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# Supporting Online Material for

## Revisiting the Role of the Mother Centriole in Centriole Biogenesis

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## Material and Methods

### *Flies and Husbandry*

pUASp-SAK, pUbqGFP-SAK, pUbq-GFP-DSAS6 and pUbq-DSAS4-GFP transgenic flies were built (thebestgene.com). V32-gal4 flies were used as a driver for overexpression in female germ line (kindly provided by Daniel St Johnston). OreR stocks were used as wild type. All flies were reared according to standard procedures and maintained at 25 ºC.

### *Constructs*

pUASp-SAK, pUbq-GFP-DSAS-6 and pUbq-DSAS4-GFP constructs were made with the gateway system from Invitrogen. SAK entry vector and mycSAK construct were described in (*S1*).The pUASp destination vector was obtained from DRGC. The destination vector pUbq-GFP was kindly provided by Renata Basto.

### *RNAi and transfections*

Production of dsRNA and transfections were performed as described *(S1)*. As described in legend of Figure 4, cells were transfected with dsRNA for GFP (control), DSAS-6 or DSAS-4. One day afterwards, cells were transfected with DNA coding for myc (control) or myc-SAK. Cells were fixed 3 days afterwards and centrosome number was scored. Primers used for dsRNA production were:

*SAKF-TAATACGACTCACTATAGGGAGAATACGGGAGGAATTTAAGCAAGTC;*
*SAKR-TAATACGACTCACTATAGGGAGATTATAACGCGTCGGAAGCAGTCT;*
*DSAS-6F-TAATACGACTCACTATAGGGAGATGTAGTGCGCATGCTGAAGGAC;*
*DSAS-6R-TAATACGACTCACTATAGGGAGAGCTGCGCTGCTCGTTTATTTTG;*
*DSAS-4F-TAATACGACTCACTATAGGGAGATCTCGCGGCGCTTAGTCGTT;*
*DSAS-4R-TAATACGACTCACTATAGGGAGAGGCGCAGGATTGGGAGGTG.*

### *Antibodies*

We used the following antibodies: rabbit anti-SAK (produced and affinity purified in our own lab, 1:10(*1*)); rat anti-α-tubulin-YL1/2 (Oxford Bioscences,1:50); mouse anti-γ-tubulin-GTU88 (Sigma,1:25); rabbit anti-centrosomin (Cnn,1:300(*S1*)); rabbit anti-CP190 (*S2*) (Rb 188, 1:300); chicken anti-D-PLP (produced in our own lab 1:1000), rabbit anti-PCNA (produced in our own lab rb 3324, 1:500), rabbit anti-DSAS-4 (1:500(*S3*)), chicken anti-DSAS-6 (produced in our own lab 1:1000). The secondary antibodies used (1:250 for imunofluorescence and 1:10000 for western blot) to detect all antigens were conjugated with Rhodamine Redex, Peroxidase (Jackson Imunochemicals), FITC (SIGMA) or CY5 (Jackson Imunochemicals) and had minimal cross reactivity to other species.

### *Western blot*

Protein extracts from embryos, eggs, ovaries and tissue cultured cells were prepared by homogenizing in SDS-PAGE sample buffer, boiling for 10 minutes and spinning at high speed to clear the lysate.

### *Embryos/Eggs/Ovaries and 14 stage oocytes staining*

Embryos/Eggs from 4- to 5-day-old flies were collected at 25 ºC on small agar plates. In order to look at meiosis II stages and first mitotic divisions, shorter time collections were performed. Agar plates were changed four times, 15 min each. Embryos/Eggs were collected, after different time points as indicated. Embryos/Eggs were dechorionated in a 50 % freshly made bleach solution and washed with distilled water. Vitteline membrane was removed in a 50 % solution of methanol and heptane for 3 min with strong agitation. Eggs were fixed and stained as described (*S4*). Ovaries from 2-3 days old well-fed females were dissected in PBS and the tips of the ovaries were teased open. Gonads were fixed for 30 minutes in 4% formaldehyde in PBS; staining was performed as described *(*S5*)*. For 14 stage oocytes staining, ovaries from 2-3 days well-fed females were dissected in absolute methanol and transferred to a 14 ml plastic tube containing 3 ml of fresh methanol. About 12-24 single ovaries were prepared in this way and

then sonicated with a water bath sonicater (Branson 2000) for 5-10 cycles of 2 seconds each, until the majority of the 14 stage oocytes had lost their chorion. Staining of 14 stage oocytes was then performed as described (*S6*).

