Repurposing a Cyclin-Dependent Kinase 1 (CDK1) Mitotic Regulatory Network to Complete Terminal Differentiation in Lens Fiber Cells

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Purpose. During lens fiber cell differentiation, organelles are removed in an ordered manner to ensure lens clarity. A critical step in this process is removal of the cell nucleus, but the mechanisms by which this occurs are unclear. In this study, we investigate the role of a cyclin-dependent kinase 1 (CDK1) regulatory loop in controlling lens fiber cell denucleation (LFCD).

Methods. We examined lens differentiation histologically in two different vertebrate models. An embryonic chick lens culture system was used to test the role of CDK1, cell division cycle 25 (CDC25), WEE1, and PP2A in LFCD. Additionally, we used three mouse models that express high levels of the CDK inhibitor p27 to test whether increased p27 levels affect LFCD.

Results. Using chick lens organ cultures, small-molecule inhibitors of CDK1 and CDC25 inhibit LFCD, while inhibiting the CDK1 inhibitory kinase WEE1 potentiates LFCD. Additionally, treatment with an inhibitor of PP2A, which indirectly inhibits CDK1 activity, also increased LFCD. Three different mouse models that express increased levels of p27 through different mechanisms show impaired LFCD.

Conclusions. Here we define a conserved nonmitotic role for CDK1 and its upstream regulators in controlling LFCD. We find that CDK1 functionally interacts with WEE1, a nuclear kinase that inhibits CDK1 activity, and CDC25 activating phosphatases in cells where CDK1 activity must be exquisitely regulated to allow for LFCD. We also provide genetic evidence in multiple in vivo models that p27, a CDK1 inhibitor, inhibits lens growth and LFCD.

Keywords: CDK1, CDC25, WEE1, lens, differentiation, p27

During the life of an organism, tissues undergo cellular proliferation, differentiation, and aging. The differentiation process is particularly exaggerated in cells that discard their nucleus such as red blood cells, keratinocytes, and eye lens fiber cells. The lens of the eye must undergo this differentiation process to achieve optical clarity.

Within the lens, epithelial cells proliferate in an area anterior to the equator, where they differentiate into fiber cells. In this unidirectional process, lens fiber cells elongate, exit the cell cycle, and change their gene expression profile to primarily express crystallin proteins, similar to red blood cells synthesizing hemoglobin as their major gene product. As lens fiber cells mature, they compact in the center of the lens and lose their organelles in an ordered fashion.[1] The mechanisms by which each type of organelle is removed are still being revealed. In support of roles for autophagy in removal of some organelles, it has been observed that organelle removal can be accelerated via enhancing autophagy by suppression of MAPK/JNK and PI3K signaling in a chick lens culture model.[2,3] In the lens, removal of mitochondria, endoplasmic reticulum (ER), and Golgi requires the action of the mitophagy protein BNIP3L/NIX[4] and lysosomal enzymes.[5] However, the absolute requirement for distinct macroautophagy pathways in lens organelle removal remains unresolved as mouse models deleted for specific macroautophagy components exhibit no defect in organelle removal.[6,7]

The last organelle to be removed during fiber cell differentiation is the nucleus. This process is initiated by disassembly of the nuclear membrane and completed with DNA

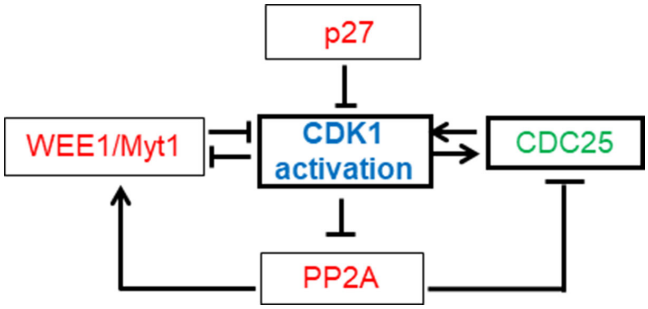

Figure 1. Regulation of CDK1 activity. Inhibitors are in *red*, and activators are shown in *green*. PP2A indirectly inhibits CDK1 by activating the inhibitory kinases WEE1 and MYT1 and inhibiting the CDK1 activating CDC25 phosphatases.

degradation by the lens-specific DNAse, DLAD/DNAse IIβ.[8] The pathways that control nuclear envelope breakdown during lens fiber cell denucleation (LFCD) have not been fully elucidated. It has recently been proposed that LFCD in the chick involves a subcellular structure called the nuclear excisosome.[9] A number of cell cycle regulatory molecules are expressed in the nondividing lens fiber cells,[10–13] and defects in cell division have been correlated with cataracts in humans,[14] suggesting that mitotic cell cycle regulators may play a role in the unidirectional processes involved in establishing lens clarity. During the cell cycle, the activity of the essential CDK1 controls progression through mitosis.[15] We hypothesized that vertebrate lenses utilize mitotic machinery, specifically CDK1, to direct phosphorylation of lamin and nuclear envelope breakdown during LFCD. Indeed, a lens-specific knockout confirmed a critical role for CDK1 in LFCD in the mouse.[16]

