Cohesin cleavage is insufficient for centriole disengagement in Drosophila

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Summary
Centriole disengagement is thought to act as a licensing mechanism restricting centrosome duplication to once per cell cycle [1] and to depend on cleavage of the cohesin complex by separase [1-3]. Whether this is a conserved mechanism in eukaryotic cells remains to be determined. We show that artificial cohesin cleavage in *Drosophila* embryos fails to cause detectable centriole disengagement. In contrast, inhibition of Cyclin dependent kinase (Cdk1), triggers rapid disengagement in metapase-arrested embryos. Our results raise the possibility that in these early embryonic divisions centriole engagement depends on Cdk1 activity not cohesin.

Mother and daughter centrioles are kept tightly together and in an orthogonal arrangement (engaged) from the time of their duplication until the subsequent mitosis and their disengagement occurs during later stages of mitosis. The molecular mechanisms behind centriole engagement during S-phase and their disengagement during mitotic exit are far from being understood. But it has been proposed that during mitotic exit, centriole disengagement is mediated by Separase [1, 2], a thiol protease known to promote disjunction of sister chromatids at anaphase onset [4].

Until recently, the only known target of separase was the cohesin complex, a ring-shaped multisubunit protein complex (composed of Smc1, Smc3, Rad21/Scc1 and Scc3/SA) known to entrap sister DNA molecules inside its ring and thereby promote sister chromatid cohesion until the anaphase onset [4]. Separase-mediated cleavage of cohesin's kleisin subunit (Scc1/Rad21 in mitotic cells) leads to opening of the cohesin ring and subsequent sister chromatid disjunction. The fact that separase has been implicated in the process of centriole disengagement [1, 2] led to the speculation that the cohesin complex could be the molecular "glue" that holds mother/daughter centrioles together from the time of their duplication until mitotic exit, in a way similar to how these complexes hold sister DNA molecules together. Indeed, many reports have suggested that cohesin interacts with some centrosomal proteins, that cohesin and other cohesion-proteins localize to the centrosome, and that cohesin depletion leads to centrosomal defects [Ref [5] and references therein]. Nevertheless, attempts to clearly define the role of cohesin in this process have let to conflicting results. While initial studies report that expression of a non-

cleavable cohesin complex (NC-Rad21) in HeLa cells does not prevent disengagement, suggesting that this process depends on a yet undefined separase target other than Rad21 [2], recent studies using purified centrioles from mammalian cells, suggest that centriole engagement is dependent on cohesins' integrity [3].

We have recently been able to artificially reproduce a bona-fide mitotic exit from metaphase-arrested embryos [6]. In this experimental setup, separation of sister chromatids is achieved using a system to inactivate cohesin complexes by an exogenous protease (Tabacho Ech Virus - TEV) [6, 7]. Proper mitotic exit, in turn, is driven by artificial downregulation of Cdk using high doses of the CKI p27. Cohesin cleavage and inhibition of Cdk are both necessary and sufficient to reproduce a bonafide anaphase and mitotic exit with normal kinetics of chromatid separation, proper relocation of the Chromosome Passenger Complex to the spindle mid-zone, normal inactivation of the Spindle Assembly Checkpoint and timely chromosome decondensation and nuclear envelope reformation [6].

To evaluate whether this artificially induced mitotic exit is also accompanied by proper centriole disengagement we have repeated the same experiments in embryos previously injected with mRNA coding for a fluorescent centriole marker (Sas4-EGFP). In most somatic cell types, disengaged centrioles are known to remain tightly joined by cohesion fibres during interphase, preventing centriole separation. However, this tight cohesion is usually not present in the rapid embryonic divisions in many species, including *Drosophila* syncytial division, making it an ideal system to easily visualize (dis)engaged centrioles. EGFP-Sas4 allowed us to distinguish two (disengaged) centrioles during the last states of mitosis (Fig. S1), which immediately further separate during S-phase (note that in these syncytial cycles centrosome duplication and separation occur simultaneously).

