Proteolytic control of centrosome activity by APC/C-Polo maintains oocyte fate in *Drosophila*

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Abstract:

In metazoans, the centrioles/centrosomes of the female germline are eliminated prior to the end of oogenesis. Although this phenomenon is important for reproduction, the function of the centrosomes during oogenesis and the mechanism controlling their stability is still unclear. Here we demonstrate that the multi-subunit ubiquitin ligase, the Anaphase Promoting Complex/Cyclosome (APC/C), ensures normal germline development by regulating the activity and stability of centrosomes during early *Drosophila* oogenesis. We show that perturbing APC/C activity by altering the activity of its co-factor Vihar/Ube2c causes ectopic microtubule-nucleation activity of the centrosomes, resulting in a failure of oocyte specification and decreased female fertility. Altering APC/C regulation induces precocious destruction of the key centrosome regulator, Polo Kinase, which leads to destabilization of the centrosomes and blocks their migration into the oocyte. Our study highlights the critical importance of the spatiotemporal regulation of centrosomes by the proteolytic mechanism during metazoan oocyte development.

Introduction:

The centrosome, a major microtubule organizing center (MTOC) in animal cells, is important for forming the mitotic spindle during cell division and plays a pivotal role influencing cell fate during animal development (Lattao et al., 2017). The centrosome consists of a pair of centrioles, barrel-shaped organelles characterized by singlets, doublets, or triplets of microtubules arranged in a 9-fold symmetrical array. The centrioles provide a scaffold for an amorphous proteinaceous matrix, the pericentriolar material (PCM), responsible for the spatial concentration of microtubule nucleating proteins.

Maternal centriole elimination is a universal feature of animal reproduction (Manandhar et al., 2005). Zygotic centrioles are either formed from the basal body of the incoming sperm or synthesized de novo (Engelstadter, 2008). Although centriole loss in oocytes is a fundamental biological phenomenon, an understanding of the mechanistic basis of this process and the functional significance of the centrioles is still missing. In species that need sperm to activate the egg (Manandhar et al., 2005; Pimenta-Marques et al., 2016; Von Stetina and Orr-Weaver, 2011), such as sea urchin (Miki-Noumura, 1977) or *Xenopus* (Tournier et al., 1989), the centrioles, and thus the centrosomes, are thought to be eliminated from the developing egg in order to prevent parthenogenesis. However, in *Drosophila melanogaster*, where the egg is activated prior to fertilization, forceful retention of the centrioles results in sterility and meiotic defects, suggesting that centriole elimination is needed for a different reason (Pimenta-Marques et al., 2016). Indeed, even in species of *Drosophila* with the capacity for "accidental" parthenogenesis (Eisman and Kaufman, 2007; Engelstadter, 2008; van der Kooi and Schwander, 2015), the maternal centrioles are still eliminated and formed again de novo after egg activation (Riparbelli and Callaini, 2003).

In *Drosophila*, failure to eliminate the germline centrioles results in sterility (Pimenta-Marques et al., 2016). As these centrioles are not thought to be necessary for the development of the oocyte (Stevens et al., 2007), it is puzzling that they are retained, and carefully regulated, until the end of oogenesis (Pimenta-Marques et al., 2016) (Januschke et al., 2006; Tissot et al., 2017; Zhao et al., 2012). If the centrioles/centrosomes serve no purpose, then perhaps their potential microtubule nucleating ability must to be carefully regulated in order to prevent them from disrupting development. We found that when centrosome activity and centriole stability are misregulated during early oogenesis, they do indeed disrupt egg development, which decreases female fertility. We further show that this regulation is achieved through proteolytic control of Polo Kinase, the key regulator of the centrosome, by the Anaphase Promoting Complex/Cyclosome (APC/C) ubiquitin ligase. Precise proteolytic regulation of the stability and activity of the centrosomes during oogenesis is therefore of critical importance for female fertility.

Results

Generation of new vihar/Ube2c alleles

The microtubule organizing activity and duplication process of the centrosomes are tightly coupled to cell cycle progression (Meghini et al., 2016; Prosser et al., 2012). This coupling is, in part, mediated by the APC/C, a multimeric protein complex with ubiquitin ligase (E3) activity. The APC/C temporally regulates many cell cycle proteins (including mitotic cyclins) as well as centriole biogenesis proteins (such as SAS6) by targeting them for destruction by the proteasome (Davey and Morgan, 2016; Nigg and Stearns, 2011). Therefore, to investigate the role of centrosome activity during oogenesis, we decided to perturb APC/C activity by targeting a highly-conserved APC/C-specific ubiquitin conjugating (E2) enzyme, Vihar (Vih; Ube2c in human) (Aristarkhov et al., 1996; Fumio et al., 1997; Townsley et al., 1997; Yu et al.). In both humans and flies, Vih/Ube2c is localized to the centrosomes and its cellular levels oscillate during the cell cycle, peaking during mitosis (Mathe et al., 2004; van Ree et al., 2010).

We created a series of CRISPR/Cas9 engineered *vih* alleles (Fig. 1A-B; see Supplementary Materials for detailed genetic characterization and validation). We specifically targeted the 27-amino acid N-terminus of the Vih protein, which has been shown to be necessary for negative autoregulation of Ube2c in human cells (Summers et al., 2008; Williamson et al., 2011), yet does not affect its ability to work with the APC/C to ubiquitinate substrates (Summers et al., 2008). Two *vih* null alleles (vih^{Null}) were created by frame-shifts (Fig. 1C). These alleles were homozygous lethal and caused similar cell cycle phenotypes as for previously reported null alleles; i.e., chromosomes were disorganized and there were supernumerary centrosomes in the larval neuroblasts (Fig. S1A) (Mathe et al., 2004).

In-frame deletions (*vih*$^{\Delta N}$) were also created, covering residues which are highly conserved in vertebrates (Fig. 1D). The reduced protein sizes of the two largest deletions (27 bp for *vih*$^{\Delta N\text{-}9}$ and 12 bp for *vih*$^{\Delta N\text{-}4}$, Fig. 1C), were confirmed by western blot (Fig. S1B). Homozygous females of both *vih*$^{\Delta N}$ alleles were viable and no mitotic phenotypes were observed in the larval neuroblasts (S1A). However, the *vih*$^{\Delta N}$ homozygous females showed a decrease in cumulative fecundity (total number of eggs laid over a 21-day period): *vih*$^{\Delta N\text{-}4}$ by 17% and *vih*$^{\Delta N\text{-}9}$ by 55% compared to wild-type (Fig. S1C). The relative fecundity decreased as the females aged and this decrease was greatest for the *vih*$^{\Delta N\text{-}9}$ allele, reaching 39% less than wild-type two weeks after eclosion.