CDK1 activity in mitosis is regulated in multiple ways (Fig. 1). CDK1 activity is inhibited by phosphorylation at Tyr15 directed by the WEE1 and MYT1 kinases, while CDK1 activity is promoted by the CDC25 family of phosphatases, which remove the phosphorylation at Tyr15. The PP2A family of phosphatases regulates CDK1 activity indirectly by activating WEE1 and inhibiting the CDC25 family of phosphatases.[17] These regulatory molecules have well-described roles in controlling the cell cycle and proliferation,[18–20] but a role for their activity in lens differentiation has never been explored.

WEE1 is an essential kinase as the knockout is lethal early in development,[21] and roles for WEE1 in in cancer and mitosis have been well described.[20] Overexpression of WEE1 results in a "rough eye" phenotype in *Drosophila*,[22] but a specific role for WEE1 in the vertebrate lens has not been described.

There are three members of the CDC25 family of phosphatases. While all three are known to regulate CDK function, only CDC25A is an essential gene and knockout embryos die at E5–E7,[23] suggesting CDC25 plays a role very early in development.[24] Intriguingly, CDC25B is regulated by the Alzheimer's associated protein Aβ in primary human lens epithelial cells and is necessary for the increased resistance to oxidative stress stimulated by Aβ.[25] Furthermore, there is case report of an individual with a natural knockout of CDC25B who presented with a cataract.[26] Although there is no report of a lens phenotype in CDC25B knockout mice, these data suggest that CDC25B specifically may be important in maintenance of the lens.

PP2A is a family of phosphatases with multiple A, B, and C subunits and regulates multiple signaling pathways in the cell. Knockout of either the Cα or Aα subunits is lethal between E6 and E10.[27] There is a report of knockdown of PP2A in developing goldfish impairing lens development,[28] suggesting that regulation of phosphorylation, potentially of CDK1, may be important in lens differentiation.

CDK1 activity is also inhibited by direct binding of the CDK inhibitor, p27(Kip1/CDKN1B).[29] p27 regulates cell cycle progression through its inhibition of CDK1.[30] Additionally, p27 is involved in CDK-dependent control of autophagy and apoptosis.[31] However, the regulation of cell migration by p27 is independent of its ability to inhibit CDK.[32] Increased levels of p27 were correlated with impaired LFCD in several mouse models of cataract.[33,34]

Here we used mammalian and avian models to test the hypothesis that there is a dynamic CDK1 regulatory loop including positive regulation by the CDC25 family of phosphatases and negative regulation by WEE1, PP2A, and p27 that controls the nuclear disassembly and terminal differentiation process in lens fiber cells.

Methods

Animals

All animals were bred and housed according to National Institutes of Health (NIH), Tufts University Institutional Animal Care and Use Committee (IACUC), and ARVO guidelines. Mice expressing a doxycycline-inducible p27 transgene were described previously.[35] Pregnant mice were treated for 2 or 5 days before birth to induce p27 expression during lens differentiation. Offspring were collected at day P1. Whole heads were fixed in 4% paraformaldehyde and subsequently dehydrated in sucrose before embedding in optimal temperature cutting compound (OCT). Heads of E18.5 embryos of wild-type (WT), *Skp2*$^{-/-}$, and p27197AA knock-in mice[36] were embedded in paraffin, sectioned, and stained with hematoxylin and eosin by the Tufts Medical Center histology core facility.

White Leghorn chicken eggs (Charles River Laboratories, Storrs, CT, USA) were sacrificed at days E8 to E18 in compliance with NIH, Tufts University IACUC, and ARVO guidelines. Primary chicken lens organ cultures were prepared from the lenses of embryonic day 12 as described in Brennan et al.[37] Briefly, whole lenses were removed from isolated chicken eyes and placed into individual wells of 24-well plates containing Medium 199 (ThermoFisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and penicillin-streptomycin antibiotic mix (50 units/ml; ThermoFisher). Lenses were cultured for 2 hours prior to treatment to ensure only clear and undamaged lenses were used for culture studies. After 3 days of culture, lenses were collected and frozen in OCT.

