STIL is required for centriole duplication in human cells

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Accepted 6 December 2011
Journal of Cell Science 125, 1353–1362

doi: 10.1242/jcs.104109

Summary

Centrioles are key structural elements of centrosomes and primary cilia. In mammals, only a few proteins including PLK4, CPAP (CENPJ), SAS6, CEP192, CEP152 and CEP135 have thus far been identified to be required for centriole duplication. STIL (SCL/TAL1 interrupting locus, also known as SIL) is a centrosomal protein that is essential for mouse and zebrafish embryonic development and mutated in primary microcephaly. Here, we show that STIL localizes to the pericentriolar material surrounding parental centrioles. Its overexpression results in excess centriole formation. siRNA-mediated depletion of STIL leads to loss of centrioles and abrogates PLK4-induced centriole overduplication. Additionally, we show that STIL is necessary for SAS6 recruitment to centrioles, suggesting that it is essential for daughter centriole formation, interacts with the centromere protein CPAP and rapidly shuttles between the cytoplasm and centrioles. Consistent with the requirement of centrioles for cilia formation, *Stil*$^{-/-}$ mouse embryonic fibroblasts lack primary cilia – a phenotype that can be reverted by restoration of STIL expression. These findings demonstrate that STIL is an essential component of the centriole replication machinery in mammalian cells.

Key words: STIL, Centriole, Centrosome, Microcephaly, Primary cilia

Introduction

The *STIL* (SCL/TAL1 interrupting locus) gene (also known as SIL) was cloned from a common chromosomal rearrangement associated with T cell acute lymphoblastic leukemia (Aplan et al., 1991). It is ubiquitously expressed in proliferating cells and during embryonic development (Izraeli et al., 1997; Izraeli et al., 1999). Mice lacking a functional STIL protein die at mid gestation with marked growth retardation, defects in the developing neural fold and randomization of left–right asymmetry (Izraeli et al., 1999). Mutations in zebrafish *STIL* cause neuronal cell death and metaphase arrest with disorganized mitotic spindles that often lack one or both centrosomes (Pfaff et al., 2007). Immunostaining of STIL in HeLa cells suggests that it localizes to mitotic spindle poles (Pfaff et al., 2007). Similarly to the situation in zebrafish, mammalian cells without STIL seem to lack centrosomes (Castiel et al., 2011). Recently, several homozygous mutations in *STIL* were identified in autosomal recessive primary microcephaly (Kumar et al., 2009). Interestingly, the other six genes known to be mutated in this disorder – *MCPH1*, *CDK5RAP2*, *ASPM*, *CPAP*, *CEP152* and *WDR62* – also encode proteins that localize to centrosomes or mitotic spindle (Bond et al., 2002; Bond et al., 2005; Guernsey et al., 2010; Jackson et al., 2002; Nicholas et al., 2010; Thornton and Woods, 2009; Yu et al., 2010). It has thus been proposed that primary microcephaly is caused by a reduced number of neurons owing to defective mitoses of fetal neuronal precursor cells (reviewed by Thornton and Woods, 2009).

Centrioles are ninefold symmetrical, cylinder-shaped structures that are found in most animal cells. They template the nine microtubule doublets in cilia and constitute the core of the centrosome, the primary microtubule-organizing center in most eukaryotic cells (reviewed by Bettencourt-Dias and Glover, 2007). Centrosomes consist of a pair of centrioles embedded in pericentriolar material (reviewed by Bornens, 2002; Doxsey, 2001; Lüders and Stearns, 2007). An increase in centrosome number is often associated with cancer and may contribute to tumor progression (reviewed by Krämer et al., 2002; Nigg, 2002). Extra centrosomes can lead to aberrant mitotic spindles and thereby to chromosomal instability, which is characteristic of many cancer types. Centrioles are also required as templates for the growth of cilia, which are conserved structures that have diverse and essential roles in development (reviewed by Davis et al., 2006). It is crucial, therefore, that each daughter cell inherits a single centriole pair after mitosis.

To ensure that centrosome duplication occurs only once per cell cycle, centriole replication is tightly coupled to the cell cycle and is best understood in worms, where five proteins essential for this process – SPD-2, ZYG-1, SAS-5, SAS-6 and SAS-4 – have

been identified (Dammermann et al., 2004; Delattre et al., 2004; Leidel et al., 2005; Leidel and Gönczy, 2003; Kemp et al., 2004; Kirkham et al., 2003; O'Connell et al., 2001; Pelletier et al., 2006). At the initial stage of procentriole assembly, SPD-2 is required for the centriolar localization of the kinase ZYG-1 before recruitment of SAS-5, SAS-6 and later SAS-4 to centrioles (Delattre et al., 2006; Pelletier et al., 2006). SAS-5 and SAS-6 are two coiled-coil proteins essential for the formation and elongation of the centriolar central tube, whereas SAS-4 seems to be required for the assembly of microtubules onto the centriolar periphery (Pelletier et al., 2006). For four of these proteins, homologs have been identified in flies: SAS-6 and SAS-4 (Basto et al., 2006; Peel et al., 2007; Rodrigues-Martins et al., 2007) and ANA-2 and SAK, which are thought to be functional orthologs of SAS-5 and ZYG-1, respectively (Bettencourt-Dias et al., 2005; Stevens et al., 2010a). Whereas SPD-2 is not required for centriole duplication in *Drosophila* (Dix and Raff, 2007; Giansanti et al., 2008), flies have one additional duplication factor, Asterless (Blachon et al., 2009; Dzhindzhev et al., 2010), which has not been identified in worms. Human homologs of SPD-2, ZYG-1, SAS-4 and SAS-6 have been identified as CEP192 (Andersen et al., 2003), PLK4 (Bettencourt-Dias et al., 2005; Habedanck et al., 2005), CPAP (Hung et al., 2000) and SAS6 (Leidel et al., 2005), respectively. Using siRNA and immunoelectron microscopy approaches, a putative centriole assembly pathway with initial activation of PLK4 kinase on the surface of the parental centriole and subsequent recruitment of SAS6, CPAP, CEP135 and γ-tubulin to the procentriole formation site has been proposed for human cells (Kleylein-Sohn et al., 2007). In addition, it has been shown that CEP152, the human ortholog of *Drosophila* Asterless, interacts with and is necessary for centrosomal loading of PLK4 and CPAP and thereby is required for centriole duplication (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010; Hatch et al., 2010).

Here, we identify STIL, a protein recently shown to be mutated in primary microcephaly, as an additional essential component of the mammalian centrosome replication machinery.

