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Cohesin cleavage and Cdk inhibition trigger formation of daughter nuclei

Raquel A. Oliveira, Russell S. Hamilton, Andrea Pauli, Ilan Davis, and Kim Nasmyth

Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU; United Kingdom.

Abstract

The metaphase-anaphase transition is orchestrated through proteolysis of numerous proteins by an ubiquitin protein ligase called the Anaphase-promoting complex or cyclosome (APC/C)¹. A crucial aspect of this process is sister chromatid separation, which is thought to be mediated by separase, a thiol protease activated by the APC/C. Separase cleaves cohesin, a ring-shaped complex that entraps sister DNAs^{2, 3}. It is a matter of debate whether cohesin-independent forces also contribute to sister chromatid cohesion^{4–6}. Using 4D-live-cell imaging of *Drosophila* syncytial embryos blocked in metaphase (via APC/C inhibition) we show that artificial cohesin cleavage⁷ is sufficient to trigger chromosome disjunction. This is nevertheless insufficient for correct chromosome segregation. Kinetochore-microtubule attachments are rapidly destabilized by the loss of tension caused by cohesin cleavage in the presence of high Cyclin-dependent kinase 1 (Cdk1) activity, as occurs when the APC/C cannot destroy mitotic cyclins. Metaphase chromosomes undergo a bona-fide anaphase when cohesin cleavage is combined with Cdk1 inhibition. We conclude that only two key events, opening of cohesin rings and down regulation of Cdk1, are sufficient to drive proper segregation of chromosomes in anaphase.

Correct attachment of sister DNAs to microtubules during mitosis depends on a force that actively holds them together. It is a matter of ongoing debate whether cohesin-independent forces (be they sister DNA concatenation^{8–11} or proteinaceous linkages provided by ORC¹² or condensin¹³) contribute to sister chromatid cohesion in metaphase cells^{4–6}. If cohesin alone resists spindle forces prior to anaphase, then artificial opening the cohesin ring by an exogenous protease should trigger sister chromatid disjunction in the absence of separase activity. If, on the other hand, cohesin were just one of several mechanisms holding sisters together, then cleavage should not suffice. This key experiment has so far only been performed in yeast, where cleavage of cohesin's α -kleisin subunit by the Tobacco Etch virus protease (TEV) in cells arrested in metaphase initiates disjunction of most sister DNAs but not those of repetitive rDNAs within the nucleolus^{14, 15}. It is nevertheless argued that the larger chromosomes of animal and plant cells, or indeed other fungi, have very different properties. To address this, we used strains of the fruit fly *D. melanogaster* whose α -kleisin subunit Rad21 has been replaced by a version containing TEV cleavage sites (Rad21^{TEV})⁷. TEV protease injection causes catastrophic mitotic failure in syncytial embryos using Rad21^{TEV} (Supplementary Information, Fig. S1). When injected before or during early stages of mitosis, it causes sister chromatids to disjoin as soon as the nuclear envelope breaks down. This precocious loss of sister chromatid cohesion is accompanied by a

Correspondence and requests for materials should be addressed to K.N. (kim.nasmyth@bioch.ox.ac.uk).

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prometaphase delay and is eventually followed by highly abnormal anaphases with extensive chromatin bridges. These results are in agreement with previous studies on cohesin depletion in a variety of metazoa^{7, 16-19} and are consistent with the notion that cohesin is essential for sister chromatid cohesion. However, such experiments do not address cohesin's role during prometaphase or metaphase when only centromeric cohesin can be detected on chromosomes²⁰. The loss of sister chromatid cohesion during mitosis could be a secondary effect caused by a lack of cohesin during DNA replication or G2. In other words, might cohesin's role in holding sister chromatids together largely be confined to S and G2 cells during which much larger amounts are associated with chromosomes?