**Imaging**

Embryos/Eggs, ovaries and 14 stage oocytes were all analysed in a confocal scanning head (model 1024: Bio-Rad Laboratories) mounted on an Optiphot microscope (Nikon) and in Leica SP-2 confocal scanning. Images shown are the maximum-intensity projections of optical sections acquired at 0,5 µm. An axiovert 200M microscope and a leica DMRA2 were used for observation and countings of tissue culture cells. Images on these microscopes were acquired with a Photometrics Cool SNAP HQ camera and the image analysis software Metamorph®. To calculate the area of the egg/embryo occupied by centrosomes, images from embryos/eggs overexpressing SAK were exported to image analysis software ImageJ where we used the option "Measure Area". The ratio between Area of Free Asters/ Area of Total Embryo was then determined. The following categories were obtained for embryos/eggs overexpressing SAK: 0-2 %, 2-20 %, 20-60 % and >60 %. To estimate the numbers of centrosomes present in embryos and eggs, images representative of each time collection were exported to Metamorph® (n=10). The option "Counting Number of Cells" was used.

**Transmission electron microscopy**

2-3 hr wild type and embryos/eggs overexpressing SAK were collected on small agar plates at 24 ºC. Embryos/eggs were dechorionated in a 50% bleach solution and washed with distilled water. Dechorionated embryos/eggs were treated with 25% glutaraldehyde in PBS with an equal volume of heptane for 3 min. Embryos/eggs were then transferred for 30 min to 2,5% glutaraldehyde in PBS and the vitelline envelope was removed with tungsten needles. The devitellinized embryos/eggs were incubated overnight at 4°C in glutaraldehyde 2,5%. After rinsing in PBS, the samples were post-fixed in 1% osmium tetroxide for 2 hr at 4°C and dehydrated in a graded series of alcohols, embedded in an Epon-Araldite mixture and polymerized at 60°C for 48h. Random and serial sections cut using an LKB Nova ultramicrotome were collected on copper grids and stained with uranyl acetate and lead citrate. Preparations were observed and photographed with a Philips CM10 electron microscope.

**Immuno-electron microscopy**

2-3 hr wild type and pUbqGFP-SAK embryos were dechorionated as above and the vitelline envelope was removed manually after incubation for 20 min in a solution of paraformaldehyde, distilled water and heptane. Devitellinized embryos were fixed for 10 min in 4% paraformaldehyde and 1% Triton-X. Embryos were cut longitudinally and incubated 1 hr at room temperature in PBS with 0,1% BSA. CP190 antigen was detected by incubation for 4 hr at room temperature with the polyclonal antiserum Rb188 diluted 1:400. pUbqGFP-SAK was localized in embryos after incubation overnight at 4°C in either mouse monoclonal (Roche, 1:150) or rabbit polyclonal (Abcam, 1:100 dilution) antibodies against GFP. Samples were then incubated for 1hr with secondary antibody coupled to 5-nm gold particles (BioCell, Cardiff), diluted 1:20. Samples were fixed overnight in a solution of 2% glutaraldehyde and 3% paraformaldehyde in PBS and post-fixed for 1 hr in osmium tetroxide and processed as above for inclusion in Epon-Araldite and sectioning. Controls were performed with omission of primary antibody.