Results

STIL localizes to centrosomes from late G1 phase to metaphase

Based on weak sequence similarity, it has recently been suggested that STIL might be the human ortholog of *C. elegans* SAS-5 and *Drosophila* ANA-2 (Stevens et al., 2010a). To confirm that STIL is a centrosomal component, we raised a mouse monoclonal antibody against the protein and generated U2OS cells stably expressing GFP–STIL in a tetracycline-dependent manner. In immunoblots, the antibody recognized endogenous STIL (supplementary material Fig. S1A) and after immunofluorescence staining specifically labeled centrosomes predominantly during mitosis with 89.0±1.7% of mitotic cells being positive (Fig. 1A). Whereas virtually all prophase and metaphase cells stained positive, the centrosomal STIL decoration was already diminished in anaphase (supplementary material Fig. S1B). Because faint centrosomal staining was also seen during interphase (supplementary material Fig. S1C), the predominant mitotic antibody staining pattern is best explained by STIL expression mainly during G2 phase and mitosis (Campaner et al., 2005; Izraeli et al., 1997; Izraeli et al., 1999) (supplementary material Fig. S1D). A centrosomal GFP–STIL signal was detectable in 68.3±4.9% of exponentially growing interphase cells 48 hours after induction. This percentage was remarkably similar to the number of interphase U2OS cells depicting a centrosomal SAS6 signal after staining with a SAS6-specific antibody (69.0±9.9%). Whereas GFP–STIL was virtually absent from centrosomes in early G1 phase, levels gradually increased during later G1 through S to G2 phase centrosomes, similarly to results described for human SAS6 (Kleylein-Sohn et al.,

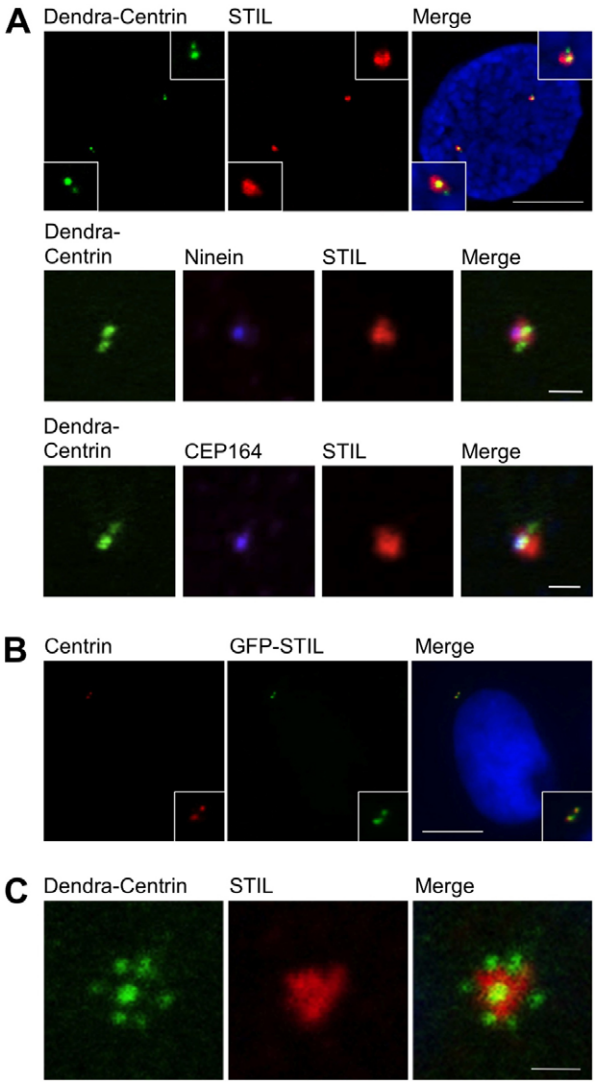

Fig. 1. STIL localizes to centrioles. (**A**) HeLa cells constitutively expressing Dendra–centrin-2 (green) were immunostained with a mouse anti-STIL antibody (red) and analyzed by immunofluorescence microscopy. DNA is stained with DAPI (blue). Centrioles are shown enlarged in insets. Scale bars: 10 µm (top panel). Middle and lower panels show HeLa cells constitutively expressing Dendra-centrin-2 (green) co-immunostained with antibodies against STIL (red) and ninein (blue, middle panel) or CEP164 (blue, lower panel). Scale bars: 1 µm. (**B**) U2OS cells conditionally expressing GFP–STIL (green) immunostained with a mouse anti-centrin antibody (red) and analyzed by immunofluorescence microscopy. DNA is stained with DAPI (blue). Centrioles are shown enlarged in insets. Scale bar: 10 µm. (**C**) Assembly of multiple procentrioles is triggered by induction of PLK4 for 11 hours in HeLa cells constitutively expressing Dendra–centrin-2 (green). After fixation, cells were stained with a mouse anti-STIL antibody (red) and analyzed by immunofluorescence microscopy. Multiple procentrioles are arranged in a typical flower-like structure around the parental centriole. Scale bar: 1 µm.

2007; Strnad et al., 2007) (Fig. 1B and supplementary material Fig. S2A). Accordingly, 2 hours after release from a nocodazole arrest, only 2.5±2.1% of U2OS cells conditionally expressing GFP–STIL harbored GFP–STIL-labeled centrosomes. This number increased to 19.7±4.0% at 4 hours, 37.0±5.7% at 6 hours, 46.3±2.1% at 12 hours and 74.0±1.4% at 16 hours after nocodazole release. Disturbed GFP conformation by mitotic phosphorylation of STIL might be the reason for the absence of a centrosomal GFP–STIL signal during mitosis (Campaner et al., 2005). This idea is corroborated by the finding that immunostaining of U2OS cells expressing GFP–STIL with an antibody against GFP led to restoration of the GFP–STIL signal during metaphase (supplementary material Fig. S2B).

Immunostaining of STIL in mitotic cells expressing Dendra–centrin-2 revealed accumulation of STIL around the parental centriole (Fig. 1A). Also, in HeLa cells conditionally overexpressing PLK4 (Cizmecioglu et al., 2010) and constitutively expressing Dendra–centrin-2, STIL accumulated around the parental centriole within the flower-like structures induced by PLK4 overexpression, similarly to localization described for PLK4, CEP135, γ-tubulin and SAS6 (Kleylein-Sohn et al., 2007) (Fig. 1C). In addition, accumulation of STIL around the parental centriole was further proven by co-immunostaining with CEP164 and ninein as markers for mature centrioles (Graser et al., 2007; Mogensen et al., 2000) (Fig. 1A). Localization of STIL within the pericentriolar material surrounding the centriolar surface was confirmed by immunoelectron microscopy (supplementary material Fig. S3). Together, these data confirm that STIL is a centrosomal protein.

A fraction of STIL rapidly shuttles between centrosomes and cytoplasm

For *C. elegans*, rapid continuous shuttling of SAS-5 between the cytoplasm and centrosomes has been demonstrated (Delattre et al., 2004). To determine the dynamics of STIL recruitment to centrosomes, fluorescence recovery after photobleaching (FRAP) experiments were conducted in U2OS cells conditionally expressing GFP–STIL. First, in exponentially growing cells harboring two GFP–STIL-labeled centrosomes, one centrosome was photobleached at different cell cycle positions as judged by the distance between centrosomes (2.0–19.6 mm), and subsequent fluorescence recovery was assayed using four-dimensional confocal microscopy. Rapid recovery of about 50% of the pre-bleach fluorescence, which was independent of the distance between centrosomes was observed, leading to the conclusion that a fraction of STIL continuously shuttles between the cytoplasm and centrosomes, in a similar manner to the movement of SAS-5 in *C. elegans* (Fig. 2A,B and supplementary material Movie 1). To address whether GFP–STIL shuttling is cell cycle dependent, we next performed FRAP experiments in U2OS cells synchronized in G1, S or G2 phase by release from nocodazole arrest and easily identifiable by the presence of only one faint, two adjacent or already slightly separated GFP–STIL-labeled centrosomes. Independently of the cell cycle position, rapid recovery of about 50–70% of the pre-bleach fluorescence was observed (supplementary material Fig. S4). In addition, we noticed a difference between GFP–STIL fluorescence recovery rates during G1 phase on the one hand and S and G2 phases on the other hand (Fig. 2C).

