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# The anaphase-promoting complex is required in both dividing and quiescent cells during zebrafish development

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## Abstract

The anaphase-promoting complex/cyclosome (APC/C) regulates multiple stages of the cell cycle, most prominently mitosis. We describe zebrafish with mutations in two APC/C subunits, Cdc16 and Cdc26, whose phenotypes reveal a multifaceted set of defects resulting from the gradual depletion of the APC/C. First, loss of the APC/C in dividing cells results in mitotic arrest, followed by apoptosis. This defect becomes detectable in different organs at different larval ages, because the subunits of the APC/C are maternally deposited, are unusually stable, and are depleted at uneven rates in different tissues. Second, loss of the APC/C in quiescent or differentiated cells results in improper re-entry into the cell cycle, again in an apparently tissue-specific manner. This study is the first demonstration of both functions of the APC/C in a vertebrate organism and also provides an illustration of the surprisingly complex effects that essential, maternally supplied factors can have on the growing animal over a period of 10 days or longer.

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*Keywords:* Cell cycle; Retina; Pancreas; Ciliary marginal zone; *Danio rerio*

## Introduction

The anaphase-promoting complex or cyclosome (APC/C) is an essential regulator of multiple steps of the cell cycle (reviewed in Harper et al., 2002; Peters, 2002). The APC/C is an E3 ubiquitin ligase that targets proteins for degradation by the 26S proteasome. Some of the best-studied targets of the APC/C are the B-type mitotic cyclins (Glotzer et al., 1991; Hershko et al., 1991). Cyclin B levels are regulated throughout the cell cycle (Evans et al., 1983). Low levels of cyclin B are maintained during G<sub>1</sub> and cyclin B accumulation coincides with DNA synthesis. Degradation of cyclin B is important for the progression of mitosis, and its clearance is complete by the end of anaphase (Murray and Kirschner, 1989; Murray et al., 1989). This dynamic regulation of cyclin B protein levels is accomplished by APC/C-mediated proteolysis. The APC/C has multiple targets besides cyclins, most of which also regulate the cell cycle and need to be degraded in order for a cell to progress through anaphase, exit mitosis, and/or maintain G<sub>1</sub> (reviewed in Castro et al., 2005).

The APC/C has at least thirteen subunits and two activators. APC2 and APC11 make up the catalytic core with their respective cullin homology and RING-H2 finger domains (Kramer et al., 1998; Yu et al., 1998; Zachariae et al., 1998). APC3/Cdc27, APC6/Cdc16, APC7, and APC8/Cdc23 have a series of Cdk phosphorylation sites as well as tetratricopeptide (TPR) repeats, which are thought to be important for homophilic interactions between subunits (Irniger et al., 1995; King et al., 1995). Some of the APC/C subunits have unknown functions and no clear domain structure, such as APC12/Cdc26. Activity and specificity of the APC/C are regulated by phosphorylation (Kraft et al., 2003; Kramer et al., 2000) and by binding of cycle-specific activator proteins such as Cdc20/Fzy and Cdh1/Fzr (Dawson et al., 1993; Sigrist and Lehner, 1997). APC/C<sup>Cdc20</sup> is active during metaphase and anaphase, while APC/C<sup>Cdh1</sup> is required during mitotic exit and to maintain cells in G<sub>1</sub> (reviewed in Fang et al., 1999).

Work in multiple organisms has confirmed the essential role of the APC/C in regulating mitotic progression and exit. In multicellular organisms, however, phenotypes with differing degrees of severity have been observed after loss of APC/C function, even for the homologous subunit. They range from early embryonic lethality in the murine APC2 knockout to late larval

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lethality in *Drosophila* APC2 null mutants to adult sterility in *Caenorhabditis elegans* APC2 RNAi knockdowns, *Drosophila* APC2 hypomorphic mutants, and *Arabidopsis* APC2 null mutants (Capron et al., 2003; Davis et al., 2002; Kashevsky et al., 2002; Wirth et al., 2004). One key to explaining this disparity comes from studies in *Drosophila* where embryonic mitotic phenotypes are not observed in zygotic null mutants, but are observed in maternal hypomorphic mutants (Reed and Orr-Weaver, 1997). This observation suggests that the lack of strong early embryonic defects in *Drosophila* APC/C mutants is due to rescue by maternal protein. *Drosophila* develops rapidly and depends on maternal products for many of its embryonic divisions (reviewed in O'Farrell et al., 1989). In mice, the cell cycle is slow, in contrast, and zygotic transcription is observed early at the two-cell stage (reviewed in Schultz, 1993). Thus, the late-stage or tissue-specific mitotic defects observed in *Drosophila*, *C. elegans*, and *Arabidopsis* are most likely attributable to maternal rescue.

In a forward genetic screen for genes that regulate post-embryonic growth of the eye in the zebrafish, *Danio rerio*, we isolated a number of novel mutants with cell division defects in the eye (Wehman et al., 2005). Our study focused on a region of the neural retina known as the ciliary marginal zone (CMZ) that is responsible for almost all retinal growth after embryogenesis (Marcus et al., 1999). The CMZ is located at the peripheral edge of the retina and is composed of a rare population of multipotent retinal stem cells and a much larger number of rapidly dividing progenitor cells with more restricted cell-fate competence (reviewed in Harris and Perron, 1998). The CMZ is arrayed such that the newly generated progenitors are located closest to the lens, near the putative stem-cell niche. Older progenitors are found more centrally, closer to the three layers of differentiated cells (reviewed in Link and Darland, 2001). This reproducible organization makes the CMZ an ideal system in which to carry out a screen for genes that regulate the proliferation of stem cells and progenitors.

Here we report the molecular identification of two mutants, *vij* and *gog*, found to have a reduced CMZ in the prior screen (Wehman et al., 2005). The corresponding genes encode subunits of the APC/C, Cdc16 and Cdc26. We show that, as in other organisms, the APC/C is required to regulate progression through mitosis and that mitotically arrested cells undergo apoptosis. We also show that these mutants have relatively late and tissue-specific defects, due to slow and uneven depletion of maternal stores. We reveal the remarkable stability of maternally supplied APC/C that persists through embryogenesis well into larval stages. Finally, we demonstrate that the APC/C is required to prevent re-entry into the cell cycle by quiescent cells.

## Materials and methods

### Injections

Fish were maintained as in Wehman et al. (2005). For rescue experiments, ~2 nl of 0.75 ng/nl Cdc26 (wild-type or *s109*) mRNA in Tris–EDTA (TE) were injected into the embryo at the one- to two-cell stage. For cyclin B experiments, we injected ~2 nl 165 mM protein (13–110 or 13–110\*) in Tris-buffered saline (TBS) into one blastomere at the two-cell stage (gift of D. Morgan).

### Positional cloning

Linkage mapping was performed as in Wehman et al. (2005). Zebrafish Cdc16 and Cdc26 were amplified using the following primers (Cdc16: CACAGGGGAACACTCACAAA and TCCAACACAGAGGACACGAT; Cdc26: CCCATGATTCCTTCTGCTCT and CACCGTTAACCAAAGCCATAA) and the sequences were deposited in GenBank (Accession Numbers DQ356892 and DQ352177). These fragments were subcloned into pCS2+ for mRNA transcription using the mMessage mMachine kit (Ambion). To genotype *gog<sup>s109</sup>*, the following primers and restriction enzyme were used: GTCCCTGCTCTCCCCTACTT, CCATTGTTCCAGGATTAGCACT and *MlyI* (NEB). To genotype *vij<sup>s17</sup>*, the following primers and restriction enzyme were used: GATGTTGTCGTCTCCCTTGCT, CTCAGCTCTACAAGGGTTCCTA, and *BspI* (NEB).

### Histology and immunohistochemistry

Nissl and DAPI staining and immunohistochemistry were performed on sections as in Wehman et al. (2005), except rabbit anti-phosphohistone H3 (1:500; Upstate). For whole-mount immunohistochemistry, larvae were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), dehydrated in methanol and stored at –20°C. Larvae were then rehydrated and digested with 1 mg/ml collagenase for 2–3 h before staining. To examine cell proliferation, larvae were incubated in the thymidine analog 5-bromo-2-deoxyuridine (BrdU) at 2 mM in embryo medium for 6 h then returned to embryo medium without BrdU for at least 1 min before fixation. For Figs. 2K–N, larvae were incubated in 2 mM BrdU for 18 h and returned to embryo medium for 36 h before fixation. To examine mitotic spindles, larvae were fixed for 1 h in 37% FA and sectioned and stained as in Horne-Badovinac et al. (2001). To examine apoptotic cell death, whole-mount TUNEL staining was performed as in Abdelilah et al. (1996) using the ApopTag kit (Chemicon), and TUNEL staining on sections was performed as in Kay et al. (2001) using the In Situ Cell Death Detection Kit (Roche). Alcian green staining was performed as in Schilling and Kimmel (1997). Pancreas staining was performed as in Field et al. (2003a), except rabbit anti-trypsin (1:100; Chemicon) and rabbit anti-amylase (1:100; Biomedica). All cell counts were performed on the most central retina section or on the whole larvae. Student's *t*-test was performed to assess statistical significance.

