Mechanisms of Development 144 (2017) 191–200

Contents lists available at ScienceDirect

Mechanisms of Development

journal homepage: www.elsevier.com/locate/mod

Drosophila p115 is required for Cdk1 activation and G2/M cell cycle transition

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ARTICLE INFO

Article history:
Received 1 April 2016
Received in revised form 28 March 2017
Accepted 7 April 2017
Available online 8 April 2017

Keywords:
Drosophila
Golgi
p115
Cdk1
Cell cycle

ABSTRACT

Golgi complex inheritance and its relationship with the cell cycle are central in cell biology. Golgi matrix proteins, known as golgins, are one of the components that underlie the shape and functionality of this organelle. In mammalian cells, golgins are phosphorylated during mitosis to allow fragmentation of the Golgi ribbon and they also participate in spindle dynamics; both processes are required for cell cycle progression. Little is known about the function of golgins during mitosis in metazoans in vivo. This is particularly significant in *Drosophila*, in which the Golgi architecture is distributed in numerous units scattered throughout the cytoplasm, in contrast with mammalian cells. We examined the function of the ER/cis-Golgi golgin p115 during the proliferative phase of the *Drosophila* wing imaginal disc. Knockdown of p115 decreased tissue size. This phenotype was not caused by programmed cell death or cell size reductions, but by a reduction in the final cell number due to an accumulation of cells at the G2/M transition. This phenomenon frequently allows mitotic bypass and re-replication of DNA. These outcomes are similar to those observed following the partial loss of function of positive regulators of Cdk1 in *Drosophila*. In agreement with this, Cdk1 activation was reduced upon p115 knockdown. Interestingly, these phenotypes were fully rescued by Cdk1 overexpression and partially rescued by Myt1 depletion, but not by String (also known as Cdc25) overexpression. Additionally, we confirmed the physical interaction between p115 and Cdk1, suggesting that the formation of a complex where both proteins are present is essential for the full activation of Cdk1 and thus the correct progression of mitosis in proliferating tissues.

1. Introduction

The golgin p115 is a peripheral membrane protein present in transitional endoplasmic reticulum (tER) sites (where proteins are packaged into COPII (coat protein II) vesicles (Barlowe et al., 1994; Orci et al., 1991)) and cis-Golgi vesicles. This protein exists as a homodimer; each molecule contains a poorly characterized N-terminal globular head and an extended C-terminal tail region (Sapperstein et al., 1995) consisting of an acidic domain and four sequential coiled coils (Allan et al., 2000; Shorter et al., 2002).

The maintenance of Golgi organization requires p115 protein. Hence, p115 depletion in *Drosophila* S2 cells causes fragmentation of Golgi cisternae and tER sites (Kondylis and Rabouille, 2003). Moreover, inactivation of p115 in mammalian cells using specific antibodies or siRNA causes fragmentation of the Golgi ribbon and the formation of Golgi mini-stacks adjacent to endoplasmic reticulum (ER) exit sites (Puthenveedu and Linstedt, 2004, 2001; Smith et al., 2009; Sohda et al., 2007, 2005).

Cell cycle progression requires Golgi fragmentation. When this process is blocked, cells are unable to progress into prophase, revealing the existence of a G2/M checkpoint related to Golgi fragmentation (Preisinger et al., 2005; Sütterlin et al., 2002). This G2/M checkpoint has also been suggested in S2 cells (Rabouille and Kondylis, 2007).

A deeper analysis of this process showed that Myt1, an inhibitory kinase of Cdk1, connects Golgi fragmentation and cell cycle progression. This protein is located at the ER/Golgi and is inactivated during mitosis by MEK1 via p90RSK and Plk1 phosphorylation (Villeneuve et al., 2013). Cdk1 can phosphorylate other golgins such as GM130 and the Golgi stacking protein GRASP65 (Lowe et al., 1998, 2000). The phosphorylation of GM130 blocks its interaction with p115, and GRASP65 phosphorylation by Cdk1 inhibits its trans-oligomerization and association with the Golgi membrane.

Studies in mammalian cells also suggest a functional association of the golgin GM130 and the Golgi stacking protein GRASP65 with the mitotic apparatus. These proteins are proposed to regulate the structure and function of the interphase centrosome and mitotic spindle assembly (Kodani and Sütterlin, 2008; Sütterlin et al., 2005; Wei et al., 2015). p115 associates with γ-tubulin throughout the cell cycle and is required for the structure and function of the late mitotic spindle as

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http://dx.doi.org/10.1016/j.mod.2017.04.001
0925-4773/

well as for resolution of the cytokinetic bridge, indicating that it plays a role during mitosis and Golgi inheritance (Radulescu et al., 2011).

The requirement of p115 in protein trafficking in different organisms and cargoes is diverse. In mammalian cells depleted of p115 or in which the interaction of p115 with Golgi membranes is blocked, exit of vesicular stomatitis virus glycoprotein from the ER is inhibited (Seemann et al., 2000; Puthenveedu and Linstedt, 2004). However, trafficking of the transmembrane protein Delta (a Notch ligand) to the surface of *Drosophila* S2 cells appears to be unaffected by the depletion of p115 (Kondylis and Rabouille, 2003). Similarly, the secretion of soluble proteins is delayed, but not inhibited, in p115-depleted mammalian cells (Sohda et al., 2007, 2005). p115 knockdown in *Caenorhabditis elegans* does not inhibit secretion of the 170 kDa soluble yolk protein, but affects trafficking of the transmembrane yolk receptor RME-2 in oocytes (Grabski et al., 2012). Studies in Arabidopsis also suggest that p115 is dispensable for vesicle tethering during the protein trafficking between the ER and Golgi apparatus. Genetic ablation of p115 results in dwarf, but viable, plants (Takahashi et al., 2010). Thus, p115 appears to exert a modest effect on trafficking of some proteins and a more pronounced effect on other cargoes.

Here, we examined the function of the ER/cis-Golgi golgin p115 during *Drosophila* imaginal disc development. In these tissues, the Golgi is present in multiple regions as confined vesicles and tubules (Kondylis et al., 2001), which still have polarity (Yano et al., 2005) and a functional exocytic pathway. Depletion of p115 in the growing wing epithelia did not induce visible secretion phenotypes but evoked phenotypes reminiscent of those observed following partial loss of positive regulators of the G2/M transition, including the accumulation of cells in G2/M phase and the presence of DNA-re-replicating cells (Björklund et al., 2006; Neufeld et al., 1998; Weigmann et al., 1997). Moreover, p115 was required for correct formation of the bipolar spindle during mitosis in cultured S2 cells.

Cdk1 overexpression completely rescued the reduced tissue size phenotype in the wing epithelia, whereas it was not rescued by overexpression of the Cdk1 activator String (also known as Cdc25 phosphatase) and was only partially rescued by Myt1 depletion. Accordingly, Cdk1 activation was reduced upon p115 knockdown. Importantly, we established the physical interaction between p115 and Cdk1, suggesting that this interaction is essential for the complete activation of Cdk1 and correct progression through mitosis in *Drosophila* imaginal tissues. This may occur via the recruitment of Cdk1 during mitosis to specific cell compartments, such as Golgi fragments, or the centrosome to permit its full activation.

