A missense mutation in the PISA domain of HsSAS-6 causes autosomal recessive primary microcephaly in a large consanguineous Pakistani family

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Asymmetric cell division is essential for normal human brain development. Mutations in several genes encoding centrosomal proteins that participate in accurate cell division have been reported to cause autosomal recessive primary microcephaly (MCPH). By homozygosity mapping including three affected individuals from a consanguineous MCPH family from Pakistan, we delineated a critical region of 18.53 Mb on Chromosome 1p21.3-1p13.1. This region contains the gene encoding HsSAS-6, a centrosomal protein primordial for seeding the formation of new centrioles during the cell cycle. Both next-generation and Sanger sequencing revealed a homozygous c.185T>C missense mutation in the HsSAS-6 gene, resulting in a p.Ile62Thr substitution within a highly conserved region of the PISA domain of HsSAS-6. This variant is neither present in any single-nucleotide polymorphism or exome sequencing databases nor in a Pakistani control cohort. Experiments in tissue culture cells revealed that the Ile62Thr mutant of HsSAS-6 is substantially less efficient than the wild-type protein in sustaining centriole formation. Together, our findings demonstrate a dramatic impact of the mutation p.Ile62Thr on HsSAS-6 function and add this component to the list of genes mutated in primary microcephaly.

INTRODUCTION

Primary autosomal recessive microcephaly (MCPH; MIM 251200) is a rare heterogeneous developmental congenital brain disorder characterized by a reduced size of the cerebral cortex, which results in a smaller occipitofrontal circumference of the head that lies at least 3 standard deviations (SD) below the age, sex and ethnically matched mean (1–3). Primary
Microcephaly is present at birth but can already be diagnosed by 30 weeks of gestation and is nearly always accompanied by non-progressive intellectual disability (4–6). In some MCPH cases, reduced height and epilepsy have been reported, but besides the observation of simplified gyri, primary microcephaly is usually not accompanied by any other severe brain defects (4.5). Although rare in the non-consanguineous population (~1 in 1 Million), microcephaly has a higher incidence (1 in 100 000) in countries where endogamy is still an integral part of the tradition, such as Pakistan or Middle Eastern countries (6–9). In the past 10 years, twelve primary MCPH loci and genes have been reported, namely MCPH1, WDR62, CDK5RAP2, CPAP/CENPJ, CASC5, ASPM, STIL, CEP63, CEP135, CEP152, PHC1, CDK6 and ZNF335 (10–22). The vast majority of these genes encode centrosomal proteins, including five (CPAP, STIL, CEP63, CEP135, CEP152) that are required for efficient centriole formation. Mutations in these genes have an adverse effect on neuronal development, possibly by preventing proper asymmetric division of neuronal progenitor cells in the ventricular zone of the developing neocortex (23–25).

In progeny from consanguineous marriages suffering from an autosomal recessive trait, it is assumed that causative homozygous mutations are located in genomic regions that are homozygous-by-descent (HBD)—so called autozygous regions (26). HBD mapping using SNP arrays is an efficient genome-wide approach for identifying such autozygous regions (27–29). Subsequent massive parallel sequencing has made it cheaper and easier to screen these HBD regions for mutations (28).

In this study, we screened a large consanguineous Pakistani family with four patients diagnosed with autosomal recessive MCPH. After excluding all loci containing known microcephaly genes, we eventually identified in the affected family members a homozygous mutation in HsSAS-6 (spindle assembly 6 homolog of Caenorhabditis elegans), a gene encoding the centrosomal protein HsSAS-6, which is critical for centriole formation and thus for proper cell division (30,31).

