Crumbs, Galla and Xpd are required for kinesin-5 regulation in mitosis and organ growth in Drosophila

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Summary statement
Crumbs and Xpd act together to regulate the stability of mitotic kinesin-5. This finding provides a mechanism for Xpd-dependent regulation of mitosis and organ growth.
Abstract

Xeroderma Pigmentosum D (XPD) is a multi-function protein involved in transcription, DNA repair, and chromosome segregation. In Drosophila, Xpd interacts with Crumbs (Crb) and Galla to regulate mitosis during embryogenesis. It is unknown how these proteins are linked to mitosis. Here, we show that Crb, Galla-2 and Xpd regulate nuclear division in syncytial embryo by interacting with Klp61F, the Drosophila mitotic kinesin-5 associated with bipolar spindles. Crb, Galla-2 and Xpd physically interact with Klp61F and co-localize to mitotic spindles. Knockdown of any of these proteins results in similar mitotic defects. These phenotypes are restored by overexpressing Klp61F, suggesting that Klp61F is a major effector. Mitotic defects of *galla-2 RNAi* are suppressed by Xpd overexpression but not *vice versa*. Depletion of Crb, Galla-2 or Xpd results in a reduction of Klp61F levels. Reducing proteasome function restores Klp61F levels and suppress mitotic defects caused by knockdown of Crb, Galla-2 or Xpd. Further, eye growth is regulated by Xpd and Klp61F. Hence, we propose that Crb, Galla-2 and Xpd interact to maintain the level of Klp61F during mitosis and organ growth.
Introduction

Crumbs (Crb) is a conserved transmembrane protein essential for epithelial apical-basal cell polarity in Drosophila (Tepass, 2012, Pocha and Knust, 2013, Martin-Belmonte and Perez-Moreno, 2012). Crb is also required for visual function in the eye by regulating apical morphogenesis of photoreceptor cell membrane (Hong et al., 2003, Izaddoost et al., 2002, Pellikka et al., 2002). In addition, Crb is involved in the regulation of Hippo signaling for organ growth (Chen et al., 2010, Ling et al., 2010, Ribeiro et al., 2014, Robinson et al., 2010). In these processes, Crb functions as an apical cue for interacting protein partners.

Recent evidence indicates that Crb is also required for controlling mitotic processes in Drosophila (Yeom et al., 2015). Segregation of chromosomes is a key process of mitosis that is coordinated by mitotic spindles. Drosophila embryo has been extensively used to study mitosis in vivo. Embryogenesis in Drosophila begins with 13 cycles of nuclear divisions to generate multinucleate syncytium prior to cellularization. Regulation of spindle microtubules is critical for precise chromosome segregation during these nuclear division cycles. A clue for the Crb’s role in mitosis was provided from genetic interaction of Crb with Galla-1 and Galla-2, two Drosophila homologs of mammalian MIP18 implicated in chromosome segregation. Crb and Galla proteins co-localize to the region of mitotic spindles during nuclear division in early embryos, and their loss results in various mitotic defects including branched/fused spindle microtubules and incomplete segregation of dividing nuclei. Interestingly, MIP18 is a component of XPD protein complex (Ito et al., 2010). XPD is a DNA helicase that regulates DNA repair, cell cycle and transcription by forming a complex with the TFIIH transcription factor. Mutations in XPD are associated with human diseases such as Xeroderma Pigmentosum (XP), Trichothiodystrophy (TTD) and Cockayne syndromes (CS) (Egly and Coin, 2011, Zurita and Merino, 2003). MIP18 is not found in the TFIIH complex, but interacts with XPD to form a distinct protein complex called MMXD to regulate mitosis (Ito et al., 2010). It is unknown whether Galla proteins are independent from the TFIIH complex in Drosophila.

The Drosophila homolog Xpd is also required for mitosis during early embryogenesis (Chen et al., 2003, Li et al., 2010). Crb physically interacts with Galla and Xpd, and loss of these proteins results in mitotic defects with abnormal spindles (Yeom et al., 2015, Li et al., 2010). Spindle microtubules are associated with various motor proteins (Hildebrandt and Hoyt, 2000, Mazumdar and Misteli, 2005, Goshima and Vale, 2003). Among these mitotic motors, Kinesin-5 plays a critical role in the formation and maintenance of bipolar spindles (Heck et
Kinesin-5 has a characteristic bipolar structure made of four monomeric motor proteins that allows kinesin’s sliding between two anti-parallel spindle microtubules (Acar et al., 2013, Kashina et al., 1996, Kapitein et al., 2005, Cole et al., 1994, Saunders et al., 2007). Such activity leads to the separation of two spindle poles from each other, resulting in the segregation of chromosomes (Kashina et al., 1997, Heck et al., 1993). *Drosophila* Klp61F is the ortholog of vertebrate Kinesin-5 family proteins with conserved structure and function (Vale and Milligan, 2000, Waitzman and Rice, 2014, Kashina et al., 1997, Heck et al., 1993). Despite the roles of Crb-Galla-Xpd (CGX in short) proteins in mitosis, it remains a puzzle how they are involved in the mitotic process.

In this study, we demonstrate that the CGX proteins regulate mitosis by directly interacting with Klp61F kinesin. Depletion of Crb, Galla-2 or Xpd results in similar spindle defects. Genetic analysis suggests that Klp61F functions downstream to Galla-2 and Xpd. Loss of CGX proteins results in proteasome-dependent reduction of Klp61F protein level. This work provides evidence that CGX proteins are required to maintain the level of Klp61F protein for proper regulation of mitosis in embryo. Our data also suggest important roles of Xpd and Klp61F in organ growth.

**Results**

**Identification of Klp61F as a genetic modifier of Crb**

Overexpression of the intracellular domain Crb (Crb$^{\text{intra}}$) in eye disc driven by *GMR-Gal4* causes severe roughening of adult eyes by affecting the integrity of differentiating retinal epithelium (Izaddoost et al., 2002, Pellikka et al., 2002, Grzeschik and Knust, 2005) (Fig. 1D). Knockdown or overexpression of Klp61F by *GMR-Gal4* in the wild-type background did not alter the size or the morphology of adult eyes (Fig. 1A-C). However, this rough eye phenotype was strongly enhanced by knockdown of Klp61F (Fig. 1E) and suppressed by overexpression of Klp61F in all tested flies (Fig. 1F). As control, *GMR>crb^{intra}* was crossed with *UAS-GFP* to ensure that the suppression of Crb$^{\text{intra}}$ by *UAS-Klp61F* was not due to titration of Gal4 by an additional copy of UAS (Fig. 1D).
Crb and Klp61F co-localize to mitotic spindles and physically interact.