To demonstrate that the GFP–STIL reporter construct used in the FRAP experiments is functional, mouse embryonic fibroblasts (MEFs) derived from *Stil*$^{-/-}$ embryos, which lack centrosomes (Castiel et al., 2011) were transiently transfected with a GFP–STIL expression plasmid. At 72 hours after

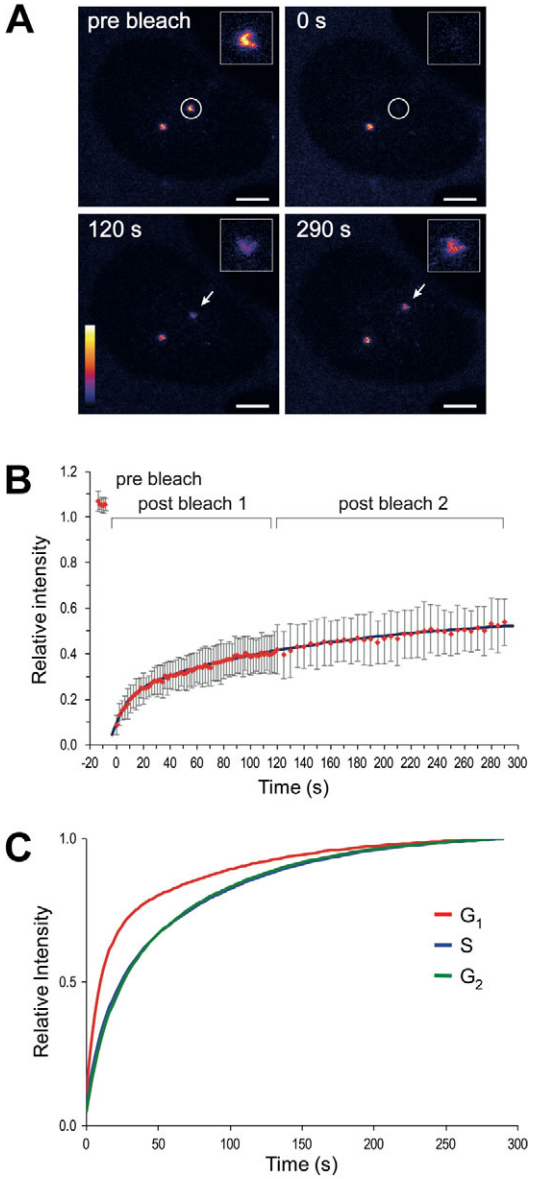

Fig. 2. STIL shuttles between centrioles and cytoplasm. FRAP analysis of GFP–STIL. (**A**) Confocal sections from a time-lapse experiment of U2OS cells conditionally expressing GFP–STIL 24 hours after induction of the transgene before photobleaching (top left), immediately after photobleaching (top right) and at two later time points (bottom). Scale bars: 5 µm. Elapsed time is shown in seconds with 0 representing the start of fluorescence recovery. The white circle indicates the targeted area. The inset shows a magnification of the centrosome region. (**B**) Quantification of fluorescence recovery of GFP–STIL at bleached centrosomes. Normalized relative pixel intensities were fitted by a double exponential equation and plotted over recovery time. Values represent mean ± s.d. from six independent experiments. (**C**) Overlay of fluorescence recovery curves of GFP–STIL at bleached centrosomes of U2OS cells conditionally expressing GFP–STIL 24 hours after induction of the transgene and 8 hours (G1 phase, red), 14 hours (S phase, blue) and 21 hours (G2 phase, green) after release from nocodazole arrest.

transfection, 44.3±16.7% of the *Stil*$^{-/-}$ MEFs but only 2.0±3.5% of untransfected *Stil*$^{-/-}$ MEFs harbored centrosomes, thereby proving the functionality of the construct used (supplementary material Fig. S5). To determine whether the centrosomal localization of STIL is microtubule-dependent, both GFP–STIL signals and anti-STIL antibody staining of endogenous STIL at centrosomes were examined after microtubule depolymerization by nocodazole or cold-treatment (supplementary material Fig. S6). Similar to what has been described for endogenous CPAP (Hung et al., 2000), the association of both GFP–STIL and endogenous STIL with centrosomes was found to be independent of the presence of microtubules in both experimental set-ups.

STIL acts upstream of SAS6 and interacts with CPAP

SAS-5 and SAS-6 in *C. elegans* and ANA-2 and SAS-6 in *D. melanogaster* interact with each other, which led to the suggestion that SAS-5 serves as a carrier that brings SAS-6 to centrioles (Leidel et al., 2005). In U2OS cells, we were unable to detect an unambiguous interaction between STIL and SAS6 by co-immunoprecipitation (data not shown). However, siRNA-mediated depletion of STIL strongly diminished the centrosomal levels of SAS6 (Fig. 3A,B and supplementary material Fig. S7). As a control for the specificity of this effect, the centrosomal levels of centrin, γ-tubulin, and pericentrin were not reduced after STIL depletion with 98.0±1.0%, 99.0±1.0% and 99.0±1.0% of the cells harboring centrosomal signals for centrin, γ-tubulin, and pericentrin, respectively, 48 hours after transfection with a STIL-specific siRNA. By contrast, siRNA-mediated depletion of SAS6 using a published siRNA sequence (Leidel et al., 2005) had no impact on the centrosomal localization of STIL (data not shown). We conclude that, in terms of recruitment to centrosomes, STIL acts upstream from SAS6.

In contrast to SAS6, STIL clearly interacts with CPAP (Fig. 3C), thereby further demonstrating its association with the centriole duplication machinery. To determine whether this interaction depends on the coiled-coil or STAN (STIL/ANA-2) domain (Stevens et al., 2010a) of STIL, a ~90 amino acid region partly conserved between human STIL and *Drosophila* ANA-2, or is affected by microcephaly mutations, co-immunoprecipitation experiments were performed using different STIL deletion mutants (Fig. 3D). As shown in Fig. 3E, the N-terminal half of STIL was sufficient for interaction with CPAP, supporting the conclusion that neither the coiled-coil nor STAN domain are necessary for this interaction, which, in addition, is also not impaired by microcephaly-specific STIL mutations.

