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Abstract

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Cell-type specific TEV protease cleavage reveals cohesin functions in Drosophila neurons

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Summary

Cohesin is a highly conserved multi-subunit complex that holds sister chromatids together in mitotic cells. At the metaphase to anaphase transition, proteolytic cleavage of the α kleisin subunit (Rad21) by separase causes cohesin’s dissociation from chromosomes and triggers sister chromatid disjunction. To investigate cohesin’s function in post-mitotic cells, where it is widely expressed, we have created fruit flies whose Rad21 can be cleaved by TEV protease. Cleavage causes precocious separation of sister chromatids and massive chromosome missegregation in proliferating cells but not disaggregation of polytene chromosomes in salivary glands. Crucially, cleavage in post-mitotic neurons is lethal. In mushroom body neurons it causes defects in axon pruning while in cholinergic neurons it causes highly abnormal larval locomotion. These data demonstrate essential roles for cohesin in non-dividing cells, and also introduce a powerful new tool to investigate protein function in metazoa.
Introduction

The investigation of non-mitotic functions of proteins essential for cell proliferation poses a major technical challenge, namely how to inactivate such proteins without compromising cell proliferation. A good example is the highly conserved multi-subunit complex called cohesin (Guacci et al., 1997; Michaelis et al., 1997) that holds the products of DNA replication (sister chromatids) together and thereby ensures their segregation to opposite poles of the cells during mitosis and meiosis (reviewed in Nasmyth and Haering, 2005 and Hirano, 2006). Cohesin forms a large tripartite ring composed of a pair of SMC (Structural Maintenance of Chromosome) proteins, SMC1 and SMC3, and an α kleisin protein, Scc1/Rad21 whose cleavage by separase causes cohesin’s dissociation from chromosomes and triggers sister chromatid disjunction at the metaphase to anaphase transition. Sister chromatid cohesion requires two other non-SMC subunits, namely Pds5 and Scc3/SA, that bind to cohesin’s α kleisin subunit. Establishment of cohesion depends on the cohesin loading complex Scc2/Scc4 and the acetyl-transferase Eco1/Ctf7.

The fact that cohesin forms a ring whose opening releases it from chromatin has led to the suggestion that it holds sister DNAs together using a topological mechanism (Gruber et al., 2003). Importantly, this type of function could also be of value in regulating aspects of chromosome organization that are independent of sister chromatid cohesion and are not directly required for chromosome segregation. It is notable in this regard that the majority of cohesin is removed from chromosome arms during prophase/pro-metaphase in most eukaryotic cells by a separase-independent mechanism (Gandhi et al., 2006; Kueng et al., 2006). Only cohesin that subsequently persists on chromosomes is cleaved by separase at the onset of anaphase (Waizenegger et al., 2000). As a consequence, there exists a large pool of cohesin ready to re-associate with chromosomes as soon as cells exit from mitosis during
telophase. Cohesin is therefore tightly associated with chromosomes for much of the cell division cycle and could have important functions on unreplicated genomes.

Much evidence has emerged recently that cohesin might have important roles in regulating gene expression (reviewed in Dorsett, 2007). About half of the cases of a multi-system developmental disorder in humans called Cornelia de Lange syndrome (CdLS), which is characterized by mental retardation, upper limb abnormalities, growth delay and facial dysmorphisms, are caused by mutations in genes encoding NIPBL/Delangin (the human Scc2 ortholog), SMC1A, or SMC3 (Deardorff et al., 2007; Krantz et al., 2004; Musio et al., 2006; Tonkin et al., 2004). Because even severe cases of CdLS appear not to be accompanied by defects in sister chromatid cohesion, it has been suggested that CdLS is caused by mis-regulated gene expression during embryonic development. Consistent with this possibility, the Drosophila Scc2 ortholog Nipped-B facilitates long-range enhancer-promotor interactions at least for certain genes whose regulatory sequences have been mutated (Dorsett et al., 2005; Rollins et al., 1999). Furthermore, mutations in mau-2, the C. elegans Scc4 ortholog, cause defects in axon guidance (Bernard et al., 2006; Takagi et al., 1997). Recently, two cohesin subunits, Rad21/Scc1 and SMC3, have been implicated in expression of the hematopoietic transcription factors runx1 and runx3 in zebrafish (Horsfield et al., 2007).

Despite these findings, it cannot be excluded that developmental “cohesinopathies” are in fact caused by “knock on” effects of compromising the establishment or maintenance of sister chromatid cohesion. In the case of CdLS, for example, haplo-insufficiency of NIPBL/Delangin might cause cell type-specific sister chromatid cohesion defects (Kaur et al., 2005) that would be overlooked by examining this process in only one type of cell. It is therefore vital to develop methods that permit observing the effects on gene expression and
development of eliminating cohesin’s function completely without interfering with cell proliferation.

To analyze cohesin’s function in a more sophisticated manner than hitherto possible in metazoa, we have used the Tobacco Etch mosaic Virus (TEV) protease to cleave cohesin’s α kleisin subunit in Drosophila melanogaster in a cell type-specific and/or temporally controlled manner. This process opens the cohesin ring and presumably abolishes its topological embrace of chromatin fibres (Gruber et al., 2003). As expected, expression of TEV protease in proliferating cells of fly embryos whose sole form of Rad21 contains TEV cleavage sites causes precocious separation of sister chromatids and has a devastating effect on chromosome segregation. More remarkably, TEV-induced Rad21 cleavage in post-mitotic neurons is lethal. It causes defects in the developmental axon pruning of mushroom body γ neurons within pupal brains and defects in cholinergic neurons that result in highly abnormal larval locomotion.

Results

A system to inactivate pre-existing cohesin complexes

To inactivate cohesin, we chose cleavage of its α kleisin subunit (Rad21). Although this does not directly affect any known functional domain of Rad21, it severs and thereby opens cohesin’s tripartite ring (Fig. 1A), leading to its rapid dissociation from chromosomes. To do this in Drosophila, it was necessary first to create a Rad21 mutant strain, second to complement the Rad21 mutation with a version of Rad21 that contains cleavage sites for a site-specific protease, and lastly to express a version of the protease that can accumulate within nuclei in a tissue specific and/or time dependent manner. We used TEV protease as it
has been used successfully for this purpose in the budding yeast *S. cerevisiae* (Uhlmann et al., 2000).

**Generation of a Rad21-mutant fly**

The *Rad21* gene (CG17436) is located within the centric heterochromatin of chromosome 3L (Markov et al., 2003), but no mutants were available. To create *Rad21* mutations, the P-element GE50159 4kb upstream of the transcriptional start of *Rad21* was remobilized by P-element Transposase. Among the homozygous lethal stocks, we identified four independent *Rad21* deletion alleles using PCR (*Rad21*ex3, *Rad21*ex8, *Rad21*ex15, *Rad21*ex16, Fig. 1C). All four alleles lack exons 1 and 2, which encode the highly conserved N-terminus of Rad21 that interacts with the ATPase head of SMC3 (Fig. 1C and S1) (Haering et al., 2002).

Homozygous mutant *Rad21* embryos develop normally during early embryogenesis (data not shown). DNA staining suggests that mitoses are normal throughout the first 16 epidermal cell divisions. Late mitoses and cell divisions in embryonic neural precursors also appear unaffected (data not shown). Maternal gene product is presumably sufficient to execute the embryonic cell division programme. Despite this, most (95%) homozygous mutant embryos die before hatching. The rare mutant larvae that hatch possess almost no motor activity and fail to grow. It is therefore conceivable that embryonic death arises from a defective nervous system.

**Flies expressing TEV-cleavable Rad21 are viable and fertile**

To rescue *Rad21* mutants we generated transgenic flies that express C-terminally myc-epitope tagged versions of Rad21 with TEV cleavage sites, which were inserted as a tandem array of three consensus recognition sequences into four poorly conserved and putatively unstructured
regions within Rad21’s central domain (Fig. 1A; for details see Fig. S1). The cleavability of these proteins was initially tested by co-transfecting tissue culture cells with vectors expressing TEV-cleavable Rad21 (Rad21\textsuperscript{TEV}) and TEV protease. This showed that all four versions of Rad21\textsuperscript{TEV} were efficiently cleaved (data not shown). Equally important, Rad21\textsuperscript{TEV} with 3 TEV sites at positions 271 or 550 as well as a version lacking TEV insertions restored full viability and fertility of homozygous Rad21\textsuperscript{ex} alleles when expressed from a tubulin-promotor (Suppl. Table T2). We were thus able to generate fly stocks that carry TEV-cleavable Rad21 as their sole source of Rad21.

**Efficient TEV-induced Rad21 cleavage in vivo**

To test whether flies can tolerate TEV protease, we created transgenic flies that express v5-epitope-tagged TEV in an inducible manner, either directly from the heat-shock promotor (hs-TEV) or under the control of the Gal4/UAS system (Brand and Perrimon, 1993) (Fig. 1B, a and b). TEV tagged with three nuclear localization sequences (NLS) accumulated within nuclei and did not cause any notable phenotypes when expressed ubiquitously or in a tissue-specific manner using a variety of different Gal4 driver lines (data not shown). Western blotting showed that TEV induction caused the appearance of cleavage fragments of the expected size from Rad21\textsuperscript{TEV} proteins but not from endogenous Rad21 or transgenic Rad21 proteins (Fig. 1D, and data not shown). Heat shock led to accumulation of TEV and Rad21 cleavage fragments more rapidly when the protease was expressed from the heat-shock promoter (hs-TEV) compared to hs-Gal4 (hs-Gal4/UAS-TEV) (data not shown). Importantly, TEV induction led to cleavage of most of the Rad21\textsuperscript{TEV} pool within a few hours.

**TEV-induced Rad21 cleavage causes chromosome missegregation**
To investigate the consequences of Rad21 cleavage in a single cell cycle, we made use of the fact that zygotic expression is specifically switched on during embryonic cycle 14. Maternal Gal4 (α4-tub-Gal4) was used to drive expression of paternally contributed UAS-TEV in embryos containing Rad21TEV as their sole source of Rad21. Western blotting confirmed that expression of TEV causes a reduction in the level of intact Rad21TEV and the appearance of a ~90 kDa TEV cleavage fragment before mitosis 14 (Fig. S2A). The residual full-length protein presumably stems from embryos (50%) that did not inherit the TEV protease-containing chromosome. These results suggest that most if not all Rad21TEV is cleaved during cycle 14.

