Interphase cohesin regulation ensures mitotic fidelity after genome reduplication

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ABSTRACT **To ensure faithful genome propagation, mitotic cells alternate one round of chromosome duplication with one round of chromosome separation. Chromosome separation failure thus causes genome reduplication, which alters mitotic chromosome structure. Such structural alterations are well documented to impair mitotic fidelity following aberrant genome reduplication, including in diseased states. In contrast, we recently showed that naturally occurring genome reduplication does not alter mitotic chromosome structure in *Drosophila* papillar cells. Our discovery raised the question of how a cell undergoing genome reduplication might regulate chromosome structure to prevent mitotic errors. Here, we show that papillar cells ensure mitotic fidelity through interphase cohesin regulation. We demonstrate a requirement for cohesins during programmed rounds of papillar genome reduplication known as endocycles. This interphase cohesin regulation relies on cohesin release but not cohesin cleavage and depends on the conserved cohesin regulator Pds5. Our data suggest that a distinct form of interphase cohesin regulation ensures mitotic fidelity after genome reduplication.**

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INTRODUCTION

When cycling cells skip chromosome separation and then reenter S-phase, the genome is reduplicated. Such cycles are referred to as endocycles. Endocycles generate polyploid cells, which are common throughout nature (Fox and Duronio, 2013; Orr-Weaver, 2015). Following developmental endocycles or in pathological conditions, some polyploid cells return to mitosis (Levan and Hauschka, 1953; Fox *et al.*, 2010; Davoli and de Lange, 2012). Division of such genome-reduplicated cells can generate genome instability through a variety of mechanisms, such as multipolar division or the formation of diplochromosomes, a mitotic chromosome structure that is a form of polyteny in which all products of replication are held together in one chromosome. Such diplochromosomes lead to mitotic errors when cells divide (Vidwans *et al.*, 2002; Hassel *et al.*, 2014; Schoenfelder *et al.*, 2014; Chen *et al.*, 2016; Stormo and Fox, 2016, 2017). Diplochromosomes have also been observed in tumor models in mice (Davoli *et al.*, 2010) and following chemotherapeutic drug treatments in human cell culture (Blakeslee and Avery, 1937; Sumner, 1998).

Previously, we developed parallel models of naturally occurring and experimentally induced endocycled *Drosophila* cell types (Stormo and Fox, 2016). One cell type, the rectal papillar precursors of the hindgut, undergo developmentally programmed endocycles before returning to mitosis (hereafter "papillar cells"). The second cell type, wing imaginal disc cells, can be induced to endocycle by transient heat-shock driven expression of the endocycle regulator Cdh1/*fizzy-related* (hereafter- "*HS>fzr* cells"). Both papillar and *HS>fzr* cells return to mitosis after endocycling, but chromosome configuration at anaphase onset is very different. In papillar cells, chromatids undergo preanaphase chromosome Separation Into Recent Sister pairs (SIRS) (Figure 1A) (Stormo and Fox, 2016). In contrast, chromatids in *HS>fzr* cells are arranged in diplochromosomes as anaphase begins (Figure 1A). Likely because of these structural differences, papillar cell mitosis is relatively error free, whereas *HS>fzr* cell mitosis is highly error prone. These results raised the question of what molecular mechanism accounts for the difference in chromosome structure between cells capable or incapable of SIRS.

One candidate regulator of reduplicated chromosome structure is the cohesin complex. Cohesins are responsible for holding sister

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Abbreviations used: BrdU, 5-bromo-2′-deoxyuridine; SMC, structural maintenance of chromosome; TEV, tobacco etch virus.

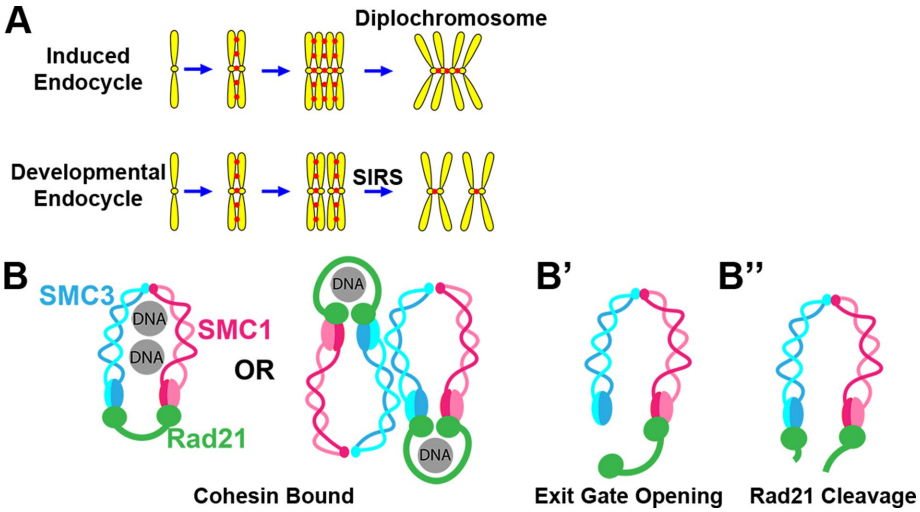

FIGURE 1: Potential cohesin regulation and impact on mitotic chromosome structure in two distinct *Drosophila* cell types that undergo endocycles. (A) Depiction of the outcome of two endocycles. In an induced endocycle, all sister chromatids are attached generating a diplochromosome. During a developmental endocycle, only recent sisters are attached at mitosis. (B) Two potential simplified depictions of the cohesin complex entrapping a pair of sister chromatids. (B′) DNA release by exit gate opening. (B″) DNA release by Rad21 cleavage.

chromatids together beginning at S-phase (when chromosomes are first duplicated) until anaphase (when chromosomes are separated). The cohesin complex consists of three main components: SMC1, SMC3, and Rad21. Several models have been developed for how cohesins bind DNA (Figure 1B) (Ivanov and Nasmyth, 2005; Haering *et al.*, 2008; Nasmyth and Haering, 2009; Eng *et al.*, 2015; Skibbens, 2016; Stigler *et al.*, 2016). The cohesin complex interacts with DNA in multiple discrete steps. Cohesins can be loaded onto chromatin throughout the cell cycle via an "entry gate" (Murayama and Uhlmann, 2015), which may be most critical in G1 (Lengronne *et al.*, 2006). G1 loaded cohesins are stabilized by replication fork passage and are maintained through G2 (Yeh *et al.*, 2008; Rhodes *et al.*, 2017). In many species, including *Drosophila,* the majority of cohesins are removed from chromosome arms early in mitosis by the prophase pathway, which opens an "exit gate" (Figure 1, B vs. B′) (Sumara *et al.*, 2002; Shintomi and Hirano, 2009; Eichinger *et al.*, 2013). Finally, any remaining cohesins, mostly at the centromere, are removed at the metaphase-to-anaphase transition by Separase-mediated Rad21 cleavage (Figure 1B″) (Uhlmann *et al.*, 1999).