Drugs used for chick lens culture included adavosertib (Selleckchem, Houston, TX, USA),[38] BN82002 (EMD Millipore, Burlington, MA, USA),[39] rubratoxin A (Cayman Chemical, Ann Arbor, MI, USA),[40] NSC663284 (Chem Cruz, Dallas, TX, USA),[41] FTY720 (Chem Cruz),[42] RO3306 (Sigma, St. Louis, MO, USA),[43] and CGP 74514[44] (Tocris Bioscience, Bristol, UK). Drugs were added at the beginning of culture and replenished daily.

Immunostaining

Chick lenses were fresh frozen in OCT prior to sectioning. Immediately prior to staining, sections were fixed

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in 4% paraformaldehyde. Nuclei were visualized by Prolong mounting media (Invitrogen/ThermoFisher). In some cases, citrate-based antigen retrieval (Vector Laboratories, Burlingame, CA, USA) was necessary to elucidate staining. Lenses were sectioned serially, and thus the presence of multiple sections on a slide indicated that the intervening slides in the series (8 or 12) also had sections with areas of denucleation. Thus, the denucleation area was calculated by measuring the distance between nuclei, the number of sections with a denucleated region, the thickness of the section, and the number of slides in the series.

Antibodies

Antibodies included p27 mouse monoclonal (BD Biosciences, Franklin Lakes, NJ, USA), rabbit polyclonal (Santa Cruz Biotechnology, Dallas, TX, USA), phospholamin A (Abcam, Waltham, MA, USA), CDC25B (Santa Cruz Biotechnology), WEE1 (Santa Cruz Biotechnology), phospho-CDK1 (Y15; Cell Signaling Technologies, Danvers, MA, USA), DNAse IIβ (kind gift from Shigekazu Nagata, Osaka University), CDK1 (BD Biosciences), Ki67 (Vector Laboratories), CDC25B (Santa Cruz Biotechnology), CDC25C (Santa Crus Biotechnology), and pCDC25C (Cell Signaling Technologies).

Results

CDK1 as a Central Hub for Regulation of Lens Fiber Cell Denucleation

We previously demonstrated that CDK1 is necessary for efficient LFCD in the mammalian lens.[16] To explore for the generalizability of this role for CDK1 in LFCD, we asked about development in an evolutionarily distant avian model. As in mice, chick lenses were surrounded on their anterior surface by a layer of epithelial cells (Figs. 2A, 2B). By embryonic day 8 (E8), mostly elongated nuclei were observed in the chick lens fibers at the center of the lens (Fig. 2A). By E10, more nuclei began to condense and became round in the central fiber cells (arrows). By day E14, all the nuclei in the center of the lens were condensed, and by E18,