Rad21TEV cleavage had no effect on progression through the first thirteen embryonic cell division cycles, during which TEV is not expressed (data not shown). In contrast, as soon as zygotic expression is switched on, TEV had a devastating effect as cells embarked on mitosis 14. DNA staining and immunolabeling of embryos with anti-tubulin revealed the absence of any normal meta-, ana-, and telophase figures (Fig. 2A). Despite formation of bipolar spindles, condensed chromosomes failed to align on a metaphase plate and were found scattered throughout cells. Cells accumulated in this metaphase-like state with high levels of Cyclin B and BubR1 concentrated at kinetochores.

These observations are consistent with the notion that Rad21 cleavage causes precocious loss of sister chromatid cohesion. This would prevent establishment of the tension at kinetochores needed to turn off the spindle assembly checkpoint and causes mitotic arrest (Logarinho et al., 2004; Tanaka, 2005). To test this, we used time-lapse microscopy to observe chromosomes marked with histone-RFP and kinetochores marked with EGFP-Cid. This revealed that upon Rad21 cleavage chromosomes condense during prophase of cycle 14, usually with paired,
presumably sister, kinetochores similar to a wild type strain (Fig. 2, t = 0-60 sec, compare Suppl. Movies 1 (wt) and 2 (Rad21-depleted)). However, during pro-metaphase, soon after bi-orientation, sister chromatids disjoin prematurely and often segregate to opposite poles. This highly abnormal process is asynchronous, with different chromosomes splitting at different times. As a result, chromosomes do not congress to a metaphase plate (Fig. 2B and Suppl. Movies 2 and 3). Exit from mitosis is delayed and cells arrest in a highly abnormal mitotic state, during which individual chromatids often lose their attachment to spindle poles, sometimes re-orient and move between poles (Fig. S2B). After about 20 minutes, chromosome de-condensation occurs abruptly and chromatids in the equatorial plane are cut by the cleavage furrow (Fig. S2C, Suppl. Movie 4). Though the mitotic arrest caused by Rad21 cleavage is only transient, mitosis nevertheless lasts about six times longer than in wild type. These results are consistent with data from previous RNAi experiments in tissue culture cells (Vass et al., 2003) and clearly show that Rad21 is essential for mitosis. We conclude that cohesin is necessary for sister chromatid cohesion in the fly, as it is in yeast and vertebrate cells.

**Cohesin binds to defined regions on polytene chromosomes**

We next used TEV cleavage to address whether cohesin has a role in holding together the multiple DNA molecules of polytene chromosomes in salivary glands. These chromosomes are generated by repeated rounds of DNA replication without intervening mitoses (endoreduplication) (reviewed in Zhimulev et al., 2004). Immunostaining of wild type polytene chromosomes squashes showed that Rad21, detected with a Rad21-specific antibody, localizes mainly to interband regions (Fig. 3A) as has been suggested in previous reports (Dorsett et al., 2005; Gause et al., 2007; Markov et al., 2003). Several lines of evidence imply that these bands genuinely correspond to cohesin. First,
immunostainings showed that myc-tagged Rad21$^{\text{TEV}}$ is bound to the same chromosomal regions as endogenous Rad21 (Fig. 3B). Second, cohesin’s other three subunits (SMC1, SMC3 and SA/Scc3) colocalize with Rad21 on polytene chromosome squashes (Fig. S3A). Third, staining by myc-, Rad21- and SMC1-specific antibodies is greatly reduced after TEV-induced cleavage of Rad21$^{\text{TEV}}$ in flies where this is the only form of Rad21 (Fig. S3B). The fact that SMC proteins are also released implies that TEV cleavage of Rad21 releases the entire cohesin complex from chromosomes. Cohesin did not co-localize with known interband-specific proteins (Z4, BEAF32, Jil1, MSL2, CTCF), and its distribution differed significantly from numerous other proteins whose localization on polytene chromosomes has been well documented (PolII, Rpb3, HSF, trx, Pc, Su(Hw), CP190, Mod[mdg4]) (Fig. S4A and B, and data not shown). The cohesin holocomplex appears to be bound to distinct but as yet undefined regions of polytene chromosomes.

**Polytene chromosomes persist after Rad21 cleavage**

To address whether cohesin holds polytene chromosomes together, we induced TEV by heat shock (from hs-TEV) in late third instar larvae surviving on transgenic Rad21 with or without TEV cleavage sites and containing morphologically normal polytene chromosomes (Fig. 4A). We noticed that some cleavage of Rad21$^{\text{TEV}}$ had taken place even before the heat shock, presumably due to low expression of hs-TEV under non-inducing conditions (Fig 4B). After heat shock, TEV caused rapid cleavage of Rad21$^{\text{TEV}}$ and its disappearance from polytene chromosomes for at least 15 hours but had no effect on Rad21 without TEV-sites and on the staining pattern of CTCF, a boundary-binding factor (Moon et al., 2005) (Fig. 4B and 4C). Surprisingly, the morphology of polytene chromosomes was unaltered by cohesin’s removal (see DAPI-stainings in Fig. 4C), even when hypotonic or non-crosslinking conditions were used during spreading that should favor their disassembly (data not shown). It is conceivable
that the chromosomes retain their integrity by virtue of the small amount of full-length Rad21<sub>TEV</sub> (Fig. 4B) that persists after TEV cleavage (either due to resistance to TEV or due to Rad21 re-synthesis). However, the simplest explanation for our results is that cohesin is not required for maintaining the integrity of polytene chromosomes.

Interestingly, cohesin is required for normal development of salivary glands. In contrast to hs-TEV, which does not cause TEV expression at 18°C, leaky expression of TEV under the control of hs-Gal4/UAS at 18°C led to smaller salivary glands (about 1/2 the size) containing thinner polytene chromosomes in 100% of wandering late third instar larvae that survived on Rad21<sub>TEV</sub>, as compared to controls (Fig. S5). Importantly, this decrease in organ size was due to smaller, not fewer, cells per gland. Similar results were obtained by expressing TEV using a salivary gland specific driver (F4-Gal4) (data not shown). These results suggest that cohesin has an essential function in non-proliferating, endocycling salivary gland cells.

**A function for cohesin in neurons?**

The finding that cohesin is required for normal salivary gland development suggests that cohesin does indeed have non-mitotic functions. Because cohesin is essential for cell proliferation, its putative additional functions would be best studied in post-mitotic cells that do not require chromosome segregation. This raises two key questions. First, is cohesin widely present in post-mitotic cells in the fly and second, is it possible to use TEV-mediated Rad21 cleavage to inactivate the complex in such cells? The answer to both questions is, yes. Immunostaining showed that Rad21 is concentrated within the nuclei of most neurons in larval brains (Fig. 5C and data not shown). Moreover, expression of TEV in neurons from Rad21<sub>TEV</sub>-rescued flies during embryonic or larval development, using the pan-neuronal drivers elav-Gal4 or nsyb-Gal4, causes developmental arrest and lethality (data not shown).
**Cohesin rings are essential for axonal and dendritic pruning**

To investigate in more detail cohesin’s function in neurons, we concentrated on post-mitotic γ neurons in the mushroom body of the fly brain. We focused on these particular cells because a recent mosaic screen for piggyBac insertions that cause abnormal pruning of γ neuron axons has implicated two other subunits of the cohesin complex, namely SMC1 and SA/Scc3 (Schuldiner et al., in press). γ neurons are a specific subtype of post-mitotic neurons in the mushroom body of the fly brain. During larval stages, the axons of γ neurons project into the dorsal and medial lobes of the mushroom body. During metamorphosis, at the time when α/β neurons are born, larval γ neuronal projections are selectively eliminated in a process called “axon pruning” (Fig. 5A) (Lee et al., 1999; Watts et al., 2003).

We first addressed whether Rad21 is normally expressed in γ neurons. Immuno-staining using Rad21-specific antibodies detected endogenous Rad21 within the nuclei of γ neurons and those of their neuronal neighbours (Fig. 5C). TEV protease can be expressed in γ neurons via specific Gal4-driver lines (e.g. H24-Gal4, Zars et al., 2000) and localizes to their nuclei (Fig. 5B). Crucially, TEV expression in Rad21\textsuperscript{TEV}-rescued flies largely eliminated Rad21\textsuperscript{TEV} from γ neurons but not from interspersed neighbouring neurons (Fig. 5C). In contrast, it had no effect on endogenous Rad21 that is not susceptible to TEV-induced cleavage.

We next analyzed the consequences of cohesin cleavage. The driver line 201Y-Gal4 is expressed in mushroom body γ neurons and has therefore been widely used in previous studies of the pruning process (Lee et al., 1999). In strains surviving on Rad21 without TEV-sites and expressing 201Y-Gal4-driven TEV, the dendrites and axons of CD8-positive γ
neurons and of FasII-positive α/β neurons were indistinguishable from wild type. The axons of γ neurons initially projected into both dorsal and medial lobes (not shown) but were pruned by 18h after puparium formation (APF) (Fig. 6A, pruned axons are indicated with open arrowheads). In Rad21^{TEV} larvae, γ neurons also projected their axons into dorsal and medial lobes (Fig. S6A), but they failed to prune these projections during pupariation (Fig. 6A, middle row). However, the absence of axons of later born α/β neurons (with high levels of FasII) in the centre of the dorsal and medial lobes at 18h APF (compare upper right to middle right panel in Fig. 6A) suggests that pupae arrest early after pupariation before α/β neurons are born. This raises the possibility that the pruning defect is in fact caused by arrest at a developmental stage that preceeds γ neuron pruning.