2. Results

2.1. p115 is required for normal tissue growth but not for exocytosis/secretion in Drosophila

To study the function of *Drosophila* p115 in the developing wing imaginal disc, we used dsRNA (double-stranded RNA) from the Vienna *Drosophila* RNAi Center (VDRC). In silico analysis of this dsRNA did not predict any off-targets (defined as other regions of the *Drosophila* genome containing a minimum of 19 nucleotides with sequence identity to the target region), suggesting its high specificity (Fig. S1A). We tested its efficiency in reducing p115 levels by RT-qPCR. Ubiquitous expression of the UAS-p115i construct using the *tubulin-Gal4* (*tub-Gal4*) driver reduced the p115 transcript level by 77% (Fig. S1B) and induced lethality before pupation (data not shown).

Interestingly, p115 knockdown in the posterior compartment of the wing (using the *engrailed-Gal4* (*en-Gal4*) driver) reduced the posterior compartment size of adult wings by 27% (Fig. 1B and G). We did not observe any disturbance of the venation or wing pattern, suggesting that the mechanisms for secreted morphogen uptake (Wingless (Wg), Fig. S2A and A') and signaling work correctly in p115-deficient tissues. The lack of an effect on secretion is in agreement with the findings in S2 cell cultures (Kondylis and Rabouille, 2003). We also analyzed the localization of a plasma membrane protein (DE-cadherin) in wing discs depleted of p115 in their posterior compartment. If p115 knockdown affects secretion, we expected E-cadherin to accumulate inside cells, which did not happen (Fig. S1C). Together, these data support the notion that *Drosophila* p115 is not essential for appropriate secretion of relevant membrane proteins and that the tissue size reduction observed in p115-knockdown flies is not a consequence of exocytosis/secretion failure.

Overexpression of p115 did not induce any recognizable phenotype (Fig. 1E and G), even though it was efficiently expressed and localized to the Golgi in different tissues, namely, hemocytes, salivary glands, and imaginal discs (Fig. S3). This suggests that p115 has a permissive, rather than an instructive, role in tissue growth. Importantly, the wing size reduction caused by p115 knockdown was enhanced when it was induced in a p115 locus-deficient background (Fig. 1D and G) or together with Dicer2 expression (43% reduction of posterior compartment size) (Fig. 1C and G). These phenotypes were reversed by expression of HA::p115 (Fig. 1F and G), which also demonstrates the specificity of the dsRNA used to knock down p115.

2.2. Cell cycle progression requires p115

The wing size phenotype could arise as a consequence of decreased cell growth, proliferation, or viability during its growing phase. To determine which of these parameters were affected, we compared control and p115-knockdown cells by analyzing various markers of cell cycle progression and cell death. For this, we depleted p115 only in the posterior compartment of imaginal discs using the *hedgehog-Gal4* (*hh-Gal4*) driver together with the UAS-GFP transgene for labeling purposes, and used the anterior compartment as a control in comparisons of cell proliferation and survival (Neufeld et al., 1998).

Bromodeoxyuridine (BrdU) incorporation assays and phospho-histone H3 (PH3) immunostaining revealed that the numbers of cells in S phase (Fig. 2G and G') and mitosis (Fig. 2A, D, and D') were higher in the p115-depleted region than in the anterior compartment, respectively. Despite the fact that p115 knockdown induced extra cell cycles, the posterior compartment of *en-GAL4 > UAS-p115i* adult wings was smaller than that of wild-type adult wings (Fig. 1B and G). Flow cytometry showed that p115-depleted cells were the same size as control cells, indicating that the decrease in tissue size was not a consequence of smaller cells (Fig. 2N). Additionally, dead cells were detected in the p115-depleted compartment (cleaved caspase-3) (Fig. 2J and J'). TUNEL showed that few cells died during mitosis (<five cells per disc, $n = 6$) (Fig. 2M and M').

DNA content analysis by flow cytometry revealed the presence of a sub-G1 aneuploid cell population (Fig. 2Q and Table S2), which were likely dead cells (Chen et al., 2007), and also a super-G2/M population. This result suggests that cells are retained in G2/M phase upon p115 knockdown. This would allow DNA re-replication and re-entry of p115-knockdown cells into the cell cycle to some extent explaining the increased numbers of BrdU positive cells, finally inducing cell death. By contrast, p115 overexpression did not influence cell size or the DNA content, indicating that it does not affect the cell cycle (Fig. S3C and Table S2).

2.3. The reduction in wing size is not a consequence of cell death

We sought to exclude the possibility that cell death is involved in the wing size phenotype and additionally to determine whether the effects on the cell cycle are a consequence of compensatory proliferation. For this, we co-expressed the baculovirus apoptosis inhibitor p35, which prevents the activation of effector caspases (DrICE) (Hay et al., 1994; Lannan et al., 2007), together with the p115 RNA interference construct. Blocking cell death in this way enhanced the reduction in tissue size (Fig. 3B and K), showing that cell death has a limited role in the growth

