The Two SAS-6 Homologs in *Tetrahymena thermophila* Have Distinct Functions in Basal Body Assembly

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Cilia and flagella are structurally and functionally conserved organelles present in basal as well as higher eukaryotes. The assembly of cilia requires a microtubule based scaffold called a basal body. The ninefold symmetry characteristic of basal bodies and the structurally similar centriole is organized around a hub and spoke structure termed the cartwheel. To date, SAS-6 is one of the two clearly conserved components of the cartwheel. In some organisms, overexpression of SAS-6 causes the formation of supernumerary centrioles. We questioned whether the centriole assembly initiation capacity of SAS-6 is separate from or directly related to its structural role at the cartwheel. To address this question we used *Tetrahymena thermophila*, which expresses two SAS-6 homologues, *TtSAS6a* and *TtSAS6b*. Cells lacking either *TtSAS6a* or *TtSAS6b* are defective in new basal body assembly. *TtSAS6a* localizes to all basal bodies equally, whereas *TtSAS6b* is enriched at unciliated and assembling basal bodies. Interestingly, overexpression of *TtSAS6b* but not *TtSAS6a*, led to the assembly of clusters of new basal bodies in abnormal locations. Our data suggest a model where *TtSAS6a* and *TtSAS6b* have diverged such that *TtSAS6a* acts as a structural component of basal bodies, whereas *TtSAS6b* influences the location of new basal body assembly.

INTRODUCTION

Cilia and flagella are microtubule based cellular appendages used for locomotion, the movement of fluids over an epithelial layer, and sensing the environment (Fliegauf *et al.*, 2007). In humans and other metazoans, cilia function in all of these capacities (Fliegauf *et al.*, 2007). Based on the presence of these organelles in basal eukaryotes as well as more complex organisms such as humans, it is believed that these structures have an ancient origin (Cavalier-Smith, 2002; Satir *et al.*, 2007). Cilia are responsible for many important developmental, cell signaling, and physiological processes; therefore, it is not surprising to find that a growing number of genetic diseases with pleiotropic symptoms are caused by mutations in genes encoding components of cilia or basal bodies (Badano *et al.*, 2006). Thus it is of significant interest to understand how these organelles are normally assembled and maintained.

Cilia are assembled from a structure called the basal body, which in mammals and other species is derived from a centriole (Sorokin, 1962) or by de novo synthesis in ciliated epithelia (Dirksen, 1971). Centrioles and basal bodies contain a barrel-shaped ninefold symmetric array of microtubules. In humans and most other species in which centrioles or basal bodies have been examined, the microtubules of the basal body are arranged in triplet bundles. However, the centrioles of *Caenorhabditis elegans* are composed of a ninefold symmetric array of microtubule singlets. Elegant electron microscopy studies of centriole assembly steps have shown that the microtubule array is organized around a hub-and-spoke structure termed the cartwheel in organisms

as diverse as ciliates, *Chlamydomonas*, *Drosophila*, and mammalian species (Dippell, 1968; Allen, 1969; Cavalier-Smith, 1974; Vorobjev and Chentsov Yu, 1982; Callaini *et al.*, 1997) or around a central tube in the case of *C. elegans* (Pelletier *et al.*, 2006).

Here we use the ciliated protozoan *Tetrahymena thermophila* to study basal body biogenesis. *Tetrahymena* have hundreds of basal bodies per cell, and before cell division many new basal bodies assemble in specific regions of the cell (Allen, 1969). Furthermore, the basal bodies of *Tetrahymena* are similar in morphology and protein composition to the basal bodies of humans (Allen, 1969; Kilburn *et al.*, 2007). From a structural standpoint, the basal body assembly process in *Tetrahymena* was meticulously documented (Allen, 1969), but few investigations have analyzed specific components of *Tetrahymena* basal bodies and their role in the assembly of this complex structure. Recently, several studies have analyzed a number of widely conserved genes that are important for centriole assembly and developed an assembly pathway. Within this group, SAS-6 has a relatively early role in assembly (Dammermann *et al.*, 2004; Habadanck *et al.*, 2005; Leidel *et al.*, 2005; Delattre *et al.*, 2006; Pelletier *et al.*, 2006; Kleylein-Sohn *et al.*, 2007; Nakazawa *et al.*, 2007; Peel *et al.*, 2007; Rodrigues-Martins *et al.*, 2007a,b; Strnad *et al.*, 2007). SAS-6 is a component of the central tube in *C. elegans* (Pelletier *et al.*, 2006) and the cartwheel in *Chlamydomonas* (Nakazawa *et al.*, 2007). In *C. elegans*, the nine microtubule singlets of the centriole cannot be organized into a centriole without SAS-6 (Pelletier *et al.*, 2006). Similarly *Chlamydomonas* or *Drosophila* SAS-6 mutants display aberrant numbers of microtubule triplets in their basal bodies (Nakazawa *et al.*, 2007; Rodrigues-Martins *et al.*, 2007a). These studies demonstrate that SAS-6 has a role in cartwheel formation. Additionally, overexpression of SAS-6 in some organisms leads to the formation of supernumerary centrioles (Leidel *et al.*, 2005; Peel *et al.*, 2007; Strnad *et al.*, 2007). These observations led us to question whether the centriole assembly initiation

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activity of SAS-6 is separate from or linked to its structural role at the cartwheel. If these activities are in fact separate functions of the SAS-6 gene, then a system that possesses two SAS-6 homologues may be able to help answer this question. *Tetrahymena* expresses two SAS-6 homologues, which provided us with a potential model system that we could use to investigate this possibility.

MATERIALS AND METHODS

Strains and Culture Conditions

Tetrahymena thermophila strains B2086, CU428, and SB1969 (gifts from Peter Bruns, Cornell University, and Eduardo Orias, University of California Santa Barbara) were used for the generation of transgenic cell lines. Cells were grown in SPP media consisting of 2% proteose peptone, 0.1% yeast extract, 0.2% glucose, and 0.003% FeEDTA at 30°C. Cells were starved in 10 mM Tris, pH 7.4. Matings between cells were performed as described in Bruns and Cassidy-Hanley (2000). *SAS6a* shutoff cells were grown in 1% SPP with 100 ng/ml CdCl₂ to activate expression of *SAS6a* or with 100 μ M EDTA to inactivate expression of the gene. In *SAS6b* overexpression experiments, cells were grown in 2% SPP with 400 ng/ml CdCl₂ and 15 μ g/ml cyclohexamide.

Identification of *Tetrahymena* SAS-6 Homologues

Possible SAS-6 homologues were identified by a BLAST search of the *Tetrahymena thermophila* predicted proteome (Eisen *et al.*, 2006) from *Tetrahymena* Genome Database (TGD) using published sequences (Dammermann *et al.*, 2004; Leidel *et al.*, 2005). We identified two predicted open reading frame (ORFs): TTHERM_00388200 corresponds to *TiSAS6a* and TTHERM_00137600 corresponds to *TiSAS6b*.

Putative SAS-6 PISA domain amino acid sequences from organisms listed in Figure 1a were aligned and used to generate a distance matrix using PROTDIST (Jones-Taylor-Thornton matrix). This distance matrix was used in the construction of a tree using NEIGHBORS and DRAWTREE (Felsenstein, 1997).

Generation of Polyclonal Antibodies to *Sas6a* and *Sas6b*

DNA encoding the first 160 amino acids of both *SAS6a* and *SAS6b* and optimized for expression in *Escherichia coli* was synthesized (Integrated DNA Technologies, Coralville, IA). This region was chosen because it contains the conserved PISA domain. These DNA fragments were cloned into N-terminal glutathione S-transferase (GST) fusion expression vector, pGEX-6P1 (Amersham, Buckinghamshire, United Kingdom), using BamHI and XhoI sites and were transformed into *E. coli* strain BL21 for expression. Recombinant *Sas6a* and *Sas6b* protein fragments were cleaved from the GST affinity tag using HRV3C protease and eluted off beads with few contaminants (Supplemental Figure S1b). Two milligrams of *Sas6a*- or *Sas6b*-enriched samples were injected into rabbits for generation of polyclonal antibodies (Affinity BioReagents, Golden, CO). Antibodies were affinity-purified against agarose-bound recombinant *Sas6a* and *Sas6b* fragments using instructions from the manufacturer (Pierce, Rockford, IL) and depleted for cross-reactivity by binding affinity-purified anti-*Sas6a* antibodies to the *Sas6b* column and vice versa. In this manner we generated antibodies specific to *Sas6a* and *Sas6b* with minimal cross-reactivity to one another. To control for the specificity of the *Sas6a* antibody, we performed Western blot analysis using this antibody on cells expressing green fluorescent protein (GFP)-*SAS6a* with or without inclusion of a 50-fold molar excess of *Sas6a* or *Sas6b* antigens. Supplemental Figure S1c shows three different cellular fractions together comprising total cellular protein. When these fractions were blotted for *Sas6a*, two bands representing the predicted molecular weights of GFP-*Sas6a* and full-length *Sas6a* were present at approximately 110 and 82 kDa, respectively (Supplemental Figure S1c, left). None of the anti-*Sas6a* reactive bands were eliminated by the addition of *Sas6b* antigen (Supplemental Figure S1c, middle). In contrast to this, all of the bands were eliminated or severely reduced by the addition of *Sas6a* antigen (Supplemental Figure S1c, right). To control for the specificity of the *Sas6b* antibody, fixed wild-type cells were incubated with *Sas6b* antibody alone or with a 50-fold molar excess of the *Sas6a* or *Sas6b* antigens (Supplemental Figure S1d, left). Inclusion of *Sas6a* antigen had no effect on the labeling pattern of the anti-*Sas6b* antibody (Supplemental Figure S1d, middle), but in the presence of *Sas6b* antigen the basal body signal was completely eliminated (Supplemental Figure S1d, right). These data show that the *Sas6a* antibody is not cross-reactive to *Sas6b* and vice versa.