Though specific for γ neurons within the central nervous system, the 201Y-Gal4 driver is also expressed in muscles (O. Schuldiner and L. Luo, personal communication). The developmental arrest might therefore be caused by inactivation of cohesin in muscles. To test this, we expressed Gal80 under control of the muscle specific myosin heavy chain (mhc) promoter (C. Winter and L. Luo, unpublished) to prevent TEV expression and hence cohesin cleavage in muscles. Remarkably, this enabled pupae to develop well beyond the stage when pruning normally occurs. FasII-positive α/β neurons were now readily detected from 18 h APF (Fig. 6A, bottom panels). Because α/β neurons are descended from neuroblasts that proliferate after giving rise to γ neurons (Lee et al., 1999), the mere presence of α/β neurons implies that neuroblast proliferation is not blocked by the cleavage of Rad21 orchestrated by 201Y-Gal4. Importantly, the pruning defect in γ neurons caused by Rad21 cleavage was still observed (Fig. 6A, bottom panels).
If the pruning defect of post-mitotic γ neurons is caused by inactivation of cohesin in γ neurons themselves and is not an indirect consequence of its inactivation in some other cell type, then expression of TEV protease under control of a different γ neuron-specific Gal4-driver should cause a similar phenotype. TEV expression via the H24-Gal4 driver confirmed that Rad21 cleavage in γ neurons causes axonal pruning defects (Fig. S6B). Furthermore, comparison of γ neuronal projections between strains with and without cohesin in H24-Gal4 positive cells revealed that γ neurons also failed to prune their dendrites upon Rad21 cleavage (Fig. S6B). Although we did not observe axon-targeting defects during larval and early pupal stages, the axonal projections of brains from late pupae (>4 d APF), which contain fully differentiated adult structures, were very often disorganized and mistargeted (Fig. S6C). Our finding that a similar pruning defect accompanies Rad21 cleavage induced by two different Gal4 drivers, whose only common (known) feature is expression in γ neurons, implies that cohesin is needed for pruning of γ neuronal axons and dendrites.

How might cohesin regulate pruning? Previous work has implicated the ecdysone receptor EcR-B1 as a key regulator of γ neuron pruning (Lee et al., 2000). Indeed, pruning defects caused by SMC1 mutations are suppressed by over-expression of EcR-B1 (Schuldiner et al., in press). The TEV cleavage system should be ideal for testing whether cohesin is needed for EcR-B1 expression in all γ neurons. We found that Rad21 cleavage caused a major drop (at 18h APF) in the concentration of EcR-B1 within nuclei from most γ neurons but not from nuclei of other interspersed neurons (Fig 6B). Only a minority of γ neurons still contained detectable levels of EcR-B1 upon Rad21 cleavage (indicated by white arrows). These data suggest that cohesin is required for cell-type specific EcR-B1 expression.

Cohesin is required in cholinergic neurons for larval locomotion
One of the advantages of the TEV system is that it enables protein inactivation in all neurons of a given type and thereby has the potential to cause changes in animal behaviour. To investigate this, we expressed TEV under control of Cha-Gal4, which expresses Gal4 specifically in cholinergic neurons (Salvaterra and Kitamoto, 2001). We noticed that this reduced the ability of Rad21TEV but not Rad21 third instar larvae to crawl up the sides of the vials. The larvae nevertheless pupariate, albeit within their food, and die as late pupae, with fully developed adult organs (data not shown). We used video imaging to compare locomotion of Rad21TEV and transgenic Rad21 third instar larvae after placing them in the center of a petridish containing non-nutritive agar. This revealed that larvae with TEV-sites in Rad21 moved less far than those without (Fig. 7A). More detailed analysis showed that larvae without TEV-sites in Rad21 moved mostly in straight lines while Rad21TEV larvae curved repetitively (Fig. 7B ii, iii), frequently turned their heads (Fig. 7B iv), and even moved backwards (Fig. 7B v; see also Suppl. Movies 5 and 6).

Three lines of evidence suggest that these dramatic changes are not caused by mitotic defects. First, chromosomes from brain cells expressing CD8-GFP from Cha-Gal4 were never positive for the mitosis-specific phoshpo-histone H3 marker (Fig. S7A), implying that Cha-Gal4 does not drive expression in dividing cells. Second, brains from larvae surviving on Rad21TEV and expressing TEV protease in cholinergic neurons do not have any detectable mitotic defects (< 1%). Cohesion defects during mitosis would greatly delay passage through mitosis and therefore cause an increase in the percentage of phospho-histone H3 positive cells. No such effect was seen (Fig. S7). Third, we were unable to detect any gross morphological defects in the pattern of cholinergic neurons marked by CD8-GFP driven by Cha-Gal4 or any reduction in their numbers, either in the central nervous system (Fig. S7B) or in peripheral sensory
organs (data not shown). We conclude that correct larval locomotion requires cohesin in cholinergic neurons.

Discussion

A new tool to study protein function in metazoa

Though it was known that TEV protease can inactivate protein function in budding yeast (Uhlmann et al., 2000), it was unclear whether TEV could be used in a complex metazoan organism. Our work shows that TEV can be expressed in a wide variety of Drosophila tissues without causing overt toxicity. More important, we show that TEV expression induces quantitative cleavage of TEV site-containing but not wild type Rad21 protein and that this is accompanied by penetrant phenotypes both in proliferating tissues and, more unexpectedly, in cells not engaged in mitosis, such as neurons and salivary gland cells.

The system we have developed has many attractive features that should make it a powerful and versatile tool for studying protein function in vivo. First, the method causes protein inactivation within a few hours and does not rely on a gradual depletion of the protein, as occurs in methods that interfere with the protein’s synthetic capacity such as recombinase mediated gene deletion or RNA interference. Second, the system is reversible. By using Gal80ts, TEV protease can be turned both on and off. Third, it is possible to be certain that phenotypes are caused by cleavage of the target protein by comparing the effect of TEV expression in animals whose target protein either does or does not contain TEV sites. Fourth, by targeting the protease to particular locations inside or even (using a secreted protease) outside cells, it should be possible to direct inactivation of the target protein to specific intra- or extra-cellular compartments. The restriction of protein inactivation to specific cellular compartments may be easier to devise using TEV than degron systems relying on the much
more complex process of ubiquitin mediated proteolysis (Dohmen et al., 1994). Unlike the MARCM system, which uses FLP/FRT-induced mitotic recombination to generate homozygous mutant clones in proliferating tissues, TEV cleavage can be triggered in all cells of a given tissue and at any stage of development, features that will greatly facilitate phenotypic and biochemical analyses. Because many eukaryotic proteins contain multiple functional domains connected by unstructured polypeptide chains, protein inactivation through TEV cleavage should be applicable to a large variety of proteins. It could also be used to clip off protein domains and thereby alter protein activity.

**Integrity of the cohesin ring is essential for sister chromatid cohesion in mitosis**

Our first priority upon developing a system to cleave Rad21 was to use it to investigate the role of cohesin during mitosis. In yeast, cohesin has a vital role in holding sister chromatids together until all chromosomes have bi-oriented during mitosis, whereupon cleavage of Scc1/Rad21 by separase triggers sister chromatid disjunction (reviewed in Nasmyth and Haering, 2005). The consequences of depleting Scc1/Rad21 from tissue culture cells using RNA interference are on the whole consistent with the above notion (Coelho et al., 2003; Vass et al., 2003). However, depletion experiments have not been able to observe directly the effects of inactivating cohesin within a single cell cycle.

We engineered a situation in which efficient cleavage of Rad21 occurred precisely as embryonic cells embarked on cycle 14, causing a devastating effect on mitosis. Chromosomes enter mitosis with paired sister kinetochores but instead of stably bi-orienting on a metaphase plate, they disjoin precociously, usually segregating to opposite poles. Importantly, these highly abnormal movements all take place prior to the APC/C-dependent activation of
separase. These observations imply that cohesin is essential for the sister chromatid cohesion necessary to resist mitotic spindle forces in metazoan organisms as well as in yeast.

Our finding that most sister chromatids (in cells with cleaved Rad21) disjoin to opposite spindle poles, albeit precociously, suggests that their chromosomes possess sufficient cohesion to establish a transient form of bi-orientation, though possibly with low accuracy. We cannot at this stage determine whether this cohesion is mediated by cohesin complexes that have survived Rad21 TEV cleavage or by an independent cohesive mechanism such as residual sister DNA catenation. We can nevertheless conclude that the latter, if it exists, is incapable of resisting spindle forces and cannot therefore maintain sister chromatid cohesion while errors in chromosome bi-orientation are corrected during a period in which the spindle assembly checkpoint (SAC) has been activated. Thus, what really distinguishes cohesion mediated by cohesin from DNA catenation is its ability to be regulated by the SAC and this may be the reason why eukaryotic cells appear to use cohesin for mitosis.

**The cohesin ring has key functions in non-mitotic cells**

Mutations in Scc2’s human ortholog as well as in SMC1 and SMC3 cause the developmental defects associated with Cornelia de Lange syndrome (CdLS) (reviewed in Dorsett, 2007). It is unclear whether these defects are caused by mitotic errors during development or by defects in non-mitotic cohesin functions. The first clue that cohesin might indeed have key roles during development besides holding sister chromatids together was the finding that mutations in *D. melanogaster* Nipped-B, the ortholog of Scc2, alters the expression of genes whose regulatory sequences have been mutated (Rollins et al., 1999).
If cohesin has non-mitotic functions during development, then these could occur in proliferating and non-proliferating (post-mitotic) cells. To analyse cycling cells, it would be necessary to restrict analysis either to a short specific cell cycle stage (e.g. the G1 period), or to develop a means of differentially inactivating cohesin complexes engaged in non-mitotic functions, leaving intact those engaged in chromosome segregation. Analysis of post-mitotic cells is easier. It is merely necessary to devise a protocol for inactivating cohesin only after cell proliferation has ceased.

Cleavage of Rad21 induced by post-mitotic pan-neuronal drivers caused lethality, suggesting that cohesin has key functions in neurons. To investigate these in greater detail, we analysed the effects of Rad21 cleavage in specific neuronal subtypes. The finding that the proliferative defects caused by a *SMC1* mutation in clones of mushroom body neuroblasts are accompanied by defective pruning of axons (Schuldiner et al., in press) led us to investigate the effects of Rad21 cleavage in γ neurons. Our results show that Rad21 cleavage abolished the developmentally controlled pruning of both axons and dendrites in γ neurons. These defects cannot have been caused by failures in cell division because cleavage had no effect on the birth of γ neurons or on their initial axonal projections.

Previous work on *mau-2* (the *C.elegans* Sec4 ortholog) has already provided a link between cohesin and axon development (Benard et al., 2004). Whereas Mau-2 was reported to act as a guidance factor required for correct axon and cell migration, investigation of γ neurons in *Drosophila* suggests that cohesin mediates elimination of axon projections and dendrites. However, our results do not rule out a function for cohesin in regulating axon-guidance because Rad21 cleavage might not be complete when γ neuronal axons start growing out in
first place. Indeed, we observed axon-projection defects in developmentally arrested late pupae.