To address cohesin's role within metaphase chromosomes themselves, we would need to observe the effect of cleaving Rad21 in metaphase cells that had entered mitosis with a normal complement of cohesin complexes. To do this, it was necessary first to devise a method for arresting the chromosome cycle of syncytial embryos in metaphase. Premature activation of the APC/C is inhibited by the Spindle Assembly Checkpoint (SAC), which promotes entrapment of the APC/C activator Cdc20 by the Mad2 protein²¹. To test whether high doses of Mad2 are sufficient to sequester Cdc20 and prevent anaphase onset, we injected into pre-blastoderm early embryos either wild type Mad2 protein or a modified version (Mad2^{L13Q}) that preferentially adopts a conformation favouring Cdc20 embrace²². Both proteins induce metaphase arrest (data not shown) but the effect of Mad2^{L13Q} is more pronounced, arresting nuclear cycles in metaphase for at least 30 minutes (Fig. 1b and data not shown). Arrest depends on Mad2-Cdc20 interaction, as a truncated version of Mad2 lacking its Cdc20-binding domain (Mad2^{ΔC})²³ has no effect on the chromosome cycle (Supplementary Information, Fig. S2). For reasons that will become apparent below, we also wished to block APC/C activity using a method that does not involve SAC. We therefore injected a catalytic dead version of the human ubiquitin-conjugating enzyme E2 UbcH10 (UbcH10^{C114S}), which should block destruction of "early" APC/C substrates such as cyclin A and Nek2A in addition to late ones such as securin and cyclin B²⁴. This also causes a robust metaphase arrest (Fig. 1c). During both types of arrest, sister kinetochores bi-orient and generate tension sufficient to turn off the SAC, as witnessed by a reduction in kinetochore-associated BubRI (Supplementary Information, Fig. S3), a SAC protein that decorates kinetochores that have not yet bi-oriented^{25, 26}.

To address cohesin's role within mitotic chromosomes, we injected TEV protease into embryos arrested in metaphase (for 5 to 10 minutes) by previous injection of Mad2^{L13Q}. TEV injection triggers anaphase-like chromosome movements within a minute (43.4 ± 16.0 sec) (Fig. 2 and Supplementary Information, Movie S2 compared to Movie S1). Sister chromatid separation depends on the presence of TEV cleavage sites in Rad21^{TEV} and on TEV proteolytic activity (Supplementary Information, Fig. S4). To compare the dynamics and fidelity of TEV-induced anaphase chromosome movements with those of cycling nuclei, we repeated the experiment with embryos derived from flies expressing a Cid-EGFP transgene²⁷, which enables visualization of centromere-specific histone H3. In all Rad21^{TEV} embryos, catalytically active TEV protease causes all eight centromere pairs to disjoin and sisters to move towards opposite poles (Supplementary Information, Fig. S5). Very similar results are obtained when the metaphase arrest is caused by UbcH10^{C114S} (Supplementary Information, Fig. S5 and S6). We conclude that cohesin cleavage is sufficient to trigger sister chromatid disjunction. A corollary is that during metaphase there are no forces resisting the tendency of microtubules to disjoin sister chromatids other than those dependent on cohesin.

Although cleavage of cohesin is sufficient to disjoin sister chromatids, chromosome segregation induced by cohesin cleavage is abnormal in a number of respects. Centromeres disjoin asynchronously (Fig. 2b) and move towards poles three to four times more slowly