Genetic interaction between crb and klp61F suggests that these two gene functions might be related in mitosis. Since analysis of mitosis in eye disc is not straightforward due to the small cell size and unsynchronized mitosis, we chose to examine nuclear divisions in syncytial embryo that has been extensively utilized to study mitotic functions of Klp61F (Cheerambathur et al., 2008, Brust-Mascher et al., 2009, Scholey, 2009, Sharp et al., 1999). Embryos were examined approximately at nuclear division cycle 11, unless stated otherwise. Previously, we have shown that Crb is detected as diffused staining in the region of chromosome segregation during nuclear division (Yeom et al., 2015). We examined whether Crb localization show any overlap with Klp61F in microtubule spindles during mitosis. Since we often found bleeding effects from Tubulin (Tub) staining, we performed immunostaining for Crb and Klp61F in the absence of anti-Tub antibody. In prophase, both Crb and Klp61F staining were enriched in the spindle poles (Fig. 2A) with similar pattern to the Tub staining (Fig. S1A). Although Crb staining appeared more diffused than Klp61F, it showed a similar pattern with Klp61F and Tubulin (Tub) in mitotic spindles of metaphase (Figs. 2B, S1B) and anaphase (Figs. 2C and S1C). In telophase, Tub was strongly localized to the midbody (Fig. S1D). Crb and Klp61F were also enriched in the region of midbody (arrows in Fig. 2D). Specificity of anti-Crb staining in syncytial embryo was shown earlier by loss of Crb staining by crb RNAi (Yeom et al., 2015). We also confirmed reduction of Klp61F levels in syncytial embryos produced from females treated with Klp61F RNAi using maternal (mat)-Gal4 (Fig. S1F). These results suggest that the anti-Klp61F antibody and Klp61F RNAi work properly.

Based on their overlapping localization, we checked whether Crb and Klp61F physically interact. In the assay using S2 cells transfected with Myc-Crb\textsuperscript{intra} and Flag-Klp61F, Flag-Klp61F was co-immunoprecipitated by Myc-Crb\textsuperscript{intra}. However, a negative control kinesin II subunit Klp64D that shares a conserved motor domain with Klp61F (41% identity) was not co-immunoprecipitated with Crb (Fig. 2E). We also performed pull-down experiments using bacterially expressed GST-fusion proteins. GST-Crb\textsuperscript{intra} could bind to Klp61F but not Klp64D (Fig. 2F), indicating their specific interaction.

Klp61F suppresses mitotic defects of Crb\textsuperscript{intra} overexpression

To examine the functional relationship between Crb and Klp61F, we compared mitotic defects caused by Crb\textsuperscript{intra} overexpression and Klp61F RNAi. Because loss of function mutations in crb
or klp61F causes lethality, we examined the effects of RNAi knockdown in the germline of mothers by using mat-Gal4. As reported previously (Garcia et al., 2009, Heck et al., 1993, Wilson et al., 1997), Klp61F knockdown by RNAi (Klp61F RNAi^{BL35804}) caused spindle defects in different phases of cortical nuclear division. Similar mitotic defects were seen by an independent RNAi line (Klp61F RNAi^{V52548}) (Fig. S2A-B). Examination of embryos at cycle 11 indicated that most embryos (79%) showing mitotic defects were in metaphase (Fig. S2J, L). Similarly, a majority of embryos (69%) with Crb\textsuperscript{intra} overexpression showed spindle defects during metaphase (Fig. S2K, L). Therefore, we focused our analysis on mitotic defects during metaphase at cycle 11. There were three major common types of spindle defects caused by overexpression of Crb\textsuperscript{intra} or Klp61F RNAi; free centrosomes with no attached spindles and chromosomes, fused/branched microtubules, multiple poles and monopolar spindles (Fig. 3A-C, S6). The most frequent phenotype of Crb\textsuperscript{intra} overexpression was free centrosomes compared with low incidents of multi-polar and monopolar phenotypes. Spindle phenotypes of Crb\textsuperscript{intra} overexpression were considerably recovered by Klp61F overexpression (Fig. 3G).

After 9\textsuperscript{th} nuclear division cycle, most nuclei migrate to the periphery of embryo to form a monolayer at the cortex prior to cellularization (Aveek Mazumdar, 2002, Foe and Alberts, 1983). Klp61F RNAi and Crb\textsuperscript{intra} overexpression often led to loss of nuclei in large patches where multiple mitotic nuclei fell into the interior of embryo. Such nuclei-free patches were found at a low frequency during nuclear division cycle 10. However, the frequency of nuclei-free patches was significantly increased later in nuclear division cycles 12-13. Because the type of spindle defects could not be determined in such regions of nuclear loss, we scored such phenotype separately based on the severity of nuclear loss. We divided the nuclear loss phenotypes into ‘mild’ and ‘severe’ phenotype which refer to embryos showing less than 10% or more than 10% of nuclei-free area, respectively (Fig. 3D-F). In wild-type control, approximately 75% of embryos were normal while 10-15% embryos showed mild or severe nuclear loss phenotype. Crb\textsuperscript{intra} overexpression resulted in the nuclear loss phenotype in about 85% embryos, and these phenotypes were partially suppressed by overexpressing Klp61F (Fig. 3H). Thus, Klp61F overexpression can suppress Crb\textsuperscript{intra} phenotypes not only in the eye but also in mitosis during early embryogenesis.
**Galla-2 knockdown is suppressed by Klp61F overexpression**

Galla-1 and Galla-2 are related *Drosophila* homologs of mammalian MIP18 associated with XPD (Ito et al., 2010). Since Galla proteins interact with Crb\textsuperscript{intra}, we tested whether Galla-1 and/or Galla-2 might also be associated with Klp61F. Both V5-Galla-1 and V5-Galla-2 expressed in S2 cells were co-immunoprecipitated with Flag-Klp61F, while Galla-1 was not co-immunoprecipitated with Flag-Klp64D negative control (Fig. 4A). Endogenous Galla-2 and Klp61F proteins were also co-immunoprecipitated in tissue extracts from wild type embryos or adult heads (Fig. S3A), suggesting their complex formation \textit{in vivo}. In GST pull-down assays, GST-Galla-2 showed direct binding to Klp61F but not Klp64D. Interestingly, GST-Galla-1 did not show detectable binding to Klp61F (Fig. 4B). Hence, Galla-2 directly interacts with Klp61F, while Galla-1 may associate with Klp61F indirectly.

Consistent with the physical interaction between Galla-2 and Klp61F, immunostaining of Galla-2 in the absence of anti-Tub antibody showed its localization to spindles in the same pattern as Tub staining (Fig. S3B). Maternal knockdown of Galla-2 strongly reduced Galla-2 levels in the spindles of dividing nuclei (Fig. S3C), supporting the specificity of Galla-2 staining and \textit{galla-2 RNAi}. We then examined the effects of Galla-2 knockdown in mitotic nuclei in early embryos. Maternal knockdown of Galla-2 by two independent RNAi lines (v110611 and BL58320) showed similar types of mitotic spindle defects like free centrosomes and fused spindles (Fig. S2C, D, S6), as seen with Crb\textsuperscript{intra} overexpression. These \textit{galla-2 RNAi} defects were strongly suppressed from 27% to 3.2% by Klp61F overexpression (Fig. 4D). \textit{galla-2 RNAi} also resulted in high levels (42% embryos) of severe nuclear loss phenotype. The nuclear loss phenotype was partially suppressed to 18% by Klp61F overexpression (Fig. 4E).