It has not so far been possible to show that γ neuron pruning defects cause changes in animal behaviour. Cleavage of cohesin in the entire population of cholinergic neurons, in contrast, has a dramatic effect, causing larvae to turn frequently, move their heads back and forth, and even crawl backwards. Importantly, the neurons clearly survive without functional cohesin and must be at least partially active because larvae are not paralyzed by cohesin cleavage, a phenotype seen when cholinergic transmission is switched off (Kitamoto, 2001). The locomotion defects are not dissimilar to those caused by mutations in scribbler (sbb) (Yang et al., 2000). Scribbler, also known as brakeless (bks) and master of thickveins (mtv), codes for a ubiquitously expressed co-repressor of transcription (Haeker et al., 2007 and references therein). Expression of a sbb transcript exclusively in cholinergic neurons is sufficient to rescue locomotion defects of sbb mutants (Suster et al., 2004). It therefore appears that the lack of scribbler and cohesin in cholinergic neurons causes similar locomotion defects. Future work will have to show whether there is a link between sbb and cohesin. Our finding that cohesin has roles in neurons that are essential for normal behaviour is consistent with the notion that the mental retardation invariably found in patients with CdLS is also due to defective neuronal function as opposed to defective cell proliferation during development.

We have shown that suppression of 201Y-Gal4-induced TEV expression specifically in muscles bypasses the early pupal arrest in Rad21TEV-rescued flies indicates that cohesin is essential in muscles as well as neurons. In addition, although cohesin does not seem to be required for the maintenance of polytene chromosome morphology, it is essential for normal progression through the endocycle in salivary glands. It is therefore conceivable that cohesin
has key functions in most post-mitotic cell types. What might this function be? Cohesin is known to be required for efficient double strand break repair as well as sister chromatid cohesion (reviewed in Nasmyth and Haering, 2005). However, it promotes repair by facilitating homologous recombination between sister chromatids. Its role in post-mitotic neurons on the other hand must be on unreplicated chromatids. We suggest therefore that cohesin’s function in neurons and other post-mitotic G0 cells is more likely to be in regulating gene expression. The finding that cohesin cleavage reduces accumulation of EcR-B1 within γ neurons is consistent with this notion. Interestingly, recent data has shown that cohesin binds to the EcR gene in several fly cell lines (Misulovin et al., 2007). Future experiments should address whether cohesin acts as a general regulator of gene expression.

In summary, we provide definitive evidence that the cohesin ring has essential functions in cells with unreplicated chromosomes. It will be important in the future to establish whether cohesin functions by trapping chromatin fibres as it appears to do in cells that have replicated their genomes.

**Experimental Procedures**

**Fly strains**

TEV cleavage experiments were performed in a Rad21-null background. Four independent Rad21\textsuperscript{ex} alleles were generated by imprecise excision of the P-element GE50159 (see Supplemental Material for details). For generation of transgenic flies expressing TEV-cleavable versions of Rad21 under control of the tubulin-promotor (Rad21\textsuperscript{TEV}), 3 TEV recognition sites were introduced into a previously generated pCaSpeR-Rad21-myc\textsubscript{10} vector. To generate a nuclear v5-tagged TEV protease expression construct, three NLS- and one v5-epitope tag were added to the coding region of TEV. For cloning details see Supplemental
Materials. Transgenic lines were produced by standard P-element-mediated germline transformation.

The fly stock $\text{Rad}21^{ex15}$, $\text{Rad}21(550-3\text{TEV})\text{-myc}$ was used as source of TEV-cleavable $\text{Rad}21$ ($\text{Rad}21^{\text{TEV}}$). The only exceptions are the Western blot experiment in Fig 1D and the characterization of the zygotic $\text{Rad}21$-mutant phenotype (Fig. 2, S2, and Suppl. Movies 1-4), for which $\text{Rad}21^{ex8}$, $\text{Rad}21(271-3\text{TEV})\text{-myc}$ and 2x $\text{Rad}21(271-3\text{TEV})\text{-myc}$; $\text{Rad}21^{ex3}$, respectively, were used as sources of $\text{Rad}21^{\text{TEV}}$. The fly stock $\text{Rad}21^{ex3}$, $\text{Rad}21\text{-myc}$ served as control (transgenic $\text{Rad}21$ without TEV-sites).

Further details on stocks can be found in Supplemental Material. A complete stocklist with all genotypes and abbreviations used in the text can be found as Supplemental Table T1.

**Immunofluorescence of embryos after TEV cleavage of Rad21**

For analysis of mitosis after TEV-induced cleavage of $\text{Rad}21^{\text{TEV}}$ in fixed samples, 3-6 h embryos were collected from the following cross: $\alpha4\text{-tub-Gal}4/2\times\text{Rad}21(271-3\text{TEV})$; $\text{Rad}21^{ex3}$ females were crossed with $\text{UAS-TEV, hs-Gal}4$, $\text{Rad}21^{ex3}/\text{TM}3$, $\text{Kr-Gal}4$, $\text{UAS-GFP}$ males. Immunofluorescence labelling of embryos was performed according to standard procedures (Knoblich and Lehner, 1993) after a pre-incubation in 0.7 µM taxol before fixation. Pictures were acquired with a Zeiss Axioplan 2 imaging system using the Zeiss AxioVision software. The following initial experiment allowed us to distinguish +TEV and –TEV embryos: $\alpha4\text{-tub-Gal}4/2\times\text{Rad}21(271-3\text{TEV})$; $\text{Rad}21^{ex3}$ females were crossed to either $\text{UAS-TEV, hs-Gal}4$, $\text{Rad}21^{ex3}/\text{TM}3$, $\text{Kr-Gal}4$, $\text{UAS-GFP}$ (+TEV) or $\text{hs-Gal}4$, $\text{Rad}21^{ex3}/\text{TM}3$, $\text{Kr-Gal}4$ (-TEV) males. Embryos were fixed during mitosis 14 and stained with anti-tubulin and a DNA stain. 50% of the embryos from the first cross displayed a drastic mitotic delay, while the other 50% were phenotypically wild-type. In contrast, all embryos from the second cross were
phenotypically wild-type. These observations indicated that +TEV embryos can be identified readily based on their severe mitotic abnormalities.

**In vivo imaging of embryos after TEV cleavage of Rad21**

For *in vivo* imaging of mitosis after TEV-induced cleavage of Rad21, Rad21^{TEV}-rescued flies were generated that contained fluorescent markers for DNA (His2Av-mRFP1) and kinetochores (EGFP-CID) as well as the maternal Gal4-driver α4-tub-Gal4 on their second chromosome. α4-tub-Gal4, His2Av-mRFP1, EGFP-Cid/2x Rad21(271-3TEV)-myc; Rad21^{ex3} females were crossed with UAS-TEV, hs-Gal4, Rad21^{ex3}/TM3, Kr-Gal4, UAS-GFP males. Embryos of this cross either displayed the characteristic severe mitotic abnormalities and were thus considered to be TEV-expressing (+TEV) or they were entirely normal and thus considered to lack the UAS-TEV transgene (-TEV). *In vivo* imaging was performed essentially as described (Schuh et al., 2007). Time-lapse confocal laser scanning microscopy was performed with an inverted Leica TCS SP1 system equipped with a 40x/1.25 oil immersion objective at 22°C-24°C. One stack of 5 frames was acquired every 15 seconds. The Leica confocal software was used for maximum projection, Gaussian filtering and contrast adjustment.

**Immunoblotting**

Pupae or dissected salivary glands were homogenized in SDS-sample loading buffer and boiled for 5 minutes. Western blotting was performed according to standard procedures.

**Immunostaining of polytene chromosome squashes**

Polytene chromosome spreads were prepared according to standard procedures as outlined in Supplemental Materials. Fluorescent images were acquired with an AXIO Imager.Z1 microscope (Zeiss) and a CoolSNAP HQ CCD camera (Photometrics) using MetaMorph
Immunostaining of brains

Immunostaining of whole-mount brains was performed as described previously (Lee and Luo, 1999). Confocal pictures were obtained using a Zeiss LSM 510 Axiovert 200M. Maximal projections of Z-stacks were generated using Zeiss software.

Antibodies

The following primary antibodies were used (WB, Western blotting; IF, immunofluorescence): guinea-pig $\alpha$-Rad21 (WB 1:3000, IF 1:600; Heidmann et al., 2004), mouse $\alpha$-myc 9E10 (WB 1:200; Sigma-Aldrich), mouse $\alpha$-myc 4A6 (IF 1:500; Upstate), mouse $\alpha$-v5 (WB 1:5000, IF 1:500; Invitrogen), mouse $\alpha$-Cyclin B (F2) (1:3; Knoblich and Lehner, 1993), mouse $\alpha$-tubulin (DM1A) (1:8000; Sigma-Aldrich), rabbit $\alpha$-BubR1 (1:2000; Logarinho et al., 2004), rabbit $\alpha$-CTCF (1:200; Moon et al., 2005), rat $\alpha$-mCD8 $\alpha$-subunit (1:100; Abcam), mouse $\alpha$-FasII (1D4) (1:50; Developmental Studies Hybridoma Bank (DSHB)), mouse $\alpha$-EcR-B1 (AD4.4) (1: 25; DSHB), rabbit $\alpha$-phospho-histone H3 (1:500; Upstate) and rabbit $\alpha$-actin (1:1000; Abcam). For WB, HRP-linked secondary antibodies (Amersham) were detected by Enhanced Chemi-Luminescence (ECL) (Amersham). For IF, Alexa-conjugated secondary antibodies (Molecular Probes) were used as 1:500 dilutions.

Larval behavior

Larval locomotion was tested essentially as previously described (Yang et al., 2000) with minor modifications. Late third instar larvae of control strains were selected based on their characteristic wandering stage. Since Rad21-depleted larvae do not crawl up the walls of food
vials, Rad21<sup>TEV</sup> larvae were considered as “wandering” based on their size and the fact that they stopped foraging within the food. “Wandering” third instar larvae were placed in the center of 90 mm diameter petridishes coated with non-nutritive 2% agar. After 1 minute of adaptation, the movement was recorded over a period of 2 min by taking images every 5 seconds with a Canon Power Shot S70 digital camera. Movies were assembled and larval movement was manually tracked using ImageJ 1.38x software. Total locomotion was measured by superimposing trails onto a 6 mm-grid and counting the number of squares through which larvae moved. For detailed analysis of locomotion behavior, higher magnification movies were taken on a dissection scope coupled to a Canon Power Shot S70 digital camera. For temporal projection of larval movement, single images were extracted from the recorded movies with a time lapse of 2 seconds. Projections of 10 images (corresponding to 20 second periods) were obtained using ImageJ software.