RESULTS

The family in this study was recruited from the urban area of Dera Ismail Khan within the Khyber Paktunkhwa province of Pakistan. The pedigree includes five generations with two affected girls of age 6 and 3.5 years (V-1 and V-3) (Fig. 1A) and two men of age 50 and 42 years (IV-7 and IV-8) (Fig. 1A), who are all progeny from consanguineous marriages (Fig. 2). All four affected individuals had a head circumference of up to 19.5 SD below the mean, which was combined with severe mental retardation and an IQ ranging between 20 and 40 (32). Accordingly, for all four patients, limited speech and pronunciation problems were reported. Otolaryngologic and ophthalmic examinations were normal in all, except for Patient V-1, who had a strabismus affecting the right eye. Strabismus has not been previously associated with microcephaly but is not uncommon in the otherwise healthy population (29). In contrast to the other affected family members, Patient V-1 was unable to walk by the age of 6 despite normal bone development. Epileptic seizures were reported for the two adult patients, Brothers IV-7 and IV-8, but not for the younger ones (see Table 1).

Computed tomography was performed on Patient V-3. This demonstrated microcephaly with poorly confined basal ganglia and missing delineation of the internal capsule. Moreover, abnormal formation of the lateral ventricles and a dysmorphic infratentorial region with hypoplasia of the vermis cerebella were also noted (Fig. 1B).

DNAs of Patients IV-7, V-1 and V-3 were sampled for genomic analysis, whereas that of Patient IV-8 could not be collected owing to aggressive behavior during sampling. Initially, we excluded all known MCPH loci in this family using short tandem repeat (STR) markers to demonstrate heterozygosity in the relevant genomic regions. We then performed a genome-wide linkage analysis assuming individuals II:5 and II:6 to be first cousins (see Fig. 2B).

Genotype data were generated for three affected individuals (IV-7, V-1 and V-3) using the Nsp1 250K SNP array from Affymetrix. We observed a single significant peak with a maximum multi-point LOD score of 3.9 on Chromosome 1 (Supplementary Material, Fig. S1). The underlying homozygous region, shared between all affected individuals included in the analysis, comprised ~20 cM on the short arm of Chromosome 1 at cytoband 1p21.3-1p13.1 (Fig. 2A). The critical interval of the new MCPH locus is defined by the two SNP markers rs555557 at position 98,912,075 bp and rs2251406 at position 117,445,365 bp, spanning a region of 18.53 Mb according to UCSC human genome assembly hg19. The segregation of the interval was confirmed by genotyping all available family members with six highly polymorphic STR microsatellite markers located between 96.8 and 111.2 Mb on Chromosome 1p (Fig. 2B). A maximum two-point LOD score of 2.8 for marker D1S495 (102.56 Mb; hg19) and multipoint LOD score of 3.53 were calculated for the area between marker D1S2671 (101.27 Mb) and D1S495 (102.56 Mb) (Supplementary Material, Table S1).

As several known MCPH genes contribute to proper cell division, we screened by Sanger sequencing four candidate genes that were known to potentially contribute to this process and that are located within the homozygous region on Chromosome 1: WDR47, PSRC1, NGF and HsSAS-6 (9,14,18,19,33–36). Only the sequence of HsSAS-6 revealed a homozygous NM_194429.2: c.185T>C (Chr1:100588878A>G) transition in a highly conserved region in exon 3 in all patients under investigation (Fig. 3A and B). As a consequence, the hydrophobic non-polar isoleucine at position 62 in the HsSAS-6 protein is exchanged by a hydrophilic polar threonine (p.Ile62Thr). Consistent with the linkage analysis, all healthy family members that were carriers of the disease allele were heterozygous for the mutation, whereas individual V-2 was homozygous for the HsSAS-6 wild-type allele (Fig. 3A). The mutation was not found in 116 unrelated, unaffected Pakistani individuals by Sanger sequencing. We further sequenced HsSAS-6 in patients of 19 additional MCPH families from Pakistan, which were previously excluded for mutations in all known MCPH genes, but did not identify further mutations in HsSAS-6.