## Results

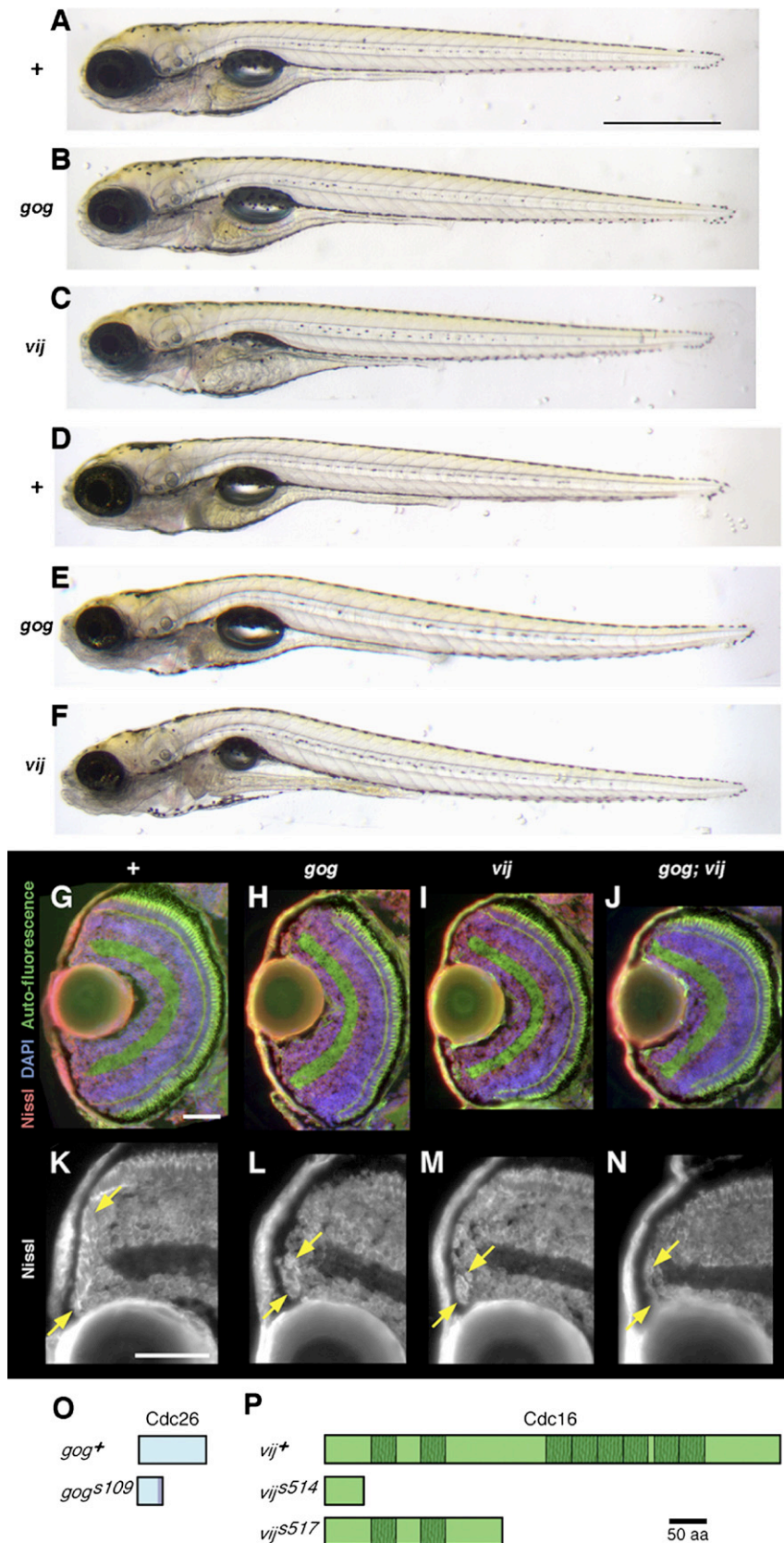
### The zebrafish genes *gog* and *vij* encode subunits of the APC/C

At larval stages, *gog* and *vij* mutant zebrafish have small eyes, but are otherwise the same size as their wild-type siblings

Fig. 1. *gog* and *vij* mutants have small eyes and carry mutations in subunits of the APC/C. (A–C) Whole-mount lateral views of live 4 dpf free-swimming larvae. In the *gog<sup>s109</sup>* mutant (B), the eyes are slightly smaller than in wild-type (A), but the animal appears otherwise normal. In the *vij<sup>s17</sup>* mutant (C), the eyes are smaller, and the swim bladder is sometimes uninflated, but the animal appears relatively normal. (D–F) Whole-mount lateral views of live 7 dpf larvae. The decrease in eye size of *gog<sup>s109</sup>* (E) and *vij<sup>s17</sup>* (F) mutants are more dramatic in comparison to wild-type (D) than at 4 dpf. *vij<sup>s17</sup>* mutants (F) begin to develop edema around the intestine, but often have inflated their swim bladder. (G–J) Horizontal sections of 4 dpf eyes stained with Nissl (red), DAPI (blue), and PFA-induced autofluorescence (green). (K–N) Magnified views of the nasal ciliary marginal zone (CMZ) (between arrows) in Nissl-stained sections. The wild-type eye (G, K) displays the laminar structure of the retina and the Nissl-bright CMZ. In *gog<sup>s109</sup>* (H, L) and *vij<sup>s17</sup>* (I, M), as well as in double mutants *gog<sup>s109</sup>;vij<sup>s17</sup>* (J, N), the eyes are normal except for a greatly reduced CMZ. All sections are oriented nasal up. (O) Structure of the wild-type and *gog<sup>s109</sup>* alleles of Cdc26. The *gog<sup>s109</sup>* allele is less than one-half wild-type length and ends with five amino acids not normally found in Cdc26 (purple box). (P) Structure of the wild-type, *vij<sup>s14</sup>*, and *vij<sup>s17</sup>* alleles of Cdc16. Both *vij* alleles lose most of Cdc16 protein, especially the TPR repeats (dark green boxes). Scale bar in panels A–F: 500 μm. Scale bars in panels G–N: 50 μm. Scale bar in panels O–P: 50 amino acids.

(Figs. 1A–F). The phenotype of *vij* (Figs. 1C, F) is slightly stronger than that of *gog* (Figs. 1B, E). The reduction in eye size is due to a decrease in proliferation from the CMZ

(Wehman et al., 2005) and becomes more dramatic with age (compare Figs. 1A–C with D–F). The CMZ is greatly reduced by morphological criteria revealed by Nissl staining



of retinal progenitors in *gog* and *vij* mutants (Fig. 1K–M), but the central retina formed during embryogenesis is fully differentiated and supports visual responses (Wehman et al., 2005) (Figs. 1G–I).

Positional cloning revealed that the *gog* and *vij* mutant phenotypes are caused by mutations in two subunits of the anaphase-promoting complex, Cdc26 and Cdc16, respectively (Supplemental Fig. 1). In *gog*<sup>s109</sup> mutants, a mutation in the 3' splice site at the beginning of the third exon of Cdc26 results in the use of a cryptic splice site two bases downstream. This aberrant splicing shifts the reading frame, causing five missense amino acids to be added before terminating prematurely, thus deleting two-thirds of Cdc26 protein (Fig. 1O). In *vij*<sup>s514</sup> and *vij*<sup>s517</sup> mutants, nonsense mutations at amino acids 52 and 241 of Cdc16, respectively, also result in severe truncations that remove all or most of the TPR repeats (Fig. 1P). These three mutations are predicted to cause null or severely hypomorphic alleles due to the extent of the protein that is lost.

If Cdc16 and Cdc26 belong to the same functional complex in zebrafish, we would expect the compound phenotype to be of similar strength to that of either *vij* or *gog* alone. To test this, we crossed *gog*<sup>s109</sup> with *vij*<sup>s517</sup>, identified double carriers using restriction enzyme-mediated genotyping, and scored the progeny of *gog*<sup>s109/+</sup>; *vij*<sup>s517/+</sup> incrosses. We did not observe any phenotypes in transheterozygous *gog*<sup>s109/+</sup>; *vij*<sup>s517/+</sup> larvae. Larvae homozygous mutant for both *gog* and *vij* were indistinguishable from *vij* single mutants judged by their eye size (Fig. 1J) and by the morphology of the peripheral retina (Fig. 1N). The double-mutant phenotype provides genetic evidence that Cdc16 and Cdc26 act in the same pathway in zebrafish. Although our original screen (Wehman et al., 2005) found five other mutants with cell proliferation phenotypes similar to *gog* and *vij*, these other genes do not seem to encode additional subunits of the APC/C or either of the two known APC/C activators (Supplemental Table 1).