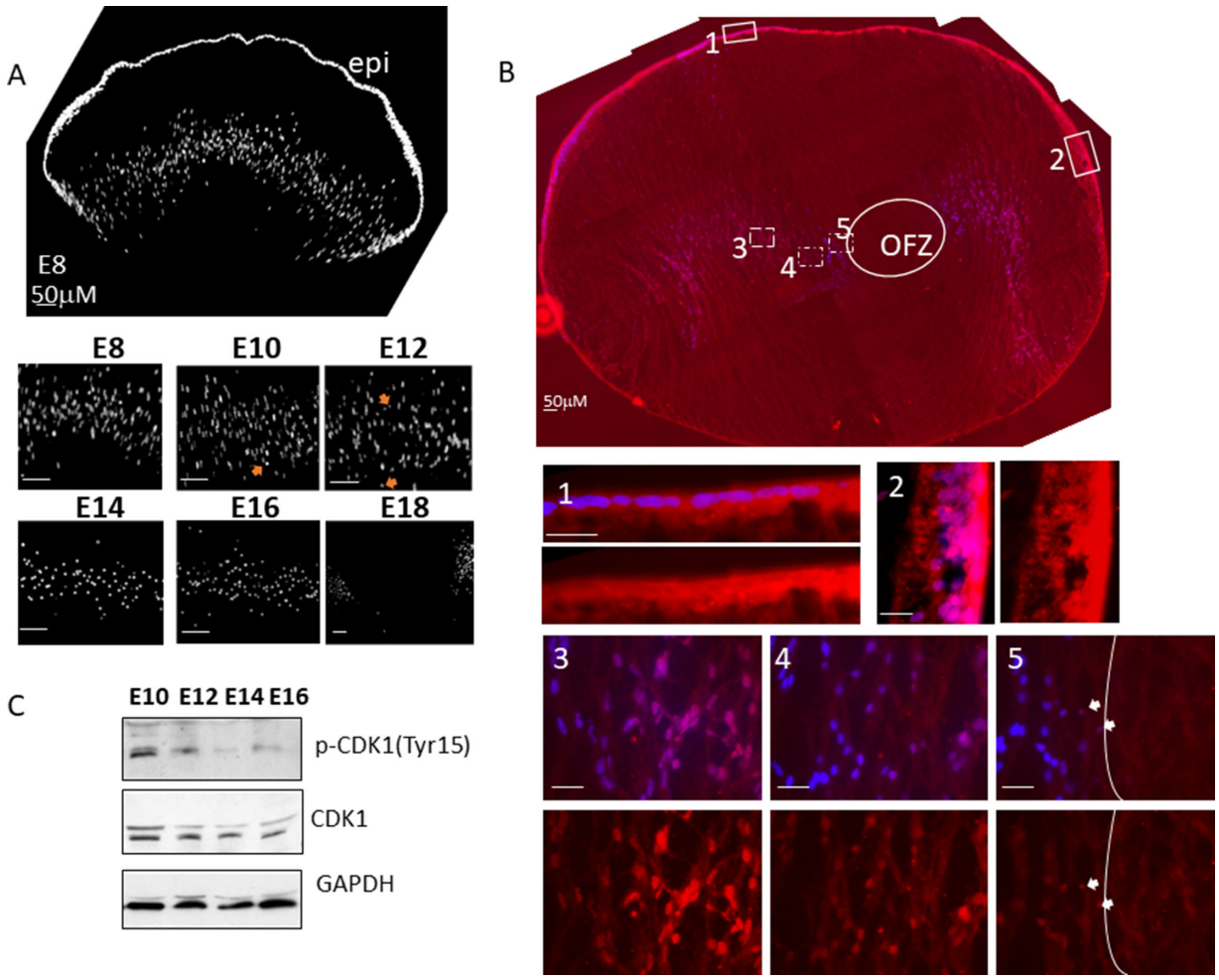

Figure 2. Nuclear morphology and CDK1 expression in chick lens development. (**A**) DAPI staining of full section of E8 chick lens showing epithelium and fiber cell nuclei. E8 to E10 show central fibers; *orange arrows* show condensed nuclei in E10 and E12 sections. Scale bars are 50 µM. Panel for E18 represents a larger region than shown for other stages. (**B**) Chick lens section from a control lens (E12) placed in culture for 3 days with an organelle-free zone (OFZ) stained with CDK1 and DAPI; composite of fourteen 20× images. The OFZ appears offset due to section angle. Regions 3, 4, and 5 are ~785 µM, ~985 µM, and ~1100 µM from left edge of equatorial epithelium, respectively. Regions 1–5, 100× images: overlay and red channel (CDK1) shown as paired images. *White line* in region 5 is edge of OFZ. *Arrows* denote CDK1 expressing nuclei at edge of OFZ. *Scale bars* in 100× images are 20 µM. (**C**) Western blot of CDK1 and pCDK1 during chick lens development.

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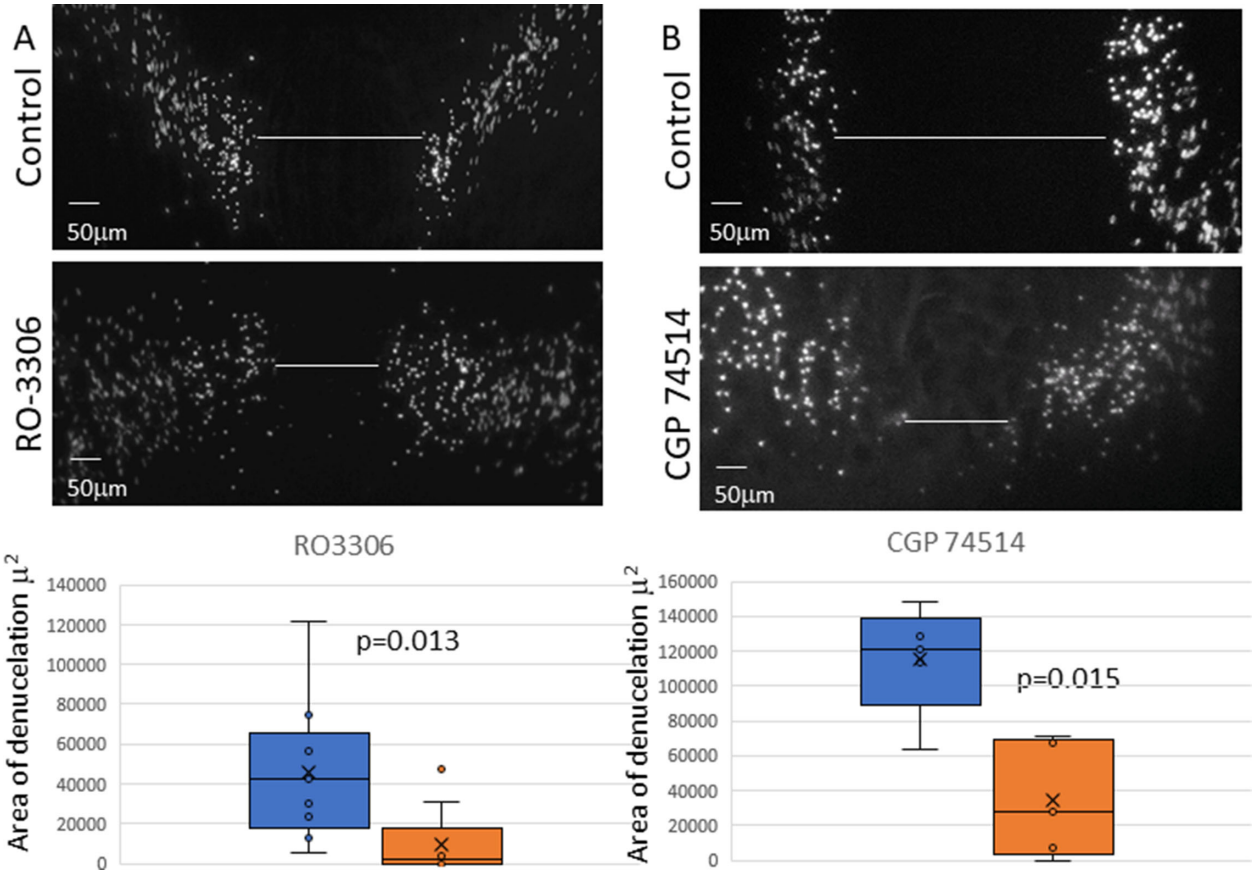