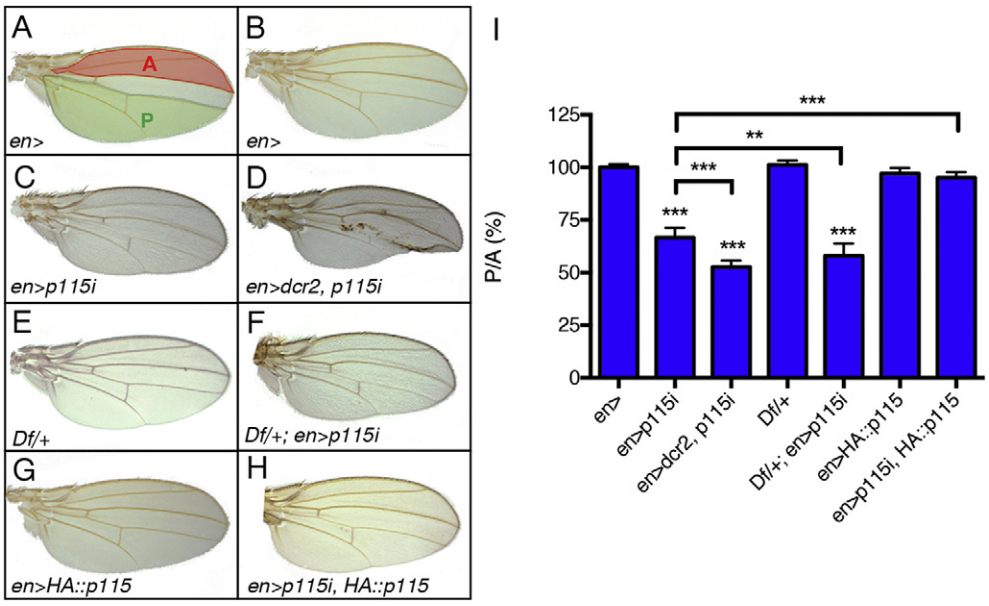


Fig. 1. p115 is required for wing tissue growth. (A–H) Female adult wings. (A) Control wing, in which the Engrailed (en) domain (posterior compartment, P) is labeled green and the anterior compartment (A) is labeled red. The area between veins L3 and L4 was ignored since the antero-posterior boundary cannot be exactly determined. (B) Control wing without the labels. Expression of p115i with the *en-Gal4* driver (C, *en > p115i*) reduces wing size. This effect is enhanced in a p115-deficient background (F, *Df/+*; *en > p115i*) and by co-expression of Dicer2 (D, *en > dcr2, p115i*). The wing size reduction is rescued by HA::p115 co-expression (H, *en > p115i, HA::p115*). (I) The graph shows the normalized posterior versus anterior (P/A) compartment size of each genotype ($n = 20$). The P/A ratio was calculated for each wing independently to avoid influence of natural variation on wing size e.g. due to nutritional conditions. Statistical significance was determined by the two-tailed Student's *t*-test with comparison to the control (*** $p < 0.001$, ** $p < 0.01$).

phenotype. Moreover, under these conditions, the numbers of cells in S phase (BrdU; Fig. 2H) and mitosis (PH3; Fig. 2A and E) were still increased in the posterior compartment.

Expression of p35 in apoptosis-inducing conditions (stress treatments or ectopic expression of pro-apoptotic genes) can generate "undead" cells. These cells are unable to proliferate but generate increased levels of growth factors, such as Wg, which can promote proliferation of surrounding cells (compensatory proliferation) (Huh et al., 2004; Pérez-Garijo et al., 2004; Ryoo et al., 2004). We could not detect ectopic Wg expression outside its normal pattern (dorsal/ventral limit and the zone that will give rise to the hinge) (Fig. S2) in our experimental settings.

Flow cytometric analysis of the effects of p35 and p115i co-expression on the cell cycle demonstrated that p115-deficient cells could become larger than control cells when cell death was blocked (Fig. 2O). Furthermore, DNA content analysis in these experimental conditions showed that aneuploid cells consistently disappeared and there was an increase in the G2/M and super-G2 population (DNA re-replication) over the G1 population (Fig. 2R and Table S2). The larger cells could correspond to the population that is normally eliminated by cell death but whose growth phase is extended under these conditions (Neufeld et al., 1998). These cell cycle parameters are similar to those observed following partial loss of function of positive regulators of the G2/M transition, such as Cdk1 or String (Björklund et al., 2006; Weigmann et al., 1997), indicating that p115 modulates their activation or localization in the cell to allow cell cycle progression.

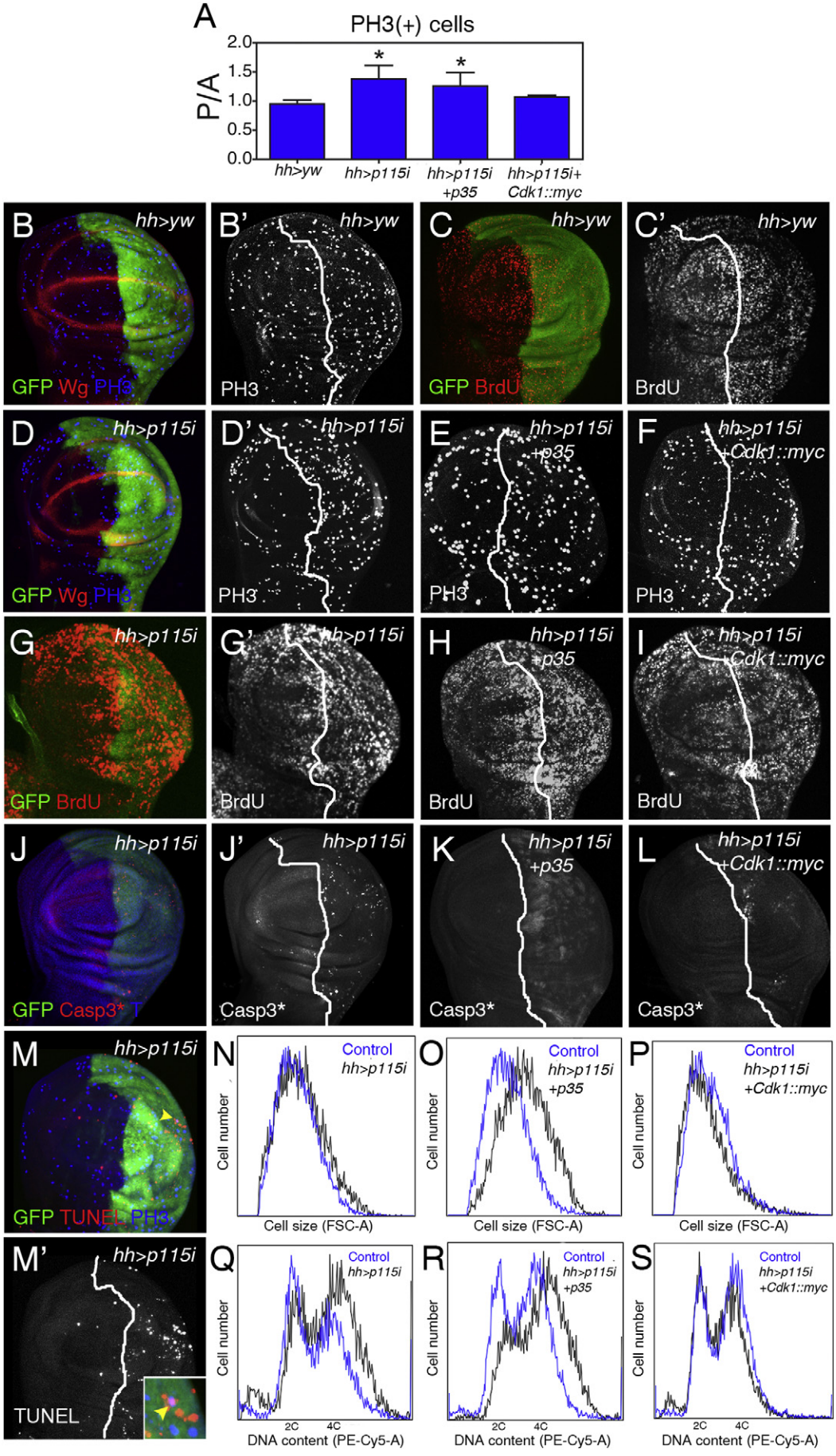
2.4. Modifiers of the phenotype induced by p115 knockdown: GRASP65 and Cdk1

To obtain insights into the mechanism or process that is affected by p115 depletion and is responsible for the impairment of cell cycle progression, we searched for modifiers of the wing size reduction phenotype, specifically testing other golgins and proteins related to the G2/M transition.