Tetrahymena Cellular Fractionation

Tetrahymena strain SB255 (mucocyst deficient, gift of Eduardo Orias, University of California Santa Barbara) was deciliated using the protocol described in Thompson *et al.* (1974). Fraction 1 is represented as 0.2% of the total cilia-containing fraction. Pellicles (fractions 3 and 4) and basal body/kinetodesmal fibers (fractions 5 and 6) were made from these deciliated cells according to the method described by Hyams and King (1985). Triton-soluble

protein (25 μ g at 1%) from the generation of pellicles and kinetodesmal fibers was loaded (fraction 2). Proteins were solubilized from pellicles and basal body/kinetodesmal fiber fractions in HpHS buffer (50 mM Tris, pH 9.0, and 500 mM NaCl). HpHS-soluble pellicle extract, 8 μ g, is represented in fraction 3, whereas an equal volume of the insoluble pellet from this extraction was loaded as fraction 4. HpHS-soluble kinetodesmal fiber protein, 2 μ g, is represented as fraction 5, and an equal volume of the insoluble pellet is represented as fraction 6.

Western Blot Analysis

Blots were blocked in 5% milk TBST (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.2% Tween-20) for 40 min at room temperature. Affinity-purified anti-*Sas6a* was used at 1:2500 in 5% milk TBST. Monoclonal anti-Atu1 antibody (12G10) was used at 1:5000 in 5% milk TBST. All incubations were performed for 1 h at room temperature or at 4°C overnight. Blots were washed three to four times for 5 min with TBST after incubation in antibody-containing solution. Western blots were scanned on a LI-COR Odyssey (Li-Cor, Lincoln, NE) using appropriate secondary antibodies (Invitrogen, Carlsbad, CA, and Rockland Biosciences, Gilbertsville, PA).

Immunofluorescence Microscopy

Cells were fixed as described in Stuart and Cole (2000), except formaldehyde fixation lasted 25 min. Centrin was labeled using 20H5 (gift from J. Salisbury, Mayo Clinic, Rochester, MN) at 1:2500 in phosphate-buffered saline (PBS), pH 6.9, 1% BSA. *Sas6a* antibody was used at 1:2500 in PBS, 1% BSA. *Sas6b* antibody was used at 1:2500 in PBS, 1% BSA. Atu1 was labeled using 12G10 (gift from Joseph Frankel, University of Iowa, Iowa City, IA) at 1:75 in PBS, 1% BSA. Cen1 antibody (Stemm-Wolf *et al.*, 2005) was used at 1:1500 in PBS, 1% BSA. GFP antibody (Rockland, Gilbertsville, PA) was used at 1:500 in PBS, 1% BSA. All incubations were performed at room temperature for 1 h or at 4°C overnight. Alexa fluor and Texas Red (Invitrogen and Jackson ImmunoResearch, West Grove, PA) secondary antibodies were used according to manufacturer's instructions. Images were acquired on a Leica DMRXA/R4/V automated microscope (Leica Microsystems, Bannockburn, IL) with a Photometrics COOL SNAP HQ2 digital camera (Photometrics, Tucson, AZ). Images were collected and projection images generated of one hemisphere of the cell using the Metamorph imaging software (Molecular Devices, Sunnyvale, CA).

Plasmid Construction and Molecular Techniques

Total RNA was extracted from log-phase cells using TRI Reagent (Molecular Research Center, Cincinnati, OH). Total cDNA was generated using oligo dT primed Moloney murine leukemia virus (MMLV) reverse transcriptase reactions (Invitrogen). We were able to amplify cDNAs for *SAS6a* and *SAS6b* of the size and sequence predicted by TGD. GFP-*SAS6a* and GFP-*SAS6b* constructs were built by cloning *SAS6a* or *SAS6b* cDNAs into pIGF vector using XhoI and ApaI sites (Doug Chalker, Washington University, St. Louis, MO). MTT-GFP-*SAS6b* was cloned into pBSMTTGFP using the Gateway system (Invitrogen and Doug Chalker, Washington University, St. Louis, MO). pMTT 6xHis-*SAS6a* was built by removing the Neo1 cassette from p4T2-1 (gift from Jacek Gaertig, University of Georgia, Athens, GA) using EcoRV and SmaI. This was inserted into pMTT-G1 (gift of Martin Gorovsky, University of Rochester) using a blunt BglII site just 5' of the MTT promoter. SalI and XhoI sites were destroyed in pMTT-G1 + Neo by cutting these sites and filling in the gap. This plasmid was then cut with HindIII and BamHI to remove the IChG1 coding region. An MCS was then cloned into this site containing Pmel and ApaI. Annealed double-stranded oligonucleotides coding for RGS6H-HHHH and a HRV3C cut site was then ligated into Pmel and XhoI sites. In this manner an inducible RGS6xHis-*SAS6a* construct was generated. A *SAS6a* knockout construct was built into the pMNB1 plasmid. Six hundred base pairs 5' of the start codon was used for the 5' arm and 1.6 kb of DNA flanking the stop codon was used for the 3' arm. The 5' arm was cloned in using ApaI and XhoI. The 3' arm was cloned into BamHI and XbaI sites. An alternative version of pMNB1-*SAS6a* was built by removing the Neo3 cassette and replacing it with the Bsr cassette (gift from Jacek Gaertig, University of Georgia, Athens, GA) by cloning into XhoI and SmaI sites. This knockout construct was used for the generation of the *SAS6a* shutoff. The *SAS6b* knockout construct was built by cloning into p4T2-1. One kilobase of DNA flanking the start codon was used for the 5' arm, and 2 kb of DNA flanking the stop codon was used for the 3' arm. The 5' arm was cloned into ApaI and XhoI sites, and the 3' arm was cloned into BamHI and NotI sites. pGEX-*SAS6a* was built by cloning the *SAS6a* minigene (Integrated DNA Technologies) into BamHI and XhoI sites present in pGEX-6P1 (Amersham). pGEX-*SAS6b* was built by cloning the *SAS6b* minigene into BamHI and XhoI sites present in pGEX-6P1. Genomic DNA was isolated from cells using the method of Gaertig *et al.* (1994). Southern blot for *SAS6a* and *SAS6b* knockouts was performed according to standard techniques. Briefly for the *SAS6a* knockout, 30 μ g of genomic DNA from CU428 (WT), *SAS6a* KO cells without 6xHis-*SAS6a* rescue, and *SAS6a* KO cells with 6xHis-*SAS6a* rescue digested with SphI and XmnI were loaded on a 0.7% agarose gel. The blot was transferred by the capillary method. The same fragment of DNA used to build the 5' arm of the knockout construct was used as a radioactive probe of the blot. For the

SAS6b knockout, 30 μ g of genomic DNA from WT, *SAS6b* macronuclear knockout, and *SAS6b* micronuclear knockout was digested with NheI and XhoI and done as above. The 5' arm of p4T2-1 *SAS6b* was used as probe in this blot. Northern blot analysis was performed on 30 μ g of total RNA from actively dividing cells. *SAS6a* or *SAS6b* cDNA, 1 ng, was used for normalization of blots. Hybridization and washing were done according to standard procedures using a formamide based buffer. Blots were scanned on a STORM PhosphorImager (GE Healthcare, Pittsburgh, PA) and analyzed using the Image Quant software (GE Healthcare).