Figure 3. Inhibition of CDK1 activity impairs lens fiber cell denucleation. Chick lenses were put into culture at E12 for 3 days in the presence or absence of inhibitors as shown. DAPI staining of central fibers. Box-and-whisker plots: control (*blue*); drug treatment (*orange*). (x) median, *line* = mean. (**A**) Treatment with 20 µM RO-3306, $n = 10$. (**B**) treatment with 100 nM CGP 74514, $n = 5$. Full-size images of lens sections and data normalized to lens diameter are shown in Supplementary Fig. S1.

we observed a nuclei-free zone, indicating that LFCD had occurred, comparable to the sequence of development in the mammalian lens.

CDK1 was highly expressed in the cytoplasm and nuclei of chick lens anterior and equatorial epithelial cells (Fig. 2B, regions 1, 2). Furthermore, CDK1 was expressed in the nuclei of fiber cells through differentiation until just before denucleation (Fig. 2B, regions 3–5; see arrows), suggesting that CDK1 may have a role in LFCD in the chick as it does in the mouse.

During mitosis, the activity of CDK1 is tightly regulated by phosphorylation and binding to inhibitory proteins.[45] Elucidation of regulation of CDK1, particularly with regard to differentiation, has never been pursued in the chick lens. CDK1 activity is inhibited by phosphorylation at Tyr15. Western blot analysis indicates that during chick lens development, the levels of phosphorylated CDK1 decrease precipitously between E10 and E14 (Fig. 2C). This upregulation of CDK1 activity is consistent both with increased proliferation in the epithelium as well promoting LFCD in fiber cells.

We sought to confirm that CDK1 activity was required for efficient LFCD in chick lenses. Chick lenses continue their usual developmental program when maintained in vitro.[2,3,37] Chick lenses were dissected at E12 and put in culture medium for 3 days in the presence or absence of CDK1 inhibitors. Inhibition of CDK1 with two different selective inhibitors, RO3306[43] and CGP 74514,[44] profoundly delayed LFCD (Figs. 3A, 3B). As gauged by region of denucleation, inhibition of CDK1 delayed LFCD by four- to eightfold (Fig. 3B, Supplementary Fig. S1). Treatment of lenses with inhibitors did not affect lens size as the diameter of the central sections was not affected by treatment (diameter of control for RO-3306, 1651 ± 191 µM; RO-3306, 1627.96 ± 118 µM; diameter of control for CGP, 74,514 = 1547 ± 121 µM; CGP, 74,514 = 1586 ± 157 µM), suggesting that treatment affects denucleation rather than growth. These inhibitors showed activity toward CDK1 in cultured cells as we observed increased phosphorylated CDK1 upon treatment with RO3306 and CGP 74514 (Supplementary Fig. S2, lanes 1, 2, 6, 7). Together, these findings indicate that as in the mouse, CDK1 controls LFCD in the chick.[16]