In order to exclude with certainty any other mutations in known microcephaly genes and in genes located in the HBD region in this family, we conducted whole-exome sequencing. We found another homozygous missense mutation, c.656C>T (p.Ser219Leu), close to the splice site of exon 8 of CAPZA1 (F-actin-capping protein subunit alpha-1). This gene encodes a protein required for the regulation of actin polymerization (38). Neither the mutation in HsSAS-6 nor the one in CAPZA1
was listed in the NHLBI Exome Variant Server (http://eversusgs.washington.edu/EVS/), but only the mutation in HsSAS-6 was predicted to have a potential pathogenic effect by the mutation prediction servers PolyPhen2 (HsSAS-6 p.Ile62Thr: score = 1.0; CAPZA1: p.Ser219Leu: score = 0.014) and ConDel (HsSAS-6 p.Ile62Thr: score = 0.847; CAPZA1: p.Ser219Leu: score = 0.001) (39,40). We further investigated whether the mutation in CAPZA1 had any adverse effect on splicing by amplifying a 242-bp product from the cDNA that covered the junction between exons 8 and 9. This showed that splicing of CAPZA1 is not affected by the mutation (Supplementary Material, Fig. S2). Moreover, previous knock-out experiments with this gene in the mouse failed to give evidence of a cell division phenotype (41). Overall, these results indicate that the mutation in CAPZA1 is not causative of MCPH in the affected individuals.

Because of this, and because microcephaly is thought to result from defects in asymmetric cell division of neuronal progenitor cells, we concentrated on the HsSAS-6 gene and its protein product, HsSAS-6, which is critical for the onset of centriole formation and thus for proper cell division (8,25,42–45). Human SAS-6 consists of 17 exons and encodes a 657-amino acid protein of 74 kDa (46,47). The onset of centriole formation relies on the oligomerization of nine SAS-6 homodimers via their N-terminal head domains into a 9-fold symmetrical ring-like structure (30,48,49). Although the crystal structure of HsSAS-6 is not available, the high-resolution crystal structure of the N-terminal head domain of SAS-6 from Danio rerio, DrSAS-6, showed that Ile62 is part of the hydrophobic core of the protein (Fig. 4A). The side chain of this residue packs against those of Leu44, Leu60, Leu70, Phe80, Phe83 and Leu139, which are strictly conserved from mammals to algae (Supplementary Material, Fig. S3). This suggested that an exchange of the hydrophobic amino acid residue at position 62 with a polar threonine might compromise proper folding and/or function of the N-terminal domain of HsSAS-6.

In order to investigate the impact of the Ile62Thr mutation on HsSAS-6 function, we generated U2OS cells expressing doxycycline-inducible EGFP-tagged (hereafter referred to as GFP) HsSAS-6, either wild type (30) or bearing the Ile62Thr mutation. The cells were induced with doxycycline for 48 h, and the subcellular localization of the fusion proteins analyzed by immunofluorescence using antibodies against GFP and the centriolar marker Centrin-2. As shown in Figure 4B and C, we found that HsSAS-6 centriolar recruitment is not altered by the Ile62Thr mutation. These results demonstrate that the Ile62Thr mutation does not impair HsSAS-6 centriolar localization and that the protein is not misfolded.

Next, we analyzed whether the Ile62Thr mutation impairs the function of HsSAS-6 in centriole formation. Wild-type HsSAS-6 fused to GFP drives the formation of centrioles in excess (43), and we found that a variant bearing the Ile62Thr mutation impairs the function of HsSAS-6 in centriole formation. These results demonstrate that the Ile62Thr mutation does not impair HsSAS-6 centriolar localization and that the protein is not misfolded.
Figure 2. Homozygosity mapping. (A) 250 K NSP SNP array reveals a homozygous 18.53-Mb stretch (blue) with identical haplotype on Chromosome 1p21.3-p13.1 restricted by SNP rs555557 and rs2251406 in all three patients. (B) The green allele between STS marker D1S2671 and D1S2726 segregates in the family in an autosomal recessive manner.