GRASP65 is a Golgi stacking protein that has been implicated in unconventional secretion in *Drosophila*, which is a type of exocytosis that occurs directly between the ER and the cell membrane, bypassing the Golgi apparatus (Schotman et al., 2008). In mammalian cells, similar phenotypes are induced by GRASP65, GM130, and p115 depletion and comprise fragmentation of the Golgi and multipolar spindle formation, suggesting that these proteins are required in related processes (Kodani and Sütterlin, 2008; Radulescu et al., 2011; Sütterlin et al., 2005). Under our experimental conditions, GRASP65 knockdown alone had no effect on wing size (Fig. 3C) and did not induce the phenotype described for *Drosophila* GRASP65 mutants (deficient adhesion between the dorsal and ventral wing epithelia and tissue shrinkage) (Schotman et al., 2008). GRASP65 expression was only reduced by 26% in the GRASP65-knockdown strain used (Fig. S4). However, functional interaction analysis showed that this partial depletion of GRASP65 together with p115 knockdown strengthened the effect on tissue size (Fig. 3D). This suggests that these two proteins function in interconnected cellular processes, as in mammals.

The G2/M transition depends mainly on regulation of the CycB/Cdk1 complex. To determine whether the impairment of cell cycle progression was due to perturbed activation of this catalytic complex, we modified its activation and analyzed the effects on cell proliferation and tissue size in p115-depleted cells. Various strategies can be used to activate the Cdk1/CycB complex; we used strains that allow overexpression of the activator phosphatase String (UAS-String) or knockdown of the major inhibitory kinase for wing imaginal disc development, Myt1 (UAS-Myt1i) (Jin et al., 2008), as well as a strain that overexpressed a tagged form of Cdk1 (UAS-Cdk1::myc).

The modifications that increase Cdk1/CycB activity did not affect wing size by themselves (Fig. 3E, G, and K). This is due to a compensatory change in the amount of time spent in G1 or G2 phase evoked by the opposite change in the other phase of the cycle. Thus, if cells spend longer in one phase of the cell cycle (e.g., G1), the other phase (G2) is shortened proportionally and hence the cell doubling time remains constant (Neufeld et al., 1998).


A
PH3(+) cells
P/A
2.0
1.5
1.0
0.5
0.0
*
*
hh>yw
hh>p115i
hh>p115i +p35
hh>p115i+ Cdk1::myc
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hh>p115i +p35
Cell number
2C
4C
DNA content (PE-Cy5-A)
S
Control
hh>p115i +Cdk1::myc
Cell number
2C
4C
DNA content (PE-Cy5-A)

Cdk1::myc overexpression rescued the reduction in wing size induced by p115 knockdown (Fig. 3J and K). Interestingly, decreasing the level of Myt1, which is a kinase required to inactivate Cdk1 that is localized in the ER/Golgi apparatus (Fig. S4), rescued the phenotype induced by p115 knockdown by 15% (Fig. 3F and K), while increasing the activities of other activators such as String did not (Fig. 3H and K). Altogether, our data suggest that p115 plays a role in the G2/M transition, specifically in the proper activation of the CycB/Cdk1 complex in *Drosophila*.

In agreement with suppression of the wing size phenotype by Cdk1::myc overexpression, we no longer observed an increase in the number of cells in S phase (BrdU; Fig. 2I) or mitosis (PH3; Fig. 2A and F) seen in p115-knockdown imaginal discs. However, a low level of programmed cell death was still observed in these conditions (cleaved caspase-3) (Fig. 2L). Additionally, flow cytometric analysis of cells from imaginal discs co-expressing p115i and Cdk1::myc in the posterior compartment showed that anterior and posterior cells were similar in size (Fig. 2P) and the cell cycle profile was similar to that of control cells (Table S2). Although the phenotypes induced by p115 knockdown were almost completely rescued, we still detected a small population of aneuploid cells (Fig. 2S). This is in agreement with the residual activated caspase-3 staining observed in these imaginal discs (Fig. 2L). Thus, we conclude that Cdk1::myc overexpression can rescue most of the phenotypes seen in p115-depleted wings.

2.5. p115 is necessary for efficient Cdk1 activation

We envision two complementary scenarios to explain how Cdk1 overexpression can rescue the phenotypes induced by p115 knockdown. In the first scenario, p115 is required for activation of Cdk1 and/or affects the levels of Cdk1 activators. Upon p115 depletion, the level of activated Cdk1 is reduced, causing the aforementioned phenotypes. In the second scenario, p115 is required for the localization of Cdk1 to sites of activation. Upon p115 knockdown, Cdk1 fails to localize properly, thereby reducing its activation.

To explore these possibilities, we first addressed whether the level of Cdk1 activation was compromised upon p115 knockdown. For this, we expressed the dsRNA against p115 in the wing pouch (central region of the wing imaginal disc that will give rise to the adult wing tissue) using the *nubbin-Gal4* (*nub-Gal4*) driver. We compared the level of phospho-Tyr15 Cdk1, indicative of the inactive configuration of Cdk1, with the total Cdk1 level by western blotting using specific antibodies. The ratio of inactive Cdk1 to total Cdk1 was increased in p115-knockdown discs, and this effect was more pronounced when the function of the RNA interference processing machinery was enhanced by co-expressing the p115-targeting dsRNA construct together with a construct encoding the RNAse III Dicer2 (UAS-dcr2) (Fig. 4A and B).

2.6. p115 is required for mitotic spindle assembly in Drosophila

Considering the functions of p115 in mammalian cells and the apoptotic cells detected in our experimental conditions, we investigated whether p115 knockdown induces the generation of multipolar spindles similar to those described previously in mammalian HeLa cells depleted of p115 (Radulescu et al., 2011) or with reduced activation of Cdk1 (Morris and Jalinot, 2005). For this, we analyzed and compared the structure of the mitotic spindle between S2 cells treated with dsRNA targeting p115 and those treated with dsRNA targeting GFP as a negative control. Although chromosomes aligned at the metaphase plate and bipolar spindles formed in most control cells, p115-depleted cells (~60%) exhibited abnormal, multipolar spindles with a range of aberrant morphologies and misaligned chromosomes (Fig. 5).

Immunostaining of α-tubulin, γ-tubulin, and PH3 were also performed in imaginal discs using *nub-Gal4* driver to express the p115 dsRNA together with Dicer2, or a Cdk1 dsRNA, to see if there is any defect in the mitotic spindles. We did not find any overt phenotype associated with the structure of mitotic spindles (Fig. S5).

2.7. Physical interaction between p115 and Cdk1

In mammalian cells, an association between p115 and γ-tubulin has been demonstrated (Golgi and centrosomes) (Radulescu et al., 2011) and Cdk1 accumulates at the centrosomes during mitosis in different cell lines (Bailly et al., 1989; Loffler et al., 2011). This suggests that p115 and Cdk1 can interact directly during the cell cycle.

We were unable to test this hypothesis by immunostaining due to the lack of suitable antibodies and the fact that overexpression of p115 (UAS-HA::p115) or Cdk1 (UAS-Cdk1::myc) may induce a broader localization of these proteins.