Egg chamber development is disrupted in vih$^{\Delta N}$ mutant females

In order to determine the cause of the reduced overall fertility, we examined the egg chambers of the homozygous flies. Oogenesis starts in the germarium and proceeds in 14 stages that are delineated by morphological changes (Fig. 1E) (Jia et al., 2016). In the germarium, after abscission from the stem cell, the germline cells undergo four rounds of mitotic division with incomplete cytokinesis to create a 16-cell syncytial cyst, where cells are connected by intracellular bridges called ring canals (Spradling, 1993). In the syncytium, one cell - marked by the synaptonemal complexes, centrosome localization and, Orb expression (Huynh and St Johnston, 2004) - ultimately becomes the oocyte, while the other 15 cells become nurse cells. The oocyte arrests in meiosis I, whereas the nurse cells undergo a specialized type of cell cycle called the endocycle, where they replicate their DNA without dividing.

The reduced fertility of the *vih*$^{\Delta N}$ females was not due to a disruption of the mitotic divisions, as we did not observe any mitotic defects in either the *vih*$^{\Delta N\text{-}4}$ or *vih*$^{\Delta N\text{-}9}$ egg chambers (Fig.

1F). There were always 16 cells in the germline cysts, and their cell membranes, marked by filamentous actin (F-actin) staining, appeared normal at early stages of oogenesis. However, after stage 3/4, we noticed that the cortical F-actin of the germline was often misplaced between two or more of the nurse cells (in 45% of *vih*$^{\Delta N\text{-}9}$ homozygous egg chambers and 16% of *vih*$^{\Delta N\text{-}9}$ heterozygous egg chambers; Fig. 1F). Instead of localizing to cell membranes, the cortical F-actin frequently formed clumps in the center of the egg chambers at later stages (Fig. 1F). This "membrane phenotype" (as it will be termed hereafter) resembles that of mutations which cause a loss of membrane integrity (Coutelis and Ephrussi, 2007; Murthy and Schwarz, 2004; Tan et al., 2014), and was never observed in control egg chambers or in marked internal control egg chambers (see Supplementary Materials). The same phenotype was observed at lower frequencies in *vih*$^{\Delta N\text{-}4}$ egg chambers (Fig. 1F). The expressivity of the phenotype increased in older females (Fig. S1D), correlating with the decline in fertility described above.

Time-lapse imaging, using the uniform fluorescent cell membrane marker CellMask, confirmed that nurse cell membranes were indeed rupturing in *vih*$^{\Delta N\text{-}9}$ egg chambers (Fig. S2A). *vih*$^{\Delta N\text{-}9}$ egg chambers without the membrane phenotype appeared morphologically normal and had no F-actin clumps. We concluded that the misplaced F-actin and rupturing membranes reflect a loss of internal structural integrity in the germline, most likely contributing to the reduced fertility of *vih*$^{\Delta N}$ females.

Centrosome transport and stability is compromised in vih$^{\Delta N\text{-}9}$ egg chambers

To investigate the cause of the membrane phenotype, we examined *vih*$^{\Delta N\text{-}9}$ egg chambers at earlier stages of oogenesis. During the mitotic divisions that form the germline cyst, the centrosomes are associated with a temporary structure called the fusome, which forms a

branching network that passes through each ring canal in the cyst (Fig. 2A) (Lin et al., 1994). After mitotic exit, the centrosomes undergo a fusome-dependent re-localization from the nurse cells to the oocyte, after which the fusome disappears (Bolívar et al., 2001; Grieder et al., 2000; Lin et al., 1994; Mahowald and Strassheim, 1970).

In control egg chambers, by the end of stage 1 of oogenesis, the centrosomes migrate into one cell, the future oocyte, where they cluster and subsequently localize with the oocyte nucleus (Fig. 2B) (Bolívar et al., 2001; Grieder et al., 2000; Mahowald and Strassheim, 1970). However, in all *vih*$^{\Delta N\text{-}9}$ ovarioles exhibiting the membrane phenotype, the majority of centrosomes failed to migrate, and remained in the nurse cells, often located near the ring canals (n>5, Fig. 2B). At later stages (4-7), the centrosomes were associated with the clumps of F-actin in the centers of the egg chambers (Fig. 2B). In ovarioles where the membrane phenotype was absent, the number of centrosomes found within the germline was similar to controls (n≤5). The tight correlation between the two phenotypes suggested that the membrane phenotype might arise from a failure to properly transport the centrosomes during the early stages of oogenesis. This failure is not due to a disruption of the fusome, as an antibody stain for the fusome structural protein Hu-li tai shao (Hts) in *vih*$^{\Delta N\text{-}9}$ egg chambers looked normal (Fig. 2B and S3A) (Yue and Spradling, 1992).

We also found that the centrosomes disappear prematurely during oogenesis in *vih*$^{\Delta N\text{-}9}$ egg chambers. In wildtype egg chambers, a cluster of oocyte nucleus-associated centrosomes is present until stage 12/13 of oogenesis (Pimenta-Marques et al., 2016), and this is what we observed (Fig. S3B). In contrast, the centrosomes were reduced after stage 6 compared to wildtype and were completely absent after ~stage 9 in all of the *vih*$^{\Delta N\text{-}9}$ egg chambers displaying the membrane phenotype (Fig. S3B), indicating that centrosome stability in these

egg chambers is reduced.