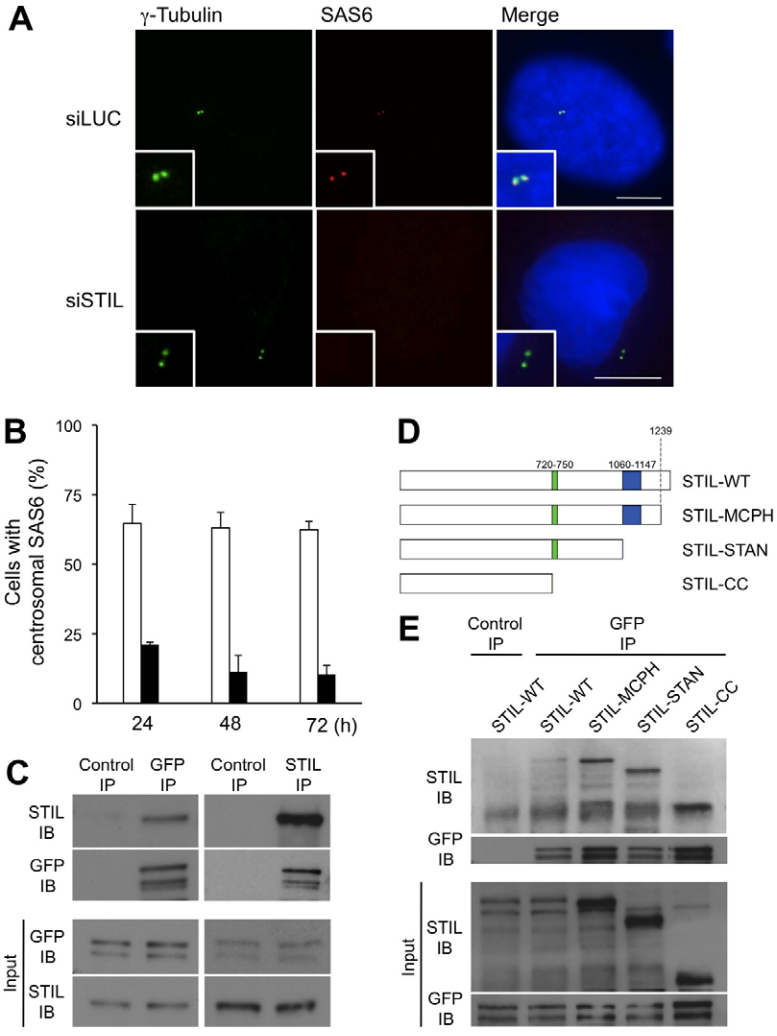

Fig. 3. STIL interacts with CPAP and is necessary for SAS6 recruitment to centrioles. (**A**) U2OS cells were transfected with control (siLUC) or STIL-specific (siSTIL) siRNA for 48 hours. After fixation, cells were stained with antibodies against γ-tubulin (green) and SAS6 (red). DNA is stained with DAPI (blue). Scale bars: 10 μm. (**B**) Mean percentages of cells with centrosomal SAS6 labeling 24, 48 and 72 hours after transfection with siLUC (white columns) or siSTIL (black columns). 100 cells for each time point were counted. Error bars represent the s.d. after combining the results of three experiments. (**C**) Transiently expressed CPAP–GFP and Strep–STIL interact with each other in vivo. CPAP–GFP was immunoprecipitated (IP, top) from HEK293T cells 48 hours after transient transfection with CPAP–GFP and Strep–STIL using an anti-GFP antibody (left). Reciprocally, STIL was immunoprecipitated using an anti-STIL antibody (right). Immunoprecipitation with an irrelevant IgG antibody served as a negative control (Control IP). The bottom panel depicts a corresponding immunoblot (IB) using antibodies against GFP and STIL of the lysates used for immunoprecipitation. (**D**) Schematic of the full-length STIL protein (STIL-WT) and C-terminal deletion constructs used. The blue region depicts the conserved STAN motif and the green region a central coiled-coil domain. Amino acid numbers defining the respective domains as well as the microcephaly mutation are given. (**E**) Immunoprecipitation experiment from HEK293T cells overexpressing the denoted STIL constructs and CPAP–GFP. CPAP–GFP was immunoprecipitated (IP, top panel) 48 hours after transient transfection with CPAP–GFP and the respective Strep–STIL constructs using an anti-GFP antibody. Immunoprecipitation with an irrelevant IgG antibody served as a negative control (Control IP). Immunoprecipitates were probed with antibodies against STIL and GFP. The bottom panel depicts a corresponding immunoblot (IB) using antibodies against GFP and STIL of the lysates used for immunoprecipitation.

Overexpression of STIL increases centrosome number

Overexpression of GFP–STIL in U2OS cells resulted in a striking multiplication of GFP–STIL-positive dots which also stained positive for centrin, γ-tubulin and SAS6 (Fig. 4A,B and supplementary material Fig. S8), indicating that they constitute centrioles or centriole precursors, similarly to what is seen after overexpression of PLK4 or SAS6 (Habedanck et al., 2005; Kleylein-Sohn et al., 2007; Leidel et al., 2005). Confirming this conclusion, an electron microscopy analysis of U2OS cells that were induced to express GFP–STIL revealed multiple structures that resembled canonical centriole cylinders (Fig. 4C). To distinguish between bona fide centriole overduplication during S phase and cell division failure as cause of supernumerary centrioles, U2OS cells conditionally expressing GFP–STIL were analyzed by fluorescence-activated cell sorting (FACS). When compared with controls, the cell cycle distribution of GFP–STIL-expressing cells was similar, without evidence of polyploidization (supplementary material Fig. S9), thereby excluding a contribution of cell division failure or a block in cell cycle progression to the centriole amplification phenotype. Also, expression of GFP–STIL did not cause multinucleation (1.7±1.2% versus 0.7±0.6% multinucleated cells without and