(Fig. 2c-e). A possible reason for slow chromosome segregation induced by cohesin cleavage is that sister chromatids are additionally connected by cohesin-independent mechanisms, such as sister DNA inter-twining (concatenation), which could hinder the disjunction process. Such resistance would be expected to produce visible anaphase bridges, which we do not observe. This suggests either that de-catenation has largely been completed in the Mad2^{L13Q} metaphase-arrested chromosomes or that Topoisomerase II (Topo II) activity is fully adequate to resolve any remaining concatenation in a timely manner as spindle forces draw sisters towards the poles. To address this, we tested the effect of inhibiting Topo II activity by ICRF-193. In agreement with previous findings²⁸, embryos injected with ICRF-193 at the beginning or during early stages of mitosis exhibit extensive chromatin bridges during anaphase (Supplementary Information, Fig. S7). In contrast, when injected shortly before TEV protease in embryos arrested in metaphase, ICRF-193 causes only modest changes in chromosome segregation dynamics despite having a pronounced effect on chromosome condensation (Supplementary Information, Fig. S8). Inhibition of Topo II has little or no effect on initiation of centromere segregation (40.2 ± 21.4 and 43.4 ± 16.0 sec after TEV injection, with and without ICRF-193, respectively) but causes the process to be even more asynchronous (Fig. 2b), possibly reflecting a greater dependence of some chromosomes on Topo II activity than others. Thus, the inhibitor often delays disjunction of the arms of larger chromosomes (see arrowhead in the right panel of Fig. 2a, at 8:00 min) and causes a ~30% reduction in velocity of centromere movements (Fig. 2c-e and Movie S3). These data imply that Topo II may have completed most de-catenation before TEV injection but that it also acts after disjunction has been triggered by cohesin cleavage.

In addition to their slower movement towards the poles, chromatids disjoined by TEV undergo highly abnormal trajectories soon after their initial disjunction. They frequently cease movement towards the original pole and move rapidly in the opposite direction (Fig. 3a). Such reversals occur more than once, with individual chromatids moving frequently back-and-forth between spindle poles (Fig. 3b and c and Supplementary Information, Movie S4). These sudden changes in the direction of chromosome movement occur stochastically and start on average 2.5 min after the initial centromere separation. The trajectories of centromeres are mostly directed towards the poles (parallel to the segregation axis, Fig. 3d) and display infrequent very fast ($> 10 \mu\text{m}/\text{min}$) movements (lower panel of Fig. 3e). Rapid changes in direction of individual chromatids do not appear to alter the trajectory of their (ex-) sisters, emphasizing that sister chromatids have fully disjoined. These observations indicate that the kinetochore-microtubule interactions established during metaphase cannot be maintained for more than 2-3 minutes after cohesin cleavage.

A key player in the de-stabilization of erroneous microtubule-kinetochore interactions during mitosis is the Aurora B kinase²⁹. At the onset of anaphase, Aurora B relocates from the centromeres to the mid-zone, which is believed to prevent re-activation of the error-correction mechanism after tension has been destroyed. The trigger for Aurora B's relocation from centromeres to midzones is still unclear, but one possibility is that loss of centromeric cohesion (or cohesin) is responsible. To test this, we compared the dynamics of EGFP-tagged Aurora B in undisturbed and Mad2TEV injected embryos. Consistent with previous studies³⁰, Aurora B-EGFP concentrates on chromatin during early stages of mitosis, accumulates at centromeres during prometaphase/metaphase, and relocates to the spindle midzone during a normal anaphase (Fig 4a, for lower magnification see Supplementary Information, Fig. S9a). Strikingly, relocation from centromeres to the midzone does not occur during the pseudo-anaphase induced by cohesin cleavage, with the kinase persisting at the centromeres of individual chromatids for several minutes after TEV injection (Fig. 4b). This proves that centromeric localization of Aurora B does not depend on cohesin, as previously suggested¹⁷. Aurora B's persistence at centromeres after cohesin

cleavage suggests that, by destabilizing kinetochore-microtubule interactions, Aurora B might be at least partly responsible for the frequent changes in the direction of chromosome movement. The abnormal chromatid movements following cohesin cleavage in metaphase-arrested embryos are accompanied by re-accumulation of BubRI at kinetochores (Fig. 4b for lower magnification see Supplementary Information, Fig. S9b). This indicates that kinetochore-mediated SAC signalling is reactivated by the loss of tension, a phenomenon that does not occur during normal anaphases.