The reduced stability of the centrosomes observed in the *vih*$^{\Delta N\text{-}9}$ egg chambers may account for the failure of centrosome transport during the early stages of oogenesis. We investigated whether further destabilization of the centrosomes increased the occurrence of the centrosome migration phenotype in *vih*$^{\Delta N\text{-}9}$ egg chambers. To this end, we recombined the *vih*$^{\Delta N\text{-}9}$ allele with an allele of *Sas4* (*Sas4*^{s2214}), a core centriole component that is necessary for duplication and for mitotic PCM recruitment (Basto et al., 2006; Conduit et al., 2015; Stevens et al., 2007). We observed no centrosome transport defects in *Sas4*^{s2214} heterozygous egg chambers (Fig. S4A). However, we found that halving the dosage of Sas4 in *vih*$^{\Delta N\text{-}9}$ egg chambers significantly increased the expressivity of the ectopic centrosome localization phenotype from 45% to 72.5% (Fig. S4A). The increased failure of centrosome migration invariably coincides with the membrane phenotype. This genetic interaction strongly suggests that altered Vih activity leads to centriole destabilization, which interferes with centrosome transport during early oogenesis, and, in turn, causes the membrane phenotype

Centrosome migration is required for the maintenance, but not the specification, of oocyte fate

In 85% of stage 3 or older *vih*$^{\Delta N\text{-}9}$ egg chambers with the membrane phenotype, all the cells of the germline cyst appeared to be endocycling (based on nuclear size). Since only the nurse cells endocycle in wildtype, this suggested that the oocyte was not being properly specified in the *vih*$^{\Delta N\text{-}9}$ germline. In addition, we have just described how centrosome migration, another marker of oocyte differentiation, is compromised in *vih*$^{\Delta N\text{-}9}$ egg chambers (Fig. 2B, (Huynh and St Johnston, 2004)). We therefore investigated whether oocyte fate is properly specified and maintained in the *vih*$^{\Delta N\text{-}9}$ egg chambers.

We first examined oocyte specification, by staining for Oo18 RNA-binding protein (Orb), an oocyte fate marker that localizes to the oocyte in the germarium region 2A (Fig. 2A) (Mahowald and Strassheim, 1970). In both *vih*$^{\Delta N\text{-}9}$ (Fig. 2C) and *vih*$^{\Delta N\text{-}9}$ egg chambers in a *Sas4* heterozygous background (Fig. S4A), Orb always initially localized to a single cell at stage 1 of oogenesis, indicating that the oocyte was indeed specified. Orb remained localized to a single cell in egg chambers lacking the membrane phenotype. However, in 95% of egg chambers with the membrane phenotype, Orb failed to remain in the posterior of the egg chamber after stage 3 (coinciding with the membrane rupture), indicating that oocyte fate was subsequently lost. In these egg chambers, Orb instead co-localized with the ectopic centrosomes in the germline (Fig. 2C and S4A), indicating that although oocyte specification *per se* does not require centrosome transport, the maintenance of Orb localization, and thus oocyte fate, may require the centrosomes to reach the oocyte.

In order to confirm the loss of oocyte fate, we also checked the expression of the meiotic cell cycle markers Dacapo (Dap) and Cyclin E (CycE), both of which are necessary for the oocyte to maintain its fate and avoid entering the endocycle, and are also needed for the oocyte centralized MTOC to form (Hong et al., 2003; Lilly and Spradling, 1996). In control egg chambers, both Dap and CycE localized to the germinal vesicle early during oogenesis and remained there until late oogenesis (Fig. S4B). However, in 86% of *vih*$^{\Delta N\text{-}9}$ egg chambers, there was neither a morphologically recognizable oocyte nucleus nor obvious Dap and CycE staining within the germinal vesicle after stage 3 (Fig. S4B), similar to the Orb staining. We conclude that if the centrosomes do not localize properly, oocyte fate is not maintained, and the oocyte nucleus slips into the endocycle.

<u>The ectopic centrosomes nucleate microtubules</u>

In order to investigate why centrosome mislocalisation disrupts egg chamber development so greatly, we analyzed centrosome activity in *vih*$^{\Delta N\text{-}9}$ egg chambers. There is a long-standing theory that the microtubule organizing potential of the centrosomes must be inactivated to enable their transport through the ring canals to reach the oocyte (Bolívar et al., 2001). However, we found that in all stage 6 *vih*$^{\Delta N\text{-}9}$ egg chambers exhibiting the membrane phenotype, microtubules appeared to grow from the multiple ectopically localized centrosome clusters, indicating that these centrosomes could nucleate microtubules (Fig. 3A). To confirm this, we performed live-imaging of stage 4/5 *vih*$^{\Delta N\text{-}9}$ egg chambers from ovarioles that did not the membrane phenotype and therefore had the oocyte specified and control egg chambers, both of which had small numbers of ectopic centrosomes present. Centrosomes were marked with Asl-YFP and growing microtubules were marked with the plus-end microtubule marker EB1-GFP (Rogers et al., 2002). We found that 29% of the ectopic centrosomes in the *vih*$^{\Delta N\text{-}9}$ egg chambers could nucleate, compared to only 8% in the controls (Fig. 3B and SMovie 1-2).

We propose that the residual nucleating ability of the centrosomes seen in *vih*$^{\Delta N\text{-}9}$ egg chambers precludes their transport to the oocyte, explaining their frequent mislocalization within the egg chamber. This would also explain why they appear to get stuck in the ring canals in stage 1 egg chambers (Fig. 2B). These centrosomes might then block the ring canals, preventing the transport of other materials to the oocyte as it grows. When present in sufficient numbers, the blockage caused by these ectopic centrosomes could disrupt the structural integrity of the egg chamber, giving rise to the membrane phenotype we observe.

<u>Centrosome misregulation is caused by ectopic APC/C-Vih activity in vih$^{\Delta N\text{-}9}$ egg chambers</u>

To understand the molecular mechanism underlying the abnormal behavior of the centrosome in *vih*$^{\Delta N\text{-}9}$ egg chambers, we next determined whether Vih$^{\Delta N\text{-}9}$ acts as part of the APC/C. To this end, we developed a reconstituted in vitro ubiquitination assay using the *Drosophila* APC/C, purified from the *Drosophila* embryos (see Supplementary Materials). We examined whether the wildtype and mutated forms of Vih could support the APC/C to ubiquitinate fission yeast Mes1 protein, a highly efficient model APC/C substrate (Kimata et al., 2008). As expected, the APC/C ubiquitinated Mes1 in the presence of wildtype Vih (Vih^{WT}), but not in the presence of a catalytically inactive form of Vih (Vih$^{\Delta CS}$) (Fig. 4A-B). The APC/C achieved a similar level of ubiquitination in the presence of Vih$^{\Delta N\text{-}9}$ as in the presence of Vih^{WT} (Fig. 4A-B), indicating that Vih$^{\Delta N\text{-}9}$ is catalytically active, in alignment with previously published results where human Ube2c lacking the entire N-terminal domain performed equally as well as the full-length protein in in vitro ubiquitination and destruction assays (Summers et al., 2008). Since disrupting the N-terminal domain has been shown to decrease the level of negative regulation of Ube2c in human cell lines (Summers et al., 2008), the 9-amino acid deletion in Vih$^{\Delta N\text{-}9}$ may result in ectopic APC/C-Vih activity within *vih*$^{\Delta N\text{-}9}$ egg chambers.