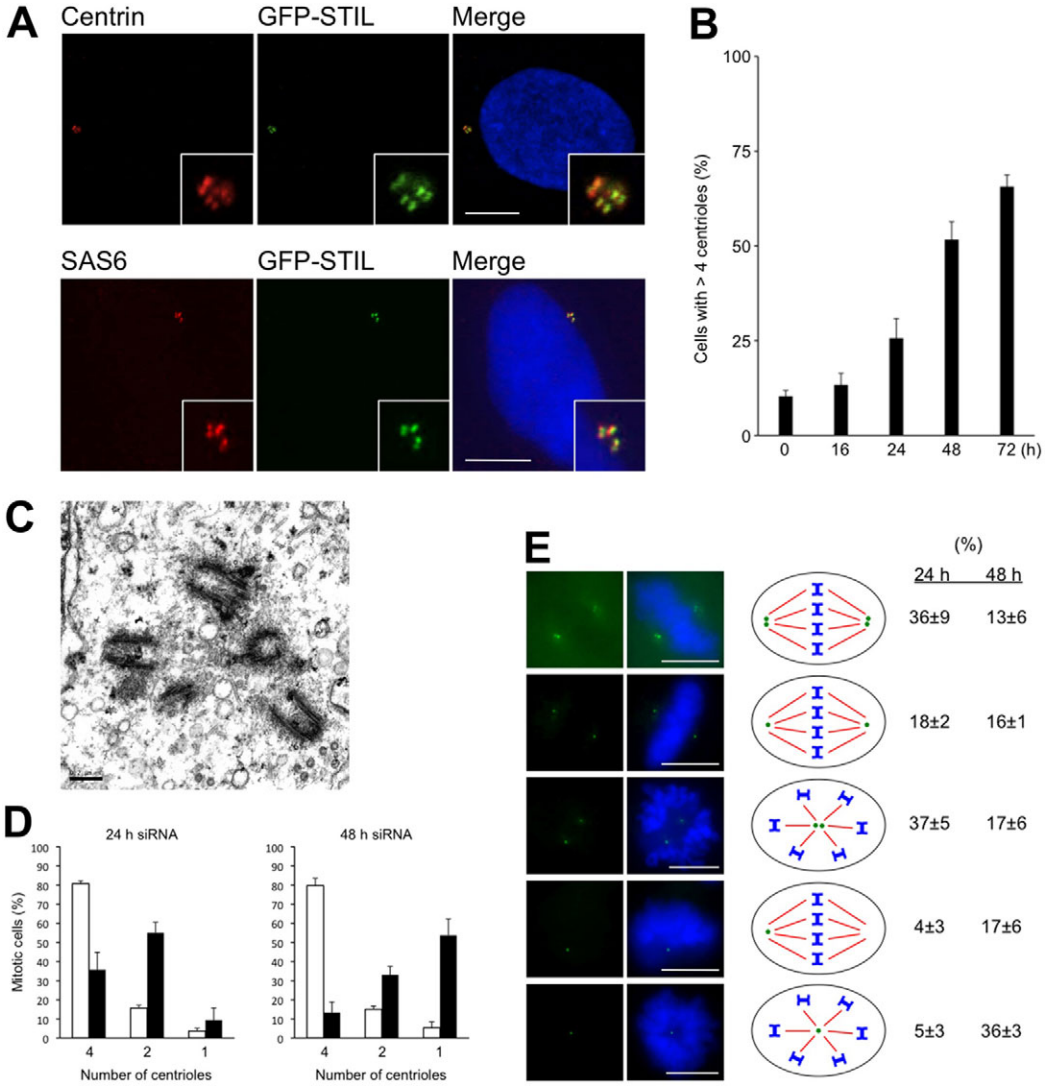

Fig. 4. STIL is required for centriole duplication. Overexpression of STIL increases centriole number. (**A**) Representative example of a U2OS cell conditionally expressing GFP–STIL (green) for 72 hours. After fixation, cells were immunostained with mouse anti-centrin (top panel) or mouse anti-SAS6 (bottom panel) antibodies (red) and analyzed by immunofluorescence microscopy. DNA is stained with DAPI (blue). Centrioles are shown enlarged in insets. Scale bar: 10 μm. (**B**) Mean percentages of cells containing more than four centrioles as judged by anti-centrin immunostaining at the given time points after induction of GFP–STIL expression. 100 cells for each time point were counted. Error bars represent the s.d. after combining the results of three experiments. (**C**) GFP–STIL expression was induced in U2OS cells for 72 hours before cells were processed for electron microscopy. (**D**,**E**) Depletion of STIL causes progressive reduction in centriole numbers. HeLa cells constitutively expressing Dendra–centrin-2 were transfected with control (LUC; white columns) or STIL-specific siRNAs (black columns) for 24 or 48 hours. (**D**) Histograms showing the percentages of mitotic cells containing one, two or four centrioles at 24 and 48 hours, counting 100 mitotic cells for each condition in three independent experiments. (**E**) Left panels show representative examples (Dendra–centrin-2, green; DAPI, blue) and models (centrioles, green; microtubules, red; chromosomes, blue) of HeLa cell phenotypes after siRNA-mediated STIL depletion. The numbers on the right indicate the frequency of each phenotype (mean ± s.d.) after 24 and 48 hours. Scale bars: 10 μm.

48 hours after induction of GFP–STIL expression, respectively). Taken together, these data demonstrate that excess STIL results in centriole overduplication.

Depletion of STIL causes progressive reduction in centrosome numbers and loss of primary cilia

To corroborate a role for endogenous STIL in centrosome duplication, loss-of-function experiments were performed. Exponentially growing U2OS cells were transfected with a STIL-specific siRNA. After 48 hours, 43.0% of mitotic cells harbored a monopolar spindle. Transfection with shRNA specific for STIL into HeLa cells led to similar results (data not shown). Counting centriole numbers 24 and 48 hours after siRNA-mediated STIL knockdown revealed, in contrast to HeLa cells treated with control siRNA, progressive aberrations in mitotic spindle morphology and a step-wise reduction in centriole numbers, which was similar to results observed after depletion of PLK4, SAS4, SAS5, SAS6 and CPAP (Fig. 4D,E) (Delattre et al., 2005; Habedanck et al., 2005; Leidel et al., 2005; Leidel and Gönczy, 2003; Tang et al., 2009).

Next, because STIL is required for mouse embryonic left–right specification (Izraeli et al., 1999), a developmental step closely associated with primary cilia function (Nonaka et al., 1998), and centrioles are necessary for cilia formation, we speculated that MEFs derived from *Stil*$^{-/-}$ embryos, which lack centrosomes (Castiel et al., 2011; Izraeli et al., 1999), might lack primary cilia. Indeed, 72 hours after serum starvation, primary cilia were detectable in 28.0±7.2% of wild-type MEFs, whereas cilia were absent (0.0±0.0%) from *Stil*$^{-/-}$ MEFs (Fig. 5A,B). Restoration of STIL expression in *Stil*$^{-/-}$ MEFs by retroviral transduction led to the reappearance of primary cilia in 25.2±11.6% of the cells. We conclude that STIL, in addition to centriole duplication, is required for primary cilia formation.

To determine whether PLK4-induced centriole overduplication requires the presence of STIL, HeLa cells conditionally overexpressing PLK4 (Cizmecioglu et al., 2010) and constitutively expressing Dendra–centrin-2 were transfected with a STIL-specific siRNA 24 hours before induction of PLK4 expression. Whereas 67.0±7.6% of cells harbored more than four centrioles 48 hours after control siRNA transfection, a STIL-specific siRNA reduced this number to 8.3±3.5% (Fig. 6A,B). To prove siRNA specificity, HeLa cells conditionally expressing PLK4 were co-transfected with siRNA targeting endogenous STIL and an expression plasmid encoding an siRNA-resistant version of GFP–STIL. Expression of siRNA-resistant GFP–STIL was able to rescue PLK4-induced centrosome overduplication in cells depleted from endogenous STIL (supplementary material Fig. S10A), thereby showing that the effect we observed is specific. We conclude that overexpression of PLK4 cannot cause centriole amplification in the absence of STIL. Similar results were obtained after siRNA-mediated depletion of SAS6 (supplementary material Fig. S10B), thereby confirming the functionality of the assay used and results by others (Hatch et al., 2010).

The STAN domain of STIL is necessary for centrosomal localization and replication

Recently, several homozygous mutations in *STIL*, which were predicted to cause C-terminal truncation of the protein, were identified in autosomal recessive primary microcephaly (Kumar et al., 2009). To test whether these *STIL* mutations abrogate its centrosomal localization or its ability to support centriole duplication, one of the homozygous nonsense mutations found in three individuals with primary microcephaly (Kumar et al., 2009), c.3715C>T/p.Gln1239X, which results in the introduction of a premature stop codon (1–1239 aa), was introduced into our GFP–STIL expression plasmid. In addition, STIL deletion mutants truncating the protein in front of the STAN motif (1–1060 aa) or the coiled-coil domain (1–720 aa) were examined (Fig. 3D). Only full-length STIL and the microcephaly mutant