To block normal mitotic exit we made use of a catalytically dead form of the E2 ubiquitin ligase Ubch10 (UbcH10^{C114S}), which stably arrests *Drosophila* embryos in metaphase[6]. Under such arrest, the centrosome cycle is blocked and metaphase bipolar spindles contain two centrosomes with engaged mother/daughter centrioles each (Fig 1A, left panels and data not shown). Due to the close proximity of engaged mother/daughter centrioles, only one Sas4-GFP focus could be detected per centrosome.

We have then artificially induced anaphase and mitotic exit in UbcH10^{C114S}–arrested embryos carrying TEV-sensitive cohesin complexes by co-injection of TEV protease (to destroy cohesins) and p27 (to inactivate Cdk1), and evaluated the effects on the distance between mother/daughter centrioles. Distances were determined by the maximal width of the signal originated from a centriole pair (*ie*, the diameter of a single Sas4-GFP foci or the maximal distance between two adjacent foci). We observed that within a few minutes of TEV+p27 injection, centrioles disengage, as judged by the increase in the width of Sas-4 signal, ultimately leading to two distinct dots (Fig 1).

The fact that centriole disengagement can be observed in our artificial mitotic exit suggests that this process is either dependent on cohesin cleavage, on Cdk down-regulation or both. To distinguish between these three possibilities we performed microinjection experiments where cohesin cleavage was induced in the absence of Cdk inhibition (TEV protease injection) and where Cdk inhibition was promoted without accompanying cohesin cleavage (p27 injection).

Consistent with our previous report, TEV-mediated cleavage of cohesin in metaphase-arrested embryos, triggers sister chromatid separation within a few minutes[6]. Upon cleavage of cohesin, however, no change in centriole structure could be observed (Fig 1) and the Sas4-GFP signal remained as a single focus without detectable change in the width of the signal (Fig 1 B,C). The simplest explanation for this finding is that cohesin has little or no role in maintaining centriole engagement. We cannot however fully exclude the possibility that additional forces preclude the detection of changes induced by cohesin cleavage or that centriolar cohesin is inaccessible to TEV protease.

Whereas cohesin cleavage alone did not produce any detectable effects on engaged centrioles, Cdk inibition, in contrast, was sufficient to induce centriole disengagment even in the absence of proper chromosome disjunction. Upon p27 injection centriole disengagement was observed with a similar kinetics to the disengagement observed in the TEV+p27 experiments (Fig. 1).

Our previous experiments revealed that Cdk inactivation in metaphase-arrested embryos was not accompanied by prompt Separase activation, as sister chromatids did not move apart during induced mitotic exit [6]. Our results therefore also raise the possibility that separase is not universally involved in centriole disengagement. In agreement, previous studies in Drosophila failed to detect any centrosome duplication defects in separase mutant embryos [8].

While Cdk inhibition was sufficient to trigger centriole disengagement, no further separation of sister centrioles could be observed. This finding suggests that even in *Drosophila* embryos, where centriole disengagement is immediately followed by centrosome separation, these are mechanistically different processes: centriole disengagment appears to depend on a drop in Cyclin B-Cdk activity whereas centrosome separation is likely to depend on a subsequent rise on cyclin B levels and/or DNA replication.

In summary, in contrast to the recent observation in mammalian cells, our experiments support the idea that centriole engagement does not depend on the integrity of the cohesin complex, at least in *Drosophila* embryos. In agreement, recent studies propose that cleavage of a novel centrosomal substrate for separase - pericentrin/kendrin - is required for centriole disengagement [9]. Importantly, our experiments further demonstrate that centriole disengagement during mitotic exit, as many other aspect of this key transition, can be negatively regulated by Cdk activity. This supports a role for Cdk1 in preventing premature centriole disengagement in *Drosophila* early embryos. Further experiments will be required to investigate whether this results from a direct Cdk-dependent phosphorylation of centrosome components or rather an indirect consequence

of changing pericentriolar organization or microtubule forces, as recently suggested [10].