If the *vih*$^{\Delta N\text{-}9}$ phenotype is indeed caused by ectopic/sustained APC/C-Vih activity, then reducing APC/C activity should ameliorate the phenotype. We therefore examined the expressivity of the membrane phenotype in *vih*$^{\Delta N\text{-}9}$ egg chambers heterozygous for an allele of *morula* (*mr*^{1}), a catalytic subunit of the APC/C. We found that only 3% of *mr*^{1}*/+; vih*$^{\Delta N\text{-}9}$ egg chambers had the membrane phenotype (Fig. S4C), compared to 45% in *vih*$^{\Delta N\text{-}9}$ single mutants (Fig. 1F), indicating that the *vih*$^{\Delta N\text{-}9}$ membrane phenotype is likely to be caused by ectopic APC/C activity conferred by Vih$^{\Delta N\text{-}9}$.

We then wondered whether ectopic/sustained APC/C activity in *vih*$^{\Delta N\text{-}9}$ egg chambers was also responsible for the premature degradation of the centrosomes (Fig. S3B). We therefore tested whether the APC/C regulates centrosome stability, by inactivating the APC/C using transheterozygous *mr* alleles. We found that 100% of *mr*$^{1/2}$ hypomorphic egg chambers appear to have greater numbers of the centrosomes at later stages (stage 9-11) of oogenesis compared to controls (Fig. 4C), and the centrosomes did not form an aggregate, suggesting that APC/C activity regulates the stability of centrosomes during oogenesis. This phenotype is similar to that of centrosome-targeted Polo overexpression (Pimenta-Marques et al., 2016).

Polo kinase mediates the regulation of centrosome stability and migration by APC/C-Vih

Having discovered a functional connection between APC/C activity and centrosome stability, we wondered which APC/C substrate was responsible for mediating the effect. Our prime candidate was Polo Kinase (Plk1 in human). Polo/Plk1 is required for PCM recruitment to the centrioles and Plk1 is a known target of the APC/C (Lindon and Pines, 2004; Visintin et al., 2008). Furthermore, it was recently shown that Polo activity in the *Drosophila* egg chamber is crucial for centrosome stability, and that lower levels of Polo enable the elimination of the centrioles by way of centrosomal PCM reduction (Pimenta-Marques et al., 2016).

Using an *in vitro* destruction assay where APC/C-dependent proteolysis can be recapitulated in *Xenopus* egg extracts (Yamano et al., 2009), we confirmed that *Drosophila* Polo is an APC/C substrate in *Drosophila*. Similar to the control APC/C substrate Cyclin B3 (CycB3), Polo was degraded in the interphase extracts in the presence of purified Fizzy-related (Fzr), an APC/C co-activator (Fig.5A-B). Moreover, the addition of recombinant Vih^{WT} or Vih$^{\Delta N\text{-}9}$, but not Vih$^{\Delta CS}$, accelerated Polo degradation in vitro, reinforcing the conclusion that Vih$^{\Delta N\text{-}9}$

is catalytically active. These results confirm that Vih cooperates with APC/C-Fzr to degrade *Drosophila* Polo.

To determine whether Polo levels are altered in *vih*$^{\Delta N\text{-}9}$ egg chambers, we carried out western blots of *vih*$^{\Delta N\text{-}9}$ ovary extracts. We found reduced Polo expression in the *vih*$^{\Delta N\text{-}9}$ ovaries compared to wildtype (Fig. 5C-F), suggesting that Polo is indeed destabilized in *vih*$^{\Delta N\text{-}9}$ egg chambers. The reduction of Polo increased with the age of the females, correlating with their observed decrease in fertility (Fig. S1C). To determine if the membrane phenotype in *vih*$^{\Delta N\text{-}9}$ egg chambers is caused by reduced Polo levels, we examined whether increasing the cellular amount of Polo, by introducing a GFP-Polo transgene, could rescue the *vih*$^{\Delta N\text{-}9}$ phenotype. We found that the two copies of GFP-Polo were able to completely rescue the membrane and centrosome transport phenotypes in the *vih*$^{\Delta N\text{-}9}$ germline (Fig. 5F). These data indicate that the membrane phenotype in the *vih*$^{\Delta N\text{-}9}$ egg chambers originates from reduced Polo levels.

With Ube2c, the APC/C is known to target its substrates in a sequential manner based on affinity (Williamson et al., 2011). Longer lasting Vih activity in *vih*$^{\Delta N}$ egg chambers, due to a lack of negative regulation on Vih$^{\Delta N}$, might therefore cause the APC/C to precociously target low-affinity substrates that it would not target at the same developmental time point in wildtype. Accordingly, if Polo is a low-affinity substrate of the APC/C, the addition of a higher affinity substrate such as Cyclin B (CycB) should prevent Polo from being targeted by saturating the APC/C. We tested this hypothesis by combining the CycB-GFP transgene with the *vih*$^{\Delta N\text{-}9}$ allele to find that CycB-GFP overexpression indeed rescued the *vih*$^{\Delta N\text{-}9}$ membrane phenotype, with only 3% of egg chambers exhibiting the phenotype (Fig. S5A), down from 45% with *vih*$^{\Delta N\text{-}9}$ alone (Fig. 1F). We also found that endogenous CycB levels were reduced in *vih*$^{\Delta N\text{-}9}$ ovaries (Fig. S5B) and that Polo levels returned closer to the wildtype levels in the

CycB-GFP overexpressing *vih*$^{\Delta N\text{-}9}$ ovaries (Fig. 5C and E). Altogether, these results identify Polo as a key factor downstream of ectopic APC/C-Vih activity and demonstrate the critical importance of the precise control of centrosome activity by the APC/C and Polo during early oogenesis in *Drosophila*.