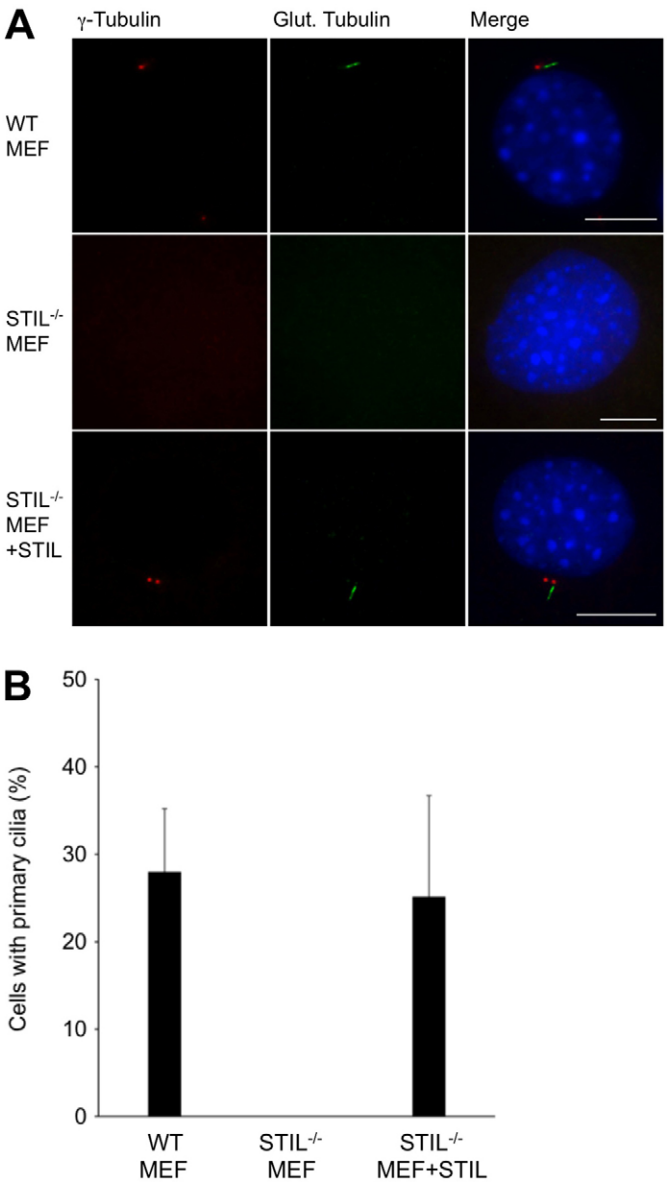

Fig. 5. STIL is required for primary cilia formation. (**A**) Mouse embryonic fibroblasts from wild-type (WT MEF) or *Stil*$^{-/-}$ embryos (SIL$^{-/-}$ MEF) as well as *Stil*$^{-/-}$ MEFs with restored STIL expression (SIL$^{-/-}$ MEF + SIL) were serum starved for 72 hours. After fixation, cells were stained with rabbit anti-γ-tubulin antibodies (red), mouse anti-polyglutamylated-γ-tubulin antibodies (green) and DAPI (blue). Representative examples of the respective cells are depicted in A. Scale bars: 10 μm. (**B**) Mean percentages of cells with primary cilia. Error bars represent s.d. after combining the results of three experiments. 100 cells were counted per experiment and cell type.

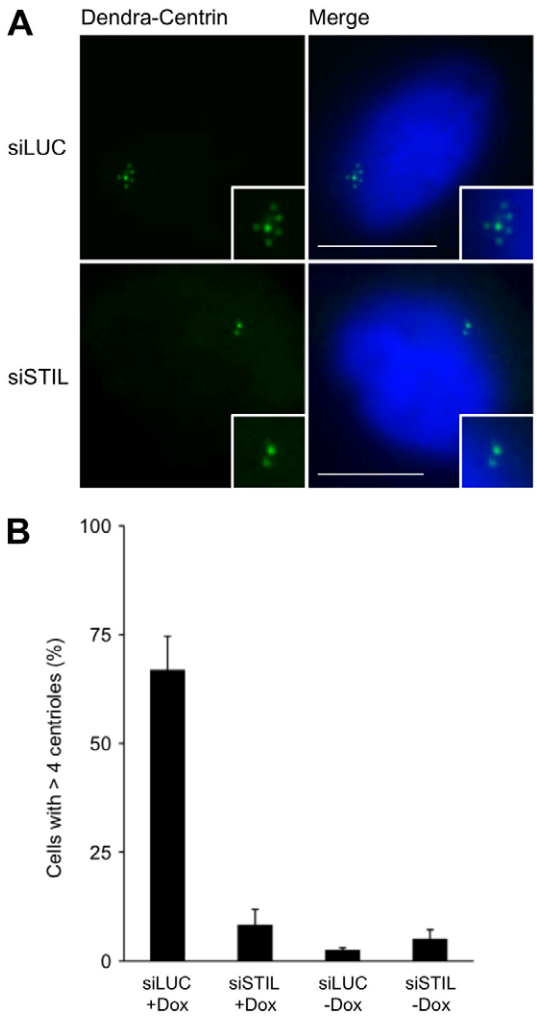

Fig. 6. STIL is required for PLK4-induced centriole amplification. (**A**) HeLa cells conditionally expressing PLK4 and constitutively expressing Dendra–centrin-2 were transfected with control (siLUC) or STIL-specific (siSTIL) siRNA 24 hours before induction of PLK4 expression. Representative examples of the respective cells are depicted in A. Scale bars: 10 µm. (**B**) Mean percentages of cells containing more than four centrioles at 48 hours after transfection, counting 100 cells for each condition. Error bars represent the s.d. after combining the results of three experiments.

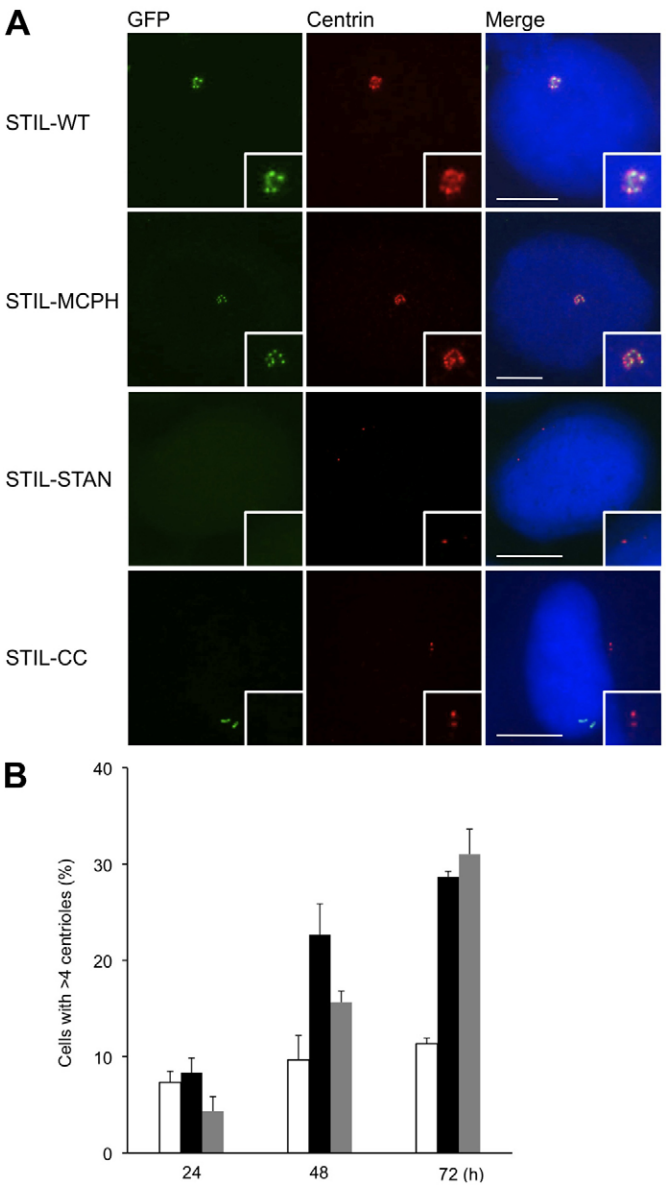

Fig. 7. STIL mutation associated with primary microcephaly does not impair its centrosomal localization or the ability of STIL to support centriole duplication. (**A**) U2OS cells transiently expressing GFP-tagged wild-type STIL (STIL-WT), microcephaly-mutated STIL (STIL-MCPH) as well as STIL deletion mutants truncating the protein before the STAN motif (STIL-STAN) or the coiled-coil domain (STIL-CC) were subjected to co-immunofluorescence with centrin (red) 48 hours after transfection. DNA is stained with DAPI (blue). Scale bars: 10 µm. (**B**) Mean percentages of cells containing more than four centrioles 24, 48 and 72 hours after transfection with empty vector (white columns), wild-type (black columns) or mutated (3715C>T) STIL (gray columns). 100 cells for each time point were counted. Error bars represent the s.d. after combining the results of three experiments.

consistently localized to centrosomes and caused centrosome overduplication after transient transfection in U2OS cells (Fig. 7A,B), supporting the conclusion that, although the microcephaly mutation analyzed here has no impact on centrosomal localization and replication, the STAN domain is necessary for these functions.