Figure 1 – A- Embryos containing TEV-cleavable Rad21 ($Rad21^{TEV}$) as their sole source of Rad21 were arrested in metaphase by injection of $Ubch10^{C114S}$ followed by injection of either TEV protease + p27 (top), TEV protease (middle) or p27 (bottom). Times (min:s) are relative to the time of TEV injection. Embryos express His2A-mRFP1 (red) and have been previously injected with mRNA coding for the centriolar marker EGFP-Sas4 (green) Insets show higher magnification (×2.5) of a centriole pairs. Scale bars, 10 µm. **B-** Quantification of the relative distances between mother/daughter centrioles over time, after co-injection of TEV with p27 or injection of TEV protease or p27. Distances were determined by the maximal width of the signal originated from a centriole pair (*ie*, the diameter of a single Sas4-GFP foci or the maximal distance between two adjacent foci) and are normalised to the distance before injection (t=0). Error bars are standard deviation. **C-** Relative centriole separation observed 15 minutes after injection of TEV+p27, TEV or p27, normalized to the distance before injection (as in B). For each experimental condition at least 100 centriole pairs, from 5 independent experiments, were measured.

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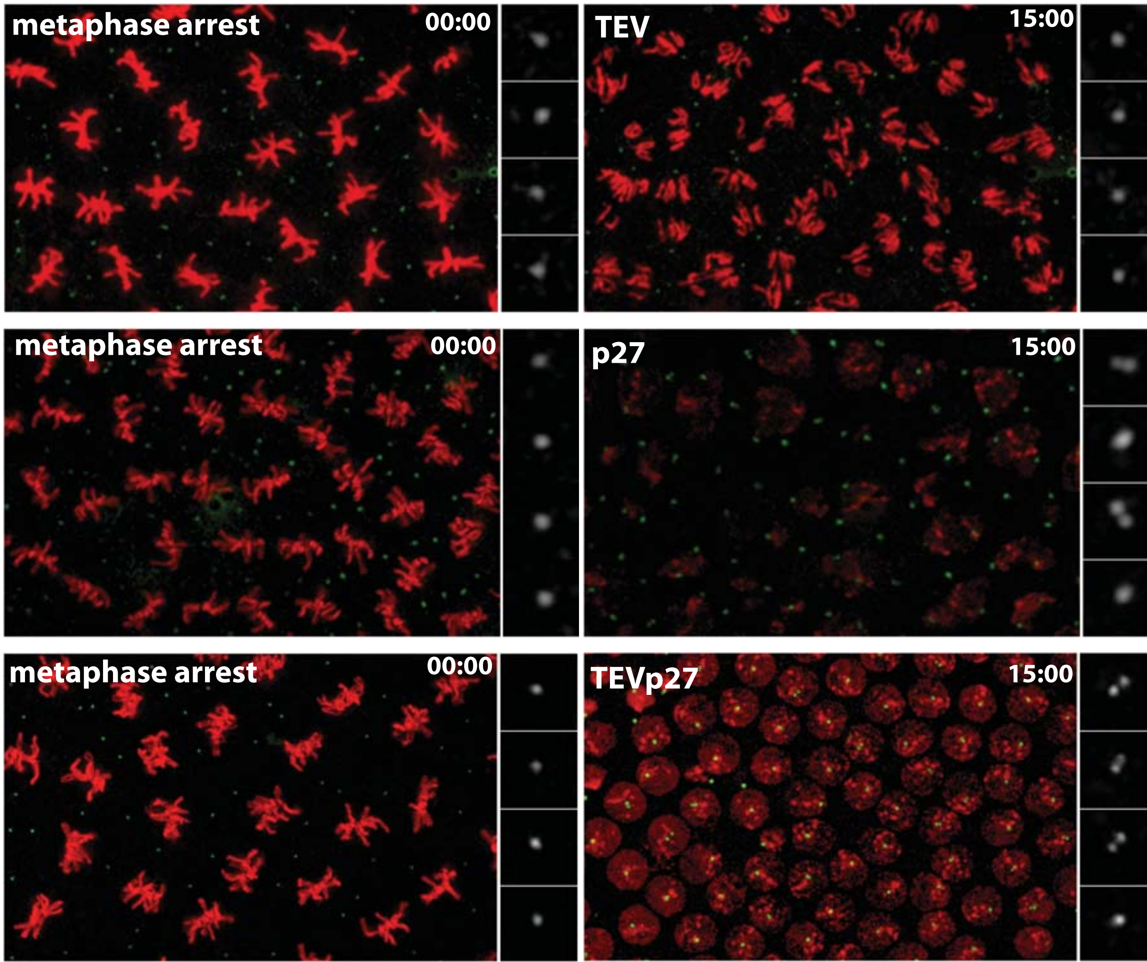
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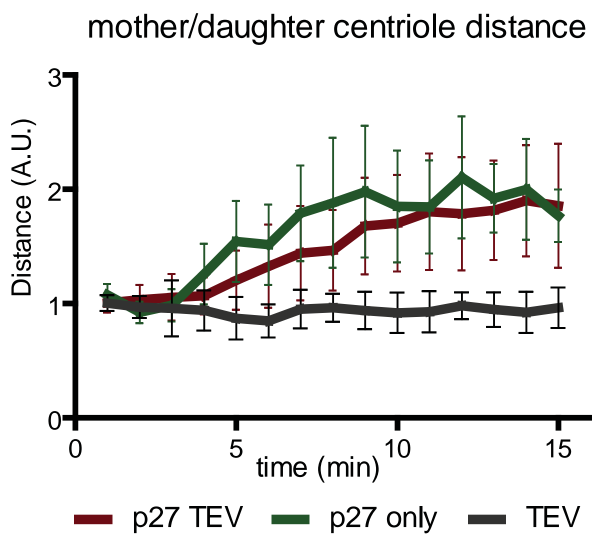
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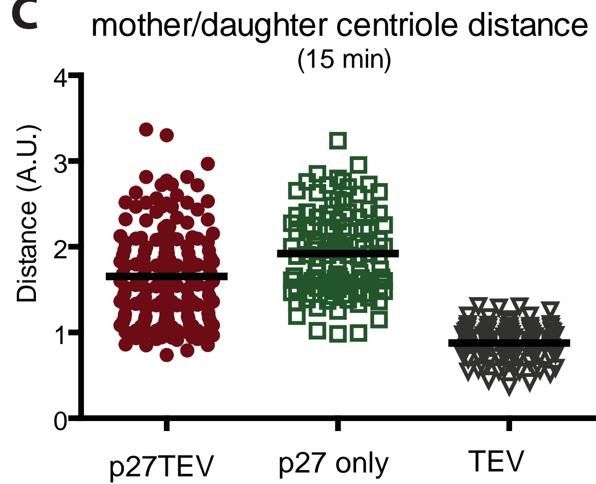
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Supplemental Information: Cohesin cleavage is insufficient for centriole disengagement in Drosophila