Table 1. Clinical data of the affected

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mutation does not (Fig. 4D and E). Furthermore, we found that \( \approx 7\% \) of cells \((N = 186)\) expressing the Ile62Thr variant exhibit less than three centrioles during mitosis, compared with \( \approx 1\% \) for cells \((N = 229)\) expressing wild-type HsSAS-6-GFP, indicative of a slight dominant-negative effect on centriole formation. Next, we set out to test whether the mutant variant can sustain centriole formation in cells depleted of endogenous HsSAS-6. To this end, we depleted endogenous HsSAS-6 using siRNAs directed against the 3' UTR \((30)\), which is absent from the GFP fusion constructs (Fig. 4D). We again analyzed cells by immunofluorescence with antibodies against GFP and centrin, but this time focusing on cells in mitosis to assay the number of centrioles at the end of the centriole duplication cycle. In control conditions, most cells had four centrioles, but \( \approx 12\% \), \( \approx 4 \) centrin foci (Fig. 4F and H). In contrast, depletion of endogenous HsSAS-6 resulted

Figure 3. (A) Sanger sequencing of the affected patients revealed a homozygous c.185T>C transition. Non-affected family members were either heterozygous for the mutation \((e.g. \text{IV-5})\) or homozygous for the wild-type sequence. (B) The PISA domain of SAS-6 in 9 organisms was aligned in Jalview 2.8 using the MAfftWS alignment and colored according to the hydrophobicity table of Kyte and Doolittle \((37)\). Red indicates conserved hydrophobic and blue conserved hydrophilic residues. The shading intensity indicates the conservation grade of the hydrophobicity \((\text{dark} = \text{very conserved}; \text{white} = \text{not conserved})\). The conservation table shows that the properties of the mutated amino acid Ile62Thr in HsSAS-6 are highly conserved among different species \((0 = \text{no conservation}; * \text{ or } 11 = \text{highest conservation}, + \text{or } 10 = \text{mutation but properties are conserved})\).
Figure 4. Impact of I62T mutation in tissue culture cells. (A) Cartoon representation of the N-terminal head domain of DrSAS-6 (PDB ID 2Y3V), illustrating the location and environment of Ile62. Selected amino acid residues are shown in sphere representation and are labeled. Ile62 is highlighted in blue. The figure was prepared using PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.5. Schrödinger, LLC). (B–C) iU2OS interphase cells expressing either HsSAS-6 (wild type) fused to GFP (B) or the corresponding I62 T mutant (C) stained for GFP (green), Centrin-2 (red) and DNA (blue). (D) Western blot analysis of protein extracts from iU2OS cells expressing the indicated constructs and treated with control siRNAs (NC) or siRNAs targeting the 3′ UTR of HsSAS-6 (3′ UTR), using antibodies against GFP (top), HsSAS-6 (middle) or α-tubulin (bottom). Note that treatment with siRNAs against the HsSAS-6 3′ UTR leads to efficient depletion of endogenous HsSAS-6 but not of the GFP-variants that lack the 3′ UTR. (E) Histogram of the average frequency of mitotic cells with more than four centrioles in control U2OS cells and in cells overexpressing HsSAS-6-GFP or HsSAS-6-GFP[I62T] for 48 h following induction with doxycycline. Data from ≥3 experiments, with > 50 cells scored in each experiment. (F–G) Histogram of the average frequencies of mitotic cells with less than four centrioles (F) or with monopolar spindles (G) in control U2OS, iU2OS HsSAS-6-GFP or iU2OS HsSAS-6-GFP[I62T] cells treated with siRNA Control (NC) or siRNA targeting the HsSAS-6 3′ UTR (3′ UTR). Data from ≥3 experiments, with > 50 cells scored in each experiment. (G–M) U2OS (H–J), iU2OS HsSAS-6-GFP (K) or iU2OS HsSAS-6-GFP[I62T] (L–M) cells in mitosis, stained for GFP (green), Centrin-2 (red) and DNA (blue). In (L–M), doxycycline induction for 48 h was concomitant with siHsSAS-6 3′ UTR (3′ UTR) treatment. Note monopolar spindles in J and M. Scale bars: 5 μm.
in ∼95% of cells having <4 centrin foci (Fig. 4F and I), with the majority of cell exhibiting monopolar spindle assembly (Fig. 4G and J). This centriole duplication phenotype was rescued to ∼20% of cells having <4 centrin foci by the expression of wild-type HsSAS-6-GFP (Fig. 4F and K). In contrast, ∼68% of cells still exhibited <4 centrin foci upon the expression of HsSAS-6[Ile62Thr]-GFP (Fig. 4F and L). As anticipated also, monopolar spindle assembly was observed in a substantial fraction of cells expressing HsSAS-6[Ile62Thr]-GFP (Fig. 4G and M). We conclude that the Ile62Thr mutation severely, although not completely, impairs HsSAS-6 function. These data demonstrate that the c.185T>C mutation in the human HsSAS-6 gene has a drastic impact on centriole formation and thus on normal cell division.