Discussion

In spite of centriole elimination from the female germline being universal across metazoans, the function of the centrosomes during oogenesis and the mechanism regulating their stability and activity had not been fully investigated. Using unique alleles of *vih*$^{\Delta N}$, which allows ectopic APC/C activity, we have demonstrated that the evolutionarily conserved APC/C-Polo pathway is a critical regulator of the centrosome during early *Drosophila* oogenesis. We uncovered a novel developmental role for Polo in enabling the migration of the centrosomes into the future oocyte through the regulation of their activity during the early stages of oogenesis. This centrosome regulation is necessary to maintain oocyte fate and prevent the centrosomes from disrupting egg chamber development. A previous study suggested that Polo levels are regulated transcriptionally during oogenesis (Pimenta-Marques et al., 2016), and we provide strong evidence that Polo levels are also post-translationally regulated by APC/C-dependent proteolysis. We therefore propose a model where Polo levels are carefully controlled over the course of *Drosophila* oogenesis to ensure proper development: sufficient levels of Polo are required to regulate centrosome transport during early egg chamber development, yet Polo must be reduced by the APC/C during later stages enabling centrosome elimination from the germline (Fig. 5G). The temporal regulation of this switch of Polo stability and the exact action of Polo on the centrioles or PCM during egg chamber development need further investigation.

It has previously been shown that the centrioles/centrosomes are dispensable for oogenesis and indeed are also not essential for the development of many other tissues in *Drosophila* (Basto et al., 2006; Stevens et al., 2007). However, if they are forcefully retained within eggs, they cause sterility (Pimenta-Marques et al., 2016), indicating that centrosome regulation is of paramount importance in this animal. In fact, there are many situations in which, if the centrosomes are gone or are too many, there are serious developmental consequences, such as ciliopathies and microcephaly (Arquint and Nigg, 2014; Kumar et al., 2009; Nigg and Raff, 2009). Additionally, when they are ectopically present or behave abnormally within a cell or tissue, they can lead to tumorigenesis (Basto et al., 2008; Castellanos et al., 2008), which has also been linked to an increase in Ube2c activity (van Ree et al., 2010). Here we have demonstrated an important developmental aspect of centrosome behavior: if they are ectopically localized and active, then they disrupt the female germline development, resulting in female sterility. We have provided a novel demonstration of a system, the *Drosophila* egg chamber, in which to study the importance of the spatiotemporal regulation of centrosomes, which may hold parallels to other systems that require similar regulation of centrosome activity.

The relationship between centrosome elimination and fertility has recently been demonstrated in *Drosophila* (Pimenta-Marques et al., 2016). Since we have shown that centrosome activity, stability, and elimination is regulated at least in part by the APC/C, through its regulation of Polo levels, APC/C activity may also be involved in eliminating centrosomes during the late stages of egg formation. It is important to note that the inability of *D. melanogaster* to form centrioles upon egg activation is not a consequence of a lack of materials – they have all the necessary components to produce centrioles de novo (Foe et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1993) – and therefore some extra barrier to

centriole formation must exist. It is tempting to speculate that, as an upstream mediator of centriole stability, perhaps the APC/C is not only key to maintaining fertility but is also one of the developmental barriers preventing de novo centriole formation, and thus parthenogenesis, in unfertilized eggs.

Supplementary Material:

Includes Materials and Methods 5 Figures and 2 Movies.

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A

vih genomic region target-site

ATG/14bp/CCCCGAGCAAAGTGGTGGAGCAG...

B

Vih protein domains:

N-terminal region

UBC Domain

Destruction Boxes

178 amino acids (19.8 kDa)

C

Novel *vih* mutant alleles

Genomic	TCAGCCCCGAGCAAAGTGGTGGAGCAGAAAGTGGTGG
vihar$^{\Delta N-9}$	TCAGCCCCG----------G
vihar$^{\Delta N-4}$	TC----------AGTGGTGGAGCAGAAAGTGGTGG
vihar^{Null-1}	TCAGCCCCG----AAGTGGTGGAGCAGAAAGTGGTGG
vihar^{Null-2}	TCAGCCCCG-GCAAAGTGGTGGAGCAGAAAGTGGTGG

D

Protein homology in the *vih*$^{\Delta N-9}$ mutation region:

D. mel	MA-QNISPEQSGGAGGGGSKHSDDSMPVKD
H. Sapiens	MASQNRDPAATSVAAARKGAEPSGGAARGP
M. musculus	MASQNRDPAAASVAAVRKGAEPCGGAARGP
D. rerio	MASQNMDPAAASTATL-KGSETSVTASKGS
	** **^ * * ^^

*Conserved residues ^Initiation for destruction motif

E

Germarium

Stage 3

Stage 6

Stage 9

Stage 14

Germline Stem cell

Germline cell

Presumptive oocyte

Nurse Cell

Oocyte

Oocyte Nucleus

F

DAPI/Phalloidin

Control

vih$^{\Delta N-4}$

vih$^{\Delta N-9}$

0% (n=50, N=2)

Heterozygous: 10% (n=30, N=2)

Homozygous: 28% (n=60, N=2)

Heterozygous: 16% (n=45, N=2)

Homozygous: 45% (n=55, N=2)

Germarium-Stage1

Stage 3-4

Stage 6

Stage 9

Figure 1: *vih* allele design and membrane phenotype in the egg chamber **A)** CRISPR/Cas9 genomically engineered gRNA target-site design within the coding region of the gene. The red arrow denotes the cut site, the red residues denote the protospacer adjacent motif (PAM) sequence, and the green residues denote the gRNA complementary sequence. **B)** Protein schematic and sequence for Vih. **C)** Four examples of vih mutant alleles that were created. **D)** Protein homology in vertebrates for the region that we altered in our *vih*$^{\Delta N}$ alleles. **E)** Schematic of egg chamber development. **F)** Development time-series of phenotypic presentation for control, *vih*$^{\Delta N-4}$, *vih*$^{\Delta N-9}$ and egg chambers, stained for DNA (DAPI) in blue and F-actin (Phalloidin) in red (arrows indicate the membranes and asterisks the oocytes). All magnification bars equal to 10 μm.