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Supplemental Results

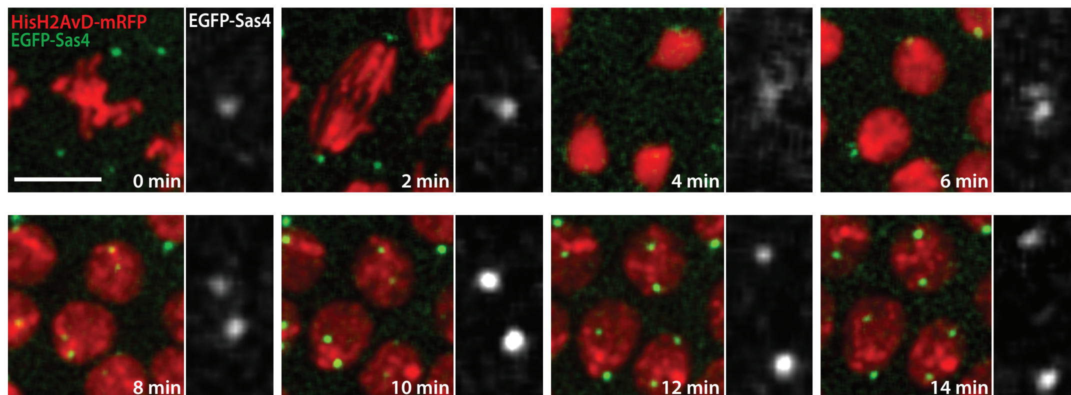

Figure S1 – EGFP-Sas4 during mitosis in Drosophila syncytial divisions. Embryos were pre-injected with mRNA coding for EGFP-Sas4 and imaged 1h after mRNA injection. EGFP-Sas4 (in green) labels centrioles throughout the cell cycle; DNA is labelled in red by the His2A-mRFP. While in metaphase engaged centrioles appear as single Sas-4 dot, at later stages of mitosis the signal splits into two discernable dots that is accompanied by a short drop in Sas4 signal (t=4min). Scale bars are 10μm and insets are 2.5x magnified. Times are relative to the last metaphase picture.