Current research efforts aim to understand the diversity of cohesin regulation across different cell types and developmental stages (Nasmyth and Haering, 2009; Skibbens, 2016). How cohesin regulation is adapted for genome reduplicated cells is poorly understood. Diplochromosomes (e.g., *HS>fzr* cells) form in cells that have undergone two rounds of replication and cohesion establishment with presumably no intervening removal of the cohesins (Vidwans et al., 2002; Stormo and Fox, 2016). It is unknown how papillar cells can avoid mitotic chromosome separation defects, as they also have diplochromosome-like polytene chromosomes prior to undergoing SIRS.

Here we investigate the role of cohesins on the structure of chromosomes in cells undergoing endocycles, using our two model cell types. Unlike other endocycled cells, such as the *Drosophila* salivary gland, these two cell types return to mitosis, which allows direct visualization of chromosome structure and the effects of cohesin regulation on mitosis. We find that in SIRS-capable papillar cells, cohesin exit gate opening during endocycles prevents formation of diplochromosomes. This interphase cohesin exit gate opening depends on the conserved cohesin regulator Pds5. These findings reveal new interphase cohesin regulation during endocycles and shed light on the structural regulation of chromosomes in genome reduplicated cells.

RESULTS

Cohesin cleavage is sufficient to separate reduplicated chromatids

Previous studies in genome-reduplicated cells have found cohesins to be dispensable for chromosome structure (Pauli *et al.*, 2008). However, these studies focused on nonmitotic cells. Our previous work (Stormo and Fox, 2016) showed a major difference in mitotic fidelity between genome-reduplicated cells that are capable of SIRS and those that are not. We showed that tetraploid cells that are SIRS-deficient retain conjoined diplochromosomes at metaphase. However, we did not explore whether differential regulation of cohesins is responsible for the decreased mitotic fidelity in such cells with persistent diplochromosomes. We therefore examined the role of the cohesin complex in chromosome structure of mitotic polyploid cells, using our two previously established models.

We first tested whether cleavage of the Rad21 cohesin subunit is sufficient to dissociate the conjoined diplochromosome configuration found in *HS>fzr* wing cells. To do this, we took advantage of an established system that enables heat-shock-inducible Rad21 cleavage (*Rad21^{TEV}*, *Materials and Methods*). We first confirmed that heat shock (Figure 2, A and G) and Rad21^{TEV} alone has no effect on chromosome structure (Figure 2, B and G). TEV-protease expression also has no effect on diploid or polyploid mitotic chromosomes when Rad21 is wild type (Figure 2, C and G). We next combined induced endocycles and cohesin cleavage by driving expression of both *HS>fzr* and *HS>TEV* transgenes using a single heat shock in a *rad21^{TEV}* animals. In these animals endocycling still occurs, resulting in tetraploid cells, but these chromosomes lack cohesion between sisters and instead unpaired chromatids are visible (Figure 2, D and G). These data strongly suggest that diplochromosomes are held together by cohesin in the same manner as wild-type mitotic chromosomes. Further, we find that cohesin cleavage is sufficient to dissociate the conjoined chromatids found in diplochromosomes.

We next performed a similar experiment in papillar cells, which lack conjoined metaphase chromosomes. It was possible that these cells are able to undergo SIRS because they lack standard cohesins. We tested whether papillar chromatid pairs are held together by cohesins. As in the wing disc, following Rad21^{TEV} cleavage all chromosome cohesion is lost, and we observe individual chromatids (Figure 2, E vs. F and H). These data show that in both cells with induced endocycles, which result in diplochromosomes, and in papillar cell endocycles, which result in paired sisters, Rad21 cleavage is sufficient to separate the products of replication at metaphase.

Opening the SMC3-Rad21 exit gate is required for SIRS and subsequent mitotic fidelity

Cohesion regulation during mitotic cell cycles ensures that chromosomes are attached specifically to their sisters and not to other