Though cohesin cleavage triggers sister chromatid disjunction, it is insufficient for traction of chromatids towards poles at normal velocities, for maintaining kinetochore-microtubule interactions, for re-localization of Aurora B, or for inhibiting SAC re-activation. What then besides cleavage of cohesin is required for these phenomena, all signs of a bona fide anaphase? In other words, upon destruction of what proteins by APC/C^{Cdc20} are the above events dependent? Obvious candidates are mitotic cyclins³¹, which are the only APC/C targets besides securin whose destruction is known to be essential, at least in yeast^{32, 33}.

If the role of mitotic cyclin destruction is merely to inactivate Cyclin dependent kinase 1 (Cdk1), then it should be possible to reproduce this event by inhibiting the kinase. To test whether Cdk1 inactivation alone can rescue the abnormalities observed during TEV-induced chromatid segregation we injected together with TEV protease human p27, a natural inhibitor of Cyclin-dependent kinases^{34, 35}. We first addressed the effectiveness of p27 in blocking entry into mitosis. Embryos injected with p27 during late S phase fail to undergo mitosis (Supplementary Information, Fig. S10a), indicating that human p27 can inhibit *Drosophila* Cdk1. To test whether p27 is also effective in embryos that have been arrested in metaphase, Mad2^{L13Q}-injected embryos were injected with p27, which causes an abrupt mitotic exit, as judged by chromosome decondensation. In most experiments, chromosome decondensation is accompanied by re-formation of a single nucleus but not by sister centromere separation (Supplementary Information, Fig. S10b). Decondensation is occasionally accompanied by formation of two chromatin masses, possibly as a consequence of spindle forces. Crucially, even under these circumstances, sister chromatids (centromeres) remain associated and move towards the same pole (Supplementary Information, Fig. S10b). The lack of sister centromere disjunction suggests that separate and by implication APC/C^{Cdc20} is not activated after Cdk1 inhibition.

We next tested the effect of Cdk1 inhibition in metaphase embryos triggered to disjoin sister chromatids by cohesin cleavage, by co-injecting TEV protease and p27 into embryos previously arrested in metaphase (via Mad2^{L13Q} or UbcH10^{C114S} injection). Remarkably, co-injection triggers anaphases that resemble those of undisturbed embryos (Fig. 5 and Supplementary Information, Movies S5, S6 and S7). Sister chromatids now disjoin synchronously (Fig. 5b) and move with nearly normal velocities to opposite poles (Fig. 5c-e) where they remain until decondensation and formation of daughter nuclei. Both anaphase segregation movements and chromosome decondensation take place with kinetics similar to undisturbed embryos. In addition, co-injection of p27 along with TEV induces timely re-localization of Aurora B from centromeres to spindle midzones and prevents re-activation of the SAC due to loss of tension (lower panels in Fig. 4a and b). These observations imply that cohesin cleavage triggers normal anaphase-chromosome movements in cells arrested in metaphase by Mad2^{L13Q}- or UbcH10^{C114S}-mediated inhibition of APC/C^{Cdc20} if and only if cleavage is combined with Cdk1 inhibition.

Our finding that cohesin cleavage is sufficient to trigger sister chromatid disjunction in animal cells excludes the existence of cohesin-independent forces capable of holding sister DNAs together within metaphase chromosomes. We suggest that cohesin alone and neither DNA-DNA concatenation⁹⁻¹¹ nor ORC¹² nor condensin¹³ exerts the counter-force opposing

microtubules. Our finding that Topo II inhibition hinders segregation induced by cohesin cleavage implies that DNA-DNA concatenation co-exists with cohesin-mediated connections within metaphase chromosomes. However, due to Topo II, this catenation cannot provide a significant counterforce. According to the ring model, interactions between cohesin's Smc1, Smc3, and kleisin subunits provide the forces that bear the brunt of opposing those exerted by microtubules. Once cohesin is cleaved and sister DNAs are pulled apart, any catenation persisting on mitotic chromosomes will produce (very) transient invisible anaphase bridges that generate tension, favouring de-catenation. The key point is that even if molecules other than cohesin contribute to the proximity of sister DNAs within metaphase chromosomes, they do not do so in a manner that prevents kinetochore-mediated disjunction once the cohesin ring has been opened. Crucially, it is the APC/C-separase pathway leading to cohesin cleavage that is responsive to the SAC.