A

| | % Non- developed embryos | |
|---|---|---|
| T ºC | WT | V32-gal4/UAS-SAK |
| 25 | 8 | 100 |
| 29 | 9 | 100 |

B

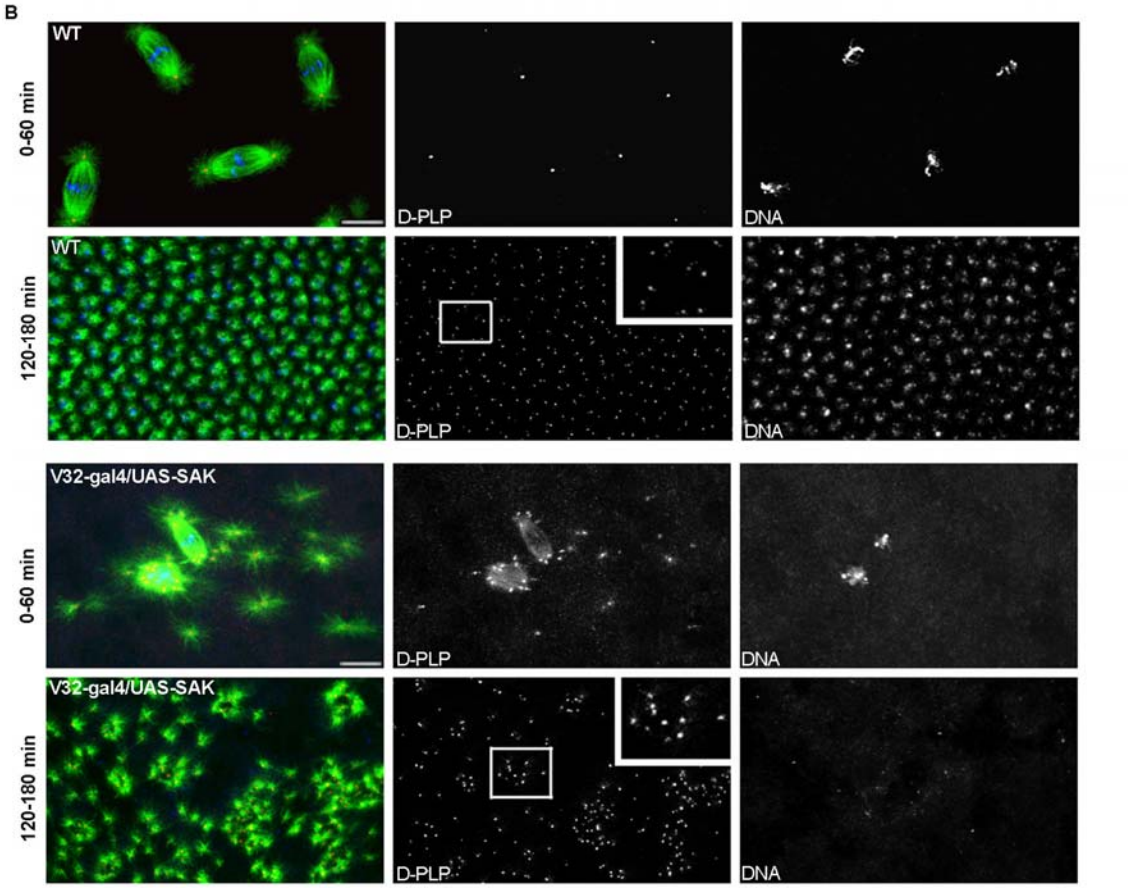


**Figure S1- Embryos overexpressing SAK do not develop and show amplified centrosomes containing D-PLP**. (A) Embryos from V32-gal4/UAS-SAK females fail to develop at both 25 and 29 ºC (n=100) as larvae do not eclode. (B) Free centrosomes contain D-PLP foci. 0-60 min old embryos and 120-180 min old embryos were analized. D-PLP (red), α-tubulin (green) and DNA (blue). Scale bar 10 μm.