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Figure Legends

Figure 1. Outline of the TEV cleavage system

(A) Schematic of the cohesin complex containing TEV-cleavable Rad21 (green), SMC1 (red), SMC3 (blue) and Scc3/SA (yellow). Cleavage of Rad21 by separase occurs in the flexible linker region. Arrowheads indicate the sites of insertion of TEV-recognition sequences (numbers refer to amino acid positions).

(B) Outline of the TEV cleavage system showing two alternative methods to express TEV in vivo in flies: (a) UAS-TEV is controlled by the UAS/GAL4 system, enabling TEV expression by specific Gal4 driver lines. (b) TEV directly fused to the heat shock promotor allows its ubiquitous induction in a time-specific manner. (c) Once expressed, catalytically active TEV protease cleaves Rad21\textsuperscript{TEV}.

(C) Representation of the genomic region of the Rad21 locus. The Rad21 gene (CG17436) resides in the centric heterochromatin of chromosome 3L. The exon-intron structure of the Rad21 mRNA is shown in bold. EST-based transcript-predictions of neighbouring genes are depicted in lighter grey. The EP-element GE50159 4kb upstream of the transcriptional start of Rad21 is represented by a red triangle. The four independently generated imprecise excision mutants of Rad21 lack the chromosomal intervals indicated by solid red lines. The Rad21 locus is missing in the $\gamma$-ray induced Deficiency Def 2-66 (dashed line). Scale bar, 10 kb.

(D) Pupal protein extracts were prepared before ($t = -0.75$ h) and at different time-points after a 45-minute heat shock at 37°C (red arrow). Western blot analysis with antibodies against endogenous Rad21 (left panel) or myc (right panel) shows full-length Rad21\textsuperscript{TEV} (arrow) and the C-terminal TEV cleavage product (arrowhead) as well as gRad21 (asterisk). V5-tagged TEV protease is detected by probing with v5 antibodies (open circle). Actin was used as loading control. A Molecular Weight Marker (in kDa) is shown on the left.
Figure 2. Cleavage of Rad21<sup>TEV</sup> during cycle 14 causes precocious sister chromatid separation and transient mitotic arrest.

(A) Cycle 14 embryos that survived on Rad21<sup>TEV</sup> and expressed maternally contributed Gal4 were fixed and double-labeled (top rows) with anti-α-tubulin (Tub) and a DNA stain (DNA), or triple-labeled (bottom row) with DNA stain (blue), anti-BubR1 (green) and anti-Cyclin B (red). +TEV indicates the additional presence of the UAS-TEV transgene. Scale bars, 50µm (top left), 10µm (top right), 10µm (bottom).

(Top) Most cells in –TEV embryos have already completed mitosis 14 (arrowhead in whole embryo views). Dividing cells (arrow) during various mitotic stages (pro-, meta-, ana-, telophase) are shown in the high magnification view. In +TEV embryos, the entire dorsolateral epidermis is arrested in mitosis.

(Bottom) In –TEV embryos, high levels of BubR1 and Cyclin B are only observed during metaphase (m), whereas anaphase (a) cells do not stain for BubR1 and Cyclin B. Arrested cells of +TEV embryos are Cyclin B positive and have high levels of BubR1 on separated sister kinetochores.

(B) Embryos surviving on Rad21<sup>TEV</sup> and expressing either only maternal Gal4 (-TEV) or maternal Gal4-driven TEV protease (+TEV) were used for time-lapse imaging. DNA is marked with H2Av-mRFP1, kinetochores with EGFP-Cid. The onset of chromosome condensation was set to zero. Time points are indicated in seconds. While top two rows represent Z projections, the bottom rows show single confocal sections. Scale bars, 2µm.

(- TEV) Chromosomes congress into a metaphase plate (t = 180) followed by anaphase (t = 210) and telophase (t = 315). (+ TEV) Chromosomes fail to congress into a metaphase plate and sister chromatids separate prematurely (t = 75-105). Note the substantial mitotic delay (t = 630).
Figure 3. Cohesin binds to distinct regions on polytene chromosomes.

(A) Polytenes of wild type flies (w^{1118}) were stained with Rad21 antibodies (green) and DAPI (DNA, red). The lower panel shows a higher magnification (2.5x). The strongly DAPI-stained heterochromatic chromocentre (arrow) is devoid of Rad21-staining. Scale bars, 20µm.

(B) Polytenes from flies expressing myc-tagged Rad21^{TEV} in addition to endogenous Rad21 were co-immunostained with antibodies against Rad21 (green) and myc (red). DNA was visualized with DAPI (blue). In the right two frames, part of one chromosome arm is shown at higher magnification with split Rad21- and myc-channels. Scale bars, 20µm (left 4 frames), 10µm (right 2 frames).

Figure 4. Cohesin is not required for maintenance of polytene chromosome morphology.

(A) Outline of the TEV cleavage experiment in salivary glands.

(B) Western blot analysis of salivary gland extracts prepared either before (t = -0.75 h) or at various time-points after heat shock (red arrow) from GFP-negative larvae. The last lane shows a sample of salivary glands from Rad21^{TEV}-expressing flies that do not contain hs-TEV. Blots were probed with antibodies against myc (detecting full-length transgenic Rad21 (arrow) and the C-terminal TEV-cleavage fragment (arrowhead)) and v5 (detecting TEV protease (open circle)).

(C) Representative polytenes of 3rd instar larvae that carry hs-TEV and express either transgenic Rad21 (left panel) or Rad21^{TEV} as their only source of Rad21 were prepared before (t = -0.75 h) and at various time-points after heat shock (red arrow). Polytenes were co-immunostained with antibodies against myc (recognizing Rad21) and CTCF. The morphology of the polytenes was visualized by DAPI staining.
Figure 5. Cohesin is expressed in γ neurons and can be selectively destroyed by TEV cleavage.

(A) Schematic representation of axonal projections of γ (green) and α/β (red) neurons of wild type and pruning-defective mutants at three characteristic time-points during development. Only the right hemisphere is shown. α’/β’ neurons are omitted from the scheme. In 3rd instar larva, γ neuron axons are bundled in the peduncle before they bifurcate to project into the dorsal (d) and medial lobes (m) (filled green arrowheads). At 18h after puparium formation (APF), the dorsal and medial projections from wild type γ neurons are selectively eliminated (“pruned”, open green arrowheads). In a pruning mutant, γ neuron axon-projections and dendrites persist (filled green arrowheads). α/β neurons project into the dorsal and medial lobes. In late pupae/adults, axons of wild type γ neurons grow out again towards the midline. In a pruning mutant, larval axon-projections of γ neurons persist in the dorsal and medial lobes.

(B) H24-Gal4 was used to drive expression of v5-tagged nuclear TEV protease and mCD8 in γ neurons of the mushroom body. Third instar larval brains were immunostained with antibodies against mCD8 (green) and the v5-epitope (red). Images show Z projections of single confocal sections of the right brain hemisphere. Scale bar, 20µm.

(C) H24-Gal4 was used to drive expression of TEV and mCD8 in γ neurons of the mushroom body from flies that expressed endogenous Rad21 (gRad21, top) or Rad21^{TEV} as their sole source of Rad21 (bottom). Brains were stained with antibodies against mCD8 (green) and Rad21 (red). Images show a single confocal section in the plane of γ neuronal cell bodies.
Note that there is no overlap between the mCD8 and Rad21 stainings after TEV cleavage in γ neurons from Rad21\textsuperscript{TEV} flies. Scale bars, 20µm.

**Figure 6. TEV cleavage of Rad21 in γ neurons causes a defect in pruning.**

201Y-Gal4 was used to drive expression of TEV and mCD8 in γ neurons of the mushroom body from flies that survived on transgenic Rad21 with or without TEV cleavage sites. Scale bars, 20µm.

(A) Brains were dissected at 18h APF and stained with antibodies against mCD8 (green) and FasII (red). Z projections of single confocal sections of the right brain hemisphere (left 3 panels). Single FasII-stained slice in the plane of α/β neurons (right panel). Absence/presence of γ neuronal projections (open/filled green arrowheads); dendrites (green arrow); α/β neurons (red arrows). In the bottom row, expression of Gal4 was suppressed in muscles by mhc-Gal80 in Rad21\textsuperscript{TEV} flies.

(B) Brains of Rad21\textsuperscript{TEV} flies, in which Gal4-expression in muscles was suppressed by mhc-Gal80, were dissected at 18h APF and stained with antibodies against mCD8 (green) and EcR-B1 (red). Images show single confocal sections in the plane of γ neuronal cell bodies. A higher magnification (10x) of the white-boxed area is shown on the right.

**Figure 7. TEV cleavage of Rad21 in cholinergic neurons induces severe locomotion defects in third instar larvae.**

(A) Wandering third instar larvae expressing TEV under the control of Cha-Gal4 and surviving on transgenic Rad21 with and without TEV-sites were tested for motility. (Rad21: Cha-Gal4/+; Rad21\textsuperscript{ex3}, Rad21-myc/Rad21\textsuperscript{ex3}, UAS-TEV; Rad21\textsuperscript{TEV}: Cha-Gal4/+; Rad21\textsuperscript{ex15}, Rad21\textsuperscript{TEV}/Rad21\textsuperscript{ex3}, UAS-TEV). Larval movements were tracked and superimposed to a grid. Locomotion was measured by the number of grid squares each larva traveled through. The
number of larvae that traveled through the indicated number of squares (1–5, 6–10, etc.) is shown as percentages of the total number of larvae tested (54 and 48 for strains containing Rad21 and Rad21\textsuperscript{TEV}, respectively).