A Regulatory Loop of CDC25, PP2A, and WEE1 Regulates CDK1 and LFCD

CDK1 phosphorylation, and thus activity, is controlled by a network of kinases and phosphatases (Fig. 1). Thus, we asked if these regulators were also involved in LFCD. The family of CDC25 phosphatases removes the Tyr15 phosphorylation on CDK1 and promotes its activity.[17] Immunofluorescence analysis indicates that CDC25B is colocalized with CDK1 in nuclei just outside the organelle-free zone in chick lenses (Fig. 4A), suggesting that CDC25B regulates the activity of CDK1 in these cells. Additional support for a role for CDC25B in LFCD comes from the observation

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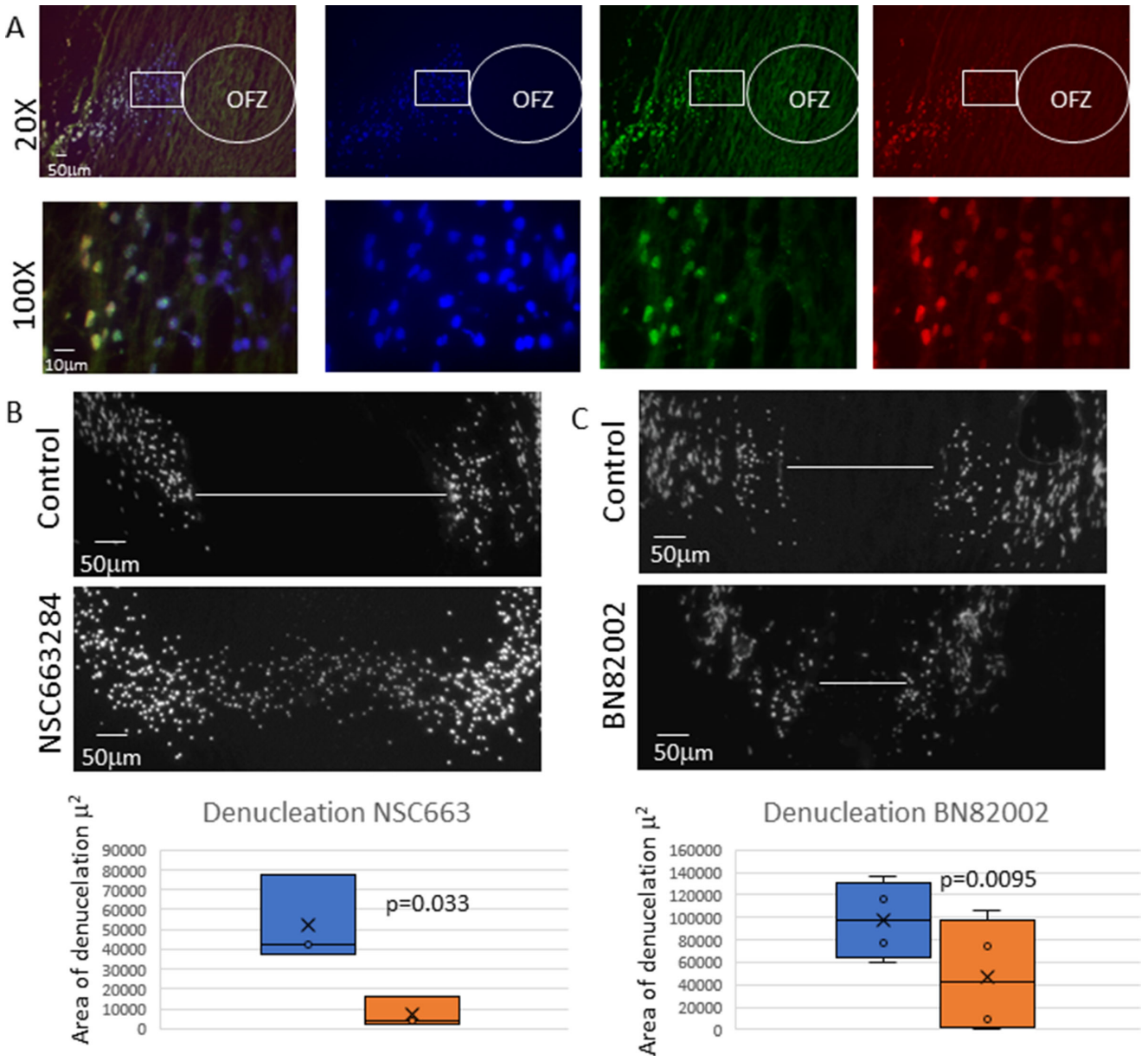