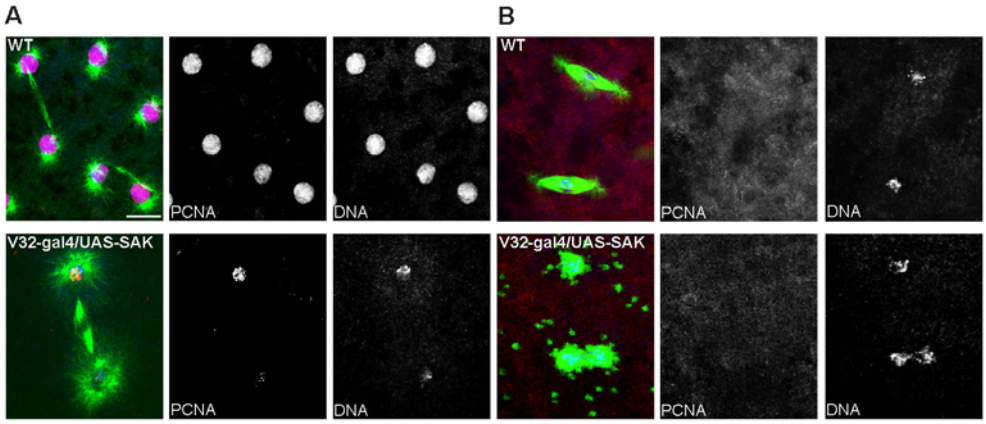


**Figure S2- Embryos overexpressing SAK are not in S-phase.** The cell cycle of wild type embryos alternates between S and M phases. There are no gap phases, such that S-phase markers start to be seen at the end of mitosis. **(A)** PCNA stains DNA at beginning of S-phase (late telophase) in the WT embryo. In very young embryos overexpressing SAK, as in the WT, PCNA is visible at the end of first mitosis. **(B)** The DNA localized in mitosis of wild type embryos or in arrested spindles of SAK overexpressing embryos does not contain PCNA staining, suggesting it is not in S-phase. PCNA (red), α-tubulin (green) and DNA (blue). Scale bar 10 μm.

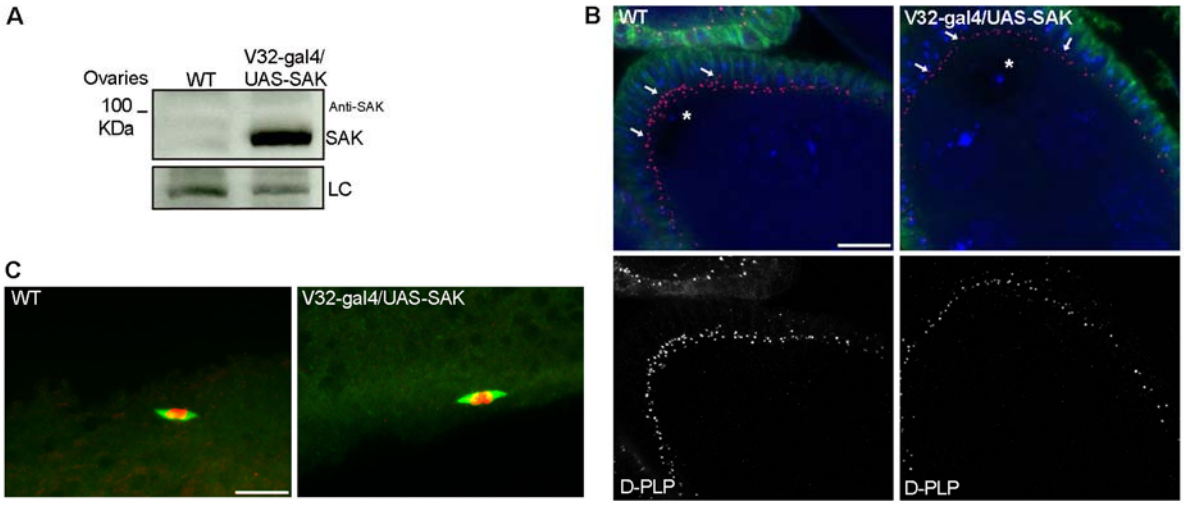


**Figure S3- No centrosome amplification is detected during late oogenesis and meiosis I in females overexpressing SAK in the germline. (a**, **b)** Overexpression of UAS-SAK in ovaries using the maternal driver V32-gal4. LC- Loading control. D-PLP (red), α-tubulin (green) and DNA (blue). * Oocyte nucleus. Arrows indicate centrioles from follicle cells. Scale bar 10 μm. **(c)** Meiosis I occurs normally in eggs from *V32-gal4/UAS-SAK* females. PLP (red), α-tubulin (green) and DNA (red). Scale bar 10 μm. Note that no free centrosomes are visible at these stages.