Discussion

Centriole formation is best understood in *C. elegans* where only very few proteins seem to be required for the process (Dammermann et al., 2004; Delattre et al., 2004; Leidel et al., 2005; Leidel and Gönczy, 2003; Kemp et al., 2004; Kirkham et al., 2003; O'Connell et al., 2001; Pelletier et al., 2006). In mammalian cells, orthologs have been described for most but not all of these proteins (Andersen et al., 2003; Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Hung et al., 2000; Leidel et al., 2005). Using both gain- and loss-of-function experiments, we show here that STIL, in addition to PLK4, CPAP, SAS6, CEP192, CEP152 and CEP135, is required for the reproduction of centrosomes and primary cilia formation in mammalian cells.

STIL starts to accumulate in the pericentriolar material surrounding the mother centriole around mid G1 phase. Whereas depletion of the protein causes a progressive loss in

centrosome number, overexpression results in excess centriole formation. Also, we demonstrate that STIL is necessary for PLK4-induced centriole duplication. Because STIL is required for mouse embryonic left–right specification (Izraeli et al., 1999), a developmental step that is closely associated with primary cilia function (Nonaka et al., 1998), and centrioles are necessary for cilia formation, we show that mouse embryonic fibroblasts derived from *Stil*$^{-/-}$ embryos lack primary cilia, which could be restored by exogenous STIL expression in *Stil*$^{-/-}$ MEFs by retroviral transduction.

Recently, based on weak sequence similarities, it was proposed that STIL constitutes the mammalian ortholog of *C. elegans* and *D. melanogaster* centriole replication proteins SAS-5/ANA-2 (Stevens et al., 2010a). In *C. elegans*, SAS-5 and SAS-6 physically interact and are dependent on one another for their presence at centrioles (Leidel et al., 2005). Moreover, SAS-5 shuttles between the cytoplasm and centrosomes (Delattre et al., 2004), suggesting that SAS-5 chaperones SAS-6 to the emerging centriole, where SAS-6 contributes directly to centriole architecture (Gopalakrishnan et al., 2010; Kitagawa et al., 2011; Peel et al., 2007; Rodrigues-Martins et al., 2007; Stevens et al., 2010a; Stevens et al., 2010b; van Breugel et al., 2011). The SAS-5/SAS-6 complex in turn is needed for SAS-4 loading and subsequent microtubule addition. ANA-2, the *D. melanogaster* ortholog of SAS-5, similarly interacts with SAS-6 (Stevens et al., 2010a). By contrast, in human cells, STIL seems not to interact with SAS6, although it is required for the recruitment of SAS6 to centrosomes. In fact, we found that SAS6, which is transiently recruited to nascent procentrioles but subsequently degraded (Kleylein-Sohn et al., 2007; Strnad et al., 2007), decorates each of the amplified centrioles after STIL overexpression.

Although not described for SAS-5 or ANA-2, we present evidence for an interaction between STIL and CPAP, the mammalian ortholog of SAS-4. CPAP is required for PLK4-induced centriole amplification and induces the assembly of centriolar microtubules after the recruitment of SAS6 to the proximal end of the procentriole (Tang et al., 2009). In flies, it has recently been shown that cytoplasmic SAS-4 provides a scaffold for pre-assembled cytoplasmic centrosome protein complexes before tethering of the complexes to centrosomes (Gopalakrishnan et al., 2011). Because a large fraction of STIL, similarly to *C. elegans* SAS-5, rapidly shuttles between the cytoplasm and centrioles, it will be interesting to see whether STIL is part of such complexes and might serve to shuttle them from the cytoplasm to centrosomes. Interestingly, the exchange rate of STIL at G1 phase centrosomes is much faster than it is in S and G2 phases, suggesting that most extensive shuttling occurs at the time of procentriole formation. In conjunction with our results, it might be concluded that STIL, which interacts with and probably recruits CPAP to centrosomes, enables the assembly of sufficiently complete centriole cylinders to provide novel docking sides for SAS6 to allow for another round of procentriole formation.

Primary microcephaly is a neurodevelopmental disorder characterized by significantly reduced brain size. Patients show hypoplasia of the cerebral cortex with a generalized reduction in the size of the entire brain (reviewed by Thornton and Woods, 2009; Woods et al., 2005). All genes found to be mutated in this disorder to date – *MCPH1*, *CDK5RAP2*, *ASPM*, *CPAP*, *CEP152*, *STIL* and *WDR62* – encode proteins that localize to the centrosomes (reviewed by Bettencourt-Dias et al., 2011). Additionally, mutations in *CEP152*, *CPAP* and *PCNT* (pericentrin) have been described in certain forms of primordial dwarfism, including Seckel syndrome and microcephalic osteodysplastic primordial dwarfism type II, which are characterized by microcephaly and global growth failure (Al-Dosari et al., 2010; Griffith et al., 2008; Kalay et al., 2011; Rauch et al., 2008). Our data presented here suggest that microcephaly-specific mutation of STIL does not interfere with centrosomal localization of the mutated protein nor did it affect the ability of STIL to support centrosome replication and CPAP interaction. Deletions upstream of the region mutated in primary microcephaly abrogate both centrosomal localization of STIL as well as centrosome replication; it might therefore be speculated that such mutations are not observed in nature because, unlike more C-terminal deletions, they are incompatible with life as in *Stil*$^{-/-}$ mice.

Many of the proteins whose genes are mutated in microcephalic disorders have recently been shown to be part of a cytoplasmic multi-protein complex in flies that is organized by SAS-4 and seems to shuttle its constituents to centrosomes where they are involved in both centriole formation and pericentriolar matrix assembly (Gopalakrishnan et al., 2011). It is interesting to speculate that, analogous to other groups of disorders, assembly or function of this complex might be compromised by mutation of any one of its components, thereby leading to development of microcephaly.

Centrosome amplification induced by overexpression of SAK, the *Drosophila* ortholog of PLK4, can induce the formation of metastatic tumors derived from larval brain cells in flies (Basto et al., 2008). Deregulated centrosome replication resulting in supernumerary centrosomes is often observed in many tumor types (Krämer et al., 2002; Nigg, 2002). STIL is overexpressed in multiple cancers and its overexpression is associated with a metastatic phenotype and poor prognosis (Erez et al., 2004; Ramaswamy et al., 2003). In addition, knockdown of STIL in cancer cells delays entry into mitosis and induced apoptosis in several cancer cell lines (Erez et al., 2007). From these data, it might be speculated that overexpression of STIL in mammalian cells contributes to malignant transformation through the formation of supernumerary centrosomes, as observed for SAK in flies.

Taken together, the data presented here demonstrate that STIL is required for PLK4-induced centriole duplication and suggest that STIL might represent the human ortholog of *C. elegans* SAS-5 and *D. melanogaster* ANA-2.

Materials and Methods

Generation of a STIL-specific monoclonal antibody

A bacterially expressed and affinity-purified His-tagged STIL fragment (STIL-500_650aa.HIS) was used to immunize mice following a modified standard immunization protocol (Köhler and Milstein, 1975). Fusions resulted in the generation of two specific monoclonal antibodies that were subsequently subcloned. Specificity of the antibody that was eventually used for further experiments (clone #29) and which was typed as mouse IgG, was verified by using bacterially expressed recombinant protein and siRNA-treated U2OS cells for western blotting and immunofluorescence staining.