**DISCUSSION**

Microcephaly is thought to result from impaired asymmetric cell division of neuronal progenitors during the peak phase of neurogenesis in the embryo and is caused primarily by mutations in genes encoding centrosomal proteins (23), including some that are required for centriole formation (14,19,20,36,50). During the normal cell cycle, centriole formation is initiated around the G1/S transition, when a small set of centriolar proteins are recruited to the proximal end of the two parental centrioles, thus initiating assembly of one procentriole next to each parental centriole (49). Elongation continues throughout S phase and into G2, as well as into the next cell cycle, when the newly formed centrioles complete their maturation with the acquisition of appendages on their distal end (51–53).

As shown in this and in previous studies, knock-down of HsSAS-6 impairs procentriole formation, thus increasing the fraction of cells with fewer than four centrioles as well as cells with monopolar spindles (43,44,54). We have also shown that such impairment of procentriole formation can be rescued by overexpression of WT HsSAS-6-GFP but only to a limited extent by that of HsSAS-6[Ile62Thr]-GFP, offering a strong cellular correlate to the phenotype manifested in the MCPH patients.

Our results are in line with a previous study on the Glu1235Val mutation in CPAP, which was one of the first mutations in this gene reported to cause microcephaly (14,24). CPAP is related to *C. elegans* SAS-4 and is also essential for procentriole formation in proliferating human cells in culture (55). CPAP localizes to the proximal part of the procentriole and centriole and has been proposed to be connected with HsSAS-6 through the bridging protein CEP135 (35). Mirroring our findings with the Ile62Thr HsSAS-6 mutant, the Glu1235Val CPAP mutant can rescue centriole formation only to a limited extent (24). These observations taken together offer a striking parallel and reinforce the notion that partial impairment of centriole formation results in MCPH.

Furthermore, studies in *Drosophila* larvae showed that compared with wild-type flies, *DmSas-6* knockout flies exhibit a significantly reduced number of centrosomes in the brain and ∼18% of centrosomes were even smaller in size (56).

In conclusion, we have demonstrated here that the homozygous c.185T>C mutation in the *HsSAS-6* gene has a drastic impact on centriole formation and thus on proper cell division, a process that is essential during neurogenesis. Furthermore, we propose that the remaining activity of the mutated HsSAS-6[Ile62T] protein enables residual asymmetric cell division and thus results in reduced brain development, causing primary autosomal recessive microcephaly.

**MATERIAL AND METHODS**

**Sample collection**

After obtaining the informed consent, blood was drawn from three affected and seven healthy family members from the Pakistani family investigated in this study and genomic DNA was isolated according to standard protocols. For cDNA analysis, blood from V-3 and VI-7 as well as from two healthy controls was collected in PAXgene RNA blood tubes (PreAnalytiX). RNA was further isolated using PAXgene RNA Kit. Moreover, blood was drawn from 116 non-affected Pakistani individuals for control analysis. The study was approved by Institutional ethical review boards of Gomal University, Dera Ismail Khan, and Quaid-i-Azam University, Islamabad, Pakistan.