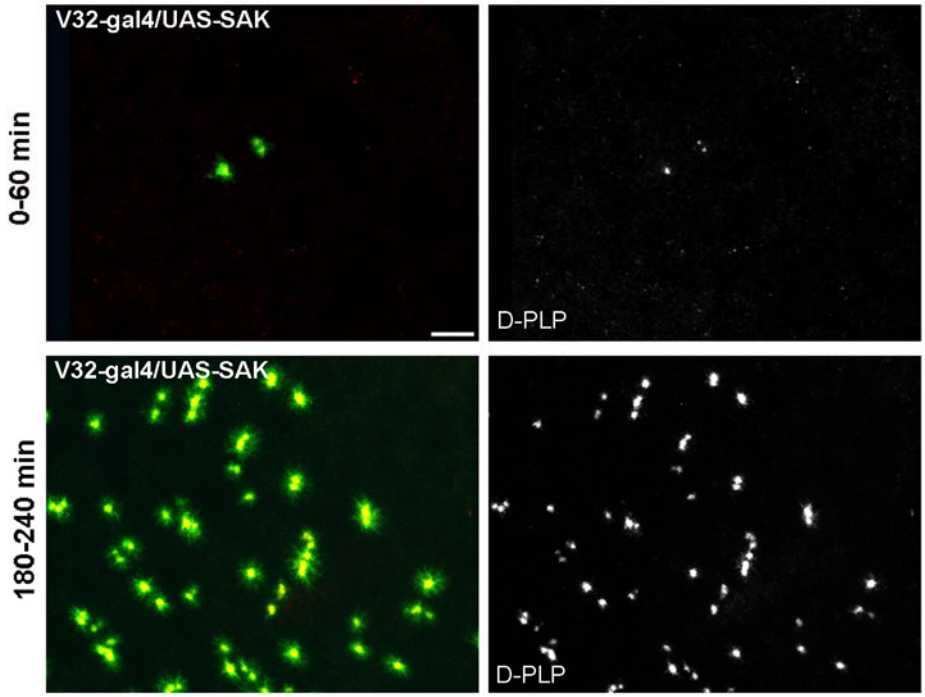


**Figure S4-** ***De novo*** **formed centrosomes contain D-PLP.** Centrosomes contain D-PLP foci. 0-60 min old eggs (top panel) and 180-240 min old eggs (bottom panel). D-PLP (red), α-tubulin (green) and DNA (blue). Scale bar 10 μm.

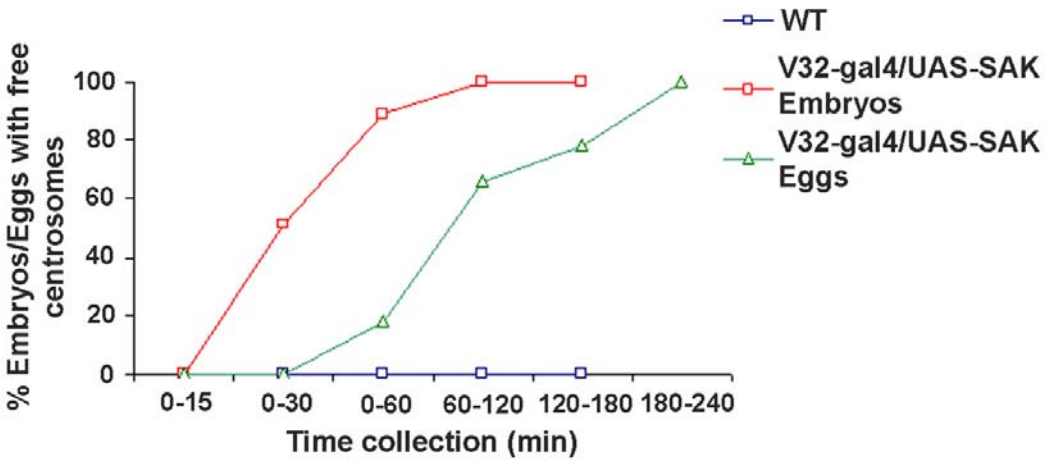


**Figure S5- Centrosome biogenesis starts later in eggs than in embryos.** *De novo* formation in eggs takes longer to be activated as compared to centrosome amplification in embryos overexpressing SAK. No free centrosomes (not associated with a spindle) were detected in wild type embryos.