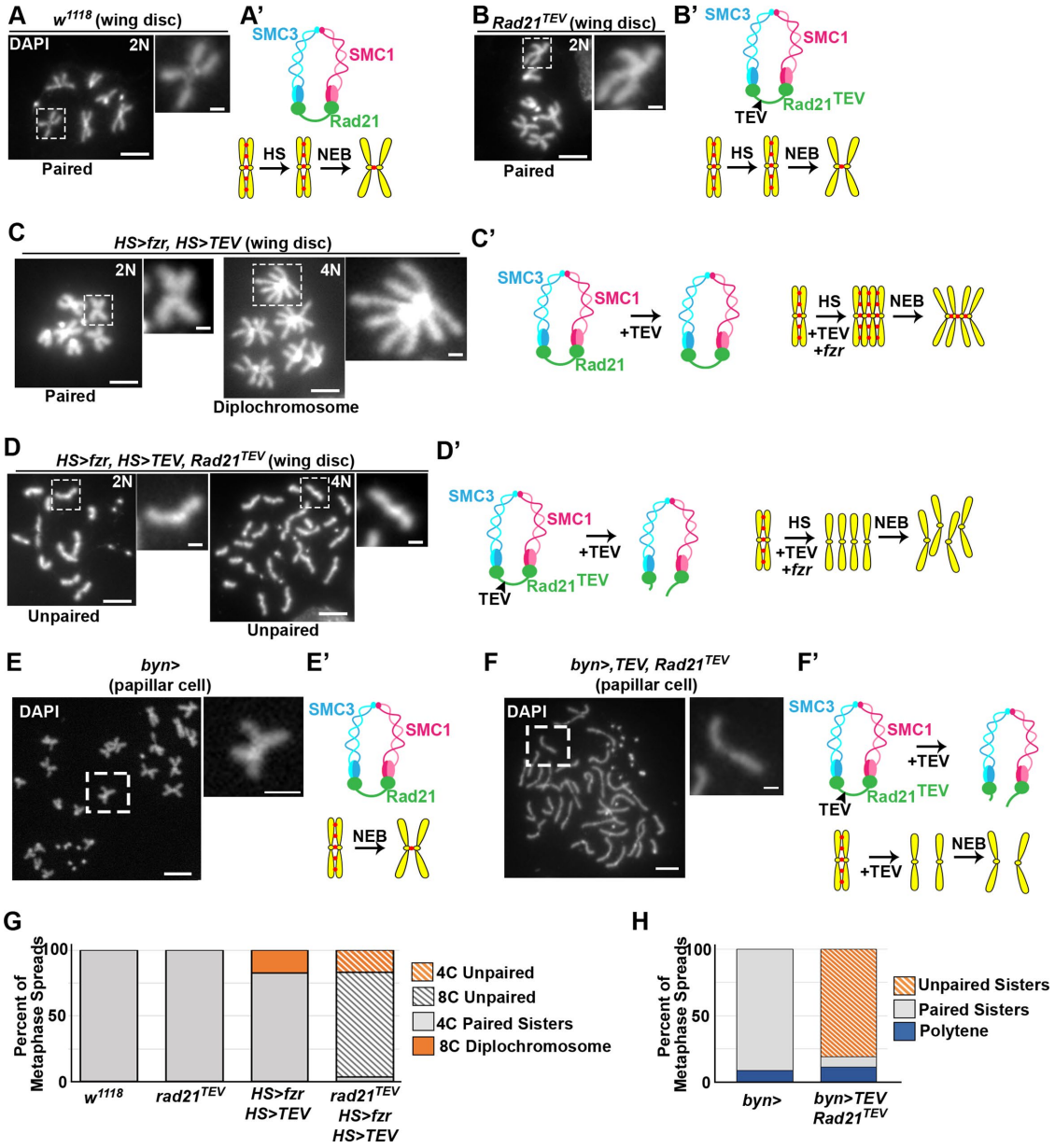

FIGURE 2: Chromatids are held together by the canonical cohesin complex in endocycled cells. Representative wing imaginal disc cell (A–D) and papillar cell (E, F) chromosome spreads. Corresponding diagrams depict cohesin state. 4′6-Diamidino-2-phenylindole (DAPI) marks DNA. Ploidy is indicated (*N* value, top right). (A) Heat-shocked wild-type (w^{1118}). (B) Cells in which $rad21^{TEV}$ is the only source of Rad21. (C) *HS>fzr* plus *HS>TEV* protease. (D) *HS>fzr* plus *HS>TEV* protease in a $rad21^{TEV}$ background. (E) Wild-type (*byn>gal4* alone). (F) *byn>gal4* driving upstream activating sequence (*UAS*)*>TEV* in a $rad21^{TEV}$ background. Scale bar = 5 µm (main images), 1 µm (insets). (G) Quantification of metaphase spreads in A–D. From left to right, *N* = 77, 11, 34, and 59 cells per genotype. (H) Quantification of metaphase spreads in E and F. From left to right, *N* = 22, 52 cells pergenotype.

chromosomes, regardless of proximity within the nucleus or homology. Moreover, following SIRS, papillar chromosomes lose polytene structure and are attached to only a subset of their sister chromatids prior to anaphase (Figure 1A). We previously suggested that papillar chromatids were attached via cohesion with only the most recent sister chromatid (Stormo and Fox, 2016), which we had evidence for based on the symmetric appearance of random or radiation-induced chromosome breaks at the same location on adjacent chromatids (Bretscher and Fox, 2016). To further examine whether papillar chromatids were attached in recent rather than random sister pairs (Figure 3A, model 1 vs. model 2), we pulse-labeled chromatids with 5-bromo-2′-deoxyuridine (BrdU) (Figure 3A, *Materials and Methods*). Our data are consistent with papillar chromatids pairing with their recent sisters (Figure 3B).

We next sought to uncover the mechanism that enables papillar chromatids to establish cohesion with only their most recent sister. We hypothesized that, during papillar endocycles, cohesin is removed between each sister chromatid after each round of replication. Such interphase cohesin regulation could occur through one of two pathways. First, cohesins could be removed from papillar chromosomes during each endocycle by Rad21 destruction, similarly to its destruction at anaphase (Figure 3C, "Rad21 Cleavage"). Second, cohesins could be removed during each endocycle by exit gate