Our finding that cohesin cleavage in metaphase cells causes highly anomalous chromatid movements demonstrates that chromosome segregation during anaphase is not mediated merely by the loss of sister chromatid cohesion. Anaphase cells must therefore possess mechanisms not operating in metaphase cells, which ensure that kinetochores of disjoined sisters do not reactivate the SAC, move rapidly to opposite spindle poles, and remain stably attached to microtubules until they reach this destination. Our experiments co-injecting p27 and TEV demonstrate that the “switching on” of these anaphase-specific mechanisms can be (and normally probably is) mediated by Cdk1 down regulation, which is usually caused by cyclin proteolysis. The APC/C has many substrates¹ while separase might cleave proteins besides kleisins^{36, 37}. Our observations imply that APC/C triggers mitotic cells to form daughter nuclei largely if not solely by activating separase and by inhibiting Cdk1. Likewise, opening the cohesin ring may be separase's sole function during this crucial transition. This conclusion is in agreement with (but due to the kinetic data goes well beyond) the finding that deletion of genes encoding securin (separase inhibitor) and a B-type cyclin permit yeast cells to undergo anaphase in the complete absence of the APC/C activator Cdc20³². If an ability to “reconstitute” a complex developmental transition is an important measure of our understanding of its underlying chemistry, then the process normally orchestrated by the APC/C and separase is simpler than we had supposed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Injection of Mad2^{L13Q} or UbcH10^{C114S} prevents anaphase onset in *Drosophila* syncytial embryos

Embryos expressing His2A-mRFP1 were (a) not injected, (b) injected with 25 mg/ml of active Mad2 (Mad2^{L13Q}) or (c) injected with 30 mg/ml of a dominant negative form of the human E2 ubiquitin-conjugating enzyme (UbcH10^{C114S}); scale bars are 10 μ m; bottom rows show higher magnifications (3x) of a single nuclear division. In all experiments performed (n = 25 embryos) both Mad2^{L13Q} and UbcH10^{C114S} induced a stable metaphase arrest.

Figure 2. Cleavage of cohesin is sufficient to trigger sister chromatid disjunction

Embryos containing TEV-cleavable Rad21 (Rad21^{TEV}) as their sole source of Rad21 were either unperturbed (control) or arrested in metaphase by injection of Mad2^{L13Q}, followed by injection of either TEV protease alone (Mad2^{L13Q}-TEV) or ICRF-193 and TEV protease (Mad2^{L13Q}-ICRF-TEV). **a**) Images show selected frames of His2A-mRFP1 expressing embryos; times (min:sec) are relative to the last metaphase figure (control) or time of TEV injection; scale bars are 10 μm; insets show higher magnification (2.5x) of a single nuclear division; **b**) asynchrony of sister chromatid disjunction measured by the duration of anaphase onset, calculated as the time between separation of the first and last kinetochore pairs of a single metaphase (n=30 metaphases for each experimental condition); **c**) representative kymographs showing centromere (Cid-EGFP) separation; each kymograph shows all centromeres from a single metaphase; vertical scale bars are 2 μm and horizontal scale bars correspond to 30 sec; **d**) kymograph analysis of averaged centromere movement. Thin lines represent the average movement of all centromeres from individual experiments (n=30) and thick lines show the average of all experiments; **e**) average segregation speeds for each experimental condition; error bars show standard deviation; *** $P<0.0001$ and ** $P<0.001$ (two-tailed t-test).