Oligonucleotides

SAS6a ORF: forward, ATGGATAGTTTATCTTAAAAAGTCTGGAAGAAGCTAGC; reverse, TCACTAATTTTTTGTGGATCAGCATATTTTATTGGAAC; *SAS6b* ORF: forward, ATGGCTGAGTACTAACCTTCATACAGAAAG; reverse, TCAGTTTAAATTTGGATTTTTGGTTAGCGG; *SAS6a* 5' KO arm: forward, GTTTATAAAGTACATGCTCTAAACAAATGC; reverse, CTTATTAAGTCTACTATTATATATGATATGCGAGG; *SAS6a* 3' KO arm: forward, CC-AATAAAATATCGTGCATCCAAACAAAAAATTAG; reverse, CACTAC-AAGTATAGAAGATGGTCTCCGAGC; *SAS6b* 5' KO arm: forward, GCATATTATTCAGCTTTCAGCAGAAGAG; reverse, CTTTATAAGTTTGTAT-GCTTATTAACCTG; and *SAS6b* 3' KO arm: forward, CCGCTAACCAAAAA-TCCAAATTAACAACTGA; reverse, CTTTATCATACATTCCTCAATAAATGCG.

Tetrahymena Strain Construction

Tetrahymena micro- and macronuclear integrants were transformed by biolistics (Bio-Rad, Hercules, CA) as described in (Bruns and Cassidy-Hanley, 2000). The *SAS6a* shutoff was generated by transforming pMTT-6xHis-*SAS6a* into the B2086 background. This inducible allele integrates at one of the two β -tubulin (*BTU1*) loci in *Tetrahymena*. This strain was then transformed with the blasticidin resistance variant of the pMNB1-*SAS6a* plasmid, pBLAST-*SAS6a*. 6xHis-*SAS6a* localizes to basal bodies and was considered to be functional based on this result (data not shown). The knockout allele was then selected for by growing cells in increasing concentrations of blasticidin (Invitrogen, Carlsbad, CA) in the presence or absence of induction of 6xHis-*SAS6a*. 6xHis-*SAS6a* was induced using 500 ng/ml CdCl₂. When a concentration of blasticidin was reached at which cells were unable to divide anymore (5 mg/ml), then selection was halted, but these cells continued to be grown with or without induction of 6xHis-*SAS6a*. A flow chart for how this strain was generated is shown in Supplemental Figure S2a. Only when expression of the *SAS6a* allele at the *BTU1* locus was induced were we able to completely eliminate endogenous *SAS6a* from the macronucleus (Supplemental Figure S2b). This experiment demonstrated that *SAS6a* is an essential gene and that we had generated a conditional null allele of *SAS6a*. We were now able to effectively repress transcription of the sole source of *SAS6a* by omitting CdCl₂ from the media or chelating the available CdCl₂ from the media using EDTA (Shang *et al.*, 2002b). The *SAS6b* knockout was generated by transforming a mating culture of strains B2086 and CU428 with p4T2-1 *SAS6b*. Micro-nuclear integrants were homozygosed by mating to B*VI. *SAS6b* knockout heterokaryon cell line was outcrossed to CU428. Different mating types of heterozygous paromomycin-sensitive clones were homozygosed for the *SAS6b* knockout allele by mating to B*VI. *SAS6b* knockout heterokaryon cells of different mating types were mated to each other and mated cells were selected based on resistance to paromomycin. *SAS6b* was absent from these cells based on immunofluorescence microscopy, PCR, and Southern blot. *SAS6b* macronuclear knockouts were generated by transforming starved B2086 cells with p4T2-1 *SAS6b*. Increasing copies of the knockout allele were selected for by growing cells in increasing concentrations of paromomycin until cells could no longer survive (4 mg/ml). Cells were taken from next lowest concentration and assayed for the presence of *SAS6b* by immunofluorescence and Southern blot.

Cellular Measurements

Basal bodies were counted based on the number of foci of colocalized centrin and α -tubulin present along a cortical row. Three to six rows in 40–100 cells were counted for each condition. The number of Cen1/Atu1 spots present was divided by the length of the ciliary row (μ m) on which they were present. This number was multiplied by 10 and rounded to the nearest tenth to yield the number of basal bodies per 10 μ m. The graphs represent the percentage of the population exhibiting the given density range of basal bodies was observed. Cilia were measured from the basal body to tip. Cilia from at least 20 cells ($n = 140$) were counted for each condition and were measured only if the signal was longer than the centrin signal to which it associated. Cilia lengths were rounded to the nearest tenth and grouped as shown in the figure. The ratio of α -tubulin foci associated with Cen1 foci was determined by performing linescans along a ciliary row and counting the number of times a Cen1 signal coincided with an α -tubulin signal from projection images of *Tetrahymena* cells in the indicated conditions. These numbers were converted to a ratio for each ciliary row examined. This ratio was rounded to the nearest tenth, and the number of times this ratio was observed was quantified in terms of percentage. All measurements were performed using the Metamorph imaging program (Molecular Devices). Microsoft Excel (Redmond, WA) was used for all of the calculations and statistical analysis.

Protein Extractions

One milliliter buffer/1 $\times 10^6$ *Tetrahymena* cells was used to solubilize cells in indicated conditions. Cells were resuspended in an appropriate amount of HBS (50 mM HEPES, pH 7.4, 110 mM KOAc, 150 mM NaCl, 2 mM MgCl₂, and 0.1% Tween-20) or HBST buffer (HBS with 1% Triton X-100), sonicated briefly, and incubated at 4°C with gentle agitation for 10 min. These extracts were spun down for 10 min at 6000 $\times g$ at 4°C, and the supernatant was removed. The HBST supernatant is represented as fraction 1 in Supplemental Figure S1c. The pellet from this step was resuspended in an appropriate amount of HpHS buffer (Tris, pH 9.0, and 500 mM NaCl), sonicated, incubated, and spun down as above. The supernatant is represented as fraction 2 in Supplemental Figure S1c, and the pellet from this fraction is represented as fraction 3 in Supplemental Figure S1c. In the *SAS6a* knockout experiments, 8 μ g of HpHS-soluble protein and an equivalent volume of the pellet are shown in this blot. *Sas6a* was not present in the HBST fraction in either condition. Extractions were carried out in the presence of leupeptin and pepstatin (10 and 1 μ M final concentrations, respectively).

Electron Microscopy

Tetrahymena cells were either chemically fixed (Allen 1969) or high-pressure frozen (Stemm-Wolf *et al.*, 2005). Briefly, *Tetrahymena* cells were pelleted, fixed in 3% glutaraldehyde, postfixed in 1% OsO₄, and embedded in Epon resin. A BAL-TEC HPM-010 high-pressure freezer (Boeckeler Instruments, Tucson, AZ) was used to freeze pelleted cells. These cells were freeze-substituted in 0.25% glutaraldehyde and 0.1% uranyl acetate in acetone and embedded in Lowicryl HM20 resin. Sections at 80 nm were cut, stained with uranyl acetate and lead citrate, and viewed in a Philips CM10 electron microscope (Mahwah, NJ). For immunoelectron microscopy, 55-nm serial sections were produced and labeled with either anti-*Sas6a* (1:50), anti-*Sas6b* (1:50), or anti Cen1 (1:200) primary antibodies (described above) after blocking with 1% nonfat milk PBS/Tween 20. Twenty-five-nanometer gold-conjugated goat anti-rabbit secondary antibody (Ted Pella, Redding, CA) was used to visualize the antigens. Grids were stained with uranyl acetate and lead citrate.

RESULTS

Identification, Cloning, and Expression of Tetrahymena SAS-6 Homologues

Two possible *Tetrahymena thermophila* SAS-6 homologues were identified by BLAST search of the TGD (Eisen *et al.*, 2006) using published and predicted SAS-6 sequences (Leidel *et al.*, 2005). We named the two putative ORFs, *TtSAS6a* and *TtSAS6b*, and reported the identity of *TtSAS6a* in the *Tetrahymena* basal body proteome (Kilburn *et al.*, 2007). Hereafter we will refer to *TtSAS6a* as *SAS6a* and *TtSAS6b* as *SAS6b*. Members of the SAS-6 gene family have two common features: a central coiled-coil domain and an N-terminal PISA domain (Leidel *et al.*, 2005), both *SAS6a* and *SAS6b* have these sequence motifs. Although the function of the PISA domain is unknown, it is the region of highest sequence similarity and identity among SAS-6 family members (Leidel *et al.*, 2005). On the basis of this, we used an alignment of the PISA domains of known and putative SAS-6 homologues to construct the phylogenetic tree in Figure 1a. Organisms with similar basal body morphologies, i.e., triplet microtubule bundles, appear to be more closely related to each other on this dendrogram, whereas those organisms with centriole and basal body morphologies that are less similar, i.e., microtubule singlets or doublets, have higher degrees of sequence divergence. Interestingly, *Paramecium* also has more than one SAS-6 homologue, which points to a gene duplication event somewhere in the ciliate lineage. Although the *Tetrahymena* SAS-6 homologues are most closely related to one another, they are only 59% identical across the 53-amino acid PISA domain and 34% identical across the entire protein. The magnitude of the amino acid differences between *Sas6a* and *Sas6b* lead us to speculate that these proteins may have functionally diverged as well.