#### *Loss of the APC/C in dividing retinal progenitors results in mitotic arrest and apoptosis*

Given the known role of the APC/C in regulating mitosis, we asked whether there was evidence of a mitotic arrest in the CMZ of *gog* and *vij* mutants. We used phosphorylated histone H3 (PH3) as a marker for cells in late G<sub>2</sub> through M phase. In a typical wild-type retinal section, one to three PH3-positive cells were observed in each CMZ on either side of the lens. These cells were typically scattered along the full extent of the CMZ from the lens to the photoreceptor layer (Fig. 2B). In *gog* and *vij* retinal sections, we consistently observed an increased number of PH3-positive cells at the retinal margin (wild-type: 4±1; *vij*: 9±1, n=8, p<0.005), although the PH3-positive cells were typically clustered closer to the lens (Fig. 2G). The two-fold increase in PH3-positive cells likely reflects a mitotic arrest in retinal progenitors. Alternatively, a greater number of cells might be cycling in *gog* and *vij* mutant retinas.

In order to rule out that more cells are dividing, we asked how many cells were in S phase using BrdU labeling. A complete cell cycle in the zebrafish CMZ at an early larval stage

is thought to be 6 to 8 h (Li et al., 2000); so we incubated fish in BrdU for 6 h to get an estimate of the number of cycling cells. When stained for BrdU incorporation at 4 days post-fertilization (dpf), we observed that the entire CMZ is labeled in wild-type larvae and that few cells with a differentiated morphology are labeled (Fig. 2A). In *vij* and *gog* mutants, only a few cells (approximately 10–20% of wild-type) are BrdU-positive, reflecting a decrease in the number of cells passing through S phase (Fig. 2F). These few BrdU-positive cells are in the same position, close to the lens, as the CMZ remnant revealed by Nissl staining (Figs. 1H, I) and are therefore likely to be young retinal progenitors. We also see a consistent reduction in BrdU-labeled cells at later larval stages such as 6 dpf and 9 dpf (Fig. 2M) (Wehman et al., 2005; data not shown). We interpret this reduction in the number of progenitor cells in the CMZ as a result of reduced division by these progenitors. The reduced number of BrdU-positive cells in combination with the observed increase in the number of PH3-positive cells is consistent with a cell cycle arrest in the CMZ where each stem cell division results in a progenitor cell that cannot complete mitosis.

In order to further characterize this mitotic arrest, we compared the patterns of BrdU and PH3-labeled cells. In the wild-type CMZ, all PH3-positive cells are also BrdU-positive, and the PH3-positive population represents a small subset of BrdU-positive cells (Figs. 2C, O). This pattern indicates that any cell in M phase has passed through S phase in the previous 6 h. In *gog* and *vij* mutants, in contrast, most of the BrdU-positive cells in the CMZ are also PH3-positive (arrowhead in Fig. 2H), but there is not a significant increase in the total number of BrdU-positive, PH3-positive cells (wild-type: 4±1, *vij*: 5±1, n=8, p>0.1). More strikingly, we also regularly observed PH3-positive cells that were BrdU-negative in *gog* and *vij* mutants (*vij*: 4±1, n=8) (arrow in Fig. 2H). We did not observe any PH3-positive, BrdU-negative cells in wild-type larvae (n=8, p<0.001). These PH3-positive, BrdU-negative progenitors must have completed S phase before the BrdU treatment (6 h earlier). Given that G<sub>2</sub> is thought to be relatively quick, it therefore seems likely that these progenitors have been stalled in M phase for at least 6 h. These cells were typically at a position in the CMZ most distant from the lens, where the oldest progenitors reside. This staining pattern is best explained by a mitotic arrest of retinal progenitors, which subsequently fail to undergo their characteristic amplifying divisions.

We then investigated what happened to the retinal progenitors after the mitotic stall. The stall could result in cell death, or arrested progenitors could eventually escape the prolonged mitosis and differentiate normally. To test this, we used TUNEL labeling as a marker of apoptosis. In wild-type retinal sections, TUNEL-positive cells in the CMZ were occasionally observed (wt: 0.4±2, n=8) (Fig. 2D). In *gog* and *vij* mutant retinas, we observed a clear increase in TUNEL-positive cells (*vij*: 2.6±0.3, n=8, p<0.0001) (Figs. 2I, O). This increase in TUNEL cells confirms that more cells are undergoing apoptosis in the CMZ of *gog* and *vij*. We then compared the TUNEL staining to the pattern of

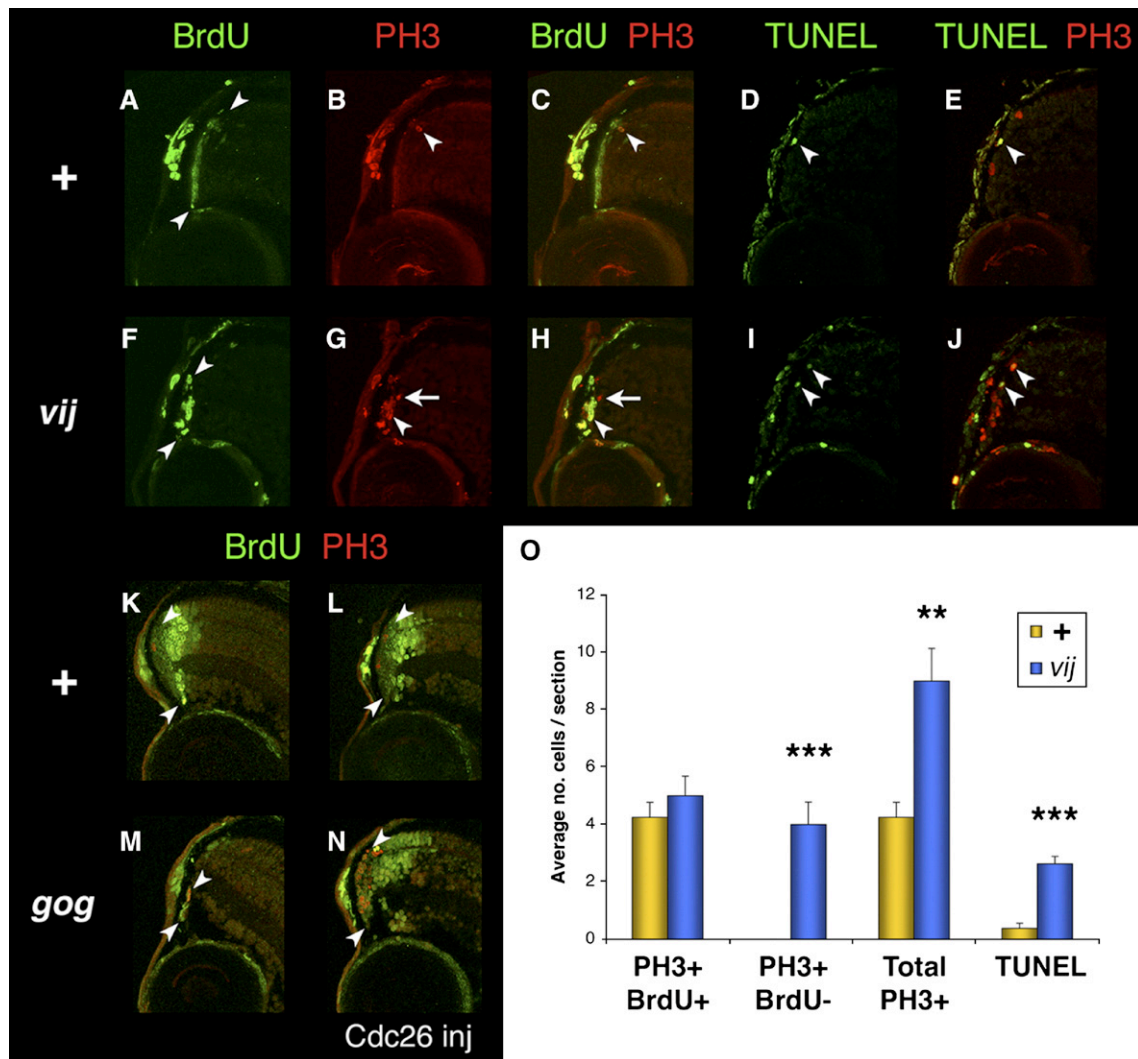


Fig. 2. Loss of the APC/C in dividing retinal progenitors results in mitotic arrest and apoptosis. (A–J) Horizontal sections of 4 dpf eyes stained for BrdU (green, after 6-h incubation) and PH3 (red), or TUNEL (green) and PH3 (red) (D–E, I–J). Only the nasal quarter of the eye is shown. Paired arrowheads in (A, F) show the extent of the CMZ. Most or all cells in the wild-type CMZ are labeled with BrdU (A), a small subset of which is also PH3-positive (B, C, arrowhead). Rarely, a TUNEL-positive cell is observed in wild-type (D, arrowhead), and this TUNEL-positive cell can also be PH3-positive (E, arrowhead). In *vjv*<sup>s517</sup>, fewer cells are labeled with BrdU and the CMZ is smaller (F, between arrowheads). There are more PH3-positive cells in *vjv*<sup>s517</sup>, and they reside close to the lens (G, arrow and arrowhead). In *vjv*<sup>s517</sup>, most of the BrdU-positive cells are also PH3-positive (H, arrowheads), but there are also PH3-positive cells that are not BrdU-positive (H, arrow), which is not observed in wild-type. In *vjv*<sup>s517</sup> mutants, more TUNEL-positive cells are observed (I, arrowheads). Some of these TUNEL-positive cells are also PH3-positive (J, arrowheads). (K–N) Horizontal sections of 5 dpf eyes stained for BrdU (green) and PH3 (red) 1 day after an 18-h incubation in BrdU. Paired arrowheads mark the extent of the CMZ. In wild-type (K), many cells in the CMZ and differentiated cells are labeled with BrdU and a couple PH3-positive cells are observed. Wild-type larvae injected with Cdc26 RNA (L) are identical to uninjected wild-type larvae. In *gog*<sup>s109</sup> mutants (M), very few cells are labeled with BrdU and PH3 and reside close to the lens. In *gog*<sup>s109</sup> mutants injected with Cdc26 RNA (N), the number of BrdU-labeled cells is increased to wild-type levels, but most of the cells in the CMZ also label with PH3 due to the transient rescue. Sections are oriented nasal up. (O) Graph of cell counts (with standard error) from retinal sections as in (A–J) (\**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001).

