulated HsSAS-6 Levels Ensure Formation of a Single Procentriole per Centriole during the Centrosome Duplication Cycle

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SUMMARY

Centrosome duplication involves the formation of a single procentriole next to each centriole, once per cell cycle. The mechanisms governing procentriole formation and those restricting its occurrence to one event per centriole are poorly understood. Here, we show that HsSAS-6 is necessary for procentriole formation and that it localizes asymmetrically next to the centriole at the onset of procentriole formation. HsSAS-6 levels oscillate during the cell cycle, with the protein being degraded in mitosis and starting to accumulate again at the end of the following G1. Our findings indicate that APC^{Cdh1} targets HsSAS-6 for degradation by the 26S proteasome. Importantly, we demonstrate that increased HsSAS-6 levels promote formation of more than one procentriole per centriole. Therefore, regulated HsSAS-6 levels normally ensure that each centriole seeds the formation of a single procentriole per cell cycle, thus playing a fundamental role in driving the centrosome duplication cycle and ensuring genome integrity.

INTRODUCTION

The centrosome is the main microtubule organizing center of animal cells and comprises a pair of centrioles surrounded by pericentriolar material (Chretien et al., 1997; Kuriyama and Borisy, 1981; Paintrand et al., 1992; Vorobjev and Chentsov, 1982). Proliferating cells are born with a single centrosome, which duplicates once per cell cycle to generate two centrosomes that direct bipolar spindle assembly during mitosis, thus ensuring faithful segregation of the genetic material. Failure of centrosome duplication can result in monopolar spindle assembly; excess centrosome duplication, in multipolar spindle assembly. Therefore, just like DNA replication, centrosome duplication must occur once per cell cycle to ensure genome integrity.

Centrosome duplication begins at the G1 to S transition when a single procentriole forms orthogonal to the proximal end of each centriole (Chretien et al., 1997; Kuriyama and Borisy, 1981; Paintrand et al., 1992; Vorobjev and Chentsov, 1982). Each growing procentriole remains tightly connected to its centriole until the end of mitosis when the two disorient from one another. Each daughter cell thus inherits two centrioles, which are dubbed the mother and the daughter centriole, and which for simplicity are referred to as centrioles in the remainder of this manuscript. The mechanisms that enable procentriole formation from the proximal end of each centriole are not understood. Furthermore, the mechanisms restricting the occurrence of procentriole formation to one event per centriole have remained elusive.

A few proteins have been reported to be required for procentriole formation in human cells, including the EF-hand protein centrin-2 and the Polo-like kinase Plk4 (Habedanck et al., 2005; Salisbury et al., 2002). Proteins of the centrin family localize to the distal lumen of centrioles and procentrioles throughout the cell cycle and are detected in procentrioles already in early S phase (Paoletti et al., 1996; Piel et al., 2000; Salisbury et al., 2002). However, it is not known whether the recruitment of centrin next to the centriole defines where procentriole formation occurs, or whether a distinct protein is recruited earlier to exert such an instructive function. Plk4 also associates with centrioles and procentrioles throughout the cell cycle and may be important for initiating procentriole formation, since overexpression of FLAG-tagged Plk4 results in the formation of several electron-dense patches on each centriole (Habedanck et al., 2005). These patches harbor centrin and presumably elongate further into mature procentrioles (Habedanck et al., 2005).

Another component that may be particularly important is HsSAS-6, a coiled-coil protein that belongs to an evolutionarily conserved family named after its founding

member SAS-6, which is required for centriole formation in *C. elegans* (Dammermann et al., 2004; Leidel et al., 2005). Depletion of HsSAS-6 from U2OS cells results in an increased frequency of monopolar spindles, suggesting that HsSAS-6 is needed for an aspect of the centrosome duplication cycle, although which one in particular is not known (Leidel et al., 2005). Conversely, overexpression of GFP-HsSAS-6 leads to multiple foci bearing centriolar markers and an increased frequency of multipolar spindles (Leidel et al., 2005). Although this could be due to overexpression of a fusion protein, which may have an altered function compared with the endogenous protein, these initial findings taken together suggest that HsSAS-6 may be important for proper progression through the centrosome duplication cycle, a possibility that we investigated in this study.