To verify that these are indeed functional genes, we isolated RNA from actively dividing cells and checked for

Figure 1. Conservation and localization of *Tetrahymena* Sas6 proteins. (a) Dendrogram showing relative distances between SAS-6 genes based on PISA domain alignment. Tt, *Tetrahymena thermophila* THERM_00388200 (TtSAS6a) and THERM_00137600 (TtSAS6b); Pt, *Paramecium tetraurelia* GSPATP00011824001 (PtSAS6.1); GSPATP00008149001 (PtSAS6.2), GSPATP00005603001 (PtSAS6.3), and GSPATP00024268001 (PtSAS6.4); Hs, *Homo sapiens* GI:35038001; Mm, *Mus musculus* GI:62511043; Cr, *Chlamydomonas reinhardtii* GI:161727419; Ce, *C. elegans* GI:1754348; D, *Drosophila melanogaster* GI:62511095. (b) Sas6a localizes to basal bodies and kinetodesmal fibers in whole cells and to foci in isolated cilia preparations. Cells are oriented in this and all fluorescent images with anterior side up. Fixed wild-type *Tetrahymena* cells were labeled with antibodies to centrin (left panel, green in merge) and Sas6a (red in merge) and DAPI stain to mark DNA (blue in merge). The asterisk indicates the oral apparatus, the white arrow shows centrin and Sas6a basal body localization, and the unfilled arrow shows Sas6a kinetodesmal fiber localization. In cilia fluorescent images, fixed isolated cilia preparations were labeled with antibodies to α -tubulin (Atu1, left panel, green in merge) and rabbit IgG or Sas6a (middle panels, red in merge). The gray arrowheads show Sas6a foci present along the length of the cilia. (c) Sas6b is enriched at unciliated and assembling basal bodies. Fixed wild-type *Tetrahymena* cells were labeled with antibodies to Atu1 (left panel, green in merge), Sas6b (middle panel, red in merge) as well as DAPI stain to mark nuclei. The white arrow shows an example of an assembling basal body, the unfilled arrow shows an unciliated basal body, and the white arrowhead shows a ciliated basal body.

the presence of *SAS6a* and *SAS6b* transcripts by RT-PCR. We were able to amplify cDNAs, as predicted by TGD, for both *SAS6a* and *SAS6b*. Northern blot analysis of these same total RNA pools showed that *SAS6a* is expressed at ~ 1.3 times the level of *SAS6b* (data not shown).

Sas6a and *Sas6b* Share Basal Body Localization, But Differ in Other Subcellular Distributions

We have previously reported that GFP-Sas6a localized to basal bodies (Kilburn *et al.*, 2007) and found GFP-Sas6b to have a similar, but not identical localization pattern (Supplemental Figure S1a). However, we were interested in confirming the expression and localization of both proteins at endogenous levels. For this reason we generated polyclonal antibodies to fragments of Sas6a and Sas6b with no detectable cross-reactivity to each other (see *Materials and Methods* and Supplemental Figure 1, c and d). Using centrin as a marker for basal bodies, Sas6a localizes to all three major sites of basal bodies in *Tetrahymena*: the cortical row basal bodies (Figure 1b, arrows inset), the oral apparatus basal bodies (Figure 1b, asterisk), and the developing oral apparatus basal bodies (data not shown). Sas6a was also found at kinetodesmal fibers, observed as discontinuous lines closely associated with basal bodies (Figure 1b, unfilled arrow, and Figure 2d, white arrows). Interestingly, GFP-SAS6a does not localize to kinetodes-

mal fibers (Supplemental Figure S1a). Kinetodesmal fibers are non-microtubule-based fibers attached to the anterior side of cortical row basal bodies only after they have been inserted into the plasma membrane and as such are markers of the mature organelle (Allen, 1969). In addition to these sites, Sas6a also localized to foci in isolated cilia preparations (Figures 1b, gray arrowheads, and 2e). Western blot analysis confirmed the presence of Sas6a in this cilia preparation (Supplemental Figure S1e, fraction 1).

Like Sas6a and centrin, Sas6b localized to all basal bodies present in *Tetrahymena*, but with varying intensity compared with the more uniform intensity of Sas6a (Figure 1c, inset). Using an antibody to α -tubulin (Atu1) to label cilia and basal bodies, we found Sas6b to be specifically enriched at actively assembling basal bodies (Figure 1c, white arrow) and at unciliated basal bodies (Figure 1c, unfilled arrow), compared with mature ciliated basal bodies (Figure 1c, arrowhead). *Tetrahymena* cortical row basal bodies assemble near the anterior side of an existing basal body (Allen, 1969; Nanney, 1975), therefore the more intense Sas6b signal in the inset of Figure 1c (white arrow) likely represents an actively assembling or a recently assembled basal body. The differences of localization patterns between Sas6a and Sas6b, suggests that the two proteins do not function completely redundantly in *Tetrahymena*.

Figure 2. Sas6a and Sas6b localize to all of the same structural domains within the basal body. (a) Sas6a localizes to the cartwheel hub, spokes, electron-dense lumen, and periphery of the basal body at the proximal end. Immunoelectron micrographs showing examples of Sas6a localization observed in whole cell preparations. The Sas6a antibody was visualized using a 25-nm gold-conjugated secondary antibody. The white asterisk indicates the electron-dense lumen, the arrow points to the hub of the cartwheel, the arrowhead shows cartwheel spoke to “A” tubule attachment point, and the black asterisk shows the basal body periphery associated Sas6a. (b) Sas6b shares all of the same localizations as Sas6a. The Sas6b antibody was visualized using a 25-nm gold-conjugated secondary antibody. Immunoelectron micrographs of whole cell preparations showing examples of Sas6b localizations, the basal body structural domains are indicated as in panel a. (c) Sas6a and Sas6b localize at nearly the same frequency to the same structural domains within the basal body. The schematic shows a basal body in longitudinal section (left drawing) as well as a view of the electron-dense lumen in cross section (right, top drawing) and the cartwheel (right, bottom drawing). Structural domains were counted as indicated in the drawings (1, cartwheel hub and spokes; 2, electron-dense lumen; 3, periphery of the basal body). The chart shows the frequency (n) gold particles were observed for each antibody at each position. (d) Sas6a localizes to the kinetodesmal fiber. The white arrows indicate anti-Sas6a particles found along the kinetodesmal fiber associated with a basal body in cross section (right). (e) Sas6a localizes to the periphery of cilia. The arrow indicates the position of a 25-nm gold particle.

Sas6a and Sas6b Localize to the Cartwheel Hub and Spoke Tips, Electron-dense Lumen, and Site of New Assembly

Sas6a and Sas6b were both found at basal bodies, but the resolution of light microscopy was insufficient to determine which specific structure(s) within the basal body contain these two proteins. We therefore used immunoelectron microscopy of whole cells to determine the high-resolution localization of both proteins at basal bodies. All mature *Tetrahymena* basal bodies contain three recognizable structures in the lumen of the basal body: the cartwheel (most proximal), the electron-dense material (midpoint), and the transition zone (most distal; Allen, 1969; Kilburn *et al.*, 2007). Previous work from our lab showed that overexpressed GFP-Sas6a localized to the region of the basal body occupied by the hub of the cartwheel (Kilburn *et al.*, 2007). Additionally, a recent report on SAS-6 in *Chlamydomonas* showed an identical ultrastructural localization pattern (Nakazawa *et al.*, 2007). We found that Sas6a, which like its GFP-tagged counterpart localized to the cartwheel hub (Figure 2a, arrow) but surprisingly also to the spoke attachment points to the “A” tubule of the microtubule triplets (Figure 2a, arrowhead). Sas6a was also found in the electron-dense material in the basal body lumen (Figure 2a, white asterisk) and on the periphery near the base (Figure 2a, black asterisk). The frequency of these basal body localizations are quantified in Figure 2c (1: cartwheel localization, 2: electron-dense lumen, 3: area surrounding proximal end of basal body).

Sas6b shared all of the same basal body localizations with Sas6a: the cartwheel hub (Figure 2b, arrow) and spoke attachment points (Figure 2b, arrowhead), the electron-dense luminal material (Figure 2b, white asterisk), and the area surrounding the proximal end of the basal body (Figure 2b, black asterisk). Both proteins were observed in the same locations in the basal body at approximately equal frequencies (Figure 2c). These data combined with our fluorescence data suggest that *SAS6a* and *SAS6b* may have evolved overlapping as well as distinct roles in basal body assembly and function.