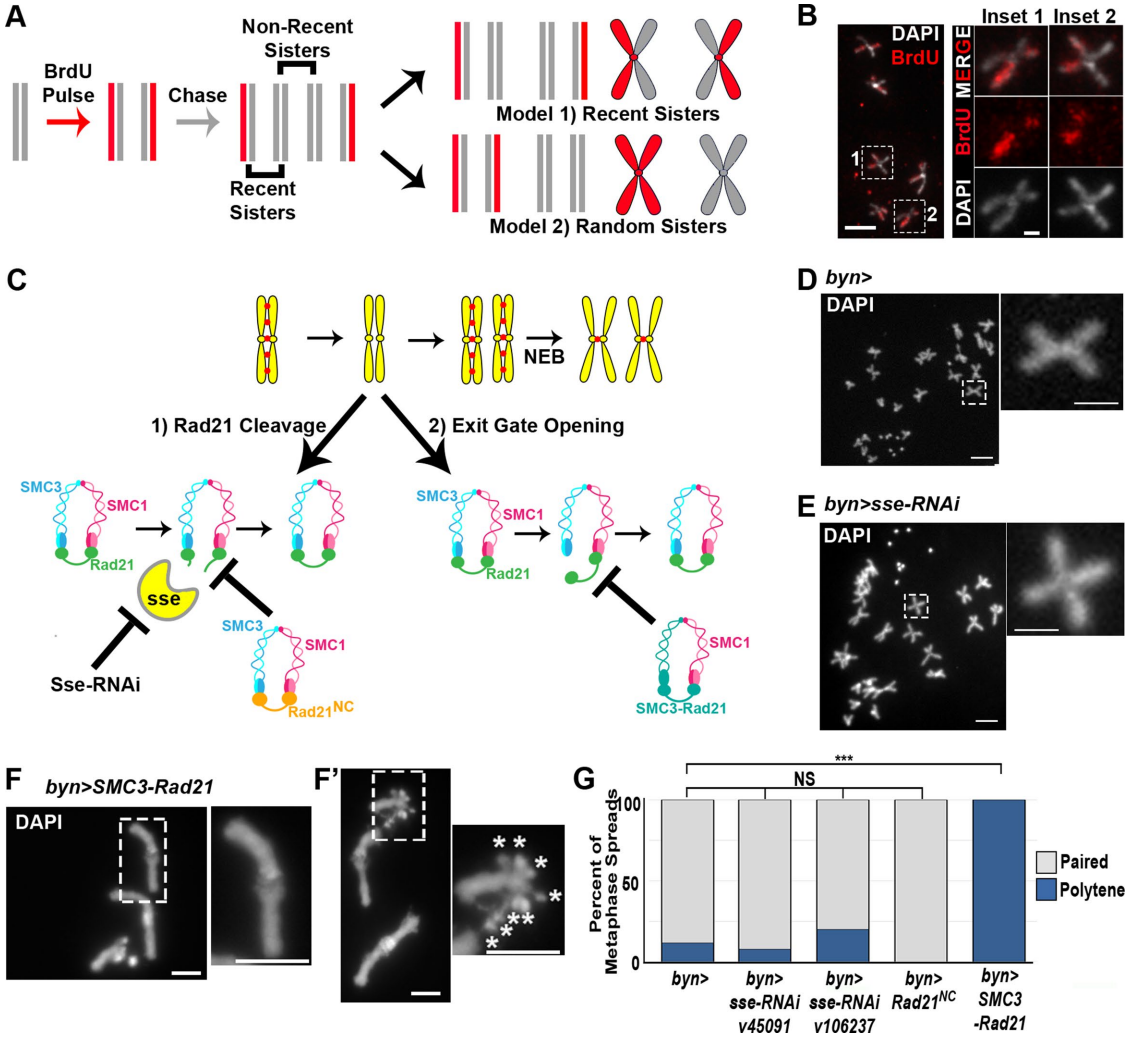

FIGURE 3: Cohesin exit gate opening is required for SIRS. (A) Model of BrdU labeling scheme in papillar cells. Vertical lines, DNA strands. X-shapes, chromatids. Gray, unlabeled DNA, red, BrdU labeled DNA. If papillar chromosomes are composed of recent sisters, then each chromosome would have one labeled chromatid and one unlabeled chromatid (model 1). If sister chromatids are arranged randomly at metaphase, then chromosomes within the same cell would be composed of labeled and unlabeled chromosomes (model 2). Also see *Materials and Methods*. (B) Representative group of labeled papillar chromosomes. DAPI (DNA, white), BrdU, red. Insets, close ups of 2 chromosomes. Inset locations indicated by hatched rectangles in low magnification image for all panels in this figure. (C) Model depicting when we hypothesize cohesins are removed in papillar cells and two potential mechanisms: exit gate opening and Rad21 cleavage. Experimental methods to block these two mechanisms are shown. (D–F) Representative metaphase chromosome spreads of papillar cells. (D) *byn>gal4* control. (E) *byn>gal4* plus *separase-RNAi* driven throughout development. (F) *byn>gal4* plus *SMC3-Rad21* driven throughout development. Asterisk denotes X-chromosome centromeres. (G) Quantification of percentage of metaphase spreads in each class of the indicated genotypes. From left to right, $N = 17, 64, 10, 8, 10$ cells per genotype.

opening (Figure 3C, "Exit gate opening"), similarly to how nonpericentric cohesin is removed in prophase.

We first tested whether Rad21 destruction takes place during papillar endocycles. Normally, Rad21 is cleaved by Separase at the onset of anaphase. Our previous work found no evidence of mitosis during papillar endocycles (Fox *et al.*, 2010), but we reasoned that it was possible for Separase to be regulated in a noncanonical manner in papillar cells, so that it was active during endocycles. To test this hypothesis, we first knocked down *separase* using two separate RNA interference (RNAi) lines (*Materials and Methods*). We then examined the structure of chromosomes in those cells during the first mitosis after papillar endocycles and SIRS. We find that in the absence of Separase, papillar chromosomes undergo SIRS normally, as chromosomes are arranged in pairs at metaphase that are indistinguishable from wild type (Figure 3, D vs. E and G). To ensure that knockdown of *separase* was successful, we performed live imaging on *separase RNAi* papillar cells undergoing mitosis. *separase RNAi* papillar chromosomes form a normal metaphase plate with neatly aligned pairs of chromatids similarly to wild-type cells (Figure 4, A vs. B, –2:00). However, mitosis is aberrant in *separase RNAi* cells because, as expected, chromatid separation fails, resulting in a DNA bridge (Figure 4, A vs. B, 2:00, 8:00, and D). As an alternative approach, we expressed a previously established noncleavable Rad21 that lacks the Separase cleavage site (*UAS>rad21^{NC}*; *Materials*