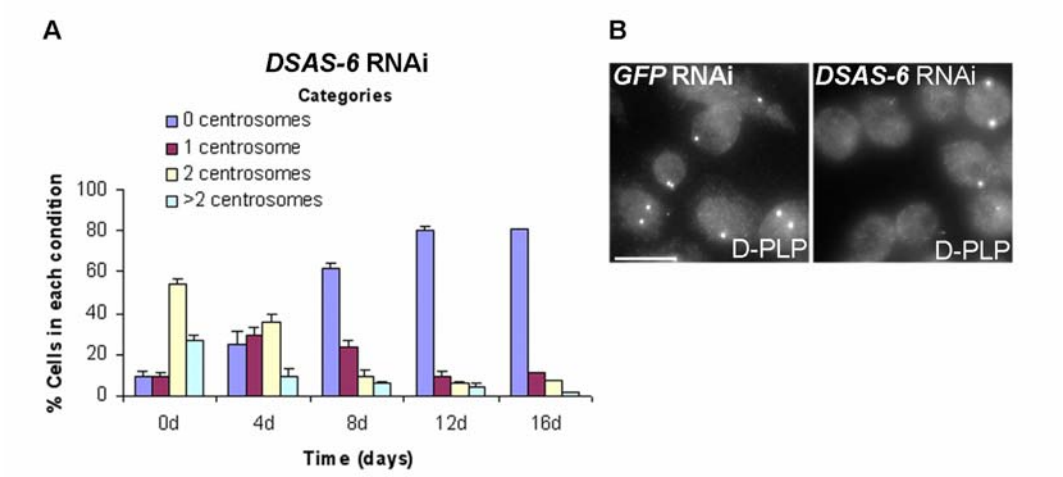


**Figure S6- DSAS-6 is required for centriole duplication in *Drosophila* cells**. **A)** Serial transfections of cells with DSAS-6 dsRNA, followed by fixation and staining for a centrosome and centriolar marker (D-PLP), show dilution of centrosomes with number of divisions. Error bars indicate standard error of mean. **B)** *DSAS-6* RNAi leads to the absence of D-PLP foci in interphase cells. Image taken after the first transfection. Scale bar 10 μm.

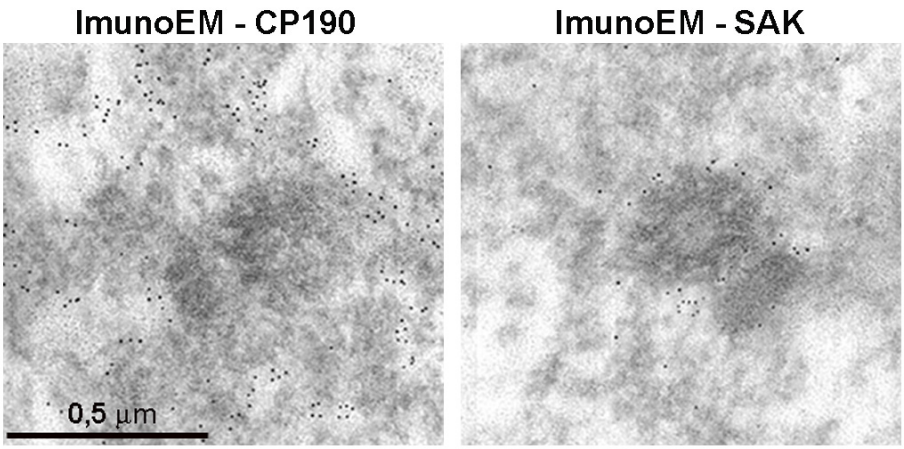


**Figure S7- SAK localizes close to the centrioles.** SAK localizes close to the centrioles whereas CP190 localizes to pericentriolar material in wild type embryos, as shown by Immunoelectron microscopy.