**Xpd RNAi phenotypes are suppressed by Klp61F overexpression**

Galla-2 physically interacts with Xpd (Yeom et al., 2015). We also found that Myc-Xpd co-localizes with GFP-Klp61F in mitotic spindles of dividing S2 cells (Fig. S1G). Hence, Xpd might function together with Klp61F in mitosis. Immunoprecipitation assays indicated that Myc-Xpd forms a complex with Flag-Klp61F in S2 cells (Fig. 5A). Further, GST-pulldown assays showed direct binding between MBP-Klp61F and GST-Xpd protein (Fig. 5B). In these interaction assays, Xpd did not show physical interaction with the negative control Klp64D in co-IP or GST pulldown tests (Fig. 5A-B).
Xpd knockdown by mat-Gal4 using two Xpd RNAi lines (v106998 and v41021) led to similar spindle defects in early embryos (Fig. S2E, F, S6). A majority of the spindle defects were free centrosomes. Spindle defects by Xpd RNAi were restored to the level of control (mat>GFP/GFP) by overexpressing Klp61F (Fig. 5C). As in gala-2-depleted embryos, Xpd RNAi resulted in large regions of nuclear loss in early embryos. The nuclear loss phenotype was also weakly suppressed by Klp61F overexpression (Fig. 5D). Although Klp61F overexpression did not change the level of total defective embryos (77% to 75%), it significantly reduced the number of severely defective embryos (60% to 41%). Taken together, mitotic defects caused by Xpd RNAi can be partially compensated by Klp61F overexpression.

**Knockdown of Crb, Galla-2 or Xpd reduces Klp61F level through proteasome**

Since mitotic defects from Crb\textsuperscript{intra} overexpression or knockdown of CGX genes can be suppressed by Klp61F overexpression, these genes may be involved in the regulation of Klp61F protein levels. To test this possibility, first we attempted to examine Klp61F levels by using Western blot experiments. Tissue extracts were prepared from syncytial embryos collected for two hours. Immunostaining of Western blots showed that Klp61F levels were variably reduced by knockdown of Crb, Galla-2, Xpd or Crb\textsuperscript{intra} overexpression. Variable reductions were probably due to heterogeneity of collected embryos that show different severity of nuclear loss. Quantitative data from 20 Western blot experiments showed that maternal knockdown of Crb, Galla and Xpd resulted in about 55% reduction in the relative Klp61F level normalized to tubulin. Overexpression of Crb\textsuperscript{intra} also showed similar levels of Klp61F reduction (Fig. S5A, Table S2). Since Western blot results can be affected by variable degrees of nuclear loss, we also checked the level of Klp61F in mitotic spindles of syncytial embryos. Compared with mat>GFP control embryos (Fig. 6A), embryos with maternal overexpression of Crb\textsuperscript{intra} showed reduced levels of Klp61F in anaphase spindles (Fig. 6B). Similarly, knockdown of Galla-2 or Xpd significantly decreased the level of Klp61F staining, respectively (Fig. 6C and D). These data suggest that Crb/Galla-2/Xpd are important for the maintenance of Klp61F during nuclear division.

Our results above raised a possibility that Klp61F may be downregulated in CGX-depleted embryos by proteasome-dependent degradation. Hence, we examined whether relative Klp61F levels normalized to Tub can be recovered by partially impairing the
proteasome function. For partial loss of proteasome function, we utilized a mutation in Rpt5, a regulatory subunit of the proteasome complex. As shown in Fig. 7B, Crb\textsuperscript{intra} overexpression or knockdown of Galla-2 reduced the level of Klp61F. However, Rpt5\textsuperscript{504210b/+} heterozygous mutation resulted in considerable elevation of Klp61F levels (Fig. S5B). Similarly, knockdown effects of Klp61F RNAi were suppressed by Rpt5\textsuperscript{504210b/+}, thus increasing the Klp61F level (Fig. S5B). Quantification of 20 Western blot assays showed that reduced Klp61F levels caused by Crb\textsuperscript{intra} overexpression, \textit{galla-2} RNAi or Klp61F RNAi were significantly increased by Rpt5\textsuperscript{504210b/+} to 1.4-1.8-fold higher levels than the control (\textit{mat}>GFP/GFP) level (Fig. S5B, Table S3).

Next, we asked whether mitotic defects of \textit{galla-2} RNAi can be suppressed by reducing the proteasome function. Spindle defects caused by \textit{galla-2} RNAi was strongly suppressed by Rpt5\textsuperscript{504210b/+} heterozygous mutant condition (Fig. 7A, C). Rpt5\textsuperscript{504210b/+} heterozygosity also suppressed mitotic defects of Klp61F RNAi (Fig. 7A, D), consistent with the recovery of Klp61F levels (Fig. S5B, Table S3). We also examined the effects of mutation in a different proteasome component Pros\beta.6. Mitotic defects from \textit{galla-2} RNAi or Crb\textsuperscript{intra} overexpression were strongly suppressed by Pros\beta\textsuperscript{+} heterozygous condition (Fig. 7B, E, F).

\textit{Galla-2 RNAi} defects are suppressed by Xpd but not \textit{vice versa}  

As shown earlier, mitotic defects of \textit{galla-2} RNAi and Xpd RNAi are suppressed by overexpression of Klp61F (Figs. 4 and 5), suggesting that Klp61F may act downstream to Galla-2 and Xpd. However, functional relationship between Galla-2 and Xpd has not been tested. Interestingly, we noted that Galla-2 levels are strongly reduced in mitotic nuclei of Xpd RNAi embryos (Fig. S4C). However, Galla-2 overexpression had little effects of restoring the spindle defects from Xpd RNAi (Fig. 8A). Conversely, Xpd overexpression strongly suppressed spindle defects caused by \textit{galla-2} RNAi (Fig. 8B). Hence, although Galla-2 protein levels are affected by Xpd, Galla-2 function in mitosis depends on Xpd but not \textit{vice versa}.

Since we found genetic interaction between Crb and Klp61F in the eye (Fig. 1), we tested whether Xpd and Klp61F have similar functional relationship in eye development. Xpd RNAi using \textit{ey-Gal4} resulted in significant reduction of the eye size. This eye phenotype was fully rescued by Xpd overexpression, suggesting the specificity of Xpd RNAi (Fig. 8G-H). In contrast,
Galla-2 overexpression or *galla-2 RNAi* did not show any noticeable defects in adult eyes (Fig. 8E-F). Thus, Galla-2 is essential for nuclear division in embryo but may be dispensable for development of adult eye. Due to the lack of eye phenotype by *galla-2 RNAi*, we could not test whether Xpd overexpression can suppress *galla-2 RNAi* phenotype as in embryo (Fig. 8B). However, using the *Xpd RNAi* eye phenotype, we asked whether Galla-2 overexpression can suppress the *Xpd RNAi* eye defects. In this test, Galla-2 overexpression failed to suppress the *Xpd RNAi* eye phenotype (Fig. 8J), consistent with the relationship in embryo (Fig. 8A).