SAS6a Is Required for Generation of New Basal Bodies and Maintenance of Cilia

To test the hypothesis that *SAS6a* has evolved an essential function separate from that of *SAS6b*, we designed an experiment to test the requirements of *SAS6a* for cell viability and basal body assembly (see *Materials and Methods* and Supplemental Figure S2a). We could only generate a complete assortment of a *SAS6a* deletion allele when an inducible 6xHis-tagged version of *SAS6a* was expressed from a different locus (Supplemental Figure S2b). This experiment demonstrated that *SAS6a* is required for cell viability and that we had constructed a strain in which we could control expression of *SAS6a*. It also showed that *SAS6b* alone is not sufficient for viability. Under conditions where *SAS6a* expression was repressed, 86% of cells divided three times or less compared with 6% of wild-type cells under identical conditions (Supplemental Figure S2c). Based on these data, *SAS6a* is essential for cell division, and the SAS-6 genes in *Tetrahymena* are not redundant for this essential function.

SAS-6 is essential for centriole and basal body assembly in *C. elegans*, *Chlamydomonas*, *Drosophila*, and mammalian cell culture (Dammermann *et al.*, 2004; Leidel *et al.*, 2005; Nakazawa *et al.*, 2007; Rodrigues-Martins *et al.*, 2007a; Vladar and Stearns, 2007). We therefore predicted that *SAS6a* would be essential for basal body duplication in *Tetrahymena*. *Tetrahymena* cells assemble new basal bodies anterior to existing ones and at semiregular intervals along the anterior-posterior axis of the cell (Allen, 1969; Nanney, 1975). Before cell division, the basal bodies along the middle and posterior of the cell duplicate at a higher frequency than the basal bodies at the cell anterior (Nanney, 1975). *Tetrahymena* divide along the cell equator, and thus both daughter cells possess basal bodies and their associated cilia from the parent. A defect in new basal body assembly would result in larger than normal gaps between existing basal bodies as well as fewer basal bodies at the anterior or posterior end of a cell that had just divided. Alternatively, if *SAS6a* were required for maintenance of existing basal bodies, then we may expect to see all

basal bodies disappear as was observed in γ -tubulin (*GTU1*) and centrion (*CEN1*) null mutants (Shang *et al.*, 2002a; Stemm-Wolf *et al.*, 2005). Figure 3a shows cells labeled for centrion (*Cen1*) and α -tubulin (*Atu1*) to mark basal bodies, in which the *SAS6a* gene has been induced or repressed for 2 d. In cases in which *SAS6a* has been induced (+*SAS6a*), we found that all of the cells were ciliated (Figure 3a, arrow inset) and that *Cen1* and *Atu1* colocalized at basal bodies as was expected with this control (Figure 3a, arrowhead). Instances of *Cen1* and *Atu1* colocalization indicate either fully formed basal bodies or basal bodies that have developed beyond the early intermediates, regardless of the presence of a cilium. When the *SAS6a* gene was repressed using EDTA (−*SAS6a*) cilia were severely truncated or absent (Figure 3a, inset arrow). In addition to the short cilia observed upon repression of *SAS6a*, *Cen1* labeling did not always colocalize with *Atu1* labeling (Figure 3a, gray arrowhead). The lack of *Atu1* signal at *Cen1*-positive locations indicates there were fewer bona fide basal bodies per cell than *Cen1* signal alone indicated. Western blot analysis confirmed that cells grown in EDTA lacked detectable *Sas6a* (Figure 3b). We used instances of *Cen1* and *Atu1* colocalization to quantify the number of basal bodies present in each condition. On average, cells expressing *SAS6a* (+*SAS6a*) had 5.7 ± 1.1 basal bodies per $10 \mu\text{m}$ ($n = 282$) and cilia $5.5 \pm 1 \mu\text{m}$ long ($n = 140$), whereas cells with repressed *SAS6a* expression (−*SAS6a*, + EDTA) had an average of 3.9 ± 1 basal bodies per $10 \mu\text{m}$ (Figure 3c, $n = 240$, $p < 0.01$) and cilia $2 \pm 1 \mu\text{m}$ long (Figure 3d, $n = 140$, $p < 0.001$). As evidence that the presence of EDTA in the media suppresses expression of *SAS6a* more so than absence of CdCl_2 from the media, cells grown in media lacking CdCl_2 (−*SAS6a*) displayed cilia of intermediate length, $3.2 \pm 1.1 \mu\text{m}$ long (Figure 3d, $n = 140$, $p < 0.001$).

To quantify the frequency that we observed *Cen1* foci lacking *Atu1* signal, we counted the number of *Cen1* foci and the number of *Atu1* foci that colocalized with these spots. These numbers were converted to a ratio for each ciliary row counted. Cells expressing *SAS6a* had a tubulin-to-centrion ratio of 1 ± 0.05 ($n = 282$), indicating that the vast majority of *Cen1*-positive foci represented fully formed basal bodies. In contrast to this, repression of *SAS6a* resulted in an average tubulin-to-centrion ratio of 0.85 ± 0.17 (Figure 3e, $n = 240$, $p < 0.001$), indicating that a significant portion of *Cen1*-positive locations likely did not represent fully formed basal bodies.

We next tested whether loss of *SAS6a* affected the localization of *Sas6b*. In a manner indistinguishable from WT cells, *Sas6b* and *Atu1* colocalized at unciliated and most ciliated basal bodies when *SAS6a* was expressed (+*SAS6a*, Figure 4a). When relative fluorescence intensity was quantified, we found that 2.9-fold more *Sas6b* signal was present at unciliated and assembling basal bodies than at ciliated basal bodies ($n = 149$). In contrast to this, cells in which *SAS6a* was repressed (−*SAS6a*) had only 1.2-fold more *Sas6b* fluorescence signal at unciliated basal bodies than was present at ciliated basal bodies ($n = 151$). These cells also more frequently displayed *Sas6b* foci in the absence of an *Atu1* signal (Figure 4a, arrowheads). +*SAS6a* cells usually had *Sas6b* present at *Atu1* foci, with an average *Atu1* foci-to-*Sas6b* foci ratio of 0.99 ± 0.13 ($n = 107$). In contrast to this, −*SAS6a* cells had significantly more *Sas6b* foci per ciliary row than *Atu1* foci, with an average ratio of 0.73 ± 0.12 (Supplemental Figure S3b, $n = 120$, $p < 0.001$). On the basis of the observation that *Sas6b* localizes to presumptive sites of new assembly independently of *Sas6a* and our differential localization data, we suggest that *Sas6b* may in fact act

upstream of or parallel to *Sas6a* in the basal body assembly pathway.

Although Western blot analysis showed undetectable levels of *Sas6a* when expression was repressed (Figure 3b), we wanted to determine whether *Sas6a* could be detected by immunofluorescence. In cells in which *SAS6a* expression was induced (+*SAS6a*) the presence of an *Atu1* signal correlated with the presence of *Sas6a* signal (Supplemental Figure S3a, arrow). Not surprisingly, cells in which *SAS6a* expression was repressed (−*SAS6a*) showed significantly reduced levels of *Sas6a* (Supplemental Figure S3a). These cells also displayed basal bodies where *Sas6a* was absent (Supplemental Figure S3a, arrowhead). Furthermore, cells expressing *SAS6a* (+*SAS6a*) had an *Sas6a* signal associated with centrion signal (Supplemental Figure S2d, arrow inset). However, cells where *SAS6a* expression was repressed (−*SAS6a*) often displayed centrion only foci (Supplemental Figure S2d, arrowheads inset) in addition to the normal basal body associated *Sas6a* (Supplemental Figure S2d, arrow inset). The lingering presence of *Sas6a* at some basal bodies indicates that either some *Sas6a* is being expressed in these cells or that some fraction of the protein remains stably associated with basal bodies. Although *Sas6a* signal is absent from most basal bodies, it remains at the basal bodies found at the anterior end of the cell. These basal bodies are among the oldest present in the cell (Nanney, 1975). Our data shows that *SAS6a* is required for new basal body assembly and maintenance of cilia length, but not for maintenance of existing basal bodies. Additionally, we show that endogenous levels of *SAS6b* are not able to rescue loss of *SAS6a* in basal body assembly or cilia length maintenance.