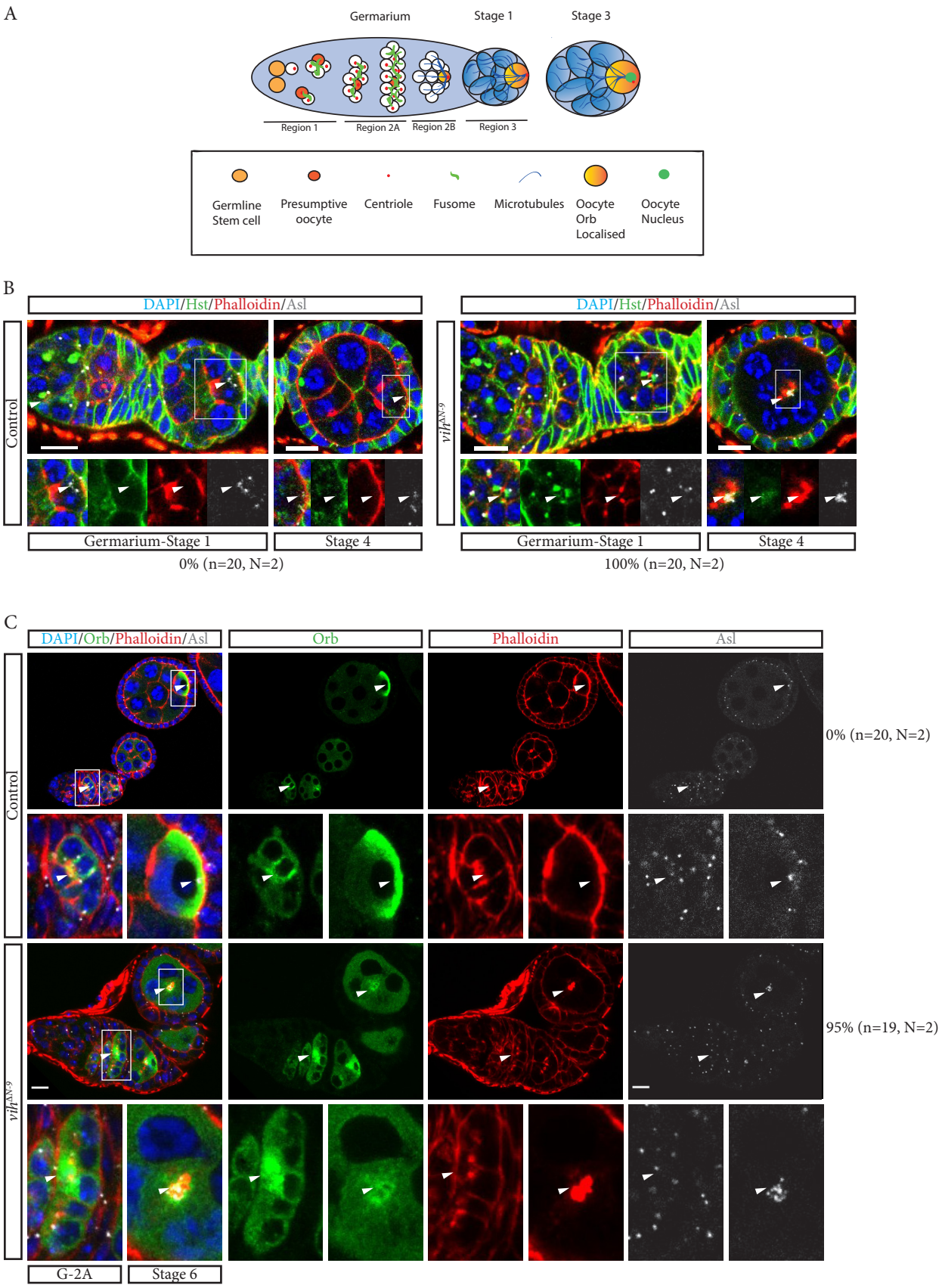

Figure 2: *vih*$^{\Delta N\text{-}9}$ egg chamber centrosomes and oocyte fate ***A)*** Schematic of the germarium (G) and early egg chamber development. **B)** germarium - Stage 1 and Stage 4 control and *vih*$^{\Delta N\text{-}9}$ egg chambers stained for DNA (DAPI) in blue, fusome (Hts) in green, and F-actin (Phalloidin) in red, and centrioles (Asl) in white (arrows indicate centrioles). **C)** Germaria-stage 6 control and *vih*$^{\Delta N\text{-}9}$ egg chambers stained for DNA (DAPI) in blue, Orb in green, F-actin (Phalloidin) in red, and centrioles (Asl) in white (arrows indicate centrioles). All magnification bars equal to 10 µm.