We then checked whether the eye phenotype resulting from *Xpd RNAi* can be ameliorated by overexpression of Klp61F. Although Klp61F overexpression did not affect eye growth in the normal condition (Fig. 8D), it strongly suppressed the *Xpd RNAi* eye phenotype (Fig. 8I), consistent with the suppression of mitotic phenotype of *Xpd RNAi* by Klp61F overexpression in embryo. These data suggest that Klp61F overexpression can bypass the defects of Xpd in two distinct developmental contexts, mitosis in syncytial embryo and eye growth.

**Discussion**

Mitotic segregation of chromosomes is crucial for genome stability in all eukaryotes. Kinesin-5 family mitotic motor proteins play a key role to form bipolar spindles necessary for chromosome segregation during mitosis. In this study, we have provided evidence that Crb, Galla-2 and Xpd proteins regulate Klp61F for proper chromosome segregation during nuclear division in early embryogenesis.

**Crb, Galla-2 and Xpd regulate Klp61F levels**

Crb, Galla-2 and Xpd proteins are enriched in mitotic spindles and show physical interaction. Syncytial embryos with reduced function of Crb, Galla-2, or Xpd have similar defects in mitotic spindles. These mitotic defects can be suppressed by maternal overexpression of Klp61F, indicating that Crb, Galla-2 and Xpd are required for the function of Klp61F in mitosis. Our data lead us to propose that CGX proteins are required for the maintenance of bipolar spindles by regulating the level of Klp61F through proteasome. This idea is supported by two observations: firstly, Klp61F protein levels are restored by reducing the function of proteasome subunits. Secondly, mitotic defects resulting from knockdown of Galla-2 or Klp61F can also be recovered by reducing proteasome function.
Interestingly, it has been reported that Crb interacts with and stabilizes Myosin V, a motor protein, to regulate rhodopsin transport during retinal differentiation (Pocha et al., 2011). Hence, it could be possible that Crb, together with Galla and Xpd, might be involved in regulating the stability of multiple proteins in diverse cellular processes. An alternative possibility is that CGX proteins might be involved in the regulation of Klp61F synthesis. In this case, proteasome-dependent degradation may facilitate the depletion of Klp61F since its synthesis is impaired. Under this condition, inhibition of the proteasome function may help maintain Klp61F levels for a limited time.

Physical interaction of CGX proteins with Klp61F raises the possibility that CGX may be required for stable association of Klp61F with spindles. This possibility is consistent with the results that *galla-2* or *Xpd RNAi* significantly reduces the level of Klp61F on spindles (Fig. 6). Furthermore, overexpression of Klp61F is sufficient to overcome mitotic defects caused by impaired CGX protein function (Figs. 3-5). This suggests that overexpressed Klp61F proteins are functional even under the CGX RNAi condition. If CGX proteins are required to activate the function of Klp61F, overexpressed Klp61F alone would be insufficient to suppress CGX RNAi phenotypes. Therefore, CGX proteins may be required to stabilize Klp61F protein rather than to promote its activity. However, since we analyzed the effects of Klp61F overexpression in a partial knockdown of CGX genes, we cannot exclude the possibility that residual CGX proteins might be able to promote the activity of overexpressed Klp61F. Studies with maternal null condition for CGX proteins may help understand the function of their physical interaction with Klp61F.

**Galla-2 is required for Xpd function but not vice versa**

Galla-1 and Galla-2 are related homologs of mammalian MIP18. MIP18 is part of the MMXD complex involved in chromosome segregation (Ito et al., 2010), but its functional relationship with XPD is not well understood. Despite their sequence similarity, XPD preferentially binds to Galla-2 (Yeom et al., 2015), consistent with strong genetic interaction seen between Galla-2 and Xpd. In embryonic nuclear division, *galla-2 RNAi* phenotypes can be suppressed by Xpd overexpression, but *Xpd RNAi* phenotypes cannot be restored by Galla-2 overexpression (Fig. 8A and J). This indicates that Galla-2 is required for the function of Xpd in syncytial embryos but not *vice versa*. However, Galla-2 levels in mitotic spindles are also reduced by knockdown of Xpd or Klp61F (Fig. S4). Therefore, although Galla-2 seems to act upstream to Xpd and Klp61F, its levels are influenced by downstream factors. A recent study has shown that
_Drosophila_ MMS19, a Galla partner, binds to Xpd to free Cdk-activating kinase (CAK) complex, thus activating Cdk1 for mitotic progression (Nag et al., 2018). Since Galla-2 is a partner of MMS19, Galla-Xpd interaction might also affect CAK to regulate the stability of Klp61F and spindle microtubules.

An intriguing question is how Crb is related to Galla and Xpd. Maternal knockdown of Crb reduces the level of Galla-1, and _crb_ RNAi mitotic phenotypes are suppressed by Galla-1 overexpression (Yeom et al., 2015). Hence, Crb seems to be required to maintain the level of Galla proteins. Conversely, Crb levels are also reduced by knockdown of Galla-2 or Xpd (Fig. S8), indicating that the level of CGX proteins are inter-dependent. Crb loss-of-function and overexpression share similar phenotypes in nuclear division (Yeom et al., 2015). In Hippo signaling, Crb\textsuperscript{intra} overexpression leads to downregulation of Expanded (Ex), an upstream regulator of Hpo signaling, thus enhancing the Yki transcriptional activity (Ribeiro et al., 2014). In contrast, Ex cannot be recruited to Crb in the absence of Crb. Hence, Crb\textsuperscript{intra} overexpression and loss of Crb both results in overgrowth through distinct mechanisms. _crb_ RNAi and Crb\textsuperscript{intra} overexpression in syncytial embryo also cause similar mitotic phenotypes with a decrease in Klp61F levels (Fig. S5A). Hence, although the underlying mechanism is currently unknown, proper level of Crb seems to be critical for the regulation of Klp61F levels.

The intracellular domain of Crb interacts with Stardust (Sdt) and Par-6 complex proteins for apical basal epithelial cell polarity (Hong et al., 2001, Bachmann et al., 2001, Nam and Choi, 2003). Maternal knockdown of Sdt or Par-6 did not significantly affect mitotic nuclei in syncytial embryos, suggesting that Crb may not function together with these proteins for syncytial mitosis (Fig. S7). However, apical localization of Crb in embryonic epithelia is regulated by endosomal trafficking (Roeth et al., 2009). Because endosomes are known to play roles for the organization of astral microtubules and chromosome alignment during mitosis (Capalbo et al., 2011, Das et al., 2014; Hehnly and Doxsey, 2014), it would be worth investigating whether endosomes might be involved in linking Crb with spindles during mitosis.