**Autozygosity mapping, haplotype and linkage analysis**

All three affected individuals, IV-7, V-1 and V-3, were genotyped on Affymetrix GeneChip Human Mapping 250K NspI Arrays at the ‘Center for Medical Research’ at the Medical University of Graz.

Genome-wide linkage analysis of the family was performed with 20 044 selected SNP markers. LOD scores were calculated with ALLEGRO (57). Data handling, evaluation and statistical analysis were performed as described previously (19).

For haplotype and local linkage analysis, a total of six highly polymorphic STR markers covering the autozygous region of interest were selected for fine mapping and segregation analysis, including D1S206 (101.6 Mb) and D1S2726 (111.18 Mb) from the ABI Prism Linkage Mapping Set v2.5, as well as D1S2719 (96.81 Mb), D1S2739 (98.93 Mb), D1S2671 (101.67 Mb) and D1S495 (102.56 Mb) selected from the UCSC browser mapping track (build 37/ hg19) (47). PCRs were performed with ABI Prism True Allele PCR Premix (Applied Biosystems) and amplicons were denaturated using HiDiFormamide with Gene Ruler 500-Liz Size Standard (both from Applied Biosystems). Genome scan data were generated on the ABI3130xl and analyzed with Peak Scanner Software v1.0 (Applied Biosystems). For linkage analysis, an autosomal recessive trait with full penetrance and a disease allele frequency of 0.001 were assumed. The two-point LOD score was calculated using the online version of Superlink (http://bioinfo.cs.technion.ac.il/sup erlink-online), and for multipoint LOD score calculation, Merlin was used (58). Sex-averaged recombination rates between markers were obtained from Rutgers map (build 37, patch 4) (59).

**Whole-exome sequencing**

We fragmented 1 µg of DNA using sonification technology (Covaris, Woburn, MA, USA). The fragments were end-repaired and adaptor-ligated including incorporation of sample index barcodes. After size selection, the library was subjected to the enrichment process. For that we chose the SeqCap EZ Human
Exome Library v2.0 kit from NimbleGen (Roche NimbleGen, Madison, WI, USA). The enriched library was subsequently sequenced on an Illumina HiSeq 2000 sequencing instrument using a paired-end 2 × 100-bp protocol.

This resulted in 8.4 Gb of mapped sequences, a mean coverage of 89-fold, a 30× coverage of 87% and a 10× coverage of 97% of target sequences. For data analysis, the Varbank pipeline v.2.3 and filter interface was used (unpublished, https://varbank.ccg.uni-koeln.de/). Primary data were filtered according to signal purity by the Illumina Realtime Analysis (RTA) software v1.8. Subsequently, the reads were mapped to the human genome reference build hg19 using the BWA (60) alignment algorithm. GATK v1.6 (61) was used to mark duplicated reads, to do a local realignment around short insertion and deletions, to re-calibrate the base quality scores and to call SNPs and short indels.

Scripts developed in-house at the Cologne Center for Genomics were applied to detect protein changes, affected donor and acceptor splice sites and overlaps with known variants. Acceptor and donor splice site mutations were analyzed with a Maximum Entropy model (62) and filtered for effect changes. In particular, we filtered for high-quality (coverage > 15; quality > 25) rare (MAF < 0.005) homozygous variants (dbSNP build 135, the 1000 Genomes database build 20110521, and the public Exome Variant Server, NHLBI Exome Sequencing Project, Seattle, build ESP6500). We also filtered against an in-house database containing variants from 511 exomes from epilepsy patients to exclude pipeline-related artifacts (MAF < 0.004).