(B) Representative images and temporal projections of movements from larvae that express TEV in cholinergic neurons and survive on either transgenic Rad21 (i, i’) or Rad21\textsuperscript{TEV} (ii-v’; same genotypes as in A). i, ii, iii, iv and v show the initial position of the larvae. H indicates the position of the head. i’, ii’, iii’, iv’ and v’ show the temporal projections of the images taken over a 20 second interval (images taken every 2 seconds). Note that controls move mostly straight, whereas larvae in which Rad21\textsuperscript{TEV} has been cleaved in cholinergic neurons show frequent episodes of turns, moving head and backwards motion.
References


Figure 3

(A) \( w^{1118} \) (gRad21)

(B) gRad21 + myc-tagged Rad21\(^{TEV} \)
Figure 4

Click here to download high resolution image
Supplemental Material

Cell-type specific TEV protease cleavage reveals cohesin functions in Drosophila neurons

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Supplemental Experimental Procedures

Fly stocks

The line $P\{w^{+}, EP\}GE50159$ was obtained from GenExel, Korea. Deficiencies in heterochromatin 3 and the $\alpha 4\text{-tub-Gal4}$ driver line are available from the Bloomington stock center. Transgenic lines with $His2Av-mRFP1$, $EGFP-Cid$ (Schuh et al., 2007), $gSMC3-HA$ (Heidmann et al., 2004), $201Y-Gal4$ (Yang et al., 1995), $H24-Gal4$ (Zars et al., 2000) and $Cha-Gal4$ (Salvaterra and Kitamoto, 2001) have been described previously. A $mhc-Gal80$ fly stock (C. Winter and L. Luo, unpublished) was kindly provided by Liqun Luo. A complete stocklist with all genotypes and abbreviations used in this paper can be found in Supplemental Table T1.

Generation of Rad21 alleles

The insertion site of the homozygous viable $P\{w^{+}, EP\}GE50159$ line 4 kb upstream of the transcription start site of Rad21 was confirmed by inverse PCR according to a standard protocol. Imprecise excisions were generated by crossing the GE50159 line to flies expressing a stable source of the P-element transposase. Out of 500 excision events, 23 homozygous lethal lines were isolated. 4 independently generated deletions...
affecting Rad21 were subsequently identified by PCR (Rad21\textsuperscript{ex3}, Rad21\textsuperscript{ex8}, Rad21\textsuperscript{ex15}, Rad21\textsuperscript{ex16}) and confirmed by sequencing DNA fragments spanning the breakpoints.

**Generation of transgenic flies expressing TEV protease**

To generate a NLS- and v5-epitope-tagged TEV protease expression construct (nuclear TEV protease), one N-terminal consensus sequence of the SV40 nuclear localization signal (NLS) followed by one v5-epitope tag were introduced by PCR at the 5’end of the coding sequence of TEV-NLS\_2, using the yeast vector YIplac204 (Uhlmann et al., 2000) as template. Primer sequences are listed below. The PCR product NLS-v5-TEV-NLS\_2 was cloned as EcoRI/NotI fragment into the pUAST (Brand and Perrimon, 1993) or pCaSpeR-hs (Thummel, 1992) transformation vectors to obtain UAS-NLS-v5-TEV-NLS\_2 or hs-NLS-v5-TEV-NLS\_2, respectively.

Transgenic lines were produced by standard P-element-mediated germline transformation (Rubin and Spradling, 1983) and either recombined to the Rad21\textsuperscript{ex3} allele (Rad21\textsuperscript{ex3}, UAS-TEV/TM3, Sb, Kr-Gal4, UAS-GFP) or crossed into the Rad21\textsuperscript{ex3} background (hs-TEV; Rad21\textsuperscript{ex3}/TM6B, Tb).

**Generation of flies surviving on transgenic Rad21\textsuperscript{TEV}**

C-terminally 10xmyc-tagged Rad21 was created based on the EST clone LD14219 obtained from the Berkeley Drosophila Genome Project (BDGP). The coding sequence of the 10xmyc-tag was amplified by PCR from the plasmid gthr-myc (Leismann et al., 2000) and cloned as Bst-BI fragment into the unique Bst-BI site in LD14219, located 12 nucleotides upstream of the Rad21 translational stop codon. The sequence encompassing Rad21-myc\textsubscript{10} was excised as a 2790 bp Eco-RI//Kpn-I
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fragment and cloned into pUAST (Brand and Perrimon, 1993) to obtain pUAS-Rad21-
myc10.

To generate TEV-cleavable versions of Rad21 (Rad21^{TEV}), SpeI-restriction sites were
introduced into the coding region of Rad21 after amino acids 175, 197, 271 or 550 by
site-directed PCR-mutagenesis, using pUAS-Rad21-myc10 as template (primer
sequences see below). An AvrII/Nhel restriction fragment encoding 3 tandem arrays
of the TEV-recognition sequence ENLYFQG (kindly provided by Stephan Gruber,
for details on the sequence see below) was inserted into the newly generated SpeI-site.
The Rad21(3TEV)-myc10 fragment (EcoRI/KpnI-blunt) was introduced into the
multiple cloning site (MCS) of a modified EcoRI/blunt cut pBS vector containing a
MCS and 3’UTR flanked by FRT-sites (<). Next, the sequence comprising
<Rad21(3TEV)-myc10-3’UTR< was excised with KpnI and inserted into a KpnI-cut
modified pCaSpeR transformation vector (derived from 10xUAS, G. Dietzl), in which
the 10xUAS-sequence had been replaced with the sequence of the tubulin-promotor
(derived from plasmid M>P2, Casali and Struhl, 2004) (final vectors:
tubpay<Rad21(3TEV)-myc10-3’UTR<SV40).

To generate vectors expressing myc-tagged Rad21 without TEV-cleavage sites, the
open reading frame of Rad21-myc10 was PCR-amplified from pUAS-Rad21-myc10 and
introduced into the same modified vector (final vector: tubpay-Rad21-myc10-SV40).

Transgenic lines were produced by standard P-element-mediated germline
transformation (Rubin and Spradling, 1983). Transgenes were tested for their ability
to rescue the lethality of Rad21 null mutations. Transgenes with TEV-cleavage sites
at positions 271 or 550 and a transgene without TEV-sites were functional.
Immunoblotting

For the preparation of embryonic extracts, dechorionated embryos were homogenized in SDS-sample loading buffer 3-6 hours after egg deposition. Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The blot was probed with mouse anti-myc 9E10 (1:15, Sigma-Aldrich) and mouse anti-α-tubulin (DM1A) (1:20000, Sigma-Aldrich) using ECL detection (Amersham).

Protein extracts from pupae, 3rd instar larval salivary glands and 3rd instar larvae without salivary glands were prepared after dissection in PBS essentially as described in Experimental Procedures.

Immunostaining of polytene chromosome squashes

Salivary glands from wandering 3rd instar larvae were dissected in PBS, permeabilized in PBX* (PBS, 0.1% Triton X-100, 3.7% formaldehyde) for 30 seconds, fixed in 45% acetic acid/3.7% formaldehyde for 5-7 minutes and squashed according to standard procedures. Slides were blocked for 1 hour at room temperature in PBST + 5% BSA (PBS, 0.01% Tween20, 5% BSA) and incubated with primary antibodies (diluted in blocking solution) overnight at 4°C. After washing in PBST (3x10 minutes), slides were incubated with Alexa-conjugated secondary antibodies at room temperature for 1.5 hours, washed as before and mounted using VECTASHIELD mounting medium containing DAPI (Vector Laboratories).

The following (additional) primary antibodies were used: guinea-pig anti-SA (1:100, Dorsett et al., 2005), rabbit anti-SMC1 (1:100, Dorsett et al., 2005), rat anti-SMC1 (1:500, Malmanche et al., 2007), mouse anti-HA 16B12 (1:250, Covance), mouse anti-polymerase II 8WG16 (1:20, Covance), rabbit anti-trx (1:50, Chinwalla et al.,
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1995), rabbit anti-Pc (1:200, Zink and Paro, 1989), rabbit anti-HSF (1:80, Andrulis et al., 2000), rabbit anti-BEAF32 (SCBP) (1:50, Zhao et al., 1995) and mouse anti-Z4 (1:1, Saumweber et al., 1980).

**Primer and DNA sequences**

Restriction enzyme sites are shown in lower case, the start codon ATG in bold, the v5-epitope sequence in italics, a NLS sequence is underlined.

**NLS-v5-TEV-NLS**

Restriction enzyme sites are shown in lower case, the start codon ATG in bold, the v5 sequence in italics, the NLS sequence is underlined.

<table>
<thead>
<tr>
<th>Primer/Sequence</th>
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<td><strong>AP2f</strong></td>
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<tr>
<td><strong>pUAS-Rad21-myc</strong></td>
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<td><strong>AP24-271SpeIf</strong></td>
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AATTCGATTG-3’
AP25-271SpeI 5’-actagtAGGCGAAGGGACATTATGAATATT-3’
AP26-550SpeIf 5’-TCAAGGAGACTCAACGactagtCCAGCTGGGTTGGATC
ATGGTC-3’
AP27-550SpeI 5’-actagtCGTTGAGTCTCTTTGATTTAAA-3’

3 TEV recognition sites (3TEV)
c/ctagGGCTAGAGAATTGTATTTTCAAGGAGGCTTCGTTCTGAAAACCTTTACT
TCCAAGGAGAGCTCGAAAATCTTTATTTCCAGGGAg/ctagc
protein sequence: “-RAR
ENLYFQGASENLRYFAQGLENLRYFAQGAS-”

Supplemental References


Supplemental Tables

Table T1: Strains used in this study

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Abbreviation</th>
<th>Source</th>
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<td>w1118</td>
<td>w</td>
<td>Bloomington stock centre</td>
</tr>
<tr>
<td>w; lethal/CyO; TM2, {Δ2-3}/Sb, {Δ2-3}</td>
<td>P-element transposase</td>
<td>kindly provided by Frank Schnorrer</td>
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<tr>
<td>w; P[w+, EP]GE50159 (III)</td>
<td>GE50159</td>
<td>GenExel, Korea</td>
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Gal4 driver

| w; act-Gal4/CyO | act-Gal4 | Barry Dickson lab stocks |
| w; hs-Gal4 (III) | hs-Gal4 | Barry Dickson lab stocks |
| w; P[w+, mata4-tub-Gal4-VP16] | a4-tub-Gal4 | Bloomington stock centre |
| w*; P[w+, GawB]F4 (II) | F4-Gal4 | Weiss et al., 1998 |
| w; elav-Gal4 (3AF) (III) | elav-Gal4 | Barry Dickson lab stocks |
| w; nsyb-Gal4/CyO | nsyb-Gal4 | kindly provided by Julie Simpson |
| y, w; 201Y-Gal4, UAS-mCD8-GFP/CyO; MKRS, Sb/TM6B, Tb | 201Y-Gal4, UAS-mCD8-GFP | Liquin Luo lab stocks |
| w; Pin/CyO; H24-Gal4, UAS-mCD8-GFP | H24-Gal4, UAS-mCD8-GFP | Liquin Luo lab stocks |
| w; Cha-Gal4 (II) | Cha-Gal4 | Salvaterra and Kitamoto, 2001 |