**Related roles of CGX and Klp61F in eye and syncytial embryo**

An initial finding in this study was a strong genetic interaction between Crb and Klp61F in the eye. It is an open question whether the functional relationships between CGX proteins and Klp61F are similar in the two distinct developmental processes: the eye development and nuclear division in syncytial embryo. As described earlier, Crb function may be mediated sequentially through Galla-2 and Xpd to regulate the Klp61F level for nuclear division in
syncytial embryo. Since the small eye phenotype of Xpd RNAi is suppressed by overexpression of Klp61F, the relationship between Xpd and Klp61F seems to be conserved in both eye and embryo development. As in the embryo, Galla-2 overexpression is unable to suppress Xpd RNAi phenotype in the eye.

Despite the similar relationship between Galla-2, Xpd and Klp61F in embryo nuclear division and eye development, there is an interesting difference in their genetic interaction with Crb\textsuperscript{intra} overexpression. Crb\textsuperscript{intra} eye phenotype is suppressed by Klp61F overexpression, similarly to the rescue effect seen in the embryo. Remarkably, the same Crb\textsuperscript{intra} eye phenotype is suppressed by reducing Galla-2 (Yeom et al., 2015), suggesting an interesting outcome that Crb\textsuperscript{intra} overexpression has an opposite relationship with Galla-2 in eye development. This apparent discrepancy may be partly explained by the differences in tissues. Crb\textsuperscript{intra} overexpression induces overgrowth in imaginal discs by inhibiting Hippo signaling (Chen et al., 2010, Ribeiro et al., 2014) and also impairs epithelial integrity (Tepass et al., 1990). Crb\textsuperscript{intra} may not have such effects in syncytial embryo since epithelia have not yet formed. Because the Crb\textsuperscript{intra} eye phenotype is suppressed by reducing Galla-2, the dominant effects of Crb\textsuperscript{intra} may depend on eye-specific interaction with Galla-2.

This work provides evidence for the roles of Crb, Galla-2 and Xpd in regulating the level of Klp61F in mitosis and for their functions in eye development. Klp61F and its mammalian homolog Eg5 are conserved motor proteins that play a key role for bipolar spindle formation in mitosis. It remains to be seen whether MIP18 and XPD of the MMXD complex might participate in the regulation of Eg5 protein levels for chromosome segregation and organ growth.
Materials & Methods

Fly genetics. All Drosophila strains were grown and maintained at room temperature unless stated otherwise. For overexpression of Crb\textsuperscript{intra} in differentiating eye, UAS-Crb\textsuperscript{intra} was crossed with GMR-Gal4 (Bloomington) at 25 °C. Two UAS-Klp61F RNAi lines (v52548, BL35804) are from the Vienna Drosophila Resource Center (VDRC) and the Bloomington Drosophila stock center (BDSC), respectively. Klp61F-GFP (BL35509, BL35510) and klp61F\textsuperscript{06345} (BL32012), klp61F\textsuperscript{07012} (BL11710) mutant lines are from the BDSC. UAS-Klp61F line was generated by injecting pUASTattB-Klp61F (Bestgene, USA). gala-2 RNAi lines are from VDRC (v110611) and BDSC (58320). Xpd RNAi lines (106998, 41021) are from VDRC. For knockdown of Xpd, UAS-Xpd RNAi was crossed with ey-Gal4 (BDSC). For maternal knockdown, crb RNAi (BL40869, 38903), Xpd RNAi, gala-2 RNAi, sdt RNAi (v29844) or par-6 RNAi (v108560, v19732) lines were crossed with mat-Gal4 (BDSC 7062, 7063). Proteasome mutants Rpr\textsuperscript{504210b} (BL11625) and pros\textsuperscript{β0'} (BL6182) are from BDSC.

Generation of Klp61F and Galla2 antibodies. An antibody against Klp61F was raised in rabbits with GST-Klp61F\textsuperscript{801-1066} (GST tagged amino acids 806-1066), and anti-Galla-2 antibody was raised in rats with GST-Galla-2\textsuperscript{1-156} expressed in Escherichia coli by isopropyl β-D-1-thiogalactopyranoside induction. Antibody production and purification were carried out by ABclonal (China). Purified Klp61F antibody was used for immunoblotting (1:1000) and immunohistochemistry (1:200). Purified Galla-2 antibody was used for immunoblotting (1:500) and immunohistochemistry (1:100).

Genetic crosses for embryo collection. To knock down maternal gene products in syncytial embryos, maternal-Gal4 females were crossed with UAS-RNAi lines. F1 females (mat>UAS RNAi) were crossed with UAS-GFP, and F2 embryos were analyzed. RNAi phenotypes were not detected when mat-Gal4 males were used for mating. For maternal overexpression of Klp61F, F1 generation females (mat>UAS-RNAi) were crossed with homozygote Ubi-Klp61F GFP, and F2 embryos were analyzed.
Immunostaining of embryos and S2 cells. Embryos laid on grape juice egg laying plates for 2h were collected using 1X TXN (10X Embryo wash solution, 7% NaCl and 0.5% Triton X-100) buffer. Embryos were dechorionated by 50% bleach and moved into 4 ml sample vial. 1ml of heptane (Sigma) and methanol (Merck) were used to remove vitelline membrane. After removing all solutions, embryos were stored in methanol at 4 °C. For anti-tubulin staining, methanol fixation was performed to preserve microtubule structures. For embryo staining, embryos were incubated in 0.2% saponin/PBS for 10 min. Primary antibodies were diluted in 0.2% saponin/0.5% normal goat serum/PBS and incubated overnight at 4 °C. After washing with 0.2% saponin/PBS four times, embryos were incubated with secondary antibodies overnight at 4 °C.

*Drosophila* S2 cells were cultured in M3 media (Sigma) with 10% Insect medium supplement (Sigma). S2 cells were transfected with plasmid for pAc 5.1 2X Myc-Xpd and pAc 5.1 GFP Klp61F. For S2 cell staining, S2 cells were fixed in 2% paraformaldehyde/PBS for 15 min and washed with cold PBS three times. Primary antibodies were diluted in 0.1% saponin/1% normal goat serum/PBS and cells were incubated overnight at 4 °C. After washing with cold PBS two times and cells were incubated in secondary antibodies at RT for 2 hours.

The following antibodies were used for embryo and S2 cell staining at indicated conditions: mouse anti-a-tubulin at 1:100 (Sigma, T9026), rat anti-Crb at 1:50 (Bhat et al., 1999), rat anti-Galla-2 at 1:100, rabbit anti-Klp61F at 1:200, rabbit anti-Myc 1:200 (Abcam, ab9106), and mouse anti-GFP 1:100 (Abcam, ab1218). Secondary antibodies conjugated with Cy3, Cy5 or FITC were from Alexa Flour (Molecular Probes). Vectashield with DAPI (H-1200, Vector Laboratories) was used for mounting samples. Fluorescent images were acquired using Carl Zeiss LSM710 confocal microscope.