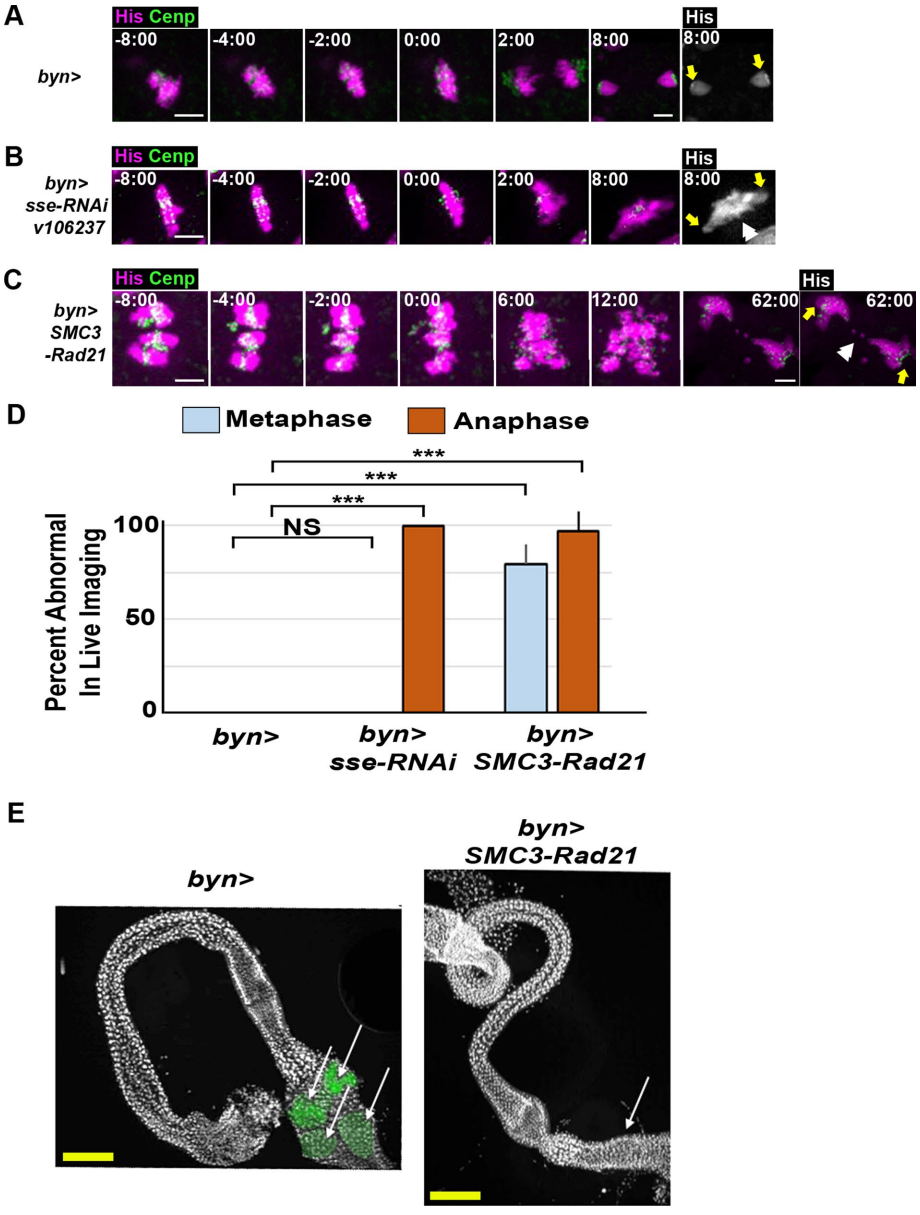

FIGURE 4: Cohesin exit gate opening is required for mitotic fidelity and organ development in papillar cells. (A–C) Time lapse of papillar cell mitosis from the indicated genotypes. His-2av-GFP, magenta; CenpC-tomato, green; time, minutes from anaphase onset. Final panel depicts histone channel only; yellow arrows show spindle pole position. White double arrowhead indicates DNA bridge. Scale bar = 5 μm (main images), 1 μm (insets). (D) Quantification of the percentage of cells with aberrant metaphase and anaphases of the indicated genotypes (+SEM, from left to right, $N = 12, 8, 27$ metaphase and $N = 12, 11, 39$ cells per genotype). (E) Representative images of the adult hindgut of animals of the indicated genotypes (from $N = 12$ control and 8 *SMC3-Rad21* animals). DAPI (DNA, white); papillae pseudocolored (green). Arrows indicate the location (or absence) of papillar structures. Yellow scale bar = 12.5 μm.

and Methods; Urban *et al.*, 2014). Here, too, chromosome structure at the first mitosis is unaffected (Figure 3G). Together, these data strongly suggest that papillar cells do not require Rad21 cleavage to remove cohesion during endocycles.

As an alternative to cohesin cleavage, we reasoned that cohesin turnover via exit gate opening could account for the interphase cohesin regulation that we hypothesize is responsible for paired sister chromatids at papillar metaphase. To test this hypothesis, we used a previously established transgenic construct in which Rad21 and SMC3 are fused via a linker region (Eichinger *et al.*, 2013) (Figure 3C). This fusion can load onto chromosomes normally but cannot be removed from chromatin because the fusion closes the exit gate. This construct is still cleaved normally at anaphase.

To test whether exit gate opening during endocycles was required for normal papillar chromosome structure, we expressed *UAS>SMC3-Rad21* and looked at chromosomes during the first metaphase after SIRS. Chromosome structure is often substantially altered in these cells. Specifically, chromosomes persist as polytene chromosomes, suggesting SIRS does not take place (Figure 3, F, F′, and G). These chromosome phenotypes do not appear to disrupt papillar endocycles, as in metaphase spreads where the X centromeres are separate (as we described before for papillar cells; Stormo and Fox, 2016), we can count 8 X centromeres, indicating these cells are octoploid (Figure 3F′, asterisks in inset). The finding that cohesins can persist on papillar chromosomes without disrupting multiple endocycle S-phases is consistent with the observation that cohesin can remain associated with chromosomes during DNA replication (Rhodes *et al.*, 2017). The chromosomal phenotype in papillar cells of *SMC3-Rad21* animals suggests cohesin exit gate opening, most likely during endocycles, is important for SIRS.

We were surprised that, at the first metaphase post-SIRS, *SMC3-Rad21* papillar cells displayed the haploid number of observed distinct chromosomes, as opposed to the diploid number. This implies that blocking cohesin exit gate opening can also promote ectopic homologue–homologue pairing. We had instead expected that homologues would remain separate at mitosis despite persistent sister-chromatid cohesion, because homologous chromosomes are not normally cohesed but are instead associated by somatic pairing mechanisms. In *Drosophila,* somatic homologue pairing is antagonized by condensins (Smith *et al.*, 2013). At mitosis, condensins overcome the attractive forces of somatic pairing to drive homologues apart, but sisters remain attached by cohesins. We suspect *SMC3-Rad21* expression antagonizes this condensin activity. Interestingly, cohesin and condensin II can antagonize each other's functions in alignment of sister chromatids in cultured *Drosophila* cells (Senaratne *et al.*, 2016).