SAS6a Is Required for Assembly of the Cartwheel

Tetrahymena basal bodies are different from mammalian basal bodies in that every mature basal body possesses a cartwheel and electron-dense lumen (Allen, 1969). The basal body is physically separated from the cytoplasm by its microtubule walls, the cartwheel at the proximal end, and the transition zone at the distal end. We were interested whether the instances of an *Atu1* signal lacking a *Sas6a* signal (Supplemental Figure S3a, −*SAS6a*) represented abnormal basal bodies. Therefore, we examined +*SAS6a* and −*SAS6a* cells by electron microscopy to determine if there were specific basal body structural defects associated with loss of *SAS6a*. Cells expressing *SAS6a* (+*SAS6a*) displayed all of the normal features associated with *Tetrahymena* basal bodies: the cartwheel (Figure 4b, unfilled arrows), electron-dense luminal material (Figure 4b, arrowheads), and ribosomes excluded from lumen of the basal body (Figure 4b, white arrows), illustrating the physical separation of the basal body interior from the cytoplasm. In basal bodies of −*SAS6a* cells, the cartwheel was absent in 67% of basal bodies examined (Figure 4b, unfilled arrow, $n = 36$) and the lumen of the basal body where the electron-dense material would normally reside was instead replaced by what are presumably ribosomes in 40% of basal bodies examined (Figure 4b, white arrow, $n = 173$), showing that the cytoplasm had infiltrated the lumen of the basal body. Examination of serial sections through adjacent basal bodies in the −*SAS6a* cells (Figure 4b and Supplemental Figure S4) shows that loss of the cartwheel (unfilled arrows) is correlated with appearance of ribosomes in the lumen of the basal body (white arrows). Both of the basal bodies in cross section display the ninefold symmetry typical of normal basal bodies and are also associated with markers of mature *Tetrahymena* basal bodies, postciliary microtubules (Figure 4b, PC), and kinetodesmal fibers (Figure 4b, KF). We therefore conclude that *SAS6a* is

Figure 3. *SAS6a* is required for new basal body assembly. (a) *SAS6a* is required for microtubule incorporation into basal bodies and for maintenance of cilia length. Fixed whole cells either grown in the presence (+*SAS6a*) or absence (−*SAS6a*) of *SAS6a* were labeled with antibodies to Cen1 (left panels, red in merge) and Atu1 (middle panels, green in merge). The white arrows show examples of a ciliated basal body, the white arrowhead shows an unciliated basal body, and the gray arrowhead shows a putative incomplete basal body: Cen1 signal is present, but Atu1 signal is absent. (b) *SAS6a* expression is effectively repressed by inclusion of 100 µM EDTA in the media of the *SAS6a* conditional null strain. Western blot showing levels of Sas6a and Atu1 in the indicated fractions for the indicated conditions. Equal total protein was loaded in this blot. Fraction 3, protein soluble in HphS buffer (see *Materials and Methods*), and fraction 4, the insoluble pellet from this treatment. Sas6a was not found in fractions 1 and 2 in either condition (data not shown). (c) Graphical representation of the basal body assembly defect seen upon activation or repression of *SAS6a*. Basal bodies were counted based on instances of Cen1 and Atu1 colocalization. Graph shows the frequency of basal body density observed in a population of cells grown with (+*SAS6a*) or without (−*SAS6a*) *SAS6a*. −*SAS6a* cells have significantly fewer basal bodies per 10 µm than +*SAS6a* cells. (d) Cells grown without induction of *SAS6a* display shorter cilia than cells grown with induction of *SAS6a*. Graphical representation of the distribution of cilium length seen in a population of cells grown with active repression of *SAS6a* (−*SAS6a*, +EDTA), incomplete repression of *SAS6a* (−*SAS6a*), or with expression of *SAS6a* (+*SAS6a*). Cilia were measured from the base to the tip and plotted as the frequency of range of lengths observed in the population. (e) Loss of *SAS6a* results in a significant increase of aberrant basal bodies. Graphical representation of the frequency at which Cen1 foci were observed with Atu1 foci shows that cells grown in the absence of *SAS6a* (−*SAS6a*) had fewer instances of Cen1 and Atu1 colocalization than cells grown with *SAS6a* (+*SAS6a*). The number of Cen1 foci colocalizing with Atu1 foci were counted along individual ciliary rows for these calculations.

Figure 4. SAS6a is required for the maintenance of the cartwheel structure. (a) Fixed cells grown in the presence (+SAS6a) or absence (−SAS6a) were labeled with antibodies to Atu1 (left panels, green in merge) and Sas6b (middle panels, red in merge). Sas6b signal at unciliated or assembling basal bodies (arrowheads) was on average 2.9-fold greater than Sas6b signal at ciliated basal bodies (arrows) when SAS6a was expressed ($n = 149$). When SAS6a expression was repressed (−SAS6a), this ratio decreased to 1.2 ($n = 151$). (b) Repression of SAS6a is correlated with the loss of the cartwheel from mature basal bodies and the appearance of ribosomes in lumen of the basal body. Electron micrographs of whole cell preparations showing basal bodies in longitudinal section (left panels) or cross section (right panels) grown with (+SAS6a) or without (−SAS6a). +SAS6a basal bodies have the cartwheel (unfilled arrows), the electron-dense lumen (white arrowhead), and the markers of mature basal bodies: the postciliary microtubules (PC) and kinetodesmal fibers (KF). Ribosomes (white arrows) are excluded from the basal body lumen. −SAS6a basal bodies often lacked a complete cartwheel (unfilled arrows), and loss of the cartwheel was correlated with the appearance of ribosomes in the lumen of the basal body. The percentages indicate the frequency at which basal bodies like the ones in the figure were observed (see Materials and Methods). Examination of serial sections of two basal bodies in cross section (1, most proximal; 4, most distal) demonstrates this. Structural features are indicated as in +SAS6a condition.

required for the assembly of the cartwheel and that without the cartwheel the physical separation of the basal body from the cytoplasm is compromised, which correlates with the loss of normal luminal structures.

SAS6b Influences the Timing and Location of New Basal Body Assembly

Having shown that SAS6a is an essential gene in *Tetrahymena*, we were curious as to whether or not SAS6b was also essential. Surprisingly, we discovered that SAS6b was in fact not an essential gene, as mating two SAS6b knockout heterokaryon strains to each other produced viable progeny. We were also able to generate SAS6b macronuclear null cells using paromomycin resistance as a selectable marker for deletion of SAS6b. Southern blot analysis (Figure 5b), immunofluorescence microscopy using Sas6b antibodies (Figure 5d), and PCR (data not shown) confirmed that wild-type SAS6b was completely absent from both SAS6b null strains. Furthermore, SAS6b null cells are capable of multiple rounds of cell division with little or no reduction in growth rate (data not shown). SAS6b is therefore not essential for cell division and the presence of SAS6a may be sufficient to sustain viability.

Although it appeared that SAS6b is not essential, we were curious if there was a phenotype associated with these knockout cells. Therefore, we examined SAS6b null cells by immunofluorescence microscopy using antibodies to Cen1 and Atu1 to mark basal bodies. Cells were categorized into five different phenotypic classes, examples of which are shown in Figure 5a. Sixty-three percent of cells had obvious defects in cortical organization. These

defects ranged from mild bends in the cortical rows (data not shown) to the spurring of an additional cortical row off of an exiting one (Figure 5a, top left, arrow). Twenty-two percent of SAS6b null cells possessed an abnormal existing oral apparatus (data not shown) or a prematurely assembled new oral apparatus (Figure 5a, top right). 12% of cells had visible gaps between exiting basal bodies with otherwise normal cortical organization (Figure 5a, middle left). A small fraction of cells (2%) were categorized as severely abnormal. These cells had many fewer basal bodies and lacked an oral apparatus altogether (Figure 5a, middle right). Even fewer cells (1%) were categorized as normal, despite the presence of basal body clusters along a cortical row (Figure 5a, bottom left, arrow). Cortical row basal bodies are aligned along the anterior posterior axis, and the presence of a basal body cluster such as the one shown in Figure 5a represents an abnormal situation. As was done with −SAS6a cells, the number of basal bodies per 10 μm were counted in −SAS6b and wild-type cells based on the number of Atu1 foci colocalizing with Cen1 foci. −SAS6b cells had an average of 4.6 ± 0.9 ($n = 161$) basal bodies per 10 μm compared with 5.5 ± 1.1 basal bodies per 10 μm for wild-type cells ($n = 304$, $p < 0.001$). The distribution of basal body densities observed in wild-type and −SAS6b cells is shown graphically in Figure 5c. The mild decrease in average basal body density in SAS6b null cells indicates that although the spatial arrangement of basal bodies is altered, new basal bodies are nonetheless being assembled, sometimes earlier than expected.