GST pull-down assays. For GST pull-down, IPTG-inducible R2 cells (BL21 derivative) were transformed with MBP-Klp61F, MBP-Klp64D (Vuong et al., 2014), GST-Xpd and GST-Crb\textsuperscript{intra}. Bacterial cell lysates were prepared as described (Frangioni and Neel, 1993). The buffer used for pull-down was: 20mM Tris pH 7.5, 150 mM NaCl, 0.5mM EDTA, 10% glycerol, 0.1% Triton X-100, 1mM DTT and protease inhibitor cocktail. For western blotting, rabbit anti-MBP antibody (NEB, E8030S, 1:10,000) and secondary anti-rabbit HRP antibody (Jackson, 711-035-151, 1:10,000) were used.
**Cell Culture, Transfection, Immunoprecipitation and Western blot analysis.** Transfection was carried out with Cellfectin II reagent (Invitrogen) according to manufacturer’s instructions. Total of 1-2µg of DNA was used for each transfection. For immunoprecipitation (IP), cells were lysed in 0.1% CHAPS buffer (0.1% CHAPS, 10 mM NaCl, 2mM HEPES, 0.1mM EDTA, 0.04% PMSF and protease inhibitor cocktail) and the lysates were precleared by incubating with protein G-sepharose beads (Amersham Bioscience) for 1h at 4°C. Precleared lysates were immunoprecipitated with anti-Myc (Abcam, ab9106) at 4°C overnight. The immunoprecipitates captured by protein G-sepharose were washed and subjected to SDS-PAGE as described (Vuong et al., 2014). Western blots were immunostained with anti-Flag (Sigma, F1804, 1:1,000) and anti-Myc. Blots were incubated with primary antibodies overnight at 4 °C. After washing with TBST solution three times for 10 min each, blots were incubated with mouse (Jackson, 715-035-151, 1:10,000) or rabbit anti-HRP antibody for 2h at RT.

Embryos used for IP were dechorionated with 50% bleach, frozen in 1X PBS and stored at -20 °C. To collect adult heads for IP, whole flies were frozen by liquid nitrogen, and heads were separated with blade. Heads and embryos were grinded with lysis buffer (20mM HEPES, 2.5mM EDTA, 1mM DTT, 5% glycerol, 100mM KCl, 0.05% Triton X-100, protease inhibitor cocktail). Embryo lysates were precleared by incubating with SureBeads™ protein G magnetic beads (BIO-RAD) for 1h at 4°C. Precleared lysates were immunoprecipitated with anti-Klp61F, anti-Galla-2, anti-GFP (Abcam, ab1218) at 4°C overnight. Immunoprecipitated lysates were washed with lysis buffer and subjected to SDS-PAGE. Western blots were stained with anti-Klp61F, anti-Galla-2 and anti-GFP (Abcam, ab290, 1:1,000) as described above.

Embryos for western blotting experiments were collected using the same method for IP. Embryos were lysed using homogenizer in 1X SDS sample buffer and boiled at 94 °C for 5 min and loaded for SDS-PAGE and western blotting.

Proteins extracted from S2 cells and embryos were fractionated by SDS-PAGE and transferred onto nitrocellulose membrane. Membrane was blocked by 5% skim milk (BD Biosciences). Western blots were stained with mouse anti-Flag (Sigma, F1804), rabbit anti-Myc (Abcam, ab9106, 1:1,000), mouse anti-V5 (Invitrogen, R960-25, 1:1,000), rabbit anti-Klp61F (1:1,000), rat anti-Galla-2 (1:500) as described above.
Statistical analysis

Statistical analyses were performed by unpaired one-tailed Student’s t-test using Microsoft Office Excel. All experiments performed at least three times. P-values of <0.05 were considered as statistically significant (* P<0.05, ** P<0.01, *** P<0.001).

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Figure 1. Genetic interaction between *crb* and *Klp61F*.

(A-F) Genetic interaction between *crb* and *klp61F* in the eye. Scale bar, 200µm. (A) *GMR-Gal4* crossed with *UAS-GFP* for control. (B) Knockdown of Klp61F and (C) Klp61F overexpression show normal eyes. (D) Overexpression of Crb\textsuperscript{intra} under *GMR-Gal4* results in a rough eye phenotype. (E) *Klp61F RNAi* enhances Crb\textsuperscript{intra} phenotype, resulting in smaller eyes with severely rough and blackened ommatidia (100%, n=44). (F) Overexpression of Klp61F partially suppresses Crb\textsuperscript{intra} phenotype, showing enlarged eyes with reduced roughness (100%, n=52).
Figure 2. Localization and physical interaction of Crb and Klp61F.

(A-D) Localization of Crb and Klp61F during nuclear division in syncytial embryos. Scale bar, 20μm. (A) Both of Crb and Klp61F are enriched in spindle poles during prophase (arrows in A’ and A”). (B-C) Crb and Klp61F show similar spindle staining during metaphase (B) and anaphase (C). Crb shows a diffused pattern that partially overlaps with Klp61F (arrows). (D) Klp61F is localized to midbody, and Crb is also enriched in midbody during telophase (arrows). (E) Co-immunoprecipitation of Klp61F and Crb. *Drosophila* S2 cells were transfected with
Flag-Klp64D (lane 3) or Flag-Klp61F (lane 4) and Myc-Crb\textsuperscript{intra}. Lane 1 and 2 are 15% input of Flag-Klp61F and Flag-Klp64D, respectively. Anti-Myc was used for IP and Myc-Crb\textsuperscript{intra} was co-immunoprecipitated Flag-Klp61F but not by Flag-Klp64D. (F) Direct binding between Klp61F and Crb. Lane 1 and 2 show 20% input for Flag-Klp61F and Klp64D, respectively. GST-Crb\textsuperscript{intra} pulled down Klp61F but not Klp64D.
Figure 3. Spindle defects and nuclear loss caused by Crb\textsuperscript{intra} overexpression are suppressed by Klp61F overexpression.

(A-C) Spindle defects by Crb\textsuperscript{intra} overexpression. Scale bar, 20\,\mu m. (A) Free centrosomes that are not associated with spindles and chromosomes (arrow). (B) Fusion of spindles from two adjacent nuclei (arrows). (C) Multiple poles (white arrow) or monopolar spindles (red arrow). (D-F) Nuclear loss phenotype is divided into three categories: Normal (D), mild (less than 10% loss) (E), and severe (more than 10% loss) (F). (G) Quantification of spindle defects. These
spindle phenotypes are suppressed by Klp61F overexpression. Statistical significance was tested for combined defective spindle phenotypes. (n>26) *p<0.05, p**<0.01, p***<0.001. t-test results for each phenotype are shown in Table S1. (H) Quantification of nuclear loss phenotypes. Klp61F overexpression partially suppresses Crbintra overexpression phenotypes (n>80). Scale bars: 20μm for A-C, 100μm for D-F.
Figure 4. Genetic and physical interaction between Klp61F and Galla.