As an alternative, we performed an immunoprecipitation assay using protein extracts of wing discs co-expressing tagged versions of p115 (UAS-HA::p115) and Cdk1 (UAS-Cdk1::myc) under the control of the *nub-Gal4* driver. As a control, we performed the same immunoprecipitation protocol using protein extracts obtained from discs expressing only HA::p115 protein under the control of the same *Gal4* driver. We detected an interaction between these proteins (Fig. 6). This result and the impairment of Cdk1 activation upon p115 knockdown suggest that these proteins need to interact to allow complete Cdk1 activation and mitotic progression (Fig. 7). This would also explain the inability of String to rescue the phenotype induced by p115 depletion. Thus, p115 would be necessary to recruit Cdk1 to some cell compartments, such as fragments of the Golgi, or the centrosome to permit the full activation of Cdk1.

3. Discussion

Here, we examined the function of the ER/cis-Golgi golgin p115 during *Drosophila* imaginal disc development using a dsRNA approach. This approach has some advantages over the use of mutant flies, such as tight spatial and temporal control of the analyzed protein expression levels, thereby avoiding lethality. We confirmed that the p115-targeting dsRNA efficiently reduced the p115 RNA level by RT-qPCR (Fig. S1B) Moreover, rescue of the phenotypes by p115 overexpression and increased reduction of the wing size by crosses with the deficiency demonstrated that this dsRNA specifically targeted p115 (Fig. 1).

Depletion of p115 in the growing wing epithelia did not induce obvious phenotypes associated with secretion. In mammalian cells, the fusion of Golgi vesicles is mediated by an association between the acidic C-terminal domain of p115, the Golgi-related protein giantin (no homolog in *Drosophila*), and the golgin GM130 (Nakamura et al., 1997; Sönnichsen et al., 1998). This acidic domain is not present in the *Drosophila* ortholog (Kondylis and Rabouille, 2003). This suggests that the mechanism underlying vesicle fusion and Golgi reorganization is different in *Drosophila* cells and also explains why p115 is not required in their exocytic pathway.

The phenotypes observed in the size and pattern of adult wings, cell size, the cell cycle, and apoptosis in p115-depleted wing discs are reminiscent of those observed upon partial loss of function of positive

Fig. 2. Proliferation profiles upon p115 depletion alone or together with p35 or Cdk1::myc expression. DNA replication and mitosis were estimated for *hh > yw*, *hh > p115i*, *hh > p115i + p35*, and *hh > p115i + Cdk1::myc* by BrdU incorporation (C, C', G, G', H, I) and PH3 staining (B, B', D, D', E, F), respectively. Activated caspase-3 was labeled by immunofluorescence to detect dead cells in the experimental imaginal discs (J, J', K, L). (A) Posterior versus anterior ratio (P/A) of PH3(+) cells inside the Wg outer-ring expression domain (wing pouch). Statistical significance was determined by the two-tailed Student's *t*-test (* $p < 0.01$, $n = 8$). TUNEL (M, M') was performed to show that few cells died during mitosis (<five cells per disc, $n = 6$). Flow cytometric analysis (N, O, P, Q, R, S) was performed by dissociating the wing imaginal discs of the indicated genotypes and staining with the DNA-specific probe DRAQ5. p115 knockdown increases the numbers of aneuploid cells (cell death) and super-G2 cells (endoreplication of DNA). These phenotypes are enhanced by p35 co-expression and rescued by Cdk1::myc co-expression (see cell cycle analysis in Table S2).

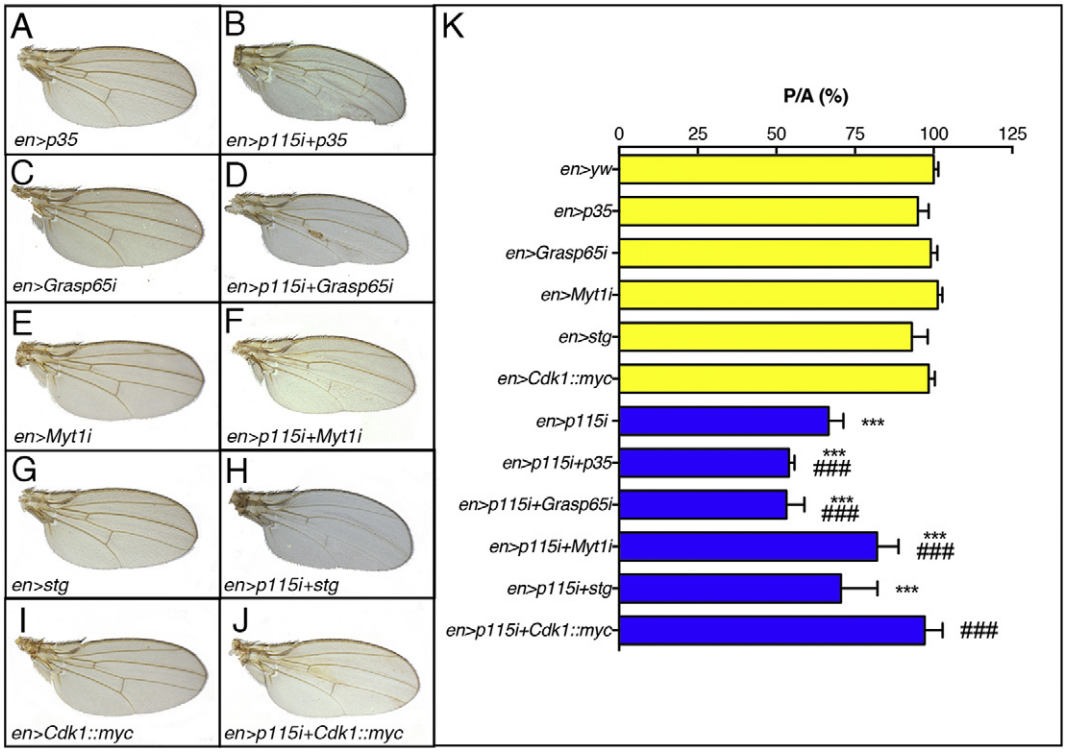

Fig. 3. The phenotypes induced by p115 depletion are enhanced by GRASP65 knockdown and rescued by Cdk1 overexpression. (A–J) Female adult wings expressing the indicated transgenes (*en = en-Gal4*) in the *Engrailed* domain. Expression of the apoptosis inhibitor p35 alone does not affect domain size (A, *en > p35*). Co-expression of p115i and p35 in this domain maintains the reduced wing size (B, *en > p115i + p35*). Co-expression of p115i and GRASP65i enhances the phenotypes, despite the fact that GRASP65 depletion by itself does not induce a recognizable phenotype (C, *en > Grasp65i*). Knockdown of the Cdk1 kinase Mytl1 partially rescues the phenotype induced by p115 depletion (F, *en > p115i + Myt1i*), but overexpression of the phosphatase String does not (H, *en > p115i + stg*), even though both manipulations can activate Cdk1. The reduction in organ size caused by p115 depletion is rescued by Cdk1::myc co-expression (J, *en > p115i + Cdk1::myc*). (K, The graph shows the normalized posterior compartment size of each genotype ($n = 15$). Statistical significance was determined by the two-tailed Student's test with comparison to *en > yw* (asterisks) or *en > p115i* (hashes) (***, ### $p < 0.001$; **, ## $p < 0.01$; *, # $p < 0.1$). (Genotypes: (A) *y w/w; en-Gal4/+; UAS-p35/+*, (B) *y w/w; en-Gal4, UAS-p115i/+; UAS-p35/+*, (C) *y w/w; en-Gal4/+; UAS-Grasp65i (TRiP)/+*, (D) *y w/w; en-Gal4, UAS-p115i/+; UAS-Grasp65i (TRiP)/+*, (E) *y w/w; en-Gal4/UAS-Myt1i*, (F) *w; en-Gal4, UAS-p115i/UAS-Myt1i*, (G) *y w/w; en-Gal4/UAS-stg*, (H) *w; en-Gal4, UAS-p115i/UAS-stg*, (I) *y w/w; en-Gal4/+; UAS-Cdk1::myc*, and (J) *w; en-Gal4, UAS-p115i/UAS-Cdk1::myc*.)