We next analyzed the consequence of the disrupted mitotic chromosome structure phenotype of *SMC3-Rad21* on papillar cell mitosis by performing live imaging. *SMC3-Rad21* papillar cells fail to form a proper metaphase plate with pairs of sister chromatids bioriented. Instead, polytene chromosomes are still evident until anaphase (Figure 4C, –2:00). Subsequently, anaphase of *SMC3-Rad21* papillar cells is highly error prone (Figure 4C, 6:00, D). Given

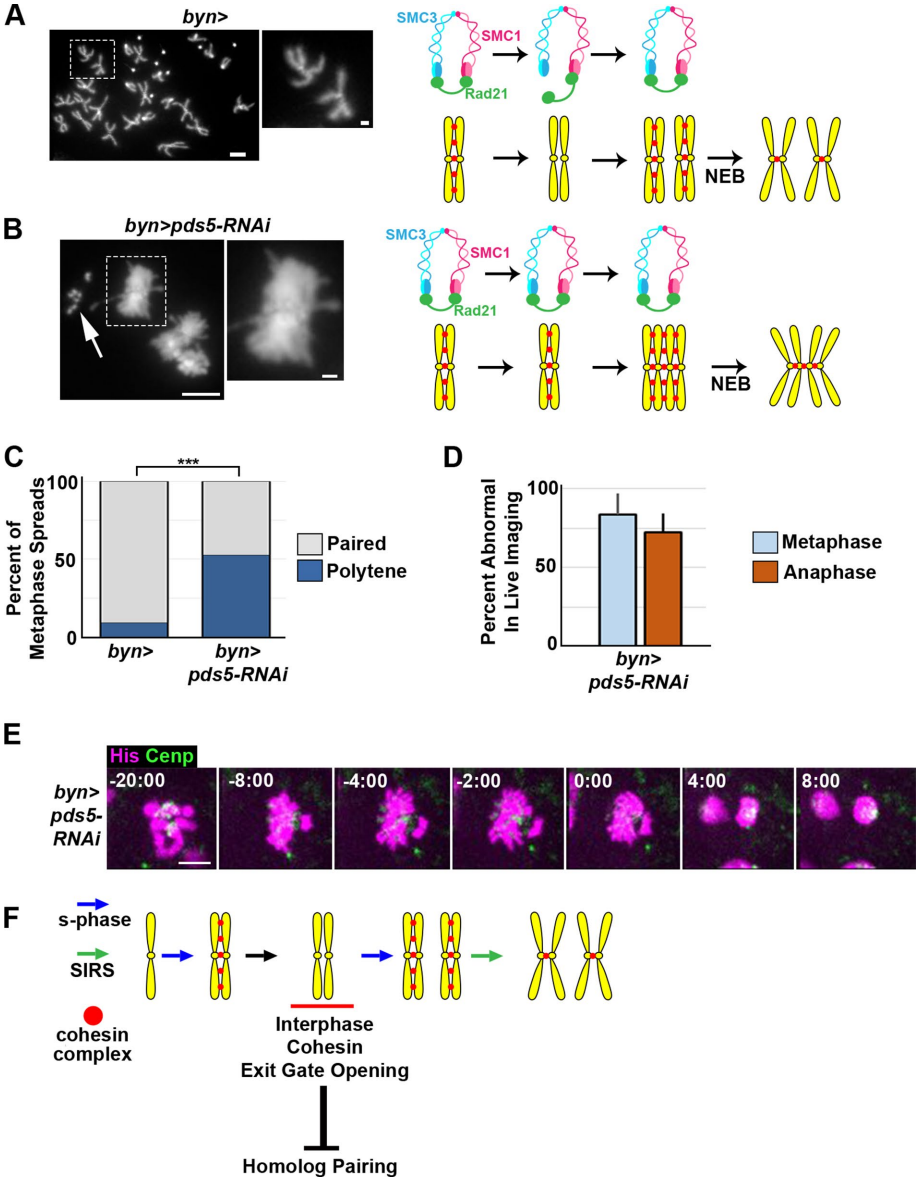

FIGURE 5: Pds5 is required for cohesin regulation during papillar endocycles. (A, B) Representative metaphase chromosome spreads of wild-type and *pds5-RNAi* papillar cells. Corresponding diagrams depict cohesin state. DAPI marks DNA. (C) Quantification of percentage of metaphase spreads in each class. $N = 44$ control and 19 *pds5 RNAi* cells. (D) Quantification of mean error rate in live imaging of *pds5-RNAi* animals (+SEM, from $N = 9$ cells). (E) Time lapse of papillar cell mitosis in a *bynGal4* plus *UAS pds5-RNAi* animal. His-2av-GFP, magenta; CenpC-tomato, green; time, minutes from anaphase onset. Scale bar = 5 μm (main images), 1 μm (insets). (F) Proposed model of cohesin regulation and function in papillar cells during endocycles, SIRS, and the first subsequent mitosis.

these mitotic defects, we examined the consequence of such error-prone divisions on tissue development by examining adult hindgut structure. Unlike in control animals, which display four rectal papillar structures in adults, *SMC3-Rad21* animals completely lack obvious rectal papillar structures (Figure 4E). This suggests that *SMC3-Rad21* cells do not survive the extremely aberrant mitotic divisions and thus fail to produce cells which are normally required for their construction (Fox *et al.*, 2010; Schoenfelder *et al.*, 2014). In contrast, the rest of the hindgut, which is formed by multiple rounds of diploid mitoses (Fox and Spradling, 2009; Sawyer *et al.*, 2017) and also expresses *SMC3-Rad21* under the same *byn>Gal4* driver, appears unaffected. We hypothesize that the lack of tissue-level phenotype in diploid cells of *SMC3-Rad21* animals is due to Separase-mediated cohesin cleavage of chromatids at anaphase in these cells. These results suggest that, in papillar cells, the distinct processes of genome reduplication followed by SIRS creates an additional chromosome structural challenge for mitotic fidelity. We propose that as these cells undergo endocycles, cohesin exit gate opening is important for later dissolution of homologue–homologue pairing, as well as for maintaining that chromatids are only cohesed with their most recent sister. Failure to properly regulate cohesins prior to mitosis leads to severe mitotic infidelity and organ malformation.