PH3 staining. In wild-type, the TUNEL-positive cells were in no consistent relationship to the PH3-positive cells (Fig. 2E). In *gog* and *vjv*, the TUNEL-positive cells were often located at a position in the CMZ most distant from the lens, where the oldest progenitors are found (Fig. 2J). This location is similar to the position of BrdU-negative PH3-positive progenitors that have been arrested for at least 6 h (Fig. 2H). This result indicates that upon loss of the APC/C, dividing retinal progenitors stall in mitosis for several hours and then die.

#### *Prolonged mitotic arrest results in aberrant spindle orientation and morphology*

To investigate the effect of APC/C disruption on dividing cells, we stained retinal sections for alpha-tubulin to observe mitotic spindles (Fig. 3). As with PH3-staining (Fig. 2), there was a two-fold increase in mitotic spindles in the CMZ of *gog* and *vjv* mutants (wt: 4 ± 1, *n* = 21; *gog*: 9 ± 2, *n* = 9; *vjv*: 9 ± 2; *n* = 10; *p* < 0.01). All cells with mitotic spindles were also positive for PH3 and the DNA appeared lined up along the

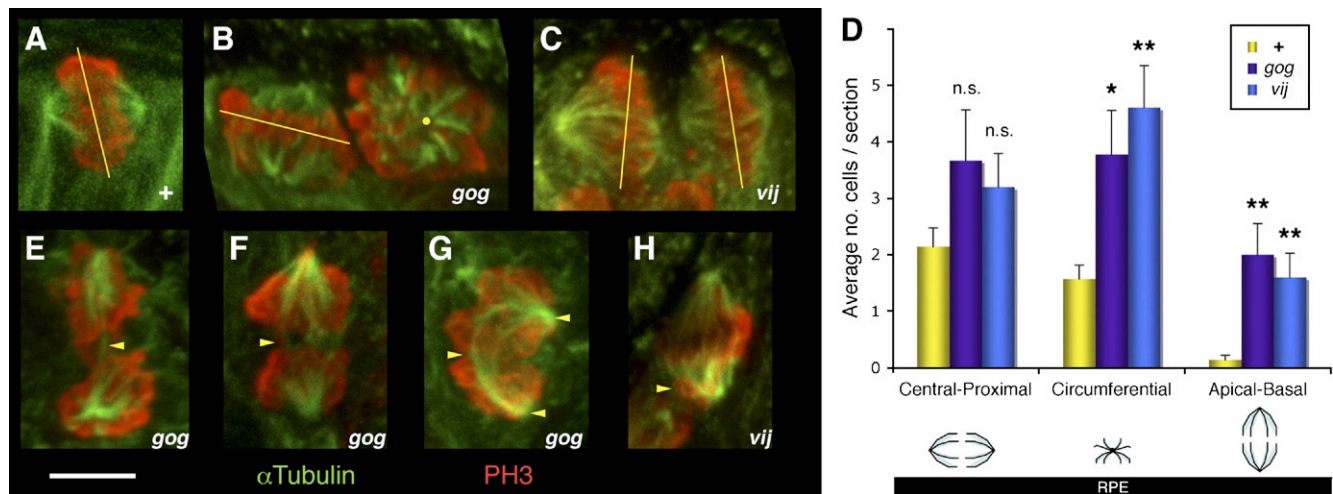


Fig. 3. Arrested retinal progenitors have spindle orientation defects and make mistakes during mitosis. (A–C, E–H) Sections stained for alpha-Tubulin (green) and PH3 (red) at 4 dpf. (A–C) CMZ progenitors oriented with the RPE up. Yellow lines mark the division plane. (A) A wild-type progenitor dividing along the central–proximal axis. (B) Two *gog*<sup>s109</sup> mutant progenitors dividing along the apical–basal axis (left) and circumferentially (right). (C) Two *vij*<sup>s517</sup> mutant progenitors dividing along the central–proximal axis. (D) Graph of cell counts (with standard error) from retinal sections as in (A–C) (\**p*<0.05, \*\**p*<0.01). (E–H) Lens and sclera progenitors display aberrant mitoses. (E) A *gog*<sup>s109</sup> mutant lens cell shows poor separation of the mitotic spindle (arrowhead). (F) There are also defects in segregation of DNA (arrowhead) in *gog*<sup>s109</sup> mutant sclera cells. (G) A *gog*<sup>s109</sup> mutant sclera cell with an elongated, curved spindle (between arrowheads). (H) A *vij*<sup>s517</sup> mutant sclera cell with chromosomes outside of the spindle (arrowhead). Scale bar: 5  $\mu$ m.

metaphase plate. This result further demonstrates that the expanded PH3-positive pool in *gog* and *vij* mutants represents cells arrested in mitosis, specifically at metaphase.

In addition to the increased number of mitotic spindles in *gog* and *vij* mutants, we observed that the orientation of spindles in the CMZ was aberrant. We cataloged the spindle orientation as described by Das et al. (2003) (Fig. 3D). In *gog* and *vij* mutants, roughly 20% of spindles were oriented perpendicular to the RPE along the apical–basal axis (*gog*: 18/85 spindles; *vij*: 16/94 spindles) (Figs. 3B–C). In wild-type, this orientation was rarely observed (wt: 3/81 spindles) and >95% of spindles were parallel to the RPE dividing either circumferentially or along the central–proximal axis (Fig. 3A). We believe that this defect in orientation is a result of the prolonged mitotic arrest and does not reflect a direct role for the APC/C in cell polarity. Similarly, in *C. elegans* hypomorphic APC/C mutants, the extent of mitotic arrest has been shown to correlate with the extent of developmental defects such as anterior–posterior axis formation (Shakes et al., 2003). The spindle orientation defects found in *gog* and *vij* mutant retinal progenitors thus provide further evidence of their prolonged mitosis.

We also observed atypical spindle and nuclear morphologies in *gog* and *vij* mutant cells from the lens and sclera (Figs. 3E–H). These defects included failure to detach the spindles (Fig. 3E), failure to separate the DNA (Fig. 3F), elongated curving spindles (Fig. 3G), and spindles missing chromosomes (Fig. 3H). These phenotypes exemplify the defects that result from misregulation of the cell cycle upon loss of APC/C function and mitotic arrest.

#### *Slow depletion of maternal APC/C protein causes larval mitotic defects*

The two APC/C mutants show no detectable abnormality in embryonic development and were grossly normal, save for their

small eyes at larval stages. A possible explanation is that Cdc16 and Cdc26 are not essential for cell division at early developmental stages in zebrafish. Alternatively, these two APC/C subunits might be required for all mitotic divisions, but maternal deposits of RNA and/or protein might be sufficient for development to proceed through embryogenesis until the maternal contribution is exhausted. Consistent with the maternal-store depletion hypothesis, RT–PCR and RNA in situ hybridization revealed that Cdc16 and Cdc26 are expressed maternally in zebrafish (Supplemental Fig. 2 and data not shown). In fact, Cdc16 and Cdc26 are expressed ubiquitously in the 12 h post-fertilization (hpf) embryo (data not shown) and become enriched in the anterior half of the embryo and larva after 1 dpf (Supplemental Figs. 2B–F). There is no apparent enrichment in RNA levels in the CMZ at 3 dpf (Supplemental Fig. 2D), suggesting that the CMZ is unlikely to have a unique requirement for Cdc16 or Cdc26. Unfortunately, protein distribution could not be determined, because antibodies directed against mammalian Cdc16 and Cdc26 do not recognize the corresponding zebrafish proteins (Wehman, unpublished observations).

If the maternal depletion hypothesis is true, we should be able to delay the onset of the proliferation defect in the retina by increasing the initial amount of Cdc16 or Cdc26 present in the fertilized egg. Following injection of Cdc26 RNA at the one-cell stage, we observed that *gog* mutants had normally sized eyes at early larval stages (4–5 dpf; Fig. 2N). This rescue was transient; *gog* mutants injected with Cdc26 RNA had smaller eyes at later larval stages (after 6 dpf) and their eyes remained intermediate in size between wild-type larvae and uninjected *gog* mutants. Injection of Cdc26 RNA bearing the *s109* mutation was not able to rescue eye size at any stage.