Figure 3. Loss of cohesin leads to unstable kinetochore-microtubule attachments

Rad21^{TEV} embryos were injected with TEV protease to trigger sister centromere disjunction in Mad2^{L13Q}-induced metaphase-arrested embryos. **a**) Selected stills from a movie showing His2Av-mRFP1 (red) and Cid-EGFP (green); arrows follow the trajectory of a single centromere after the initial pole-ward movement; times (min:sec) are relative to TEV injection; scale bar is 5 μ m; **b**) representative kymograph showing centromere behaviour for 15 minutes after TEV-induced segregation. Vertical scale bars are 5 μ m and horizontal scale bars correspond to 1 minute; **c**) example of a single centromere trajectory for 15 minutes after TEV-mediated centromere disjunction; pauses are in red and runs in blue (away from starting point) and green (towards starting point); grey line represents the segregation axis; **d**) directions of centromere movement (measured by the angle of trajectory) in Mad2-TEV experiments plotted on rose diagrams to show overall bias in run directions towards the axis of segregation (0°-180°); **e**) frequency (%) of maximum speed per run in control and Mad2-TEV embryos; top two panels show the maximum speeds per run observed during the initial segregation phase (until 2 minutes after the initial separation of sister centromeres); bottom panel shows later chromosome movements observed in Mad2-TEV experiments (from 2 minutes after separation onwards); insets show speeds >10 μ m/min enlarged (5x) on the y axis; single centromere trajectories were analysed from 80 individual centromeres (out of 5 independent experiments); note that while during the segregation phase the chromosome movements in TEV-induced pseudo-anaphases are consistently slower than in controls, later chromosome movements are still overall slower but exhibit infrequent very fast movements (>12 μ m/min).

Figure 4. Localization of Aurora B and BubRI after TEV-induced sister chromatid disjunction Early embryos surviving on Rad21^{TEV} and expressing His2A-mRFP1 (red) were injected with mRNA encoding Aurora B-EGFP (a) or EGFP-BubRI (b) (shown in green); **a) upper panel** - Aurora B localization in a control embryo undergoing an unperturbed mitosis; **middle panel** - Aurora B localization in a metaphase arrested embryo (Mad2^{L13Q}) injected with TEV protease; **lower panel** - localization of Aurora B in a Mad2^{L13Q}-induced metaphase embryo that has been injected simultaneously with TEV protease and the Cdk inhibitor human p27; **b) upper panel** - BubRI localization in a control embryo undergoing an unperturbed mitosis; **middle panel** - BubRI localization in a metaphase-arrested embryos (UbcH10^{S114S}) with TEV protease; **lower panel** - localization of BubRI in a UbcH10^{C114S}-induced metaphase embryo that has been injected simultaneously with TEV protease and p27; in all figures, times (min:sec) are relative to the last metaphase (control) or to TEV injection; scale bars are 5 μm.

Figure 5. Cleavage of cohesin and Cdk inhibition trigger formation of daughter nuclei
 Embryos surviving on Rad21^{TEV} were either uninjected (control) or arrested in metaphase with Mad2^{L13Q} or UbcH10^{C114S} and subsequently co-injected with TEV protease and the CDK inhibitor human p27. **a**) Images show selected frames of embryos expressing His2A-mRFP1 (red) and Cid-EGFP (green); times (min:sec) are relative to the last metaphase figure (control) or to the time of TEV-p27 injection; scale bars are 10 μm; insets show higher magnification (2.5x) of a single nuclear division; **b**) asynchrony of sister chromatid disjunction measured by the duration of anaphase onset, calculated as the time between separation of the first and the last kinetochore pairs of a single metaphase (n=30 metaphases for each experimental condition); **c**) representative kymographs showing centromere (Cid-EGFP) separation; each kymograph shows all centromeres from a single metaphase; horizontal scale bars are 2 μm and vertical scale bars correspond to 30 sec; **d**) kymograph analysis of averaged centromere movement. Thin lines represent the average movement of all centromeres from individual experiments (n=30) and thick lines show the average of all experiments; **e**) average segregation speeds for each experimental condition; error bars show standard deviation.