(A) S2 cells were transfected with Flag-Klp61F, Flag-Klp64D, V5-Galla-1 and V5-Galla-2. Lanes 1 and 2 are 15% input of V5-Galla-1 and V5-Galla-2, respectively. Anti-Flag was used for IP and both of Galla-1 and Galla-2 were co-immunoprecipitated with Klp61F (Lanes 3 and 4). Galla-1 was not co-immunoprecipitated with Klp64D (Lane 5). (B) Direct binding between Klp61F and Galla. Lanes 1 and 2 are 15% input of MBP-Klp61F and MBP-Klp64D, respectively. Klp61F was pulled down by GST-Galla-1 (Lane 3) and GST-Galla-2 (Lane 4). GST-Galla-2 did not pull down Klp64D (lane 5). Only Galla-2 shows direct interaction with
Klp61F. (C-C”) Galla-2 shows similar localization to spindles. Scale bar, 20μm. (D) Quantification of spindle defects by *galla-2 RNAi*. Spindle defects are suppressed by Klp61F. Statistical significance was tested for combined defective spindle phenotypes. *p<0.05, p**<0.01, p***<0.001. t-test results for each phenotype are shown in Table S1. (E) Quantification of nuclear loss phenotypes (n>75). Klp61F overexpression partially suppresses *galla-2 RNAi* phenotypes.
Figure 5. Genetic and physical interaction between Klp61F and Xpd.

(A) Co-immunoprecipitation of Klp61F and Xpd from S2 cells transfected with Flag-Klp64D (Lane 2) or Flag-Klp61F (Lane 3) and Myc-Xpd. Lane 1 is 15% input of Flag-Klp61F. Myc-Xpd is co-immunoprecipitated with Flag-Klp61F but not with Flag-Klp64D. (B) Direct binding of Klp61F and Xpd. Lanes 1 and 2 are 15% input of MBP-Klp61F and MBP-Klp64D, respectively. Klp61F (a) was pulled down by GST-Xpd (Lane 3) but not by GST (Lane 4). Klp64D shows no interaction with GST-Xpd (lane 4). (C) Quantification of spindle defects by Xpd RNAi and its suppression by Klp61F. Statistical significance was tested for combined defective spindle phenotypes. *p<0.05, **p<0.01, ***p<0.001. t-test results for each phenotype are shown in Table S1. (D) Quantification of nuclear loss by Xpd RNAi and its suppression by Klp61F (n>84).
Figure 6. Klp61F levels are reduced by Crb\textsuperscript{intra} overexpression, galla-2 or Xpd RNAi. (A-B) \textit{mat}\textsuperscript{>GFP/GFP} for control. Scale bar, 20\,\mu m. Blue is DAPI, white is Klp61F. Klp61F shows the pattern of mitotic spindles in anaphase of nuclear division in embryo (A). (B) Crb\textsuperscript{intra} overexpression by using \textit{mat-Gal4}. Level of Klp61F was reduced. (C-D) Effects of maternal knockdown of Galla-2 and Xpd. Klp61F levels were reduced by \textit{galla-2 RNAi} (C) or \textit{Xpd RNAi} (D).
Figure 7. Crb, Galla and Xpd are required for Klp61F stability.

(A) Effects of \(Rpt5^{04210b/+}\) heterozygous mutation. \(Rpt5^{04210b}\) heterozygous mutation can suppress spindle defects by \(galla-2\) RNAi or \(Klp61F\) RNAi. Statistical significance was tested for combined defective spindle phenotypes. *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\). t-test results for each phenotype are shown in Table S1. (B) Effects of \(pros\beta6^{1/+}\) heterozygous mutation on spindle defects. Spindle defects from \(galla-2\) RNAi or Crb\textsuperscript{intra} overexpression are restored by \(pros\beta6^{1/+}\) heterozygous mutation. Statistical significance was tested for combined defective spindle phenotypes. *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\). t-test results for each phenotype are shown in Table S1. (C-F) Effects of \(Rpt5^{04210b/+}\) heterozygous mutant on Klp61F level. \(Rpt5^{04210b/+}\) suppresses mitotic defects of maternal knockdown of Galla-2 (C) or Klp61F (D).
\textit{Prosβ6}/+ suppresses mitotic defects of maternal knockdown of Galla-2 (F) or Crb\textsuperscript{intra} overexpression (F). Phenotypes of \textit{galla-2 RNAi}, \textit{Klp61F RNAi} and Crb\textsuperscript{intra} overexpression are shown in Fig S2, respectively. Scale bar, 20μm.
Figure 8. Xpd RNAi phenotypes are suppressed by overexpression of Klp61F but not by Galla-2

(A) Galla-2 overexpression using mat-Gal4 has no significant effect in the embryo. Spindle defects resulting from Xpd RNAi are not significantly recovered by Galla-2 overexpression. (B) Spindle defects caused by gala-2 RNAi are rescued by Xpd overexpression. Note that overexpression of Xpd using mat-Gal4 causes spindle defects in embryo, as shown in Fig. 8B.
(C-I) Adult eye phenotypes. *ey>GFP* control (C), overexpression of Klp61F (D), Galla-2 overexpression (E) or *galla-2 RNAi* (F) show normal eyes. Scale bar, 200μm. (G) Knockdown of Xpd under *ey-Gal4* (*ey>Xpd RNAi*) shows a small eye phenotype. (H) The small eye phenotype of *Xpd RNAi* is rescued by Xpd overexpression (n=31). (I) Overexpression of *Klp61F* suppresses the small eye phenotype caused by *Xpd RNAi* (n=32). (J) Overexpression of Galla-2 cannot rescue the small eye phenotype of *Xpd RNAi* (n=30).
Figure S1. Localization of Tubulin during nuclear division and effect of Klp61F RNAi

(A-D) Pattern of Tubulin in embryos at nuclear division cycle 11. Scale bar, 20 μm. (A-D) Localization of Tubulin. (A) Tubulin is localized in spindle poles during prophase. (B-C) Tubulin shows spindle pattern in meta- and anaphase. (D) Tubulin is enriched in midbody during telophase (arrows). (E-F) Effects of Klp61F RNAi on Crb. (E-E‴) mat>GFP control embryos were stained with Crb and Klp61F in the absence of anti-Tubulin antibody. Both Crb and Klp61F show tubulin pattern. Scale bar, 20 μm. (F-F‴) Crb and Klp61F levels are decreased in Klp61F knockdown condition. (G-G‴) Localization of Klp61F and Xpd in S2 cells. S2 cells were transfected by Myc-Xpd and GFP-Klp61F constructs. Both Klp61F and Xpd show tubulin pattern.
Figure S2. Mitotic defects by knockdown of Crb, Galla-2, Xpd and Klp61F

(A-H) Mitotic defects by knockdown of Crb, Galla-2, Xpd and Klp61F by mat-Gal4. Quantification of different phenotypes such as free centrosomes and fused spindles is shown in Fig. S6. (A-B) Independent Klp61F RNAi lines (BL35804, v52548). (C-D) gala-2 RNAi
(E-F) *Xpd RNAi* lines (v106998, v41021). (G-H) *crb RNAi* lines (BL40869, BL38903). (I-K) Dividing nuclei stained for Tubulin (green) and DAPI (white). Scale bar, 20µm. (I-I’’) Wild type prophase, metaphase and anaphase. (J-J’’) Prophase, metaphase and anaphase in Klp61F knockdown condition. (K-K’’) Prophase, metaphase and anaphase in Crb\textsuperscript{intra} overexpression condition. Spindle defects were mainly detected during metaphase in both Crb\textsuperscript{intra} overexpression and *Klp61F* RNAi conditions. (L) The chart shows that majority of defective embryos are in metaphase. 70% of defective embryos with Crb\textsuperscript{intra} overexpression (n = 83) has phenotypes in metaphase. 80% of defective *Klp61F* RNAi embryos (n = 84) show phenotypes in metaphase.
Figure S3. Endogenous IP with Klp61F and Galla-2 and effects of *galla-2* RNAi.