To demonstrate rescue of cell division, we incubated the Cdc26-injected larvae and uninjected controls in BrdU for 18 h

starting at 3 dpf and fixed them at 5 dpf. In wild-type animals, this protocol labeled the CMZ and a large number of differentiated cells at the retinal periphery (Figs. 2K, L). In *gog* mutants that had not been injected with *Cdc26* RNA, we observed very few cells labeled with BrdU in the CMZ or among differentiated cells (Fig. 2M). In contrast, in injected *gog* mutants, we observed a wild-type pattern of progenitors in the CMZ and differentiated cells labeled by BrdU (Fig. 2N). Thus, complementation of the mutant with wild-type RNA efficiently rescued the cell proliferation phenotype. Furthermore, overexpression did not disrupt embryonic development in wild-type or mutant, suggesting that the cell cycle can proceed normally at widely varying levels of individual APC/C subunits.

When we further analyzed the BrdU-labeled sections, we discovered a large increase in PH3-labeled cells in injected mutants (Fig. 2N) over uninjected wild-type (Fig. 2K) and *Cdc26*-injected wild-type (Fig. 2L). The majority of BrdU-positive progenitors in the CMZ are also PH3-positive in *Cdc26*-injected mutants except for the youngest progenitors close to the lens (Fig. 2N). Given the transient nature of the rescue, this pattern of staining in injected mutants suggests that retinal progenitors rapidly arrest in mitosis while attempting to undergo their amplifying divisions. This is likely due to the level of APC/C falling below its critical threshold. Retinal stem cells, in contrast, divide rarely and may therefore be able to maintain sufficient levels of APC/C protein.

#### *The APC/C is required for embryonic cell division*

The previous results make it likely that maternal supply is sufficient for cell cycle progression during the embryonic stage of zebrafish development. To prove that the APC/C is necessary

for mitosis in embryonic cells, we decided to use a dominant-negative approach. A truncated form of sea urchin Cyclin B (amino acids 13–110) was injected as protein into one blastomere at the two-cell stage. This truncated protein is thought to out-compete endogenous APC/C targets when added in excess (Holloway et al., 1993). In over 50% of embryos injected with truncated Cyclin B, we observed a group of cells that were larger and had undergone fewer divisions (Fig. 4B) when compared to uninjected embryos at the 32- to 128-cell stage (Fig. 4A). In addition, these larger cells had often lost contacts with their neighbors (arrowhead in Fig. 4B). At 1 dpf, most of the injected embryos were shrunken and malformed or dead (not shown). If we instead injected a control protein where the destruction box (D-box) motif had been mutated (13–110\*), we observed no phenotype in injected embryos at any stage (Fig. 4C). Mutation of the D-box prevents the APC/C from recognizing Cyclin B (Holloway et al., 1993). Thus, inhibition of maternal APC/C function results in an early and severe cell division defect. Comparable phenotypes have been observed from maternal screens by several zebrafish groups (Dosch et al., 2004; Kishimoto et al., 2004; Pelegri et al., 2004) and these phenotypes may be caused by mutations in similar cell cycle regulatory genes that are maternally provided.

To further confirm that the cellular phenotype was due to mitotic arrest, we stained the injected and control embryos with DAPI to label DNA. In embryos injected with truncated Cyclin B, we observed defects in segregation of DNA during mitosis (Fig. 4E). This phenotype was not observed in uninjected or D-box mutant Cyclin B injected embryos (Figs. 4D, F). Therefore, injection of truncated Cyclin B results in mitotic defects, dependant on the presence of the D-box, the APC/C recognition motif. This is consistent with our interpretation that the larval defects in *gog* and *vij* mutants are due to the gradual loss of stable maternal APC/C protein.

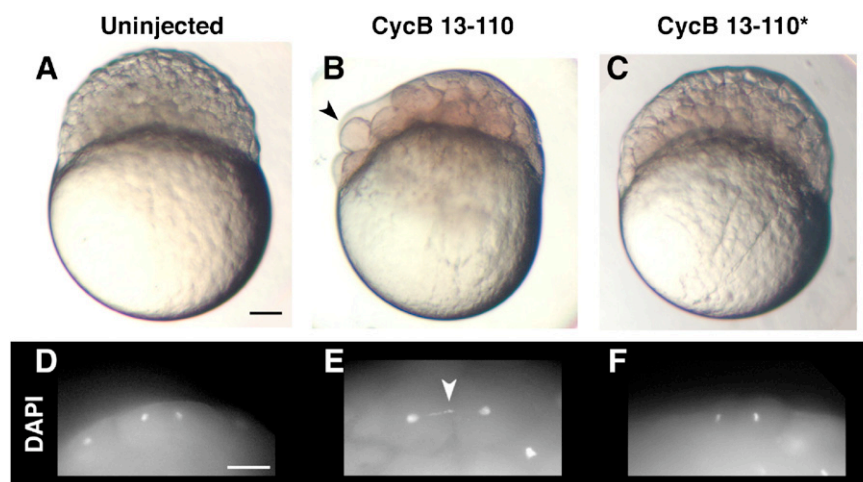


Fig. 4. Inhibiting the APC/C results in a cell cycle defect at an early embryonic stage. Embryos were injected at the two-cell stage and photographed 2 h later. (A–C) Live images of embryos. (A) In uninjected controls, the embryos has undergone 8 or 9 rounds of division and reached the 500- to 1000-cell stage by 3 h post-fertilization. (B) In embryos injected with an N-terminal truncation of Cyclin B (amino acids 13–110), there are fewer cells and the cells are larger. Several cells on one side of the embryo appear to have lost contacts with their neighbors (arrowhead). (C) Embryos injected with a mutated version of Cyclin B with two substitutions in the destruction box (13–110\*) underwent normal division and had cells with regular size and morphology. (D–F) DAPI-stained fixed embryos. (D) In uninjected controls undergoing telophase, the chromosomes are well segregated. (E) In Cyclin B 13–110 injected embryos, the DNA is not always segregated correctly (arrowhead). (F) Embryos injected with Cyclin B 13–110\* undergo normal segregation of DNA. Scale bars: 100  $\mu$ m.

*APC/C loss disrupts the exocrine pancreas and other tissues*

Given the essential function of the APC/C in dividing embryonic cells, we expected all tissues (not just the retina) to be affected by loss of Cdc16 and Cdc26. Both APC/C mutants, *gog* and *vij*, die at larval stages around 12–14 dpf. We hypothesized that this lethality could be due to a problem with food consumption or digestion because it coincides with the stage at which starved larvae typically die. To investigate the morphology of the intestine, liver, and pancreas, we crossed *gog* and *vij* into a transgenic line expressing GFP in the endodermal organs, *s161t* (Field et al., 2003b).

We observed a striking phenotype in the exocrine pancreas. At 3 dpf, there is no difference between wild-type and *gog* or *vij* mutants in the size of the exocrine pancreas. Starting at 4 dpf in *gog* mutants, the exocrine pancreas becomes progressively shorter. By 5 dpf, the exocrine pancreas no longer reaches past the posterior extent of the swim bladder in *gog* mutants (Fig. 5B). By 7 dpf, the exocrine pancreas is less than half its normal length (Fig. 5D) and by 9 dpf, only the endocrine pancreas appears to remain (Fig. 5F). In *vij* mutants, the phenotype appears earlier (3.5 dpf) than in *gog* and proceeds more rapidly. The exocrine pancreas is dramatically shorter by 4 dpf (Fig. 5J) and apparently absent by 5 dpf (Fig. 5L). We

repeated our analysis with specific markers of the exocrine pancreas such as amylase (Figs. 5O–P) and trypsin (not shown) and observed the same progressive shortening of the exocrine pancreas. Markers of the endocrine pancreas such as islet-1 and insulin appear normal at larval stages (Wehman and P.D. Dong, unpublished observation). Given the role of the exocrine pancreas in producing digestive enzymes such as trypsin, these findings could explain the lethality of *gog* and *vij* mutants.

To confirm the maternal-store depletion hypothesis for this tissue, we rescued the exocrine pancreas phenotype by RNA injection. Injection of Cdc26 mRNA at the one-cell stage was sufficient to fully rescue the pancreas in *gog* mutants at 9 dpf (Fig. 5H). This result demonstrated that Cdc26 is unusually stable, because RNA rescue of a phenotype over a week after injection is atypical. Similarly, injection of Cdc16 mRNA into *vij* mutants at the one-cell stage can delay the shortening of the pancreas by 1 or 2 days (Fig. 4N). Although it seems clear that progressive loss of the exocrine pancreas is due to loss of the APC/C, it is unclear whether the apparent retraction is due to degeneration or another developmental defect. We did not observe a dramatic increase of TUNEL-positive cells in the pancreas by whole-mount staining at 60 hpf, 3 dpf, or 4 dpf in *gog* and *vij* mutants (data not shown and Figs. 6I, K, M). These data suggest that other processes such as defects in cell adhesion