Figure 5. SAS6b is important for but not essential to vegetative cell growth and new basal body assembly. (a) Loss of *SAS6b* is correlated with appearance of cells with fewer basal bodies and/or lacking complete oral apparatus. Fixed wild-type (WT) and *SAS6b* KO cells (-*SAS6b*) were labeled with antibodies to Cen1 (left panels, red merge) and Atu1 (middle panels, green merge). Cells ($n = 300$) that resembled the cells in the images shown in this figure were counted. The percentage of cells that matched these representative images is shown in the bottom right-hand corner. The arrow in the first cell (63%) indicates a branching of a cortical row. The arrow in the fifth panel (1%) indicates a cluster of basal bodies present along a cortical row. (b) *SAS6b* is not required for vegetative cell growth. Southern blot showing genomic DNA from indicated strains digested with XhoI and NheI. The blot was probed with DNA 1 kb 5' of the *SAS6b* start site. Diagram below blot shows wild-type (WT) *SAS6b* locus and null (KO) locus. The wild-type fragment recognized by the probe is 2.3 kb and the null fragment is 1.3 kb. (c) *SAS6b* null cells have fewer basal bodies per 10 μm than wild type. (d) *Sas6b* basal body signal is absent in *SAS6b* KO cells. Fixed wild-type (WT) or *SAS6b* KO cells (-*SAS6b*) were labeled with the anti-Sas6b antibody.

Loss of *SAS6b* Does Not Significantly Affect Cartwheel Formation

Having shown that loss of *SAS6a* results in the absence of the cartwheel from basal bodies, we were curious if loss of *SAS6b* produced a similar result. If *Sas6a* and *Sas6b* both functioned as structural components of the cartwheel, then we might expect that the loss of *SAS6b* would affect the formation of the cartwheel in the same manner as loss of *SAS6a*. However, based on all of the data presented thus far,

it seemed more likely that loss of *SAS6b* would not affect the formation or maintenance of the cartwheel. To test this prediction, we analyzed *SAS6b* null cells by thin-section transmission electron microscopy. *SAS6b* null cells (-*SAS6b*) possessed all of the structural features found in wild-type basal bodies: the cartwheel at the proximal end (Figure 6b, unfilled arrow), the electron-dense luminal material (arrowhead), postciliary microtubules (PC), and kinetodesmal fibers (KF). Although all of the normal structural

Figure 6. Loss of *SAS6b* does not affect cartwheel assembly, but does result in accumulation of electron densities near the site of new basal body assembly. (a) Electron micrographs showing wild-type (WT) basal bodies in longitudinal or cross section. Arrowhead, the electron-dense lumen; unfilled arrow, a cartwheel in cross section. A new basal body (asterisk) is seen in the panel on the right just below the kinetodesmal fiber (KF) and opposite of the postciliary microtubules (PC). (b) Basal bodies from *SAS6b* null cells (*-SAS6b*) possess all of the structures associated with wild-type basal bodies. Relevant structures are indicated as in panel a. Fifty-one percent of basal bodies examined ($n = 200$) showed electron densities (arrows) at or near the site of new assembly. (c) Cen1 localizes to the site of new basal body assembly in wild-type (WT) cells. Immunoelectron micrographs using a 25-nm gold-conjugated secondary antibody showing the localization of Cen1. The arrow indicates Cen1 localization at the site of new assembly. (d) Cen1 localizes to the site of new assembly in *SAS6b* null cells (*-SAS6b*) and is associated with the electron densities. Immunoelectron micrographs labeled as in panel c. The arrows point to Cen1 association with the electron densities.

elements were present in *SAS6b* null basal bodies, 51% ($n = 200$) of basal bodies examined had electron-dense regions at or near the site of new assembly (Figure 6b, arrows), which was significantly higher than the 15% ($n = 189$) observed for wild-type basal bodies. New basal bodies form just adjacent to the kinetodesmal fiber and opposite of the postciliary microtubules (Figure 6a, asterisk). It is therefore possible that the electron-dense regions seen in the *SAS6b* null cells represent new basal body assembly intermediates. Alternatively, the electron densities seen more commonly in the *SAS6b* null cells could be distorted basal body collar regions. To help exclude this second possibility, we examined the localization of Cen1 in *SAS6b* null cells. Cen1 localizes to the site of new basal body assembly in *Tetrahymena* (Stemm-Wolf *et al.*, 2005). If the aggregates seen in the *SAS6b* null are associated with new basal body assembly events, then the localization of Cen1 to this density could reflect this possibility. As in wild-type cells, Cen1 localized to the site of new assembly in *SAS6b* null cells (*-SAS6b*). More interestingly, Cen1 was also found on the electron densities or in close proximity to them (Figure 6d, arrows). The localization of Cen1 to the electron densities may reflect a role for the protein in determining the site of new basal body assembly. It also supports the possibility that the electron densities are somehow related to new basal body assembly.

Overexpression of *SAS6b* Results in Inappropriate New Basal Body Assembly

Overexpression of SAS-6 proteins has been shown to cause the formation of excess centrioles in human cells as well as in *Drosophila* (Leidel *et al.*, 2005; Peel *et al.*, 2007; Strnad *et al.*, 2007). Overexpression of *SAS6a* in *Tetrahymena* results in aggregations of the protein along the cortex of the cell, but not in ectopic basal bodies (data not shown). However, it is possible that over the course of evolution the basal body seeding property observed for other Sas-6 proteins has been assumed by the *SAS6b* gene. In controls, all cells overexpressing GFP ($n = 200$) displayed normal localization patterns for both Sas6b and centrin: Sas6b was enriched at the developing oral apparatus, which is always found along the equator of the cell below the existing oral apparatus (Figure 7a, ++GFP). In contrast, after approximately two cell divisions cells overexpressing GFP-*SAS6b* displayed three classes of cortical organization defects when labeled with antibodies to GFP and Cen1. Forty-seven percent of cells examined ($n = 203$) had disorganized cortical row basal bodies (Figure 7b, left panel). 5% of cells had an abnormal existing oral apparatus (Figure 7b, middle panel), and 4% of cells displayed clusters of basal bodies in abnormal locations. The right panel in Figure 7b shows an example of one such cell; the arrows point to clusters of basal bodies present along a cortical row where such accumulations of basal bodies is not observed in wild-type cells. Additionally, cells with the most severe cortical architecture abnormalities had more intense GFP-Sas6b signal. As further evidence that the clusters of GFP-*SAS6b* represent bona fide basal bodies, these clusters were also labeled with Sas6a antibody (Figure 7c).

To investigate the temporal localization pattern of Sas6a and Sas6b in the same cell, we induced GFP-*SAS6b* expression in a *SAS6b* macronuclear knockout. As stated previously, new basal bodies assemble anterior to existing basal bodies adjacent to the kinetodesmal fiber. If Sas6a and Sas6b are recruited to basal bodies at the same time then new basal body assembly events would be observed as a colocalization of GFP-Sas6b and Sas6a at the new anterior basal body. If either protein is recruited to sites of new assembly in advance of the other, then this would be observed as the presence of either Sas6a or GFP-Sas6b in the absence of the other. GFP-Sas6b appears at sites of new basal body assembly in the absence of Sas6a. At higher magnification two GFP-Sas6b foci (Figure 7d, arrowheads) are seen just to the cells left of the Sas6a kinetodesmal fiber signal (Figure 7d, arrow). This data shows that GFP-Sas6b localizes to sites of new basal body assembly before Sas6a basal body incorporation.

DISCUSSION

The defining feature of basal bodies and centrioles is their ninefold symmetrical arrangement of microtubules. However, the molecular mechanism(s) by which this symmetry is faithfully established have only recently been addressed. In *C. elegans*, SAS-6 is required for the formation of the central tube, the scaffold around which the microtubules of the centriole assemble (Pelletier *et al.*, 2006). In many other eukaryotes, a central hub with nine spokes (the cartwheel) is present at the base of the basal body or procentriole. Each spoke makes contact with the "A" tubule of a microtubule triplet (Lange and Gull, 1996). These observations imply that the cartwheel is important for establishment of ninefold symmetry. *Chlamydomonas* and possibly *Drosophila* SAS-6 mutants assemble centrioles lacking the cartwheel, and these