Pds5 is required for interphase chromosome regulation

Our data suggest that cohesin removal in cells undergoing endocycles followed by SIRS is crucial for subsequent mitotic fidelity. We next sought to determine the regulation of cohesin exit gate opening. From a candidate analysis of known cohesin regulators, our most striking result was with Pds5. In multiple organisms including *Drosophila* (Dorsett *et al.*, 2005), Pds5 is required for sister chromatid cohesion. At the first metaphase post-SIRS, *pds5* animals exhibit ectopic homologue pairing and chromatids remain in a polytene configuration at metaphase (Figure 5, A vs. B and C), as in *SMC3-Rad21* animals. However, *pds5* papillar chromosomes contrasted with those of *SMC3-Rad21* flies in one important respect. In cells expressing *SMC3-Rad21*, chromosome arms are often closely aligned in a classic polytene configuration (Figure 3, F and F′). In contrast, following knockdown of *pds5*, chromosome arms separate, but all centromeres remain together, a configuration more similarl to cells with diplochromosomes (Figure 5, B and C). As with *SMC3-Rad21* flies, live imaging shows a failure to separate chromatids in *pds5* animals, as evidenced by clusters of DNA that move in tandem and a failure to form a metaphase plate (Figure 5, D and E). Also as with *SMC3-Rad21* flies, papillar ploidy appears unaffected by *pds5 RNAi*, as evidenced by our ability to count ~8 separate fourth chromosomes in otherwise polytene *pds5 RNAi* cells (Figure 5B, arrow). These results strongly suggest that *pds5*-mediated cohesin exit gate opening at centromeres is key in interphase cohesin regulation during the premitotic endocycles of papillar cells. Deficiencies in this mechanism contribute to mitotic errors in cells with genome reduplication.

DISCUSSION

Cohesins typically hold together all products of S-phase through interphase until cleavage of the complex at anaphase. Here our results imply that cohesin removal mechanisms can be repurposed in

cells undergoing endocycles to prevent subsequent mitotic infidelity. In *Drosophila* papillar cells, our results suggest that chromosomes lose cohesion and then reestablish it with the most recent sister chromatid during endocycles (Figure 5F). We thus propose that this cohesin regulation occurs during interphase but involves a repurposing of the cohesin exit gate opening mechanism normally used during prophase. Our work also reveals a role for cohesin exit gate opening in antagonizing pairing between homologous chromosomes in cells that endocycle (Figure 5F).

Does such interphase cohesin regulation that we propose to occur in papillar cells also occur in other polyploid cell types? *Drosophila* ovarian nurse cells partially separate chromatids but do not proceed to a full mitosis (Hammond and Laird, 1985; Dej and Spradling, 1999) and thus may also undergo some degree of interphase cohesin regulation. In nonmitotic polyploid cells such as salivary gland cells, the cohesin complex is present and dynamic (Gause *et al.*, 2010; Cunningham *et al.*, 2012). However, because these cells are nonmitotic, chromatids are never separated enough to observe constraint by cohesins. Our work here suggests that interphase cohesin regulation is definitely not a property of all polyploid cells, as cells with induced endocycles contain diplochromosomes, where four chromatids remain cohesed (Figure 2). The difference between naturally occurring and ectopically induced endocycles may account for mitotic defects associated with the latter. Additionally, we also uncover a surprising role for cohesin exit gate opening in antagonizing homologue pairing. While lack of this anti-pairing mechanism may contribute to the phenotypes we see in papillar cells with compromised exit gate opening, we note that cells with ectopic diplochromosomes do not pair (Figure 2) and exhibit similar mitotic defects to papillar cells with defective exit gate opening (Stormo and Fox, 2016). Future work can examine the connection between cohesin exit gate opening and known homologue pairing regulators such as condensins. Further, we note that while we heavily favor an interphase model of cohesin regulation, it is possible that only the cohesins between recent sister chromatids, and not any other cohesins, somehow resist the prophase pathway specifically during SIRS. Such a mechanism would still likely involve some differential marking (such as acetylation) of the cohesins between recent sisters, which would likely have to occur in the last endocycle (i.e., would still be an interphase mechanism).

With respect to molecular mechanisms of cohesin regulation, our results also revealed differences between *pds5* knockdown and *SMC3-Rad21* expression in papillar cells. We propose that *SMC3-Rad21* represents a situation where cohesin complexes cannot be removed by the prophase pathway at onset of mitosis, and therefore chromosome arms remain attached. In contrast, *pds5* knockdown blocks cohesin release during endocycles but not during prophase. Our results reveal a differential sensitivity for cohesin regulation at chromosome arms and centromeres in this RNAi condition. This could reflect that Pds5 in papillar cells is more essential for centromeric cohesion than for arm cohesion. Along these lines, kinase activity of polo kinase as well as Aurora B phosphorylation of SA1 and 2 participate with Pds5 and Wapl in the prophase pathway to remove arm cohesion (Sumara *et al.*, 2002; Gimenez-Abian *et al.*, 2004; Hauf *et al.*, 2005; Kueng *et al.*, 2006; Shintomi and Hirano, 2009), and it is possible that in *pds5 RNAi* animals these other arm cohesin regulators are still able to separate papillar polytene chromosome arms.

Future work can address regulation of pericentric cohesins during papillar cell endocycles. In mitotic cells, pericentromeric cohesin is not removed during the prophase pathway, because that region is protected by Shugoshin (Moore *et al.*, 1998; Lee *et al.*, 2004; Watanabe, 2005). Shugoshin directly antagonizes Wapl, a partner of Pds5 (Hara *et al.*, 2014). If Pds5 is required to remove cohesins during endocycles, then how does it bypass Shugoshin? One possibility is that Shugoshin is not present in these cells during endocycles. If so, then this would allow the prophase pathway to clear cohesins from the entire chromosome, including centromeres, during each endocycle.