Transgenes and Mutants

| w*; P[w+, His2Av-mRFP1] (II.2) | His2Av-mRFP1 | Schuh et al., 2007 |
| w*; P[w+, EGFP-Cid] (II.1) | EGFP-Cid | Schuh et al., 2007 |
| w*; P[w+, gSMC3-HA12] (III.2) | gSMC3-HA | Heidmann et al., 2004 |
| y, w; P[w+, mhc-Gal80] (III) | mhc-Gal80 | C. Winter and L. Luo, unpublished |
| Df(3L)2-66, kni[ri-1] p[p]/TM3, Sb, Ser | Df(3L)2-66 | Bloomington stock centre |

Rad21-excisions

| w; Rad21^{αx3}/TM3, Sb, Kr-Gal4, UAS-GFP | Rad21^{αx3} | present study |
| w; Rad21^{αx8}/TM3, Sb, Kr-Gal4, UAS-GFP | Rad21^{αx8} | present study |
| y, w; Rad21^{αx15}/TM3, Sb, Kr-Gal4, UAS-GFP | Rad21^{αx15} | present study |
| w; Rad21^{αx16}/TM3, Sb, Kr-Gal4, UAS-GFP | Rad21^{αx16} | present study |
### TEV protease transgenes

- **w; P[w+, UAS-NLS-v5-TEV-NLS2] (III)**  
  UAS-TEV  
  present study

- **w; P[w+, hs-NLS-v5-TEV-NLS2] (II)**  
  hs-TEV  
  present study

### Rad21-excision + TEV-protease

- **w; Rad21\(^{ex3}\), P[w+, UAS-NLS-v5-TEV-NLS2]/TM3, Sb, Kr-Gal4, UAS-GFP**  
  Rad21\(^{ex3}\), UAS-TEV/TM3, Sb, Kr>GFP  
  present study

- **w; Rad21\(^{ex3}\), P[w+, UAS-NLS-v5-TEV-NLS2], hs-Gal4/TM3, Sb, Kr-Gal4, UAS-GFP**  
  Rad21\(^{ex3}\), UAS-TEV, hs-Gal4/TM3, Sb, Kr>GFP  
  present study

- **w; P[w+, hs-NLS-v5-TEV-NLS2]; Rad21\(^{ex3}\)/TM6B, Tb, ubiquitin-GFP**  
  hs-TEV; Rad21\(^{ex3}\)/TM6B, Tb  
  present study

### transgenic Rad21 (+/- TEV-sites)

- **w; P[w+, tubpr<Rad21(550-3TEV)-myc\(_{10}<SV40\)> (III)**  
  Rad21(550-3TEV)-myc (Rad21\(^{TEV}\))  
  present study

- **w; P[w+, tubpr<Rad21(271-3TEV)-myc\(_{10}<SV40\)> (III)**  
  Rad21(271-3TEV)-myc (Rad21\(^{TEV}\))  
  present study

- **w; P[w+, tubpr<Rad21-myc\(_{10}<SV40\)> (III)**  
  Rad21-myc (Rad21)  
  present study

- **w; P[w+, tubpr<Rad21(271-3TEV)-myc\(_{10}<SV40\)> (II.3)**  
  Rad21(271-3TEV)-myc (Rad21\(^{TEV}\))  
  present study

- **w; P[w+, tubpr<Rad21(271-3TEV)-myc\(_{10}<SV40\)> (II.7)**  
  Rad21(271-3TEV)-myc (Rad21\(^{TEV}\))  
  present study

- **w*; P[w+, tubpr<Rad21(271-3TEV)-myc\(_{10}<SV40\)> (II.3), P[w+, tubpr<Rad21(271-3TEV)-myc\(_{10}<SV40\)> (II.7)**  
  2x Rad21(271-3TEV)-myc (2x Rad21\(^{TEV}\))  
  present study

### Rad21-excision + transgenic Rad21 (+/- TEV-sites)

- **w; Rad21\(^{ex15}\), P[w+, tubpr<Rad21(550-3TEV)-myc\(_{10}<SV40\)> (III)**  
  Rad21\(^{ex15}\), Rad21\(^{TEV}\)  
  present study

- **w*; Rad21\(^{ex8}\), P[w+, tubpr<Rad21(271-3TEV)-myc\(_{10}<SV40\)> (III)**  
  Rad21\(^{ex8}\), Rad21\(^{TEV}\)  
  present study

- **w; P[w+, tubpr<Rad21(271-3TEV)-myc\(_{10}<SV40\)> (II.3), P[w+, tubpr<Rad21(271-3TEV)-myc\(_{10}<SV40\)> (II.7)**  
  2x Rad21(271-3TEV)-myc (2x Rad21\(^{TEV}\))  
  present study

### Rad21-excision + Gal4 (+ TEV-protease or transgenic Rad21\(^{TEV}\))

- **w; Rad21\(^{ex3}\), hs-Gal4/TM3, Sb, Kr-Gal4, UAS-GFP**  
  hs-Gal4, UAS-GFP  
  present study

- **w*; P[w+, α4-tub-Gal4-VP16]/CyO, wg-lacZ; Rad21\(^{ex3}\)/TM3, Sb, ubx-lacZ**  
  α4-tub-Gal4/CyO, wg-lacZ; Rad21\(^{ex3}\)/TM3, ubx-lacZ  
  present study

- **w*; P[w+, α4-tub-Gal4-VP16], P[w+, His2Av-mRFP1] (II.2), P[w+, EGFP-Cid] (II.1)/CyO, wg-lacZ; Rad21\(^{ex3}\)/TM3, Sb, ubx-lacZ**  
  α4-tub-Gal4, His2Av-mRFP1, EGFP-Cid/CyO; Rad21\(^{ex3}\)/TM3, ubx-lacZ  
  present study

- **w*; P[w+, α4-tub-Gal4-VP16], P[w+, His2Av-mRFP1] (II.2), P[w+, EGFP-Cid] (II.1)/CyO, wg-lacZ; Rad21\(^{ex3}\)/TM3, Sb, ubx-lacZ**  
  α4-tub-Gal4, His2Av-mRFP1, EGFP-Cid/CyO; Rad21\(^{ex3}\)/TM3, ubx-lacZ  
  present study
**Table T2: Rescue of Rad21 excision alleles by ectopic expression of either Rad21-myc or Rad21^{TEV}-myc**

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<th>Relative Viability *</th>
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<td>♂ Rad21^{ex16}/TM3 Sb, Kr&gt;GFP</td>
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<tr>
<td>Papae#</td>
<td>Adults##</td>
</tr>
<tr>
<td>♂ Rad21^{ex16}/TM3 Sb, Kr&gt;GFP</td>
<td>0</td>
</tr>
<tr>
<td>♂ Rad21^{ex3}/TM3 Sb, Kr&gt;GFP</td>
<td>0</td>
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<tr>
<td>♀ Rad21^{ex3}, Rad21-myc</td>
<td>95.1</td>
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<tr>
<td>♀ Rad21^{ex15}, Rad21(550-3TEV)-myc</td>
<td>111</td>
</tr>
<tr>
<td>♀ Rad21^{ex8}, Rad21(271-3TEV)-myc</td>
<td>87.5</td>
</tr>
</tbody>
</table>

* Relative Viability: percentage of rescued pupae/adults, normalized to the values obtained when Rad21^{ex}/TM3 Sb, Kr>GFP males were crossed to w^{1118} females.

# Rescued pupae were identified by the absence of GFP-expression (n ≥ 400).

## Rescued adults were identified by the absence of Sb (n ≥ 250).
Supplemental Figures

Figure S1. Multiple sequence alignment of protein-sequences of metazoan Rad21-homologs.

Annotated Rad21-protein sequences or sequence fragments were aligned to each other using ClustalW (Alexander Schleiffer, unpublished data). The conserved N- and C-terminal domains (green frames), human and (predicted) *D. melanogaster* separase cleavage sites (blue and red asterisks, respectively) and the boundaries of exons 2-3 and 3-4 are indicated. Four poorly conserved regions were chosen to introduce 3 tandem arrays of TEV recognition sequences (black arrows). The amino acid position after which the TEV sites had been introduced is indicated. TEV sites that rendered a functional Rad21^{TEV} protein (271 and 550) are highlighted in purple boxes.
Figure S2. Rad21^{TEV} is cleaved by zygotically expressed TEV before mitosis.

(A) Total extracts corresponding to 30 or 10 embryos were analyzed by immunoblotting with antibodies against myc and α-tubulin 3-6 hours after egg deposition. (-TEV) Embryos surviving on myc-tagged Rad21^{TEV} express maternal Gal4, but do not contain the UAS-TEV transgene. (+TEV) Embryos surviving on myc-tagged Rad21^{TEV} express maternal Gal4, which drives zygotic TEV.
expression. The arrowhead indicates the position of full length myc-tagged 
Rad21\textsuperscript{TEV}, the asterisk that of the C-terminal TEV-cleavage product. Note that only 
50\% of the embryos used for the +TEV extracts contain the UAS-TEV construct 
(for details on genetic crosses see Supplemental Experimental Procedures). 
Therefore, the data is fully consistent with complete or almost complete cleavage 
of Rad21\textsuperscript{TEV}.

(B) Unstable kinetochore attachment after TEV expression. Frames shown were 
taken at 15 second intervals from a cell during the mitotic arrest resulting from 
zygotic TEV expression in embryos surviving on Rad21\textsuperscript{TEV}. DNA is marked with 
His2Av-mRFP1 (red), kinetochores with EGFP-Cid (red). The arrow follows the 
movement of a single kinetochore.

(C) Frames were taken at times indicated (min:sec) from a cell in a TEV-
expressing embryo surviving on Rad21\textsuperscript{TEV}. DNA is marked with His2Av-mRFP1 
(red), kinetochores with EGFP-Cid (red). After an initial mitotic arrest, 
chromosome decondensation starts abruptly (16:00) and unattached chromatids in 
the central region are cut by the cleavage furrow.
Figure S3. The cohesin complex binds to polytene chromosomes.

(A) Polytene chromosomes from wild type flies (top two rows) or flies transgenic for HA-tagged SMC3 (bottom row) were co-immunostained with antibodies against endogenous Rad21 (green) and either endogenous SA (top row), endogenous SMC1 (middle row) or the HA-epitope (bottom row) (red). A schematic of the cohesin complex is shown at the left of each row (arrow points to the subunit co-stained with
Rad21). DNA was visualized with DAPI. Scale bars, 15µm.