Figure 7. *SAS6b* helps dictate sites of new basal body assembly and acts upstream of *SAS6a*. (a) Overexpression of GFP does not affect cellular morphology. Fixed cells overexpressing GFP (++GFP) were labeled with antibodies to centrin (left panel, green in merge) and Sas6b (middle panel, red in merge). Sas6b is enriched at the developing oral apparatus (inset), which is always found immediately below the existing oral apparatus along the equator of the cell (100%, $n = 200$). (b) Overexpression of GFP-*SAS6b* causes the formation of clusters of basal bodies in abnormal locations. Fixed cells overexpressing GFP-*SAS6b* were labeled with antibodies to Cen1 (red) and GFP (green). Forty-seven percent of cells in the population had disorganized cortical rows and large gaps between existing basal bodies (left panel). Five percent of cells had an abnormal oral apparatus (middle panel), and 4% of cells had clusters of basal bodies in abnormal locations (right panel, $n = 203$). Arrows point to clusters of basal bodies in abnormal locations. (c) Sas6a colocalizes with GFP-*Sas6b* clusters. Fixed cells overexpressing GFP-*SAS6b* were labeled with antibodies to Sas6a and GFP. Arrows indicate Sas6a and GFP colocalization. (d) GFP-*Sas6b* localizes to presumptive sites of new basal body assembly before Sas6a. Fixed *SAS6b* macronuclear null cells carrying an inducible GFP-*SAS6b* allele at a different locus were induced to express GFP-*SAS6b* and labeled with antibodies to Sas6a (left panel, red in merge) and GFP (middle panel, green in merge). The arrow shows Sas6a kinetodesmal fiber localization. The arrowheads show GFP-*Sas6b* doublets representing a new basal body assembly event.

centrioles often had greater than or less than nine microtubule triplets (Nakazawa *et al.*, 2007; Rodrigues-Martins *et al.*, 2007a). Furthermore, SAS-6 has been shown to localize to the cartwheel hub in *Tetrahymena* and *Chlamydomonas* (Kilburn *et al.*, 2007; Nakazawa *et al.*, 2007). In human cells SAS-6 is only found at the immature centriole (Strnad *et al.*, 2007), the only centriole of the pair with a cartwheel (Vorobjev and Chentsov Yu, 1982). Based on the cumulative observations from all these systems, it seems likely that the central tube of *C. elegans* is analogous to the cartwheel and that SAS-6 is required for the formation of both, either as a structural component (Strnad and Gonczy, 2008) and/or as a recruitment factor for other components.

It is now clear that SAS-6 is component of the hub of the cartwheel in *Tetrahymena* and most other organisms (Kilburn *et al.*, 2007; Nakazawa *et al.*, 2007). However, endogenous Sas6a and Sas6b not only localize to the cartwheel hub, but also to the spokes and electron-dense lumen and at the site of new basal body assembly. These localizations were not observed for CrSas-6 (Nakazawa *et al.*, 2007). This discrepancy may be due to the specificity of our antibodies or it may be due to CrSas-6 being localized in biochemically isolated centrioles, whereas our study used whole cell preparations. It may also be that the noncartwheel hub localization patterns observed herein are *Tetrahymena* specific.

On repression of *SAS6a*, the cartwheel hub and basal body luminal structures were absent from mature basal bodies. This result provides confirmation in another model system that Sas6 proteins function at centrioles and basal bodies as components of the cartwheel. The presence of mature basal

body structures associated with basal bodies lacking the cartwheel combined with the overall decrease in basal body number observed in *-SAS6a* cells leaves open the possibility that after *SAS6a* depletion the protein was turned over at mature basal bodies and subsequently the cartwheel was disassembled. Recently, it has been shown that GFP-Sas6a can be recruited to the cartwheel in the absence of new basal body assembly (Pearson *et al.*, 2008), showing that there is a dynamic population of Sas6a at the cartwheel. Furthermore, in humans, the cartwheel is lost from procentrioles at the onset of mitosis concomitant with the disappearance of Hs-Sas6 (Lange and Gull, 1996; Strnad *et al.*, 2007). These observations support the possibility that loss of Sas6 from centrioles results in disassembly of the cartwheel. Our observation that Cen1 localizes to foci absent of all or significant Atu1 signal suggests that Cen1 acts upstream of Sas6a, contrary to what has been reported in human cells (Strnad *et al.*, 2007). There are a few possible alternative explanations for this observation: *Tetrahymena* may employ different assembly mechanisms than human cells, these basal bodies may have progressed beyond early assembly intermediates and then fallen apart, or basal body assembly may not proceed in a linear pathway. The last possibility seems particularly attractive as both *Drosophila* and *Chlamydomonas* SAS-6 mutants assemble triplet microtubules in the absence of the cartwheel.

Apart from its role in central tube or cartwheel assembly, overexpression of SAS-6 has been shown in a few systems to lead to the formation of excess centrioles (Leidel *et al.*, 2005; Peel *et al.*, 2007; Strnad *et al.*, 2007), suggesting a role for the

protein in the initiation of new centriole assembly. However, it is unclear as to whether or not this potential role for the protein is specifically related to or separate from its cartwheel localization. If the Sas6 proteins not only function as structural components of the cartwheel, but also as an initiation factor for new basal body or centriole assembly, then it is possible that the two SAS-6 genes present in *Tetrahymena* reflect these potential functions. On the basis of multiple lines of evidence, we conclude that the *Tetrahymena* SAS-6 genes have in fact evolved such that *SAS6b* is predominantly responsible for the timing and spatial determination of new basal body assembly, whereas *SAS6a* has more of a structural role in the basal body. Although Sas6a and Sas6b localize to all of the same structural domains within the basal body, Sas6a is a component of the kinetodesmal fiber, whereas Sas6b is not. Sas6b is specifically enriched at immature basal bodies, which implies an increased requirement for the protein at immature basal bodies, whereas the levels of Sas6a are nearly constant at all basal bodies. Loss of *SAS6b* does not seem to affect cartwheel morphology, but does affect the distribution of basal bodies within the cell. The significant increase in basal bodies with Cen1-labeled electron densities near the site of new assembly in *SAS6b* null cells provides an attractive explanation for the cortical disorganization seen in *SAS6b* null cells. It is possible that these densities represent sites of new basal body assembly from a single mother. The assembly of a new basal body at one of these sites may result in the branching off of an additional cortical row as basal body assembly proceeds, thus resulting in cortical disorganization. Additionally, GFP-*SAS6b* was found to localize to presumptive sites of new assembly in the absence of Sas6a. Furthermore, Cen1 and Sas6b do not depend on the presence of Sas6a for their localization. Overexpression of GFP-*SAS6b* caused the formation of clusters of new basal bodies in abnormal locations within the cell; no such activity for Sas6a was observed. This finding would not be unique to *Tetrahymena* as it has been observed in *Drosophila* as well as mammalian cell culture systems (Leidel *et al.*, 2005; Peel *et al.*, 2007; Strnad *et al.*, 2007). Cumulatively our observations suggest a model in which *SAS6b* normally acts to spatially and temporally dictate where new basal body assembly will occur, whereas *SAS6a* normally functions as a structural protein required for microtubule incorporation into basal bodies.

The presence of two SAS-6 homologues in *Tetrahymena* leaves open the possibility of functional redundancy between *SAS6a* and *SAS6b*. However, this functional redundancy is likely not mutual, as *SAS6a* is an essential gene, whereas *SAS6b* is not. This situation is not without precedent; for example, only one of the two α -tubulin genes, *TUB1*, is essential for viability in yeast (Schatz *et al.*, 1986). The pleiotropy of *SAS6b* null phenotypes likely results from the same cause; in the absence of *SAS6b* the spatial and temporal cues provided by Sas6b are absent and so basal bodies assemble at inappropriate locations. This could manifest in a disorganized cortex, a misplaced oral apparatus, or a complete lack of assembly. Cells derived from a division of a cell with a misplaced oral apparatus may therefore not inherit an oral apparatus. It is likely that given enough cell divisions, *SAS6b* null cells will all eventually appear as the most severe phenotype shown in Figure 5a.

Loss of SAS-6 has been shown to inhibit cilia assembly (Rodrigues-Martins *et al.*, 2007a; Vladar and Stearns, 2007). However, it was unclear whether this requirement was in the establishment of cilia or the maintenance of their length. We conclude that *SAS6a* is not only required for the generation of cilia, but also for the maintenance of their length

based on the following logic. Cells expressing *SAS6a* have wild-type cilia lengths and no defects in new basal body assembly. However, upon repression of *SAS6a* expression the cells become unable to assemble new basal bodies and there is a concomitant shortening of the cilia they do possess. If *SAS6a* was not required for the maintenance of cilia length, then cilia present before repression of *SAS6a* should not be affected by the absence of *SAS6a*. Consistent with a ciliary role for *SAS6a*, the protein was seen in cilia by immunofluorescence, immunoelectron microscopy, and Western blot.

We have presented evidence here that the *Tetrahymena* SAS-6 genes have evolved separate functions in both basal body assembly and maintenance of cilia length. *SAS6a* is required for maintenance of cilia length and cartwheel assembly, whereas *SAS6b* is important for spatially determining the site of new basal body assembly. In the future we look forward to pursuing the mechanism by which each protein performs the potentially separate functions of initiation and cartwheel assembly. Specifically, we would like to pursue the identification of complexes containing each or both proteins with the goal of linking or separating the cartwheel assembly from new basal body assembly initiation.

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