In disease, continued study of chromosome structure after genome reduplication is important because diplochromosomes are induced by common cancer therapeutics such as topoisomerase inhibitors (Hande, 1998; Sumner, 1998). Our data suggest that if cells prone to diplochromosomes regulated cohesins differently, so that only paired recent sisters were present at metaphase, then the rate of mitotic errors and aneuploidy in these cells would dramatically drop. Given our identification here of interphase cohesin regulation during papillar endocycles, papillar cells represent a valuable system for further study of cohesin regulation. Additionally, the importance of mitotic genome reduplicated cells in disease suggests that understanding chromosome structure in these cells may give insight into new therapies.

MATERIALS AND METHODS

Drosophila stocks

Stocks were obtained from the Bloomington *Drosophila* Stock Center (stock number in parentheses): w^{1118} (3605); *His-2av-GFP* (24163); vtd^{ex14} (26165); vtd^{ex8}, *rad21.271TEV-myc* (27613); *UAS>NLS-V5-TEV-NLS* (27605); *HS>NLS-V5-TEV-NLS* (27612); *pds5-RNAi* (35632, previously validated by Kusch (2015) to cause meiotic recombination phenotypes); $Gal80^{TS}$ (7018); the Vienna Drosophila Stock Center (Dietzl *et al.*, 2007): *sse-RNAi* (v45091, previously validated to phenocopy *sse* mutants in larval neuroblasts; Cipressa *et al.*, 2016); *sse-RNAi* (v106237); or were kind gifts: *tomato-Cenp-C*; *HS>fzr* (Sigrist and Lehner, 1997); *byn>gal4* (Singer *et al.*, 1996); *UAS>SMC3-vtd-GFP* (Eichinger *et al.*, 2013); $UAS>rad21^{NC}$ (Urban *et al.*, 2014).

Drosophila culture and genetics

All flies were raised on standard media (Archon Scientific, Durham, NC). All experiments involving a UAS transgene (including RNAi) were performed at 29°C to maximize Gal4-mediated transgene expression. Heat shocks to induce *fzr* or *TEV* expression were performed on third instar larvae. Animals for these experiments were heat shocked in a vial at 37°C (water bath) for 20 min. In experiments involving inducible transgenic Rad21 cleavage, endogenous Rad21 was removed using two null *rad21* mutant alleles in trans (vtd^{ex14} and vtd^{ex8}). These mutant alleles were rescued by a ubiquitously expressed *rad21* transgene containing a tobacco etch virus (TEV) cleavage site ($rad21^{TEV}$, Pauli *et al.*, 2008). This construct was then cleaved either in all cells (using a heat shock promoter, *HS>TEV)* or specifically in our cell type of choice using a UAS promoter (*UAS>TEV)*. Animals were examined 10 h after HS>fzr expression, as this is the time where we previously established that mitosis resumes after heat shock in these animals.

For papillar cell experiments, we used the hindgut specific *byn>gal4* driver to express transgenes. For all transgenes except for UAS TEV, we expressed these transgenes throughout development. To avoid prolonged UAS-TEV expression, we used previously established methods that rely on *Gal80ts* to repress *Gal4* expression (Fox and Spradling, 2009; Fox *et al.*, 2010) to confine expression of *UAS-TEV* to the period of endocycles (second larval instar) and not mitosis (which occurs much later: hours 24–48 post–puparium formation at 22°C).

Chromosome cytology

Chromosome preparations were performed as previously described (Gatti *et al.*, 1994; Fox *et al.*, 2010). We used enriched for metaphase cells by first incubating tissue in colcemid (Sigma, St. Louis, MO) at 50 µg/ml for 20 min in phosphate-buffered saline (PBS). Imaging was performed on a Zeiss Axio Imager 2 with a 63× oil immersion lens.

Live imaging

Live-imaging preparations were prepared as previously described (Prasad *et al.*, 2007; Fox *et al.*, 2010). Imaging was performed on a spinning disk confocal (Yokogawa CSU10 scanhead) on an Olympus IX-70 inverted microscope using a 60×/1.3 NA UPlanSApo Silicon oil, 488 and 568 nm Kr-Ar laser lines for excitation, and an Andor Ixon3 897 512 electron-multiplying charge-coupled device camera. The system was controlled by MetaMorph 7.7. Images were analyzed in ImageJ (Schneider *et al.*, 2012).

BrdU feeding and staining

To determine whether papillar chromatids are associated with most recent sisters, we fed 1 mg/ml BrdU dissolved in PBS + food coloring for 1 h during the second instar stage, when papillar cells endocycle twice to reach 8C ploidy. The goal of this experiment was to occasionally label only the second-to-last S-phase in these cells so that one-fourth of all DNA strands at mitosis were BrdU labeled at mitosis. If one-fourth of DNA strands contain BrdU, we would expect one half of chromatids to be labeled and each chromosome to contain one labeled chromatid if recent sisters are paired (Figure 3A, Recent Sisters). In contrast, if sister chromatids are randomly paired, then we would see chromosomes in which neither chromatid was labeled, as well as chromosomes in which both chromatids were labeled, in the same cell (Figure 3A, Random Sisters). To ensure pulse labeling of chromosomes, larvae were washed in PBS after feeding, and animals with no food coloring in their gut were discarded.

To image BrdU in metaphase spreads, chromosome cytology and BrdU antibody staining (Rat anti-BrdU, Serotec 1:100, clone 3J9) was performed based on Sullivan and Karpen (2001) with slight modifications. In brief, hour 24–48 post–puparium formation (at 22°C) animals were dissected. Dissected tissue was incubated in 0.5% sodium citrate for 15 min then fixed on a coverslip in 11:11:2 methanol:acetic acid:H_2O. Fix was removed and replaced with 10 µl of 45% acetic acid. The coverslip was then squashed on a positively charged slide (VWR, Radnor, PA) and then frozen in liquid nitrogen until the coverslip could be removed using a razor blade. Slides were then transferred to 95% ethanol at –20°C. All subsequent steps were performed directly on the slide, and tissue denaturation and BrdU antibody staining were performed as described previously (Fox and Spradling, 2009).

Statistics

All statistics were computed in Prism 7 (GraphPad, La Jolla, CA). Metaphase spreads were blinded and then scored. Metaphase spreads were compared with wild type using a chi-squared test on total counts. For live imaging, cells were averaged within animals, and then mean and standard error were calculated by averaging between animals. Means were compared using one-way analysis of variance (ANOVA). NS, not significant for $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

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