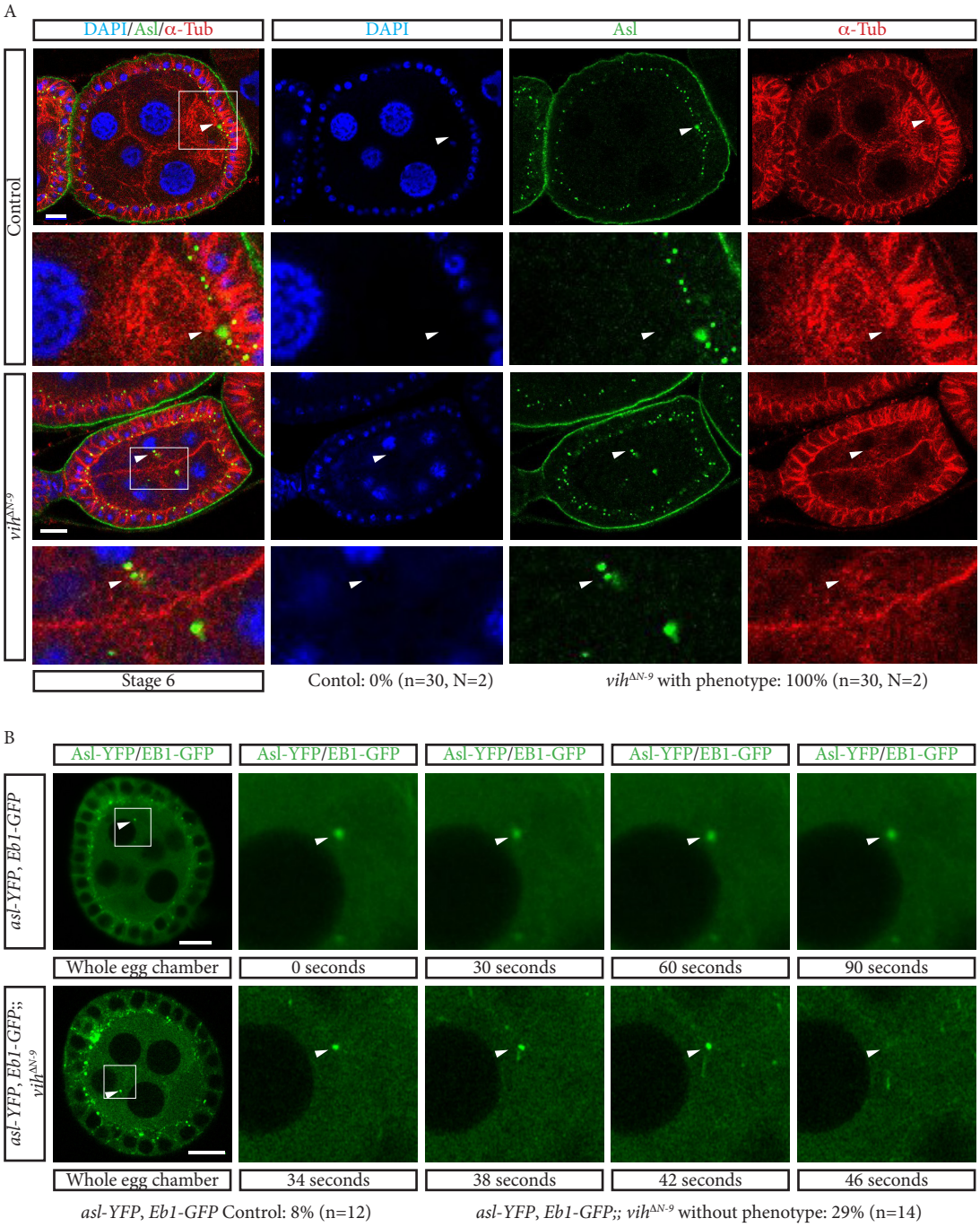

Figure 3: *vih*$^{\Delta N\text{-}9}$ causes ectopic centrosome nucleation **A)** Control and *vih*$^{\Delta N\text{-}9}$ ~stage 6 egg chambers stained for DNA (DAPI) in blue, centrioles (Asterless) in green, and microtubules (μ-Tubulin) in red (arrows indicate centrioles). **B)** Control and *vih*$^{\Delta N\text{-}9}$ stage 4/5 egg chambers with Asl-YFP and Eb1-GFP (arrows indicate centrioles). All magnification bars equal to 10 μm.