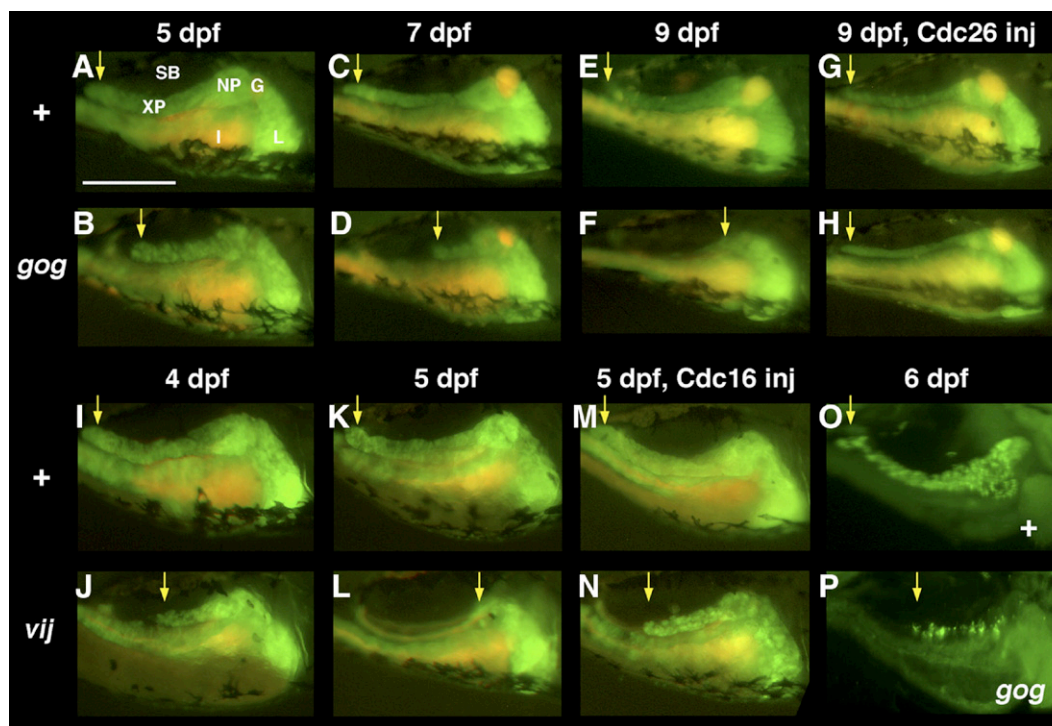


Fig. 5. APC/C deficiency results in progressive loss of the exocrine pancreas. (A–N) Right side view of endodermal organs in the *s161t* GFP line. (O, P) Right side view of larvae stained for amylase. Arrows in A–P mark the posterior extent of the exocrine pancreas. In wild-type larvae (A, C, E), the exocrine pancreas (XP) extends from the endocrine pancreas (NP) past the posterior end of the swim bladder (SB) between 5 dpf (A) and 9 dpf (E). In *gog*<sup>s109</sup> mutants (B), the exocrine pancreas no longer extends as far posteriorly as wild-type at 5 dpf. At 7 dpf, the exocrine pancreas extends less than half its normal length in *gog*<sup>s109</sup> mutants (D). At 9 dpf, the exocrine pancreas is no longer morphologically distinguishable from the endocrine pancreas in *gog*<sup>s109</sup> mutants (F). In wild-type larvae injected with Cdc26 mRNA at the one-cell stage, pancreas morphology is normal at 9 dpf (G). In *gog*<sup>s109</sup> mutants injected with Cdc26 mRNA, the progressive loss of the exocrine pancreas is rescued at 9 dpf (H). Wild-type (I, K) and *vij*<sup>s517</sup> (J, L) endodermal organs at 4 dpf and 5 dpf. In *vij*<sup>s517</sup> mutants, the exocrine pancreas has retracted to half its normal length by 4 dpf (J) and is no longer visible at 5 dpf (L). Injection of Cdc16 RNA does not alter pancreas development in wild-type larvae at 5 dpf (M), and temporarily rescues the exocrine pancreas in *vij*<sup>s517</sup> (N). Amylase staining at 6 dpf confirms that the exocrine pancreas is reduced in *gog*<sup>s109</sup> (O, P). Abbreviations: G: gall bladder, I: intestine, L: liver, NP: endocrine pancreas, SB: swim bladder, XP: exocrine pancreas. Scale bar: 500  $\mu$ m.

or morphology may also be involved in the progressive loss of the pancreas, but does not exclude that the cells of the exocrine pancreas are gradually dying.

Upon closer inspection, other tissues are also affected by loss of the APC/C. Most prominently, we observed a seven-fold increase in PH3-positive cells in the olfactory epithelium in *vij*

mutants (not shown, wt:  $2 \pm 1$ , *vij*:  $16 \pm 1$ ,  $n=8$ ,  $p < 0.001$ ). Like the retina, the olfactory epithelium is still adding many cells at 4 dpf. We also investigated whether the cartilage in the head was altered using Alcian green staining (Schilling and Kimmel, 1997). We discovered that the three posterior-most ceratobranchial arches are dramatically shorter in *vij* mutants at larval

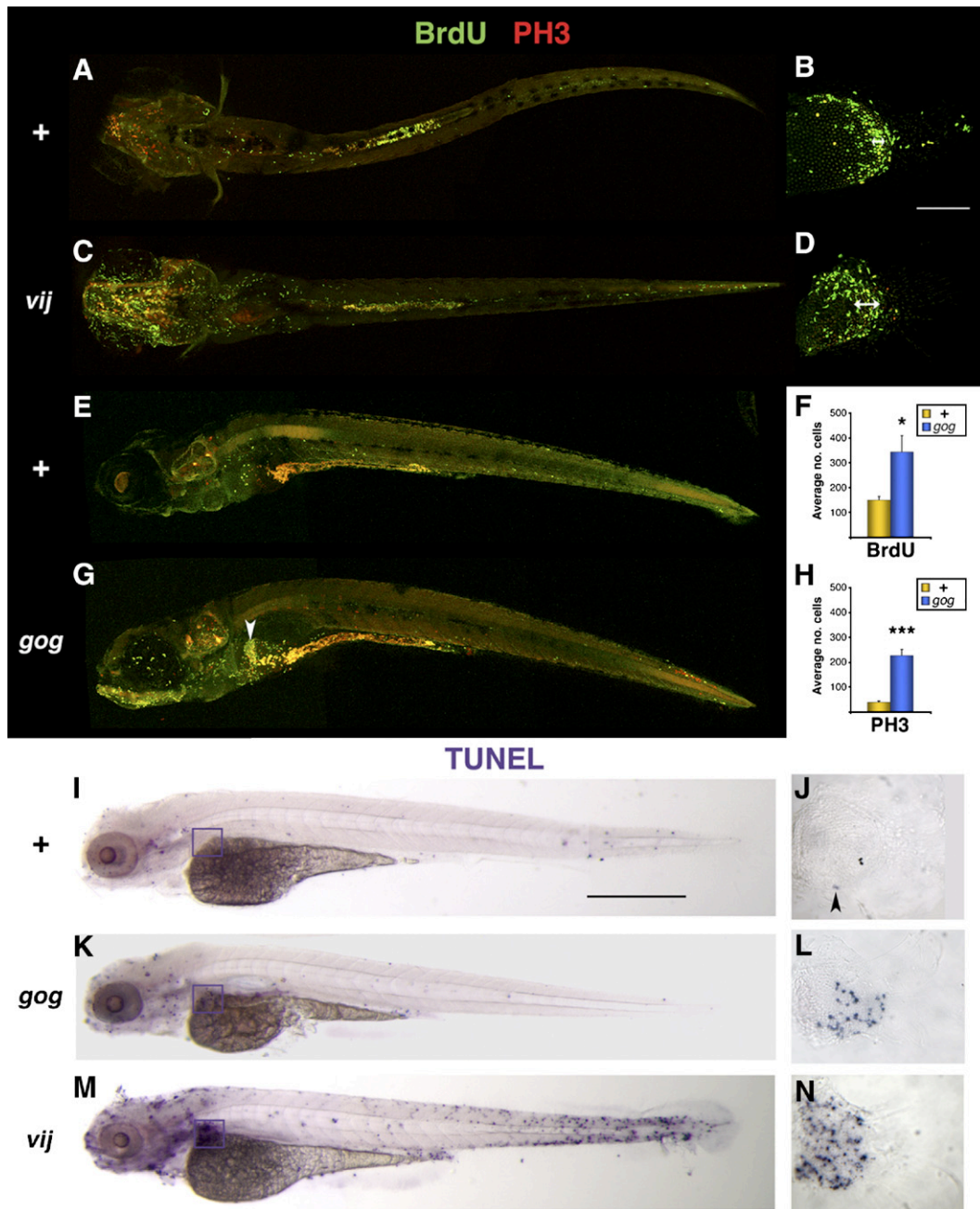


Fig. 6. Loss of the APC/C in dividing and quiescent cells results in mitotic arrest and apoptosis. (A–H) Larvae were stained in whole-mount for BrdU (green) and PH3 (red) immediately after a 6-h incubation in BrdU. Ventral and lateral views of 9 dpf larvae show that few cells divide in unfed wild-type (A, E), whereas many cells are BrdU- and PH3-positive in *vij*<sup>s514</sup> (C) and *gog*<sup>s109</sup> (G). In *gog*<sup>s109</sup>, the gall bladder (arrowhead) shows an especially striking increase in dividing cells (G). Graph of BrdU (F) and PH3 (H) cell counts (with standard error) from E, G (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ). Pectoral fin dissection shows that quiescent cells are re-entering the cell cycle at 4 dpf. In wild-type, division is mostly restricted to the distal edge of the fin (B, double arrow). In *vij*<sup>s517</sup> mutants, more cells divide and the division is more dispersed (D, double arrow). (I–N) PTU-treated larvae stained for TUNEL (purple) at 4 dpf. Lateral views of whole-mount larvae (I, K, M) and dissected pectoral fins (J, L, N) with the indicated genotype are shown. There is a developmental background of apoptosis in wild-type larvae (I), although dying cells are rarely observed in the pectoral fin (J, arrowhead). There is a significant increase in apoptotic cells in *gog*<sup>s109</sup> mutants, especially in the head and pectoral fin (K, purple box, L). Many cells are dying in *vij*<sup>s517</sup> mutants, especially in the head, pectoral fin and tail (M, purple box, N). Fins are oriented proximal to the left, distal to the right. Scale bar in B: 100  $\mu$ m. Scale bar in I: 500  $\mu$ m.

stages (Supplemental Fig. 3). These are the last of the five ceratobranchial arches to form, first appearing at 64–68 hpf (Schilling and Kimmel, 1997). These defects are consistent with the hypothesis that tissues that add many new cells at larval stages (such as the retina, olfactory epithelium, and late-forming branchial arches) are among the first tissues affected by gradual depletion of the APC/C.