Figure 4. CDC25 regulates lens fiber cell denucleation. (**A**) Immunofluorescence of chick lens showing CDC25B (*green*) and CDK1 (*red*) 20× and 100×. (**B**, **C**) E12 chick lenses placed in culture for 3 days in the presence or absence of inhibitors as shown. (**B**) 10 µM NSC663284, $n = 4$. (**C**) 10 µM BN82002, $n = 4$. Full-size images of lens sections and data normalized to lens diameter are shown in Supplementary Fig. S4.

that CDC25B is colocalized with the nuclear pore complex in central lens fiber cells, and they disappear coordinately before denucleation (Supplementary Fig. S3A). Furthermore, CDK1 and CDC25B physically interact, as demonstrated by coimmunoprecipitation with αCDK1 (Supplementary Fig. S3B). CDC25B is also expressed in fiber cell nuclei prior to LFCD in mouse lenses (Supplementary Fig. S3C) suggesting a common pathway of CDK1 regulation in the mouse and chick.

If LFCD requires CDC25, then inhibition of this phosphatase should inhibit LFCD. To determine the role of CDC25 in LFCD, we treated chick lenses with the selective CDC25 inhibitors NSC663284[41] and BN82002.[39] As expected, cells treated with these drugs show increased levels of p-CDK1, indicating inhibition of CDK1 activity through inhibition of CDC25 (Supplementary Fig. S1A, lanes 1, 3, 6, 8). Treatment with inhibitors did not affect lens growth as the diameter of the central sections was not affected (diameter of control for NSC663284, 1499 ± 245 µM; NSC663284, 1587 ± 146.76 µM; diameter of control for BN82008, 1514 ± 117 µM; BN82002, 1658 ± 118 µM). Fiber cells in lenses treated with NSC663284 failed to denucleate and BN82002 delayed LFCD markedly (Figs. 4B, 4C, Supplementary Fig. S4). Combined, the data indicate that CDK1 activation by CDC25 is necessary for efficient LFCD.

WEE1 kinase phosphorylates CDK1 on Tyr15, thereby inhibiting its activity.[17] Immunofluorescence analysis indicates that WEE1 expression overlaps with CDC25B expression (Fig. 5A), suggesting that like CDC25, WEE1 is part of the regulatory machinery of CDK1 and of LFCD. In contrast with inhibition of CDK1 and CDC25 leading to inhibition of LFCD, it was anticipated that inhibition of WEE1 would

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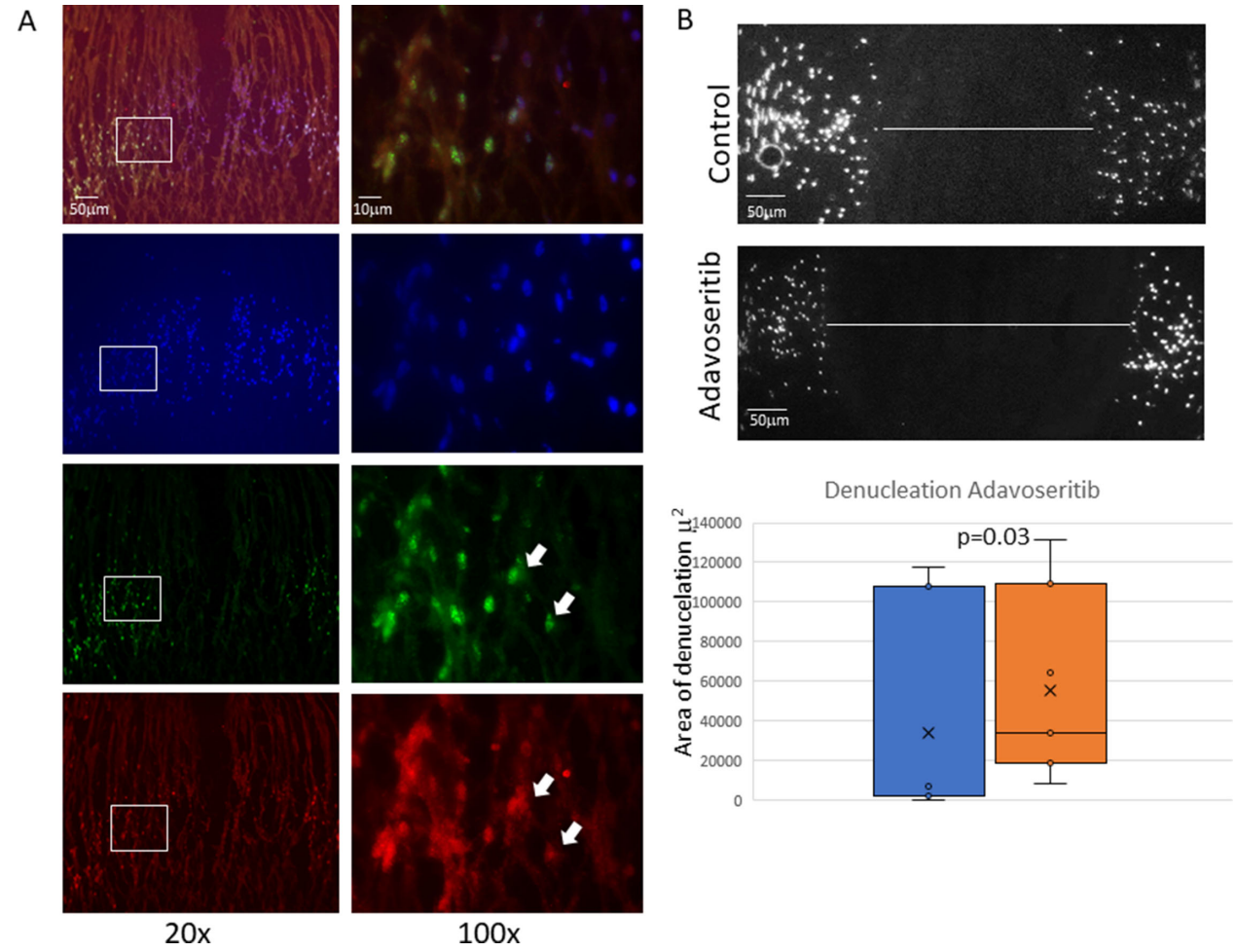