(A) Lysates from adult heads (Canton S) and syncytial embryos (*mat>GFP/GFP*) were immunoprecipitated with GFP (Lanes 1 and 3), Klp61F (Lanes 2 and 4). Galla-2 was co-immunoprecipitated with Klp61F in both tissues (Lanes 2 and 4). (B-C) Immunostaining for Galla-2 in the absence of anti-Tubulin antibody. Scale bar, 20 µm. (B-B’’) Galla-2 pattern in control embryos (*mat>GFP*). Galla-2 staining shows similar pattern as mitotic spindles. (C-C’’) In Galla-2 knockdown condition, level of Galla-2 is reduced in mitotic spindles.
Figure S4. Effects of Crb\textsuperscript{intra} overexpression, Xpd RNAi or Klp61F RNAi on Galla-2 level. (A-A’’) Galla-2 staining in spindles in wild type embryos. (B-D) Strong reduction of Galla-2 by Crb\textsuperscript{intra} overexpression (B), Xpd RNAi (C), or Klp61F RNAi (D). Scale bar, 20\textmu m.
Figure S5. Quantitative analysis of Klp61F levels in embryos of different genotypes.

(A) Effects of different genotypes on the level of Klp61F in syncytial embryos. Protein extracts from embryos of indicated genotypes were separated by SDS PAGE and western blotted. Blots were immunostained for Klp61F and Tubulin. Klp61F levels relative to Tubulin were quantified. Quantification data and t-test results for 20 blots are shown in Table S1. (A) Knockdown of Crb, Galla, and Xpd or Crb\textsuperscript{intra} overexpression resulted in about 55% of reduction of Klp61F level. (B) Effects of a proteasome mutation (Rpt5\textsuperscript{04210b/+}) on the level of Klp61F. Protein extracts were prepared from different genotypes as indicated. Western blots
were stained for Klp61F and Tubulin as in (A). Quantification of relative Klp61F levels with statistics for 20 western blots is shown in Table S2. Crb\textsuperscript{intra} overexpression, \textit{galla-2 RNAi}, and \textit{Klp61F RNAi} result in reduction of Klp61F level. \textit{Rpt5\textsuperscript{04210b/+}} heterozygous mutation increased Klp61F level about 1.4-1.8-fold higher than the control (\textit{mat>GFP/GFP}).
Figure S6. Quantification of spindle defects in different genotypes.

(A) Quantification of different mitotic phenotypes. Overexpression of Crb\textsuperscript{intra} and knockdown of Crb, Galla-2, Xpd and Klp61F by \textit{mat-Gal4} lead to spindle defects in embryos. Spindle defects are divided into three categories: free centrosomes, fused/branched spindles, and others (mono-polar or multi-polar spindles). (B) Quantification of all mitotic phenotypes shown in (A). Each bar shows the sum of different phenotypes for indicated genotypes. Error bars are s.d. n>30. **P<0.01 ***P<0.001 (C) Nuclear loss phenotype caused by knockdown of Crb, Galla-2, Xpd and Klp61F or Crb\textsuperscript{intra} overexpression. ‘mild’ and ‘severe’ means embryos showing less than 10% or more than 10% of nuclei-free area, respectively. In control \textit{(mat>GFP/GFP)}, 80% of embryos were normal while 20% of embryos showed mild or severe phenotype. About 75% of embryos showed nuclear loss phenotype in Crb\textsuperscript{intra} overexpression or indicated RNAi condition (n>85).
Figure S7. Effects of sdt and par-6 RNAi in nuclear division

(A-B) Knockdown of Sdt. A majority of embryos show relatively normal pattern of nuclear division (A-A”). Some embryos show nuclear loss phenotypes (B-B”), but cortical nuclei show normal pattern of mitotic spindles (B’’’). (C-D) Knockdown of Par-6. A majority of embryos show relatively normal pattern of nuclear division (C-C”). Some embryos show nuclear loss phenotypes (D-D”) as in (B), but cortical nuclei show normal pattern of mitotic spindles (D’’’). (E) Quantification of nuclear loss phenotypes shown in (B) and (D). Both sdt and par-6 RNAi did not show significant difference from control (mat>GFP). Scale bar, 100µm.
Figure S8. Effects of Galla-2 or Xpd knockdown on Crb level.

(A-A’’) Localization of Crb in control embryo (mat>GFP/GFP). Crb is distributed in the spindle region during metaphase. Scale bar, 20µm. (B-B’’) Crb level is reduced in mat>galla-2 RNAi condition. (C-C’’) Crb level is decreased in Xpd knockdown condition.
### Table S1. Statistical analysis

T-test results of each experiment shown in main figures. * p<0.05, **p<0.01, ***p<0.001
Table S2. Quantitative data for Figure S5A

Quantification of 20 Western blot assays. Data show that relative Klp61F levels normalized to Tubulin are reduced in Crb, gala-2 and Xpd knockdown or Crb^{intra} overexpression condition.

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Average: 1 | 0.491 | 0.4015 | 0.3955 | 0.4595 | 0.492 |
S.D: 0.181 | 0.150 | 0.143  | 0.149  | 0.120  |
Table S3. Quantitative data for Figure S5B

Quantification of 20 Western blot assays. Data show relative Klp61F levels normalized to Tubulin. Reduced Klp61F levels caused by Crb*intr* overexpression or *galla-2* and *Xpd RNAi* are recovered by *Rpt5*^{501210b/+} heterozygous mutation.

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<th>mat&gt;GFPP</th>
<th>mat&gt;crb^{intr}a</th>
<th>mat&gt;crb^{intr}a/Rpt5^{501210b}</th>
<th>mat&gt;galla-2 RNAi/GFP</th>
<th>mat&gt;galla-2 RNAi/Rpt5^{501210b}</th>
<th>mat&gt;Klp61F RNAi/GFP</th>
<th>mat&gt;Klp61F RNAi/Rpt5^{501210b}</th>
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Average 1 0.3615 1.629 0.3905 1.74 0.45 1.358
S.D 0.099 0.230 0.100 0.175 0.096 0.174