RESULTS

HsSAS-6 Is Required for Procentriole Formation

To determine the specific step in the centrosome duplication cycle at which HsSAS-6 is required, we depleted the protein from HeLa cells using siRNAs and analyzed the number of centrin foci in mitotic cells as a marker of centriole number. As anticipated, the vast majority (>90%) of cells treated with control siRNAs contained four centrin foci corresponding to two pairs of centriole/procentriole (Figures 1A and 1E). By contrast, 48 hr after transfection, 54% of cells treated with HsSAS-6 siRNAs contained only two separated centrin foci, likely corresponding to the two centrioles; such cells assembled a bipolar spindle (Figures 1B and 1E). The phenotype became more severe 72 hr after transfection, with 53% of cells containing a single centrin focus; over half (66%) of this subset of cells assembled a monopolar spindle (Figures 1C–1E). Analogous phenotypes were observed after depletion of HsSAS-6 in U2OS cells (see Figure S1 in the Supplemental Data available with this article online). We performed serial section electron microscopy (EM) to investigate whether the single centrin focus present in the monopolar configuration corresponds to a single centriole. As shown in Figure 1F, we found this to be the case and did not detect the presence of an associated procentriole or additional electron-dense material in the vicinity. Taken together, our findings demonstrate that HsSAS-6 is required for the step of procentriole formation in the centrosome duplication cycle.

HsSAS-6 Localizes to the Proximal End of the Procentriole

To characterize the distribution of HsSAS-6, we raised and affinity-purified HsSAS-6 antibodies, which labeled one dot at each spindle pole, as well as the cytoplasm to a lesser extent (Figure 2A). These antibodies also recognized a single major band of the expected size of 74 kDa in whole-cell lysates (Figure 2C). The signals observed by immunofluorescence and western blot were significantly diminished in HsSAS-6 siRNA-treated cells (Figures 2B and 2C), indicating specificity. We used these antibodies to characterize the localization of HsSAS-6 using triple-

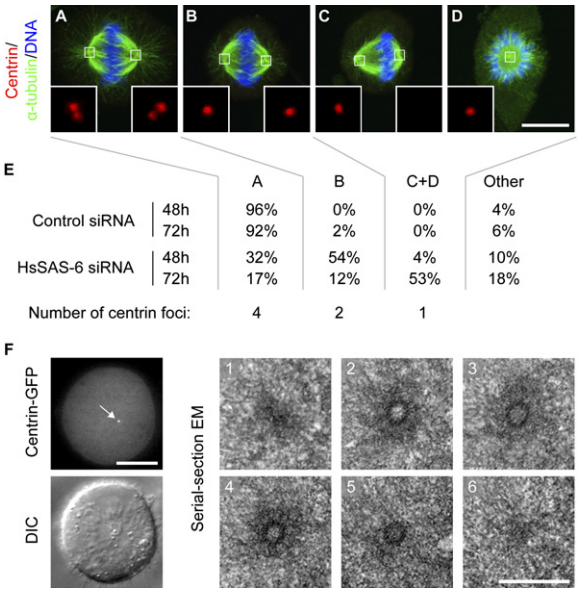

Figure 1. HsSAS-6 Is Required for Procentriole Formation

(A–D) HeLa cells transfected with control siRNAs or siRNAs directed against HsSAS-6, stained with antibodies against centrin-3 (red) and α-tubulin (green); DNA is shown in blue. Insets are magnified 5-fold. Scale bar, 10 μm. Cells were classified into the following categories: two pairs of centrin foci, bipolar spindle (A); two single centrin foci, bipolar spindle (B); one single centrin focus, bipolar spindle (C); one single centrin focus, monopolar spindle (D).

(E) Distribution of cells in the categories described above (n = 50 and n = 100 for 48 hr and 72 hr time points, respectively); Other: cells that could not be assigned to any of the four categories (~5% of cells contained more than four centrin foci, for each experimental condition, and some cells contained a pair of juxtaposed centrin foci in the case of cells targeted with HsSAS-6 siRNAs). Note that monopolar spindle assembly was also observed with an independent siRNA (Leidel et al., 2005).

(F) HeLa cells expressing centrin-GFP were treated with siRNA directed against HsSAS-6 and observed 72 hr after transfection. A cell with a monopolar spindle and single centrin focus (arrow) was first imaged by DIC and fluorescence microscopy (scale bar, 10 μm), then fixed and analyzed by serial section EM (1–6: selected sections spanning the entire centriole; scale bar, 500 nm).

labeling experiments. HeLa cells in the G2 phase of the cell cycle that expressed centrin-GFP (Piel et al., 2000) were labeled with GFP antibodies to mark the distal end of centrioles and procentrioles (Figure 2D, green) and with C-Nap1 antibodies to mark the base of centrioles (Fry et al., 1998) (Figure 2D, blue). This allowed us to estimate the position of centrioles and procentrioles (Figure 2D, gray lines). Strikingly, we found that HsSAS-6 was invariably located between the axis of the centriole and the distal end of the procentriole (Figure 2E, red). We found an analogous distribution in U2OS cells (Figure S2A). By measuring the distances between the signals in triply labeled HeLa cells (Figure 2F), we found that the focus of HsSAS-6 is located ~175 nm away from the axis of the centriole, corresponding to the proximal part of the procentriole (Figure 2G, value C). Importantly,