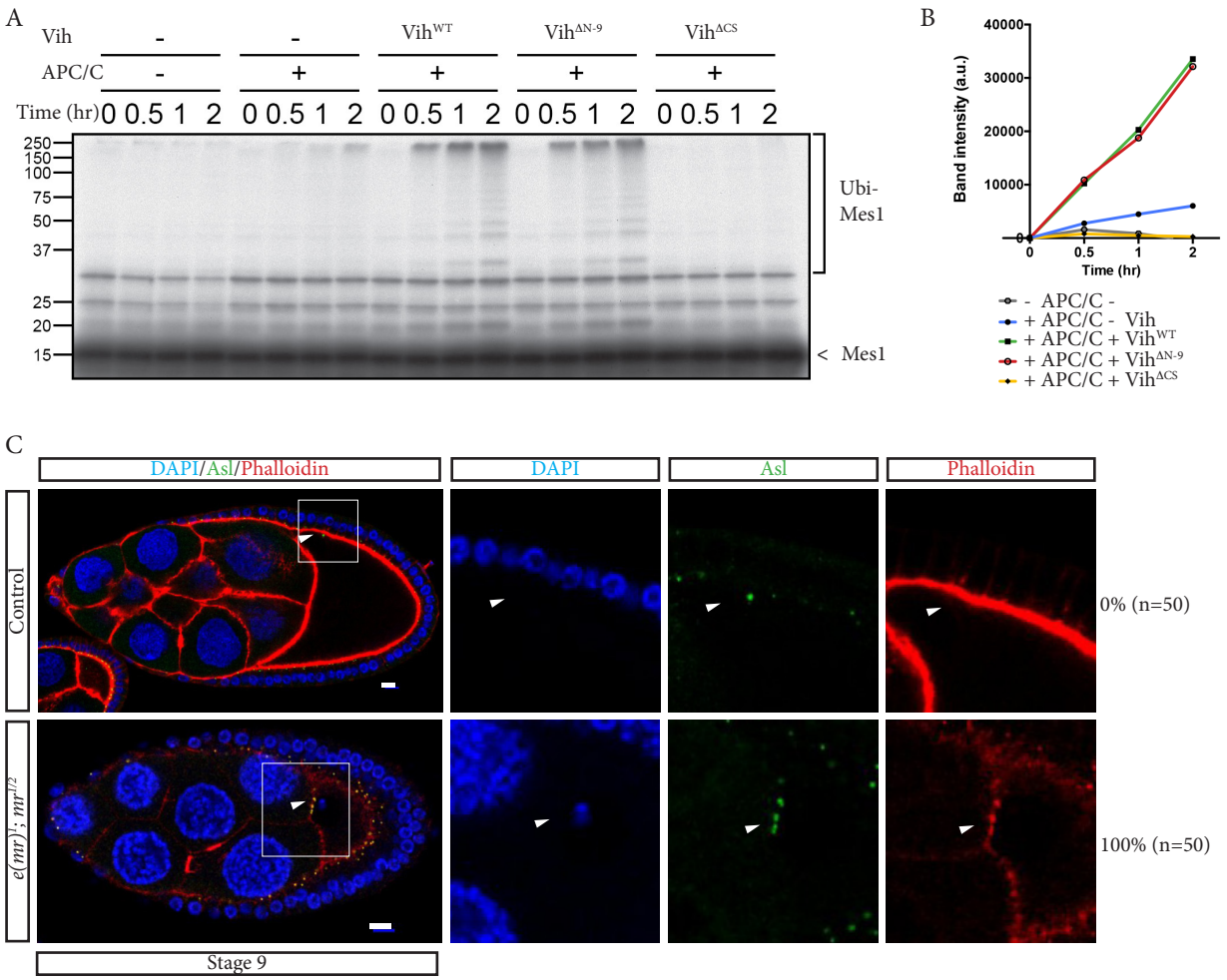

Figure 4: Vih$^{\Delta N\text{-}9}$ works with the APC/C in vitro and in vivo A) *Drosophila* reconstituted in vitro ubiquitination assay: with no Vih, Vih^{WT}, Vih$^{\Delta N\text{-}9}$, or Vih$^{\Delta CS}$. B) Ubiquitination assay quantification. C) Control and *mr*$^{1/2}$ stage 9 egg chambers stained for DNA (DAPI) in blue, centrioles (Asl) in green, and F-actin (Phalloidin) in red (arrows indicate centrioles). Magnification bars equal to 10 µm.

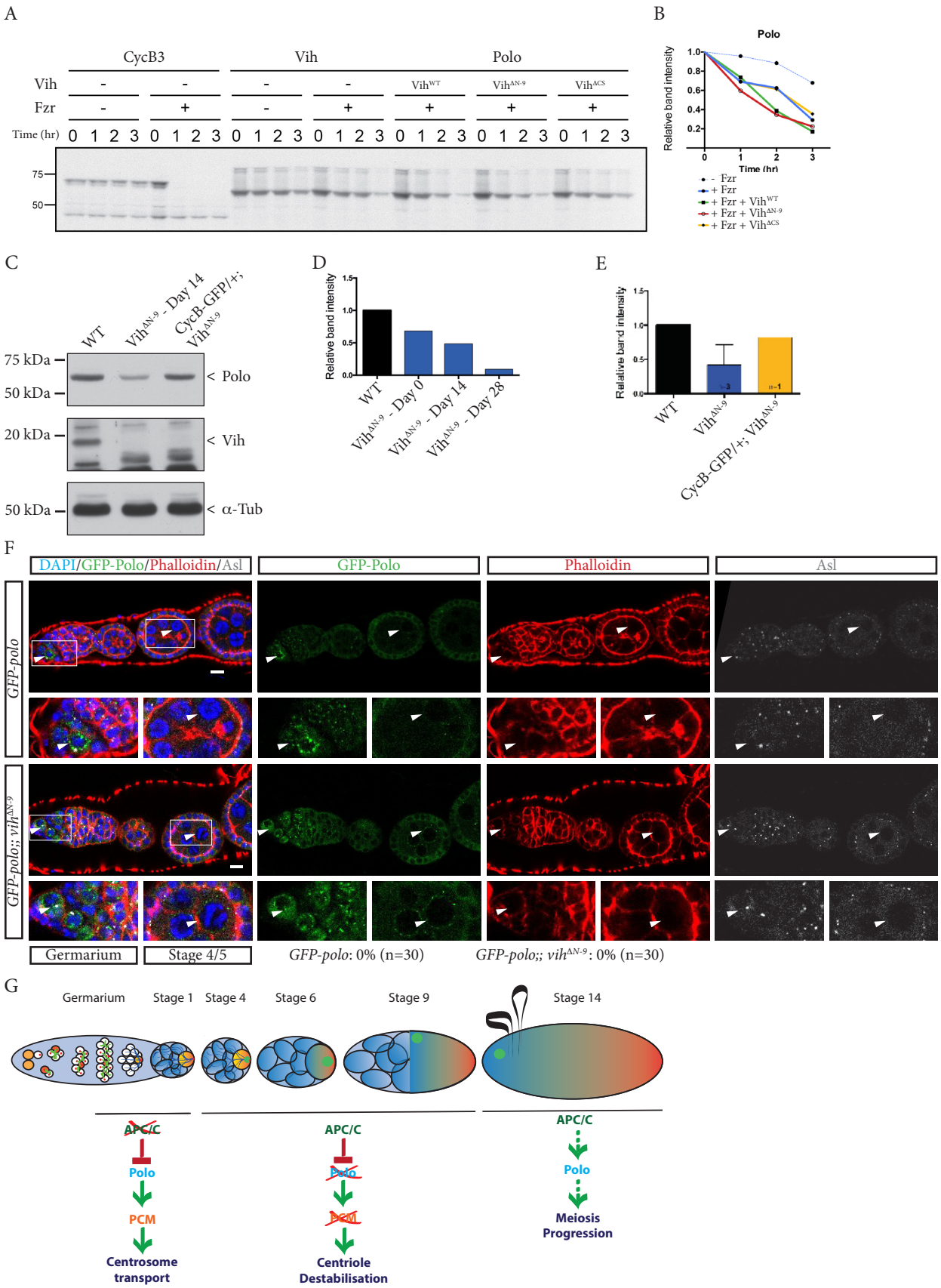

Figure 5: Polo kinase is a target of the APC/C in vitro and in vivo **A)** In vitro *Drosophila* destruction assay of Polo: with no Vih, VihWT, Vih$^{\Delta N\text{-}9}$ or Vih$^{\Delta CS}$. **B)** Polo destruction quantification. **C)** Western blot of control, *vih*$^{\Delta N\text{-}9}$, and *CycB-GFP*/+; *vih*$^{\Delta N\text{-}9}$ ovary extracts 14 days after eclosion, stained with Vih and Polo antibodies. **D)** Quantification of western blot Polo protein band intensity for control and *vih*$^{\Delta N\text{-}9}$ ovary extracts at 0, 14, 28 days after eclosion. **E)** Quantification of Polo protein band intensity from western blots for control, *vih*$^{\Delta N\text{-}9}$ flies 0, 14, and 28 days after eclosion combined, and *cycB-GFP*/+; *vih*$^{\Delta N\text{-}9}$ ovary extracts. **F)** GFP-Polo in control and *vih*$^{\Delta N\text{-}9}$ egg chambers stained for DNA (DAPI) in blue, GFP-Polo in green, F-actin (Phalloidin) in red, and centrioles (Asl) in white (arrows point to the GFP-Polo puncta in the germline stem cells and those surrounding the nurse cells in later stages). All magnification bars equal to 10 µm. **G)** Schematic of APC/C action with Polo.