regulators of the G2/M transition. These phenotypes include the accumulation of cells in G2/M phase, the presence of DNA-re-replicating cells, and apoptosis (Björklund et al., 2006; Neufeld et al., 1998; Weigmann et al., 1997). In *Drosophila*, this transition is mainly controlled by the activity of the Cdk1/CycB complex.

The dependency of the G2/M transition on Cdk1 and the relationship of p115 with this were reinforced by our search for modifiers of the phenotypes induced by p115 knockdown. The phenotypes were completely rescued by Cdk1 overexpression; however, they were not rescued by overexpression of the classical Cdk1 activator String and only partially rescued by Myt1 depletion. Myt1 expression was only reduced by 36% in the Myt1-knockdown strain used (Fig. S4) that can explain the partial rescue. Interestingly, the level of Cdk1 was normal and only its activation was reduced in p115-knockdown cells.

Lindqvist et al. (2007) proposed that Cdk1 works as a bi-stable circuit during cell cycle progression, which implies that a certain level of Cdk1/CycB activation is needed to initiate mitosis and a higher level is needed to exit mitosis. In this scenario, a reduction in the p115 level, and the corresponding decrease in Cdk1 activation, would allow cells to enter mitosis, but not to proceed through cytokinesis or to exit mitosis and thereby give rise to daughter cells. Hence, upon Cdk1 overexpression, the concentration of Cdk1 would increase and more Cdk1 would be localized in regions where it can be activated. Consequently, a larger proportion of this kinase would be activated, allowing proper mitotic progression.

The observation that String, a cytoplasmic activator of Cdk1, was unable to rescue the phenotypes induced by p115 knockdown whereas they were partially rescued by Myt1 knockdown adds another layer of complexity. This could be related to the p115-Cdk1 interaction and the subcellular localization of Myt1. Myt1 is concentrated in Golgi and ER membranes (Liu et al., 1997). During mitosis, the activity of Myt1 is reduced, favoring the activation of Cdk1 by cytoplasmic phosphatases, specifically String (Booher et al., 1997). Despite this, Myt1 and p115 may be required for Cdk1 sequestration. Myt1 and Cdk1 can interact via domains other than the kinase domain (Wells et al., 1999). In mammalian cells, an association between p115 and γ-tubulin has been described (Golgi and centrosomes) (Radulescu et al., 2011) and Cdk1 accumulates at the centrosomes in different cell lines (Bailly et al., 1989; Loffler et al., 2011). This suggests that p115 is required for Cdk1 localization during mitosis, possibly via an association with the centrosomes (Bailly et al., 1989; Lindqvist et al., 2007; Loffler et al., 2011) or the Golgi apparatus (Hölzenspies et al., 2009). In cultured S2 cells, p115 depletion caused a multipolar spindle phenotype, similar to that described in mammalian cells depleted of p115 (Radulescu et al., 2011) or in which activation of Cdk1 is reduced (Morris and Jalinot, 2005). This suggests that the mechanisms were conserved during evolution, even though the Golgi structure during interphase differs between *Drosophila* and mammalian cells.

We were unable to observe this phenotype directly in the wing imaginal disc. This may be because multipolar spindles usually join together in *Drosophila* to generate bipolar spindles, which would obscure this phenotype (Basto et al., 2008). Alternatively, it may suggest that the requirement for these proteins differs between S2 cells and *Drosophila* imaginal disc cells.

Finally, we confirmed the physical interaction between p115 and Cdk1, suggesting that p115 sequesters or re-localizes Cdk1 during

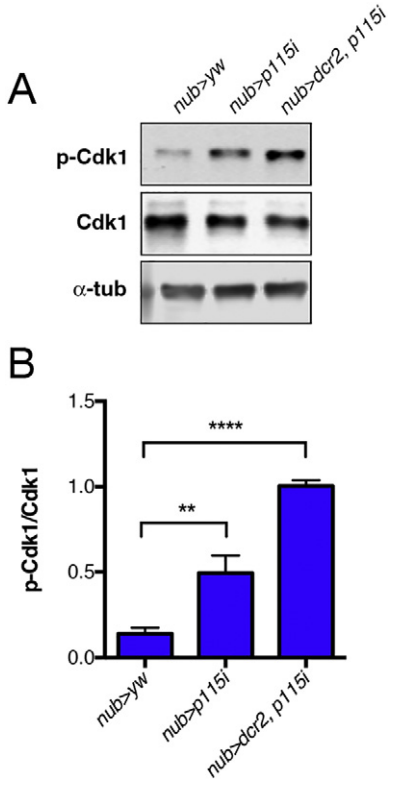

Fig. 4. p115 is required for Cdk1 activation. (A) Immunoblotting of phospho-Cdk1 (Y15), total Cdk1, and α-tubulin. (B) The graph shows the activation level of Cdk1 (phospho-Cdk1/Cdk1) determined by densitometric quantification. Statistical significance was determined by the two-tailed Student's *t*-test with comparison to *nub* > *yw* (**** $p < 0.0001$, ** $p < 0.01$). (Genotypes: ***nub*** > ***yw***: *w nub-Gal4/y w*, ***nub*** > ***p115i***: *w nub-Gal4/w*; *UAS-p115i*/+, and ***nub*** > ***dcr***; ***p115i***: *w nub-Gal4/UAS-dcr*; *UAS-p115i*/+.)

mitosis to allow the complete activation of Cdk1 and consequently correct progression through mitosis in *Drosophila* imaginal cells. These results suggest that the mechanisms responsible for the Golgi fragmentation checkpoint are tightly linked with the presence of p115 and its interaction with Cdk1. Thus, even in cells with a tubular-vesicular Golgi structure, mitotic progression may require the association between p115 and Cdk1 to attain the level of activation needed to complete the cell cycle. We propose that this mechanism of Cdk1 regulation guarantees Golgi inheritance.