(B) Polytenic chromosome spreads from 3rd instar larvae, which express heat-inducible TEV (hs-TEV) and myc-tagged Rad21^{TEV} as their only source of Rad21, were prepared after a 45 min heat shock. Spreads were co-immunostained with antibodies against endogenous Rad21 and myc (top) or myc (green) and SMC1 (red) (bottom). Note the absence of Rad21-, myc- and SMC1-staining after TEV cleavage of Rad21^{TEV}. DNA was visualized with DAPI. Scale bar, 15µm (top), 10µm (bottom).
Figure S4. Rad21 binds to distinct regions on polytene chromosomes.

(A) Polytene chromosomes from wild type flies (w^{118}) were co-immunostained with
antibodies against Rad21 (green) and BEAF (red), a well-characterized boundary-associated factor. DNA is shown in blue in merged images. Two different magnifications are shown (top row: 40x objective; bottom row: 100x objective, split channels). BEAF and Rad21 localize to distinct interband regions. Scale bar, 20µm.

(B) Polytenic chromosomes from wild type flies (w^1118) were co-immunostained with antibodies against Rad21 and (a) PolII (RNA Polymerase II), (b) HSF (Heat-Shock Factor), (c) Z4 (interband-specific Zinc-finger protein), (d) Pc (Polycomb) and (e) trx (Trithorax). DNA was visualized with DAPI. As seen in the merged images (Rad21 in green, other proteins in red), the distribution of cohesin and of the other tested factors differs significantly. White arrows point to the few regions, in which an overlap between Rad21 and the other factor tested could be detected. The insets in a1-a4 show a higher magnification (3x) of a PolII-stained chromosomal puff, which is flanked by Rad21-bound regions. Scale bars, 20µm (a and b), 10µm (c-e).
Figure S5. Cohesin is required for salivary gland development.

(A) Salivary glands were dissected from 3rd instar larvae that had been raised at 18°C throughout development. (Left) In the whole-mount preparations, DNA was stained with DAPI. (Right) Polytene chromosome spreads were immunostained with antibodies against Rad21 (green). DNA was visualized with DAPI (blue). Three different genotypes were compared: a) gRad21/Rad21<sup>ex3</sup>, hs-Gal4, UAS-TEV; b) Rad21<sup>ex15</sup>, Rad21<sup>TEV</sup>/Rad21<sup>ex3</sup>, hs-Gal4; c) Rad21<sup>ex15</sup>, Rad21<sup>TEV</sup>/Rad21<sup>ex3</sup>, hs-Gal4, UAS-TEV. Since polytene chromosome spreads from genotypes (a) and (b) are similar, only a representative spread from (a) is shown. Note that salivary glands with reduced amounts of Rad21 have smaller but not fewer cells. Scale bar, 500µm (whole...
mount salivary glands), 20µm (polytene chromosome spreads).

(B) Quantitative analysis of salivary glands average length (in mm), total number of cells per salivary gland and the average diameter per nucleus (in µm) was performed from salivary glands from 3rd instar larvae of the indicated genotypes (>10 per genotype). Larvae in which Rad21^{TEV} has been cleaved show smaller salivary glands with smaller nuclei. The number of cells per salivary glands remains unaltered.

(C) Protein extracts from a strain carrying hs-Gal4, UAS-TEV and surviving on myc-tagged Rad21^{TEV} were analyzed by Western Blotting. Extracts of larvae lacking salivary glands (L), dissected salivary glands (SG) or pupae (P) were prepared from crosses raised at 18°C. Samples from lanes 3 and 4 were prepared 1 hour after heat shock treatment (45 min 37°C). Western Blot analysis was performed with antibodies against myc (detecting full-length Rad21^{TEV}-myc (arrow) and the C-terminal TEV-cleavage fragment (arrowhead)), v5 (detecting TEV-protease) and actin (loading control). Before heat shock induction of TEV, significant levels of the protease could be detected in salivary glands (open circle). The TEV cleavage fragment of Rad21 is also observed. Neither TEV protease nor Rad21^{TEV} cleavage fragments were detected in larvae without salivary glands or pupae before TEV induction. A Molecular Weight Marker (in kDa) is shown on the left.
Figure S6. Analysis of pruning in $\gamma$ neurons with H24-Gal4-induced TEV cleavage of Rad21.

H24-Gal4 was used to drive expression of TEV and mCD8 in $\gamma$ neurons of the...
mushroom body from flies that survived on transgenic Rad21 with (Rad21\textsuperscript{TEV}) or without (Rad21) TEV cleavage sites. Shown are maximal Z projections of single confocal sections of a right brain hemisphere, stained with antibodies against mCD8 (green) and FasII (red). Scale bars, 20µm.

(A) Larval γ neurons from a Rad21\textsuperscript{TEV} brain project into the dorsal and medial lobes (filled green arrowheads).

(B) (24h APF, left) In the presence of Rad21 (control), γ neurons have pruned their medial and dorsal axon projections (open arrowheads) as well as their dendrites. In γ neurons of Rad21\textsuperscript{TEV} pupae, larval axon-projections persist in the medial and dorsal lobes (filled green arrowheads). Note also the presence of unpruned dendrites (green arrow).

(4d APF, right) In the presence of Rad21 (control), γ neurons have re-extended their axons medially towards the midline. Axons of α/β neurons in the dorsal and medial lobes are labeled with FasII (red arrows). In γ neurons of Rad21\textsuperscript{TEV} pupae, larval axon-projections as well as dendrites (filled green arrowheads and green arrow, respectively) persist in the dorsal and medial lobe. Projections of α/β neurons are normal (red arrows).

(C) In the presence of Rad21 (top), γ neurons are tightly bundled and project exclusively towards the midline at 4d APF. No γ neurons are found in the FasII-positive dorsal lobe (open green arrowhead). In the absence of Rad21 (bottom), γ neuronal projections persist in the dorsal and medial lobes (filled green arrowheads), but are often disorganized and mistargeted. Although the FasII staining for α/β neurons appears weaker than in the control, the projection pattern is normal.
Figure S7. TEV cleavage of Rad21 in cholinergic-neurons does not cause mitotic defects.

Brains from control (-TEV: UAS-CD8-GFP; Cha-Gal4/CyO; Rad21\textsuperscript{ex15}, Rad21\textsuperscript{TEV}/TM6B) and TEV-cleaved brains (+TEV: UAS-CD8-GFP; Cha-Gal4/CyO; Rad21\textsuperscript{ex15}, Rad21\textsuperscript{TEV}/Rad21\textsuperscript{ex3}, UAS-TEV) were immunostained for CD8 (green) and the mitotic marker phospho-histone H3 (PH3, red).

A) High magnification images show that there is no co-localization between the CD8 positive cells and the mitotic marker. Additionally, mitotic figures in TEV-cleaved brains look similar to the controls. Scale bars, 20μm.
B) Confocal images of brains and ventral nerve cords from both control and TEV-cleaved brains. PH3 staining reveals that there is no detectable accumulation of mitotic figures after TEV cleavage, and that there are no obvious morphological defects in the CD8 positive cholinergic neurons. Scale bars, 100µm.

**Supplemental Movies**

**Supplemental Movie 1:**
Progression through mitosis 14 of *Drosophila* embryogenesis is illustrated as observed by time-lapse *in vivo* imaging of a control embryo expressing His2Av-mRFP1 and EGFP-Cid but not UAS-TEV. A maximum projection of five confocal sections with 600 nm step size acquired at 15 seconds interval using a 100X (NA 1.4) objective is presented. Several epidermal cells enter and progress through mitosis 14. Occasional lateral jumps in the movie reflect periodic stage movements for compensation of the embryonic germband extension movements.

**Supplemental Movie 2:**
Progression into mitosis 14 after Rad21\textsuperscript{TEV} cleavage by zygotic UAS-TEV expression. Time-lapse *in vivo* imaging of an embryo expressing His2Av-mRFP1 and EGFP-Cid was performed. Technical details were as described for Supplemental Movie 1. Two cells enter mitosis soon after the start of the movie followed by premature separation of sister chromatids.

**Supplemental Movie 3:**
Chromatid behavior during the mitotic arrest in cycle 14 after cleavage of Rad21\textsuperscript{TEV} by zygotic UAS-TEV expression. Time-lapse *in vivo* imaging of an embryo
expressing His2Av-mRFP1 and EGFP-Cid was performed. Five confocal sections with 600 nm step size were acquired at 15 seconds interval using a 100X (NA 1.4) objective. Single sections are shown in the acquired sequence. One of the separated chromatids looses its orientation to the left pole and is re-oriented to the opposite pole on the right.

**Supplemental Movie 4:**
Adaptation and exit from the mitotic arrest resulting from cleavage of Rad21\textsuperscript{TEV} by zygotic UAS-TEV expression. Time-lapse in vivo imaging of an embryo expressing His2Av-mRFP1 and EGFP-Cid was performed. A maximum projection of five confocal sections with 600 nm step size acquired at 15 seconds interval using a 40X (NA 1.25) objective is presented. Epidermal cells in the upper half of the movie have already entered mitosis 14 and are in a mitotic arrest at the start of the movie. Several cells eventually adapt and exit from mitosis.

**Supplemental Movie 5:**
Movie showing the locomotion of a third instar control larva (genotype: Cha-Gal4/+; \textit{Rad21}^{ex3}, \textit{Rad21/Rad21}^{ex3}, \textit{UAS-TEV}) on non-nutritive agar.

**Supplemental Movie 6:**
Movie showing the locomotion of a third instar larva after TEV cleavage of Rad21\textsuperscript{TEV} in cholinergic neurons (genotype: Cha-Gal4/+; \textit{Rad21}^{ex15}, \textit{Rad21}^{TEV}/\textit{Rad21}^{ex3}, \textit{UAS-TEV}).
Supplemental Movie 2
Click here to download Supplemental Movies and Spreadsheets: SupplMovie2.avi
Supplemental Movie 3
Click here to download Supplemental Movies and Spreadsheets: SupplMovie3.mov
Supplemental Movie 4

Click here to download Supplemental Movies and Spreadsheets: SupplMovie4.mov
Supplemental Movie 5

Click here to download Supplemental Movies and Spreadsheets: supplementary movie 5.mov
Supplemental Movie 6

Click here to download Supplemental Movies and Spreadsheets: supplementary movie 6.mov