#### *Loss of the APC/C in quiescent cells results in re-entry into mitosis*

In addition to its role in mitosis, the APC/C is known to maintain cells in G<sub>1</sub> and prohibit immediate progression to the next S phase (reviewed in Peters, 2002). There is also recent evidence that removal of the APC/C in differentiated cells may cause them to leave G<sub>0</sub> and re-enter the cell cycle (Almeida et al., 2005; Wirth et al., 2004). If a mitotic arrest were the only phenotype occurring in *gog* and *vij* mutants, we would observe a global decrease in BrdU-labeled cells, as we have seen in the retina. However, if loss of APC/C function also causes cells to re-enter the cell cycle from a quiescent state we would expect to see the exact opposite, an increase in BrdU-labeled cells.

In order to simultaneously assay proliferation in many tissues, we performed whole-mount BrdU- and PH3-staining in *gog* and *vij* mutants after a 6-h incubation in BrdU. This protocol is identical to the one used for our analysis of the retina where we observed an increase in the number of PH3-positive progenitors and a decrease in BrdU-positive progenitors (Fig. 2). In contrast to the retina, we discovered a global increase in both BrdU- and PH3-positive cells in the mutant larvae at 4 dpf, a stage when there is normally a large amount of cell division occurring throughout the body (data not shown). This increase in BrdU incorporation is evident in most tissues, but is particularly striking in the tail and pectoral fins. In wild-type fins, new cells are mainly added at the distal edge, and few cells are observed to divide in the central and proximal parts (Fig. 6B). In *vij* mutants, BrdU-positive cells are more dispersed at the distal edge of the fin, and more labeling is observed in the central and proximal portion of the fin (Fig. 6D).

We repeated the whole-mount BrdU- and PH3-staining in *gog* and *vij* mutants at 9 dpf, a stage when few cells should divide over a 6-h period in wild-type larvae that have not been fed (Figs. 6A, E). We observed a dramatic increase in the numbers of both BrdU-positive and PH3-positive cells in *vij* (Fig. 6C) and *gog* (Fig. 6G). In *gog* mutant larvae, there were twice as many BrdU-positive cells (wt: 149±14, *gog*: 342±66, *n*=5, *p*<0.05) and six times as many PH3-positive cells (wt: 37±5, *gog*: 225±25, *n*=5, *p*<0.001). Although *vij* mutants show the same widespread increase, *gog* mutants also have a specific and dramatic increase in BrdU-positive cells evident in the gall bladder at 9 dpf (arrowhead in Fig. 6G). These phenotypes are due to the slow and uneven depletion of APC/C in our mutants.

We finally asked whether there was a concomitant increase in TUNEL-positive apoptotic cells, above the background level of developmental apoptosis in wild-type larvae at 4 dpf (Fig. 6I). We observed a global increase in TUNEL-positive

cells in both *gog* (Fig. 6K) and *vij* mutants (Fig. 6M). This increase was especially striking in the pectoral fins of both mutants (Figs. 6L, N), which also showed a substantial increase in proliferating cells (Fig. 6D). As with all other phenotypes, the extent of apoptosis was more dramatic and began at an earlier stage in *vij* than *gog*.

Although the observed increase in BrdU-positive cells could reflect a slower progression through S phase, we do not favor this explanation given the specific increase we observed in post-mitotic regions of the fin (Fig. 6D) and the gall bladder (arrowhead in Fig. 6G). The model that APC/C-depleted cells are unable to maintain G<sub>0</sub> and re-enter the cell cycle best explains the observation that both markers of proliferation, BrdU and PH3, are increased in most tissues. The observation that the increase of PH3-positive cells is always greater than the increase in BrdU-positive cells shows that the cells that inappropriately re-enter the cell cycle are also subject to prolonged mitotic arrest. Finally, the widespread increase in TUNEL-labeling suggests that mitotically arrested cells eventually die by programmed cell death.

#### Discussion

In a forward-genetic screen for zebrafish mutants with arrested eye growth, we discovered seven loci required for proliferation of retinal progenitors (Wehman et al., 2005). By positional cloning we show here that two of the genes, *gog* and *vij*, encode Cdc26 and Cdc16, respectively, two subunits of the APC/C. Based on the protein sequence changes found in the single *gog* allele and the two *vij* alleles, we conclude that all three mutations have created (zygotic) null phenotypes. The phenotype of *gog*; *vij* double mutants is not stronger than that of either single mutant, suggesting that the two subunits work in the same pathway and are part of the same functional protein complex (the APC/C) in zebrafish cells as in other organisms.

Subsequent experiments using a dominant-negative inhibitor of the APC/C demonstrate that this protein complex is likely required for all mitotic divisions in the developing zebrafish. The early embryonic function of the APC/C is obscured in *gog* and *vij* mutants by maternal deposition of RNA and/or protein into the oocyte and by unusual stability of both Cdc16 and Cdc26 protein. Uneven depletion of the maternal store leads to tissue-specific defects, which are first apparent in the eye. Other tissues, such as the late-forming branchial arches and the olfactory epithelium are also affected, possibly due to more rapid dilution of the maternal APC/C in frequently dividing cell types.

Prior to this study, several zebrafish mutants with defects in DNA replication and cell proliferation have been reported, especially from a large-scale mutagenesis screen using retroviral insertions (Amsterdam et al., 2004). Among these, mutations in MCM2 and MCM3 result in CMZ defects, similar to (although more pleiotropic than) the ones described here for *gog* and *vij* (Gross et al., 2005). Maternal stores may mask the embryonic functions of MCM2 and MCM3, much in the same way as we have shown here for the APC/C. Therefore, we may find additional mutations in cell cycle regulators in our collection of CMZ mutants (Wehman et al., 2005), although the genetic map

positions of the remaining five loci already exclude other APC/C components as candidate genes (Supplemental Table 1). In any case, the zebrafish CMZ may provide an efficient system in which to discover genes universally involved in regulation of the vertebrate cell cycle that are supplied maternally.

We used two cell-cycle markers to analyze the *gog* and *vij* phenotypes in more detail: the S phase tracker BrdU, which permanently labels cells that have undergone DNA replication during the incubation period; and the M phase marker PH3, which labels only those cells that are in late G<sub>2</sub> or in mitosis at the time of fixation. We found that, in the retina, fewer progenitor cells in the marginal zone incorporate BrdU, and more of them are PH3-positive. This suggests that progenitors with insufficient quantities of Cdc16 or Cdc26 are arrested in mitosis (and therefore continue to label with PH3) and can no longer divide (and are therefore BrdU-negative). We could further show that mitotically arrested cells are eventually removed from the retina by programmed cell death by colabeling of retinal progenitors with PH3 and TUNEL. As a combined result of mitotic arrest and apoptosis, the eye fails to grow. Similar phenotypes were seen in the olfactory epithelium and the branchial arches. Injection of RNA encoding wild-type protein into mutant embryos at the one-cell stage was able to delay the cell proliferation defect by several days and transiently normalize organ growth. Overexpression of either Cdc16 or Cdc26 did not perturb embryogenesis, suggesting that the APC/C has a permissive function, does not have an inductive role in patterning the embryo, and may operate at widely varying concentrations of its individual subunits.

A different cell cycle phenotype was observed in several other tissues, including the tail and fins of both mutants and the gall bladder of *gog*. Here, regions that in wild-type contain mainly postmitotic cells now exhibit increased BrdU incorporation and increased PH3 labeling. We explain this finding by the known role of the APC/C in maintaining cells in G<sub>1</sub> by delaying entry into S phase (reviewed in Peters, 2002). If, in a quiescent cell, the APC/C levels drops below a certain threshold this cell may re-enter the cell cycle and begin DNA replication. The cells that re-enter the cell cycle proceed normally through G<sub>2</sub> but subsequently arrest in M phase, as evidenced in our experiments by the accumulation of PH3-labeled cells, which always exceeded the number of BrdU-positive cells. This phenomenon can explain why the exocrine pancreas not only stops growing but also fails to maintain differentiated cells. In the retina, however, we did not observe ectopic BrdU-positive cells, which could mean that this tissue still maintains sufficient levels of APC/C protein. Alternatively, the reversal of the G<sub>0</sub> state after loss of APC/C may not occur in all cell types, such as retinal neurons. Cdk inhibitors are candidate factors that may work redundantly with the APC/C to keep these neurons in a postmitotic state (reviewed in Peters, 2002; see also Ohnuma and Harris, 2003).