4. Experimental procedures

4.1. Drosophila melanogaster strains and phenotypic analysis

The following UAS lines were used: *UAS-p115i* (VDRC, #103350), *UAS-Grasp65i* (*III*) (Bloomington *Drosophila* Stock Center (BDSC), #34082), *UAS-Cdk1::myc* (*III*) (Meyer et al., 2000) (BDSC, #6638), *UAS-p35* (*III*) (BDSC, #5073), *UAS-stg* (*III*) (Neufeld et al., 1998) (BDSC, #4778), *UAS-Cdk1i* (*III*) (BDSC, #40950), *UAS-Myt1i* (*II*) (VDRC, #105157) and *UAS-dcr* (*X*) (BDSC, #24648). The following Gal4 lines were also used: *nub-Gal4* (a gift from Dr. de Celis, Centro de Biología Molecular Severo Ochoa, Madrid, Spain), *en-Gal4* (BDSC, #6356), *hh-Gal4*, *UAS-GFP/TM6B*, and *tub-Gal4*. The p115 deficiency line used was *Df(1)ct4b1*, *y[1]/Binsn* (BDSC #3221).

All phenotypes were analyzed at 25 °C unless stated otherwise. Wings were mounted for examination in lactic acid-ethanol (1:1). Images were acquired on an Olympus MVX10 dissecting microscope or a Zeiss IIIRS microscope with a Leica DFC300FX digital camera and subsequently processed using Adobe Photoshop CS3 Extended. For posterior compartment measurements, the wing area below the fourth vein was considered.

All data presented are mean ± standard deviation and were subjected to a two-tailed Student's *t*-test. *P* values lower than 0.05 were deemed to be significant. At least 15 female wings were measured per genotype analyzed.

4.2. p115 construct

Because *Drosophila p115* has no introns, the HA::p115 N-terminal fusion could be constructed by amplifying the coding sequence directly from genomic DNA using the following primers: 5′-CACCATGGAGT TCCTGAAGAGTGGC-3′ and 5′-TTCCTGCTGGCGGTGCCACTTGG-3′. This amplicon was cloned into the pENTR/D-TOPO vector (Life Technologies, K240020) and sequenced. Afterward, it was subcloned using Gateway LR Clonase II technology (Life Technologies) into pTHW (The *Drosophila* Vector Gateway Collection, #1099). Standard germ cell transformation was performed to achieve at least three independent transgenic insertions of the construct (Spradling and Rubin, 1982).

4.3. Imaginal disc immunofluorescence, TUNEL, and RT-qPCR analysis

Rabbit polyclonal anti-PH3 (1:500; Upstate), mouse monoclonal anti-BrdU (1:100; Dako, M0744), and rabbit polyclonal anti-cleaved caspase-3 (1:100; Cell Signaling) antibodies were employed. The following secondary antibodies were from Molecular Probes: Alexa Fluor 488, 562, and 633 nm (1:200; Life Technologies, A11001, A11004, A21235, A11008, A11011, and A21086). Nuclei were stained with ToPro-3A (1:200; Life Technologies, T3605), and F-actin was labeled with TRITC-labeled phalloidin (1 μg/mL; Sigma, P1951). Third instar imaginal discs were dissected (110 h after egg laying (AEL)), fixed, and stained as described previously (de Celis, 1997). TUNEL was carried out with an In Vivo Cell Death Detection Kit, TMR red (Roche, #12156792910) according to the manufacturer's instructions. The permeabilization step was modified by incubating samples in solution containing 0.3% Triton X-100 and 0.1% sodium citrate for 30 min at 65 °C. Subsequent washes and incubation with the secondary antibody were performed following standard immunofluorescence protocols. Confocal images were captured using a Zeiss LSM 510 Meta confocal microscope.

For RT-qPCR, total RNA was extracted from third instar larvae or S2 cells using TRIzol reagent (Life Technologies). SuperScript III reverse transcriptase (Life Technologies) was used for reverse transcription. Quantitative PCR was conducted using a QuantiTect SYBR Green PCR kit (Qiagen). A list of the primers used for the analysis is presented in Table S1.

4.4. Cell culture, dsRNA treatments, and immunostaining

S2 cells were grown in Schneider's medium supplemented with 10% fetal bovine serum (FBS; Invitrogen). To generate the dsRNAs, regions of the genes were amplified from genomic DNA by PCR using the T7 promoter sequence fused with gene-specific primers (Table S1). An Ambion MEGAscript T7 kit was used for the transcription reaction following the manufacturer's instructions. Thereafter, the dsRNAs were quantified and stored at −20 °C. The dsRNA treatment and cell immunostaining were performed as described previously (Bettencourt-Dias and Goshima, 2009). Briefly, S2 cells were centrifuged, resuspended in serum-free media, and plated in 96-well plates together with 1 μg of dsRNA for 1 h, and then media containing 15% FBS was added. At the end of the dsRNA treatment (day 4), cells were resuspended, transferred to coverslips treated with 0.1 mg/mL Concanavalin A, and allowed to adhere for 2.5 h before fixation. Cells were fixed in 4% paraformaldehyde for 10 min, permeabilized with PBS supplemented with 1% BSA and 0.1% Triton X-100 (PBT) for 1 h, incubated overnight at 4 °C with rat monoclonal anti-α-tubulin (1:100; Sercotec), mouse monoclonal anti-γ-tubulin (1:500; Sigma), and rabbit polyclonal anti-