**Sanger sequencing**

Primers covering the coding exons and splice sites of *HsSAS-6, NGF, PSCR1* and *WDR47* were designed with Primer3 (http://www-genome.wi.mit.edu/genome_software/other/primer3.html) (63). A list of all oligonucleotides can be found in Supplementary Material, Table S2. CAPZA1_cDNA_fov 5′ GGAAGTTCAC CATCACACCA and CAPZA1_cDNA_rev 5′ GGCCTTGAAT TGTTGTATCTGA primers were used to investigate the effect of the splice site mutation in exon 8 of CAPZA1 on mRNA level. PCR was performed using HotStarTaq Master Mix Kit (Qiagen) with the following cycling conditions: 94°C for 15 min, followed by 35 cycles of 95°C for 25 s, 57°C for 30 s and 72°C for 1 min with no final elongation step. The Sanger sequencing reaction was set up with the Big Dye v3.1 cycling sequencing kit (Applied Biosystems) according to the manufacturers’ protocol. Dye remnants were removed with Centri-SepTM columns (Applied Biosystems). Bidirectional DNA sequencing was conducted on the ABI3130xl (Applied Biosystems). Bidirectional DNA sequencing was conducted on the ABI3130xl (Applied Biosystems). The Sanger sequencing was then performed according to the manufacturer’s protocol to generate the expression plasmid pEBTet-HsSAS-6-GFP and pEBTet-HsSAS-6-I62T, which were sequence verified.

**Cell culture and transfections**

U2OS cells were obtained from the ECACC and maintained in McCoy’s 5A Glutamax medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) for U2OS cells or tetracycline-negative FBS (Brunschwig) for the inducible episomal cell lines (iU2OS). iU2OS cell lines were generated by transfecting U2OS cells with pBEt-HsSAS-6-GFP or pEBTet-HsSAS-6-I62T-GFP using Lipofectamine2000 (Invitrogen). Transfected cells were selected with 1 μg/ml of puromycin 1 day after transfection and amplified. For the expression of the GFP-fused constructs, early passage cells were induced with 1 μg/ml of doxycycline for 48 h.

Endogenous HsSAS-6 was depleted using a Stealth RNAiTM siRNA (Invitrogen) targeting the 3′ UTR of HsSAS-6 (5′ GAG CUGUUUAAAGACUGGAAUUA 3′) (30). Stealth RNAiTM siRNA-negative control LO GC (Invitrogen) was used as a control.

**Cell-extract preparation and biochemical assays**

Cells were collected, washed in PBS and lysed on ice for 1 h in lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5 mM
EDTA 0.5% NP-40, Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics)]. Lysates were cleared by centrifugation for 10 min at 12,000 × g and 4°C before the supernatant was collected. SDS–PAGE was performed using 10% polyacrylamide gels (BioRad), followed by transfer on nitrocellulose membrane (Amersham). The membrane was probed with mouse HsSAS-6 antibody (Santa Cruz, 1:1000) or mouse α-tubulin antibody (Sigma, 1:10000), followed by incubation with HRP-conjugated secondary (Promega) and the signal detected with Chemiluminescence (Roche).

**Immunofluorescence and microscopy for human cells**

U2OS cells grown on glass coverslips were fixed for 7–10 min in –20°C methanol, washed in PBS and blocked for 15–30 min in 1% bovine serum albumin and 0.05% Tween-20 in PBS. Cells were incubated 2 h at room temperature with primary antibodies, washed three times for 10 min in PBST (0.05% Tween-20 in PBS) incubated for 45 min at room temperature with secondary antibodies, stained with ~1 μg/ml of Hoechst 33258, washed three times in PBST and mounted. Primary antibodies were 1: 4000 mouse centrin (20H5; gift from Jeffrey L. Salisbury) and 1: 500 rabbit GFP (gift from Viesturs Simanis). Secondary antibodies were 1: 1000 goat anti-rabbit coupled to Alexa 568. For quantification of centrioles, mitotic cells (prophase to metaphase) with similar cytoplasmic GFP expression were used; highly expressing cells that often harbored GFP aggregates were not retained for analysis. Imaging was done on a Zeiss LSM710 confocal microscope. Optical sections were acquired every 0.12 μm, and planes containing centrioles were projected together. Images were processed using ImageJ and Adobe Photoshop, preserving relative image intensities within a series.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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