Together, these experiments lead us to propose the following model of APC/C function in zebrafish. A functional APC/C is required for the correct progression from M phase through G<sub>1</sub> in dividing cells, as well as for maintenance of G<sub>0</sub> in quiescent and possibly differentiated cells (Fig. 7A). When APC/C function is

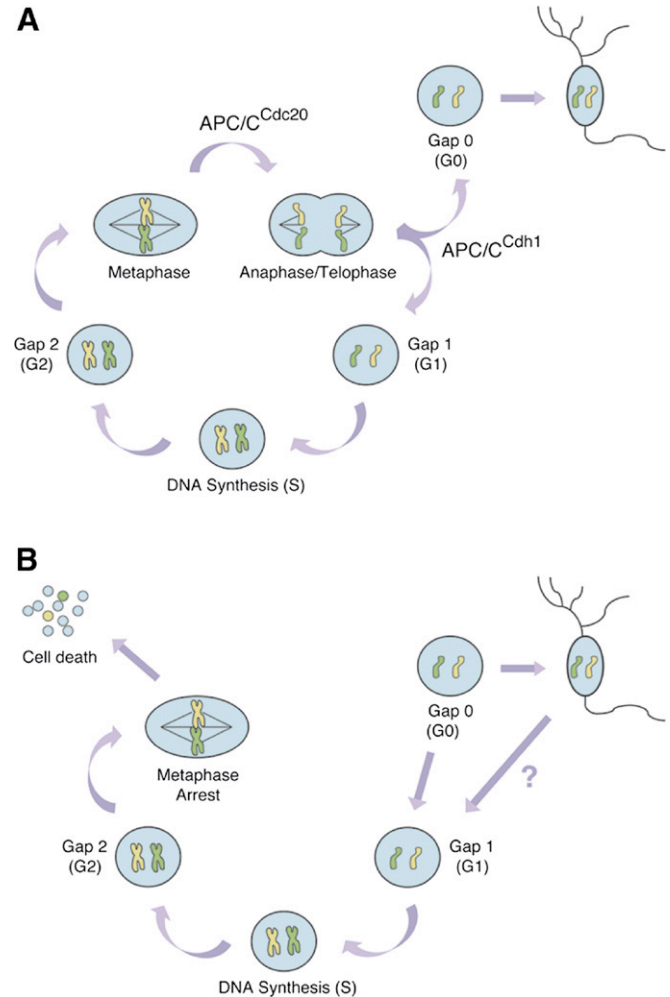


Fig. 7. Model of cell cycle progression with and without APC/C function. (A) In normal cells, the mitotic cell cycle progresses from G<sub>1</sub>, when cells prepare to replicate their genome, to S, when DNA synthesis occurs. After duplicating each chromosome, they progress to G<sub>2</sub>, when they prepare for M. Finally, in M, the cells line up their chromosomes and the mitotic spindle ensures that each daughter will receive one copy of the genome before cytokinesis occurs. The daughters then have the choice to re-enter the cell cycle (G<sub>1</sub>) or to exit the cell cycle and enter into G<sub>0</sub>. Quiescent cells in G<sub>0</sub> typically go on to differentiate. APC/C<sup>Cdc20</sup> is required for progression from metaphase to anaphase, while APC/C<sup>Cdh1</sup> function is required for correct regulation of mitotic exit and to maintain cells in G<sub>0</sub> and G<sub>1</sub>. (B) In cells that lack functional APC/C protein, all phases of the cell cycle are affected except S and G<sub>2</sub>. Cells that are in G<sub>1</sub> are hurried into S and cells that are in G<sub>0</sub>, whether quiescent or differentiated, re-enter G<sub>1</sub>. After completing S and G<sub>2</sub>, cells line up their chromosomes in metaphase, but then arrest. Sister chromatid separation does not occur and the cell is trapped in M for hours. Finally, the arrested cell undergoes apoptosis.

lost in a dividing cell, it results in mitotic arrest and apoptosis (Fig. 7B). When APC/C function is lost in a quiescent or differentiated cell, it results in re-entry into the cell cycle, before the cell also arrests in mitosis and undergoes apoptosis (Fig. 7B). To our knowledge, this report is the first demonstration of the dual role of the APC/C in both dividing and quiescent cells in a living vertebrate. These two functions could only be revealed because of the maternal supply of Cdc16 and Cdc26 in zebrafish embryos. Maternal deposits of these proteins: (1)

rescue zebrafish APC/C mutants from an early mitotic arrest; (2) are stable, lasting through embryonic development and, in some tissues, to larval stages; and (3) are depleted unevenly among different cell types leading to tissue-specific phenotypes.

Although precedents exist for both APC/C functions discovered here, the field has not agreed on a unifying model of its role in multicellular organisms. In *Drosophila* APC2 null mutants, the imaginal discs are small and mitotic neural precursors arrest in mitosis and fail to differentiate, consistent with the APC/C functioning in dividing cells (Reed and Orr-Weaver, 1997). The authors propose, as we do here, that the absence of defects until larval stages is due to maternal rescue of embryonic mitotic divisions. They also report that in APC2 hypomorphic mutants, the germline is defective and embryos from mutant mothers display an early embryonic mitotic defect. These defects support the hypothesis that gradual loss of maternal APC/C leads to relatively late phenotypes. It is unclear whether these tissues are specifically affected or whether similar defects would be observed in other tissues if whole animal BrdU, PH3, or TUNEL assays were performed as we have done in this study. Although *gog* and *vij* mutants were originally identified for having a slightly smaller eye, we discovered defects in every tissue we analyzed, i.e. retina, tectum, olfactory epithelium, pancreas, liver, branchial arches, pectoral fins, and tail. We believe this reflects a universal requirement for the APC/C in all cells. We explain the disparity between the early lethal phenotype of the APC2 mouse knockout and later larval defects in zebrafish and *Drosophila* APC/C mutants as a reflection of maternal loading. Zebrafish and *Drosophila* develop rapidly and depend on maternal products (reviewed in O'Farrell et al., 1989; Pelegri, 2003). In zebrafish, zygotic gene transcription begins at the thousand-cell stage (3–3.5 hpf) (Kane et al., 1992). In contrast, the cell cycle is slow in mice and zygotic transcription is already observed at the two-cell stage (reviewed in Schultz, 1993).

Evidence for a role of the APC/C in maintaining the quiescent state ( $G_0$ ) has been established by studies focusing on the response of individual tissues to loss of the APC/C, rather than a whole-organism view. In an elegant study making use of an inducible Cre-lox system in mice, Wirth and colleagues analyzed the role of APC2 specifically in differentiated hepatocytes and discovered that, in the majority of livers analyzed, quiescent cells aberrantly re-entered the cell cycle before arresting in metaphase (Wirth et al., 2004). The small subset of livers that did not show spontaneous re-entry were still more responsive to mitogenic inducement, but it is not clear why these livers were phenotypically distinct after the same treatments in the same genetic background. A similar study in cultured rat neurons showed that loss of the APC/C activator Cdh1 in differentiated cells also resulted in re-entry into the cell cycle and eventually apoptosis (Almeida et al., 2005). The authors go on to show that these phenotypes are due to the accumulation of cyclin B, a major target of the APC/C. While these studies demonstrate that differentiated cells can re-enter the cell cycle upon loss of APC/C, we reveal that this principle can be applied to many tissues of the developing vertebrate.

While several studies propose that maternal APC/C is able to compensate for an absence of functional zygotic APC/C, few have remarked on its stability. Temperature-sensitive *C. elegans* APC/C mutant embryos develop to adulthood when raised at the restrictive temperature, suggesting maternal protein can persist for days (Golden et al., 2000). In zebrafish, most maternal proteins are used up by 1 dpf (reviewed in Pelegri, 2003). Defects first appear around 1 dpf in zebrafish Cyclin B1 mutants, as expected for an unstable, degraded protein that regulates the cell cycle (Amsterdam et al., 2004; Wehman, unpublished observations). In *gog* and *vij* mutants, we observed a substantial number of cells continuing to divide after 9 dpf (Figs. 5C, G). In addition, injection of RNA for Cdc26 at the one-cell stage rescued growth of the eye until at least 7 dpf and delayed retraction of the exocrine pancreas to at least 9.5 dpf. Clearly, Cdc26 protein must be unusually stable to function 9 days after it is produced. Subunits of the APC/C may be stabilized by their participation in a large protein complex, but they must also be able to function at low concentration, because considerable dilution will have occurred after 9 days of growth and division from one cell to the larval stage. These aspects of the APC/C have been underappreciated by previous studies.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.10.043.

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