Figure 5. Inhibition of WEE1 potentiates lens fiber cell denucleation. (**A**) Immunofluorescence of chick lens showing localization of CDC25B (*green*) and WEE1 (*red*). *Arrows* show coordinate disappearance. Images are 20× and 100×. (**B**) E12 lenses were placed in culture in the presence and absence of 1 µM adavosertib as noted, $n = 6$. Mean line for control (*blue*) is at the bottom of the box. Full-size images of lens sections and data normalized to lens diameter are shown in Supplementary Fig. S5.

activate CDK1 and accelerate LFCD. In support of this hypothesis, treatment of lenses with adavosertib accelerated LFCD by 60% (Fig. 5B, Supplementary Fig. S5) but did not affect overall lens growth (diameter of control, 1696 ± 26 µM; adavosertib, 1649 ± 115 µM) indicating an inhibitory role for WEE1 in LFCD. Confirming its activity with regard to WEE1 regulation of CDK1, adavosertib decreased the level of p-CDK1 in cells in culture (Supplementary Fig. S2, lanes 4, 5).

PP2A is a multisubunit phosphatase that activates WEE1 and MYT1 while inhibiting CDC25 (Fig. 1); thus, we hypothesized that activation of PP2A would inhibit CDK1 and delay LFCD, whereas inhibition of PP2A would accelerate LFCD. To test this hypothesis, lenses were treated with a PP2A activator FTY720 and inhibitor rubratoxin A. As predicted, the PP2A inhibitor caused a 60% increase in LFCD (Fig. 6A, Supplementary Fig. S6). In comparison, activation of PP2A decreased LFCD 40% (Fig. 6B, Supplementary Fig. S6). Treatment of cells in culture with the PP2A activator increased p-CDK1 while the PP2A inhibitor, rubratoxin, decreased p-CDC25C[40] (Supplementary Fig. S2, lanes 6, 9–11). Treatment of lenses with either rubratoxin A or FTY720 did not affect lens growth (diameter of control for rubratoxin A, 1708 ± 154 µM; rubratoxin A, 1577 ± 131.74 µM: diameter of control for FTY720, 1699 ± 76 µM; FTY720, 1622.7 ± 133 µM).

Collectively, these results indicate that CDK1 in fiber cell nuclei interacts with an inhibiting kinase, WEE1, and an activating phosphatase, CDC25, in the region where CDK1 activity is exquisitely regulated to foster LFCD. The activating phosphatase and inhibiting kinase are themselves regulated by PP2A, and thus LFCD is regulated by PP2A activity. Furthermore, these data support a fine-tuned spatiotemporal regulation of CDK1 with positive and negative regulators of CDK1 expressed in the lens fiber cells immediately prior to denucleation.

p27 Regulates CDK1 in Postproliferative Developing Fibers and Inhibits LFCD

In mitosis, p27 inhibits CDK1 by direct binding and thus functions in a regulatory capacity different from WEE1 and CDC25. Increased levels of p27 have been correlated with impaired LFCD in several mouse models of cataract, but direct evidence for a mechanistic connection between p27 and LFCD is lacking.[33,34] Thus, we hypothesized that increased p27 would directly interfere with LFCD. We tested this hypothesis using three different mouse models.

To begin, we determined whether transgenic overexpression of p27 interferes with efficient LFCD using an inducible

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