Plasmids

CETN2 (centrin-2) cDNA (accession number NM_004344.1) was cloned into pDendra2-C (Clontech, Palo Alto, CA) containing an N-terminal Dendra2 tag. For GFP–STIL and Strep–STIL expression, *STIL* (accession number NM_001048166.1) was cloned into pEGFP–C1 (Clontech, Palo Alto, CA) and pEXPR-IBA105 (IBA, Göttingen, Germany), respectively. Subsequently, the EGFP–STIL fusion was shuttled into the tetracycline-inducible pcDNA4-TO vector (Invitrogen, Carlsbad, CA). For HIS tagging, a STIL fragment encoding STIL amino acids 500–650 was cloned into pHis-Parallel 2 vector (Novagen, Darmstadt, Germany). *CENPJ* was cloned into pEGFP-N1 (Clontech, Palo Alto,

CA). Construction of GFP–STIL and Strep–STIL mutants (STIL-MCPH, STIL-STAN and STIL-CC) was done by introducing stop codons at nucleotide positions 3715 (C to T, STIL-MCPH), 3179 (T to A, STIL-STAN) and 2158-2159 (CC to TA, STIL-CC) using standard site-directed mutagenesis.

Cell culture

U2OS and HEK293T cells and MEFs were cultured in DMEM with 10% FCS. HeLa cells were maintained in RPMI 1640 supplemented with 10% FCS. Derivatives of HeLa cells conditionally expressing PLK4 were generated as reported (Cizmecioglu et al., 2010). HeLa cells stably expressing Dendra–centrin-2 were generated by transfection with the transgene in pDendra2-C (Clontech, Palo Alto, CA) into HeLa cells conditionally expressing PLK4 (Cizmecioglu et al., 2010). Derivatives of U2OS cells conditionally expressing GFP–STIL were generated by transfection with the transgene in pcDNA4-TO (Invitrogen, Carlsbad, CA). De-repression of the transgenes was induced by culturing the cells in medium containing tetracycline (GFP–STIL) or doxycyclin (PLK4) for the duration specified in the figure legends.

Antibodies

Mouse monoclonal antibodies against human SAS6 and GFP (B-2) and rabbit polyclonal antibodies against GFP (FL), cyclin D1 and β-actin (C4) were from Santa Cruz (Santa Cruz, CA). Rabbit (T5192) and mouse (TU-30) antibodies against γ-tubulin were from Sigma (Deisenhofen, Germany) and EXBIO (Prague, Czech Republic), respectively. A mouse antibody against polyglutamylated tubulin (GT335) was from Enzo Life Sciences (Lörrach, Germany) and rabbit antibodies against ninein and Strep were from Abcam (Cambridge, UK) and IBA (Göttingen, Germany), respectively. Highly cross-absorbed secondary reagents Alexa Fluor 568, Alexa Fluor 488 and Alexa Fluor 647 were obtained from Invitrogen. An immunogold-labeled goat anti-mouse antibody was from Aurion (Wageningen, The Netherlands). A mouse monoclonal antibody against centrin and a rabbit polyclonal antibody against CEP164 were provided by Jeff Salisbury (Mayo Clinic, Rochester, MN) and Erich Nigg (Basel, Switzerland), respectively.

Microscopy

U2OS, HeLa and HEK293T cells, as well as MEFs grown on glass coverslips, were fixed in −20℃ methanol:acetone (1:1) or 4% paraformaldehyde. Immunofluorescence microscopy was performed with the combinations of antibodies specified in the figures. Fluorescence images were captured and processed using either a Zeiss LSM 710 or 780 (Zeiss, Göttingen, Germany) confocal laser-scanning microscope, equipped with Zen software (Zeiss) and a Plan Apo 63× 1.4 NA oil-immersion objective (Zeiss) or a TCS SP5 (Leica, Wetzlar, Germany) confocal laser-scanning microscope, equipped with confocal software (2.61, Leica) and a Plan Apo 63× 1.32 NA oil-immersion objective (Leica). Images were cropped and processed using Photoshop (Adobe). Matching confocal planes were analyzed in all colocalization experiments.

FRAP analysis

Images were acquired on a Leica TCS SP5 confocal microscope (Leica, Wetzlar, Germany) with a HCX PL Apo lambda blue 63× 1.4 NA oil-immersion objective. EGFP was excited at 488 nm by an argon laser and fluorescence emission was collected between 498 and 600 nm. The FRAP experiments were performed at 37℃ and 5% CO_2. First, five consecutive pre-bleach scans (image format 512×512 pixels) were acquired at 5% of the maximum AOTF value at 700 Hz (total scanning time 6.5 seconds). Then, one of the centrosome signals was bleached by five consecutive scans within a circular region of 3 µm diameter using the 'zoom in' bleaching function at 100% laser power while reducing the signal intensity to approximately 10% of the initial value. The detection pinhole was set to 2 airy units (AU). Single section images were then collected at 2 second (post bleach 1) and 5 second (post bleach 2) intervals for almost 5 minutes. Here, the laser power was again attenuated to 5% resulting in a total intensity loss by bleaching of less than 10%. Images were processed using Fiji freeware. Mean intensity values of the recovering signal were calculated within a circular region of 1.5 µm diameter around the centrosome. All recovery values [$I_{frap}(t)$] were double normalized according to published method (Phair et al., 2004) to the background [$I_{base}(t)$] and the total fluorescence intensity of the visible cell part [$I_{whole}(t)$] as well as to the initial background subtracted and averaged intensities of the cell [$I_{whole\text{-}pre}$] and the FRAP region ($I_{frap\text{-}pre}$).

RNA interference

For siRNA-mediated ablation of *STIL* and *SAS6*, the following oligonucelotide sequences were used: *STIL* siRNA, 5′-CAGUAACUCUAGCAAAUAA-3′; *SAS6* siRNA, 5′-GCACGUUAAUCAGCUACAAUU-3′ (Leidel et al., 2005); and *luciferase* siRNA, 5′-CUUACGCUGAGUACUUCGA-3′ were transfected with Oligofectamine reagent (Invitrogen, Carlsbad, CA). Alternatively, HeLa cells were transiently transfected with a vector encoding shRNA targeting *STIL* (pLKO.1, target sequence: 5′-AAGACAACTGCTGTTGAAGAC-3′) or control sequence (5′-CAACAAGATGAAGAGCACCAA-3′) (Erez et al., 2007).

Immunoprecipitation

HEK293T cells transiently expressing Strep–STIL constructs and CENPJ–GFP were lysed 48 hours after transfection with Triton lysis buffer (1% Triton X-100, 20 mM Tris-HCl, 120 mM NaCl) and protease inhibitors (Complete mini, Roche). Preclearing by incubation with secondary anti-mouse or anti-rabbit IgG antibodies conjugated to agarose beads (Sigma, Deisenhofen, Germany) was followed by centrifugation and incubation of the supernatants with affinity-purified rabbit anti-STIL (Castiel et al., 2011) or mouse anti-GFP (Roche, Grenzach-Wyhlen, Germany) antibodies overnight at 4℃. Then antibody-conjugated beads were added for an additional 1 hour. Samples were washed three times with washing buffer (0.1% Triton X-100, 20 mM Tris-HCl, 120 mM NaCl), resuspended in loading buffer, denatured at 95℃ for 5 minutes and analyzed by western blotting using anti-GFP, anti-STIL or anti-Strep antibodies for blotting. For the immunoprecipitation negative control, lysates were precipitated with IgG-conjugated beads only.

Acknowledgements

We are grateful to Ingrid Hoffmann, Jeff Salisbury and Erich Nigg for providing HeLa cells conditionally expressing PLK4 and antibodies against centrin and CEP164, respectively. This work was performed in partial fulfilment of the requirements for the PhD degree of Ahuvit David, Faculty of Medicine, Tel-Aviv University.

Funding

This work was supported by the Deutsche Krebshilfe (A.K.), a DKFZ-MOST grant (A.K. and S.I.) and the Israel Science Foundation (S.I.).

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.104109/-/DC1

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