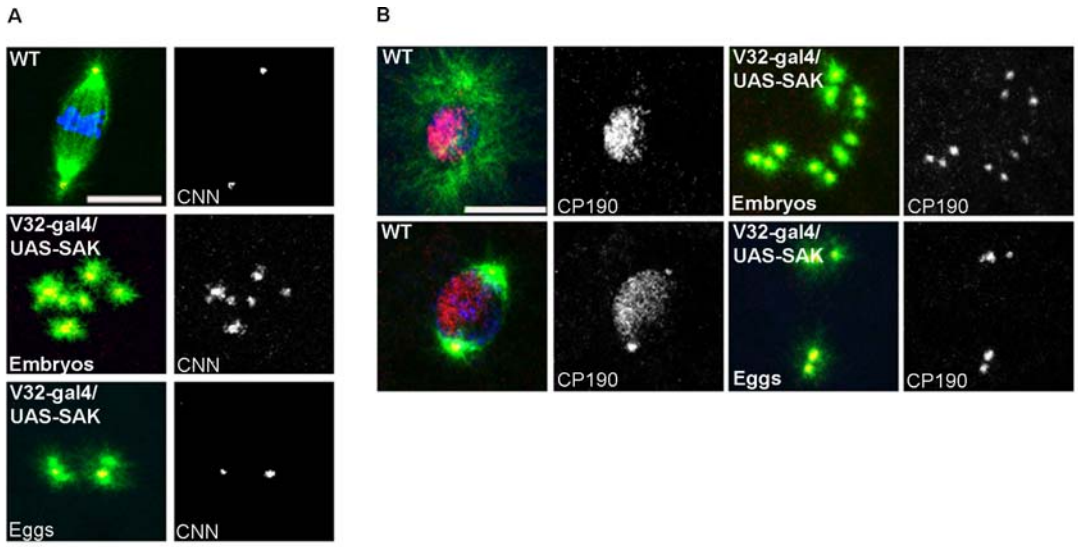


**Figure S8-** ***De novo*** **and canonical-formed centrosomes show centrosomal markers. (A, B)** Centrosomes in both 0-1 h embryos and eggs overexpressing SAK contain CNN and CP190. CNN (red), CP190 (red), α-tubulin (green), DNA (blue). Normally, CP190 shuttles from the nucleus in interphase to centrosomes in mitosis. Note that CP190 localizes to free centrosomes. Scale bar 10 μm.

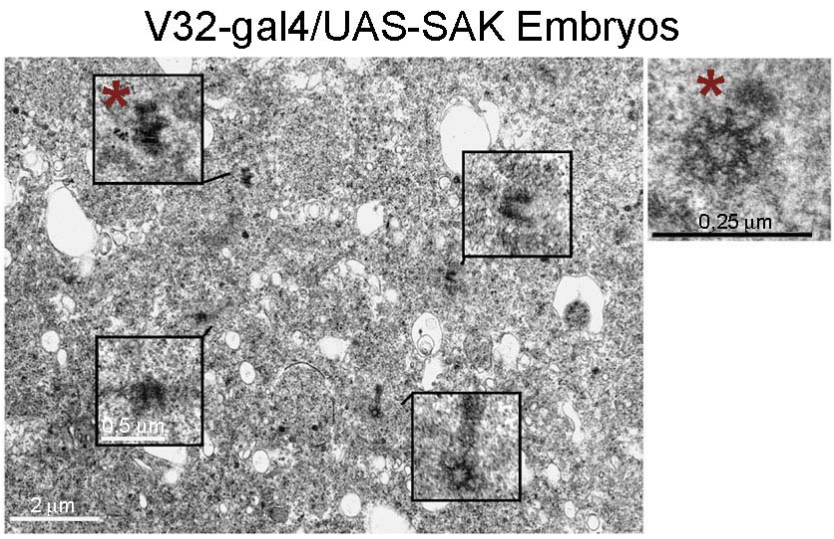


**Figure S9-** Structurally normal centrosomes are visible by electron microscopy in embryos overexpressing SAK (scale bar as indicated). Note the existence of pro-centrioles (*) close to already formed centrioles.