Supplemental Experimental Procedures

Fly stocks.

To destroy cohesin by TEV protease cleavage, Drosophila strains were used with TEV-cleavable Rad21 ($Rad21^{TEV}$) in a Rad21-null background ($Rad21^{ex15}$, *Rad21(550-3TEV)-myc*) [7]. Fly strains also expressed His2A–mRFP1 (polyubiquitin His2A–mRFP1, provided by J. Mummery-Widmer, Jürgen Knoblich laboratory, IMBA, Austria) to monitor DNA.

Microinjections.

In all experiments, 0–30 min old embryos were collected and processed as described previously [6]. Embryos were injected at the posterior pole (up to three sequential injections) using a Burleigh MIS-5000 Micromanipulator and a custom built gas injection system, using pre-pulled Femtotip I injection needles (Eppendorf). Estimated injection volumes were between 5% and 10% of the

total volume of the embryo. Embryos were injected with mRNA encoding Sas4-EGFP at 0.5 μg/μl and aged for one hour to allow for protein expression. Embryos were then injected with proteins purified from *E. coli* at the following concentrations: 30 mg/ml hUbcH10^{C114S}, 10 mg/ml TEV protease and 1.5 mg ml–1 His–p27. For all double-protein injection experiments, embryos were allowed to arrest in metaphase for 5 to 10 min before injection of TEV protease or p27 or co-injection of previously mixed TEV and p27.

mRNA synthesis.
The ORF of Dm Sas4 cDNA (a gift from Jordan W. Raff lab) was amplified by PCR using the primers 5´- ATACTAGTATGCAGGAGGCTGGCGAAAGTCCTGTTGG-3´and 5´- ATGCGGCCGCCTAATACTTGGCATAGTCTGTGTCC -3´, digested with SpeI and NotI and cloned into the site of pRNA–EGFP vector, creating the vector pRNA–EGFP-Sas4, in which the N-terminal tagged ORF of DmSas4 was flanked by 80 bp 5´ and 200 bp 3´ untranslated regions of the human globin gene. Capped mRNAs were synthesized by in vitro transcription using the mMessage mMachine T3 kit (Ambion) and purified with RNeasy columns (Qiagen). mRNA was eluted in RNase-free water and injected at ~ 0.5 μg/ml.

Microscopy.
Aligned embryos on coverslips were covered with Series 700 halocarbon oil (Halocarbon Products Corporation). Time-lapse microscopy was performed with an inverted wide-field DeltaVision microscope (Applied Precision Inc.) equipped with a 100x 1.35 oil immersion objective at 22–24°C in a temperature controlled room. One stack of 10 frames was acquired every 20 s using a Roper CascadeII backthinned 512x512 EMCCD used in conventional readout mode. Out-of-focus light was reassigned to its point of origin using SoftWoRx Resolve 3D constrained iterative deconvolution algorithm (Applied Precision Inc.). Deconvoluted images were projected using softWoRx software. Movies were assembled using ImageJ software (http://rsb.info.nih.gov/ij/) and selected stills were processed with Photoshop.

Quantitative analysis of centriole disengagement.

Time-lapse movies of EGFP-Sas4 from each experimental condition were assembled and processed using ImageJ software (http://rsb.info.nih.gov/ij/). After background and photobleachig correction, an automatic threshold was used to segment the images. The maximal width of the signal originated from a centriole pair (*ie*, the diameter of a single Sas4-GFP foci or the maximal distance between two adjacent foci) was manually measured at each time point. Each value was normalized by the width for t=0. Graphs were produced using Prim 5 (GraphPad Software, Inc). For each experimental condition, at least 100 centriole pairs from 5 independent experiments (5 different embryos undergoing mitosis 11 or 12) were used.