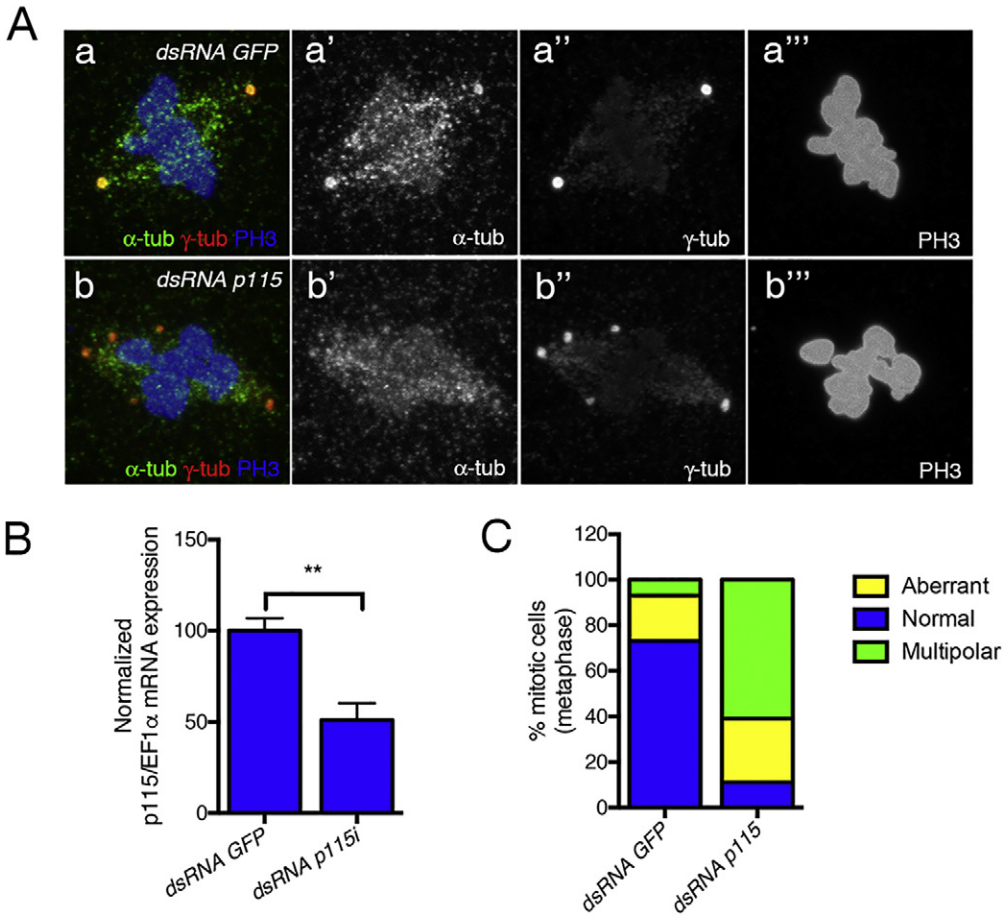

Fig. 5. Knockdown of p115 induces multipolar spindles in cultured S2 cells. (A) S2 cells were treated with 1 μg of control dsRNA against GFP (a, a', a'', a''') or p115-targeting dsRNA (b, b', b'', b''') for 4 days and immunostained with antibodies against α-tubulin, γ-tubulin, and PH3. (B) Quantitative RT-PCR analysis showing that the transcript level of p115 is lower in S2 cells treated with p115-targeting dsRNA than in S2 cells treated with GFP-targeting dsRNA ($n = 3$, $^{**}p < 0.01$). (C) Quantification of the phenotypes of cells in metaphase following treatment with control and p115-targeting dsRNAs ($n = 100$ mitotic cells per condition). Normal refers to the presence of a bipolar spindle. Multipolar refers to the presence of more than two centrosomes. Aberrant refers to other phenotypes that are observed within the normal variability of S2 cell spindles, including monopolar or monastral spindles, pole detachment, chromosomal misalignment, and longer/shorter spindles (see Goshima, 2010 for more details).

PH3 (1:1000, Upstate) antibodies diluted in PBT containing 5% donkey serum, and then stained with secondary antibodies and 5 μg/mL Hoechst 33342 (Life Technologies).

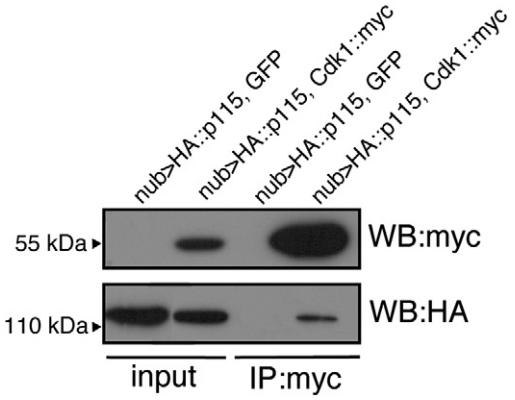

Fig. 6. Physical interaction between p115 and Cdk1. Co-immunoprecipitation was performed from wing imaginal discs overexpressing HA::p115 alone or together with Cdk1::myc using agarose beads coupled to a rabbit anti-myc antibody. Lanes marked "input" show the relative amounts of the indicated proteins in cell lysates. Lanes marked "IP:myc" show the relative amounts of protein precipitated using the anti-myc antibody. A specific interaction between Cdk1 and p115 is observed. (Genotypes: ***nub*** > ***HA::p115***, ***GFP***: *w nub-Gal4/+*; *UAS-HA::p115/+*; *UAS-mCD8::GFP/+*, and ***nub*** > ***HA::p115***, ***Cdk1::myc***: *w nub-Gal4/+*; *UAS-HA::p115i/+*; *UAS-Cdk1::myc/+*.)

4.5. Western blotting and immunoprecipitation

Cell lysates obtained from wing discs (around 300 discs per well) were cleared overnight at 4 °C using agarose beads conjugated with a rabbit anti-myc antibody (Sigma, A7470). Thereafter, the beads were washed four times with PBS containing protease inhibitors and boiled in SDS-PAGE loading buffer. The primary antibodies used for blotting included mouse anti-myc (1:500; Santa Cruz Biotechnologies, sc-40) and mouse anti-HA (1:500; Santa Cruz Biotechnologies, sc-7392), which were detected using HRP-conjugated secondary antibodies (1:2000;

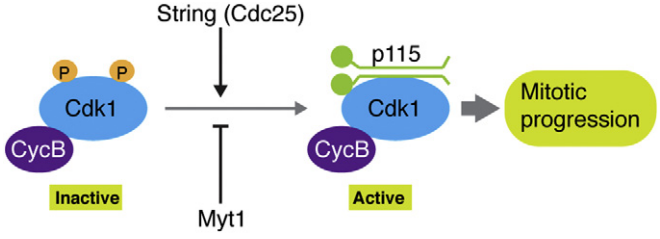

Fig. 7. Role of p115 in the G2/M transition. The G2/M transition depends mainly on regulation of the CycB/Cdk1 complex. The activity of this complex depends on phosphorylation by Myt1 kinase and dephosphorylation by String. p115 interacts with Cdk1 and aids mitotic progression, possibly by recruiting Cdk1 to areas where it is activated.

Calbiochem, DC02L and DC03L). For western blotting, rabbit polyclonal anti-Cdc2 (1:2000; Millipore), rabbit polyclonal anti-phospho-Cdc2 (Y15; 1:1000; Cell Signaling), and mouse monoclonal anti-α-tubulin (1:5000; Sigma) antibodies were used. The blotting was performed essentially as described previously (Ibar et al., 2013). The Gel Analyzer tool from ImageJ was used for densitometric quantifications.

4.6. Flow cytometry

For fluorescence-activated cell sorting (FACS) analysis, third instar larvae wing imaginal discs were dissociated in PBS Trypsin-EDTA 10× (Sigma, T4174) for 2 h and stained with 20 μM DRAQ-5 for the last 30 min (Biostatus Limited). Data were acquired using a Becton Dickinson FACS Canto flow cytometer and analyzed using the population comparison tool from FlowJo Software (with a minimum of 5000 GFP + cells). A representative histogram from three replicates for each genotype is shown in the figures.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mod.2017.04.001.

Acknowledgements

We thank Alfonso Gonzalez for helpful discussions, and Maria Rosa Bono and Daniela Sauma for their help with flow cytometric analysis. We also thank the Developmental Studies Hybridoma Bank for antibodies and the Bloomington Stock Center (NIH P40OD018537) and Vienna *Drosophila* Research Center for *Drosophila* stocks. This work was funded by FONDAP grant 15090007, PIA ACT1401 and FONDECYT grant 1140522 awarded to A.G. and a CONICYT Ph.D. Fellowship awarded to C.I.

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