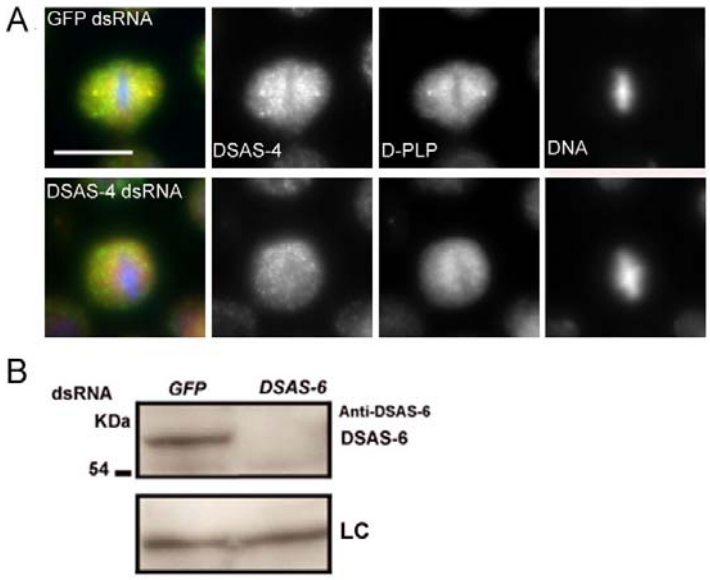


**Figure S10- DSAS-6 and DSAS-4 are efficiently depleted after RNAi. (A)** RNAi against DSAS-4 reduces the amount of endogenous DSAS-4 protein in S2 cells as no centrioles and no DSAS-4 labelling is seen at the poles of mitotic cells (because of the low levels of DSAS-4 in cells, it is difficult to detect this protein by western analysis(*S3*)). Scale bar 10 μm. **(B)** RNAi against DSAS-6 reduces the amount of endogenous DSAS-6 protein in S2 cells. LC- loading control.

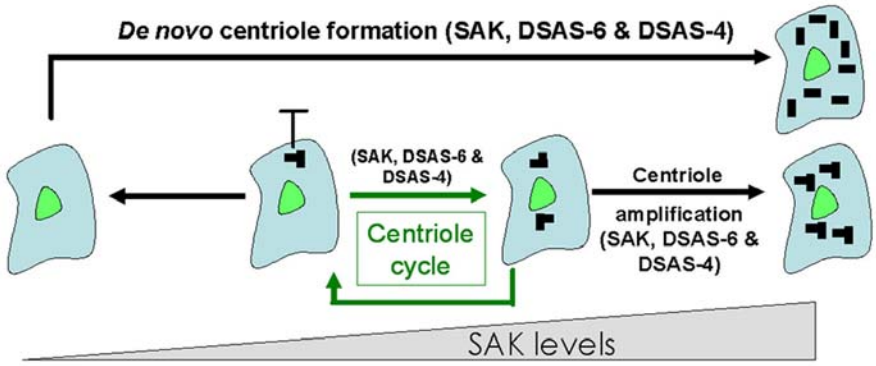


**Figure S11- Regulation of centriole formation by SAK.** Our experiments suggest a self-assembly process of centriole biogenesis requiring SAK, DSAS-6 and also DSAS-4 (requirement for SAK, DSAS-6 and DSAS4 shown in parenthesis). The regulation of the levels of SAK is crucial as its absence leads to the absence of duplication and excessive amounts lead to centriole amplification. Amplification occurs next to centrioles, suggesting they are platforms for assembly of regulatory complexes, when these structures are present. In their absence, centrioles are formed *de novo*. The establishment of such a platform where SAK can attach and promote centriole formation is probably less efficient in the absence of centrioles. As a result canonical duplication is faster then de novo (this work and Ref (*S7)*, (*S8)*). The idea of a scaffold where regulatory molecules bind in order to catalyse the self organization of macromolecular structures is not new in biological systems *(S9)*. It has been widely advocated that the self organization of the spindle relies on protein interaction gradients around chromatin, such as the one of RanGTP *(S9)*. It is not yet known whether SAS4 and SAS6 are necessary for the recruitment, assembly and/or stabilization of structural components. However, in *C. elegans*, SAS-6 is necessary for the formation and elongation of the central centriolar tube, while the assembly of the singlet MTs onto the central tube is dependent on SAS-4 *(S10)*. SAS6 may have a regulatory role in centriolar assembly, as overexpression of SAS-6 in human cells leads to centrosome amplification, similarly to SAK *(S11*). The fact that both canonical and de novo centriole formation depend on SAK, DSAS-6 and DSAS-4 suggests these as good candidates to target to control aberrant centriole formation in human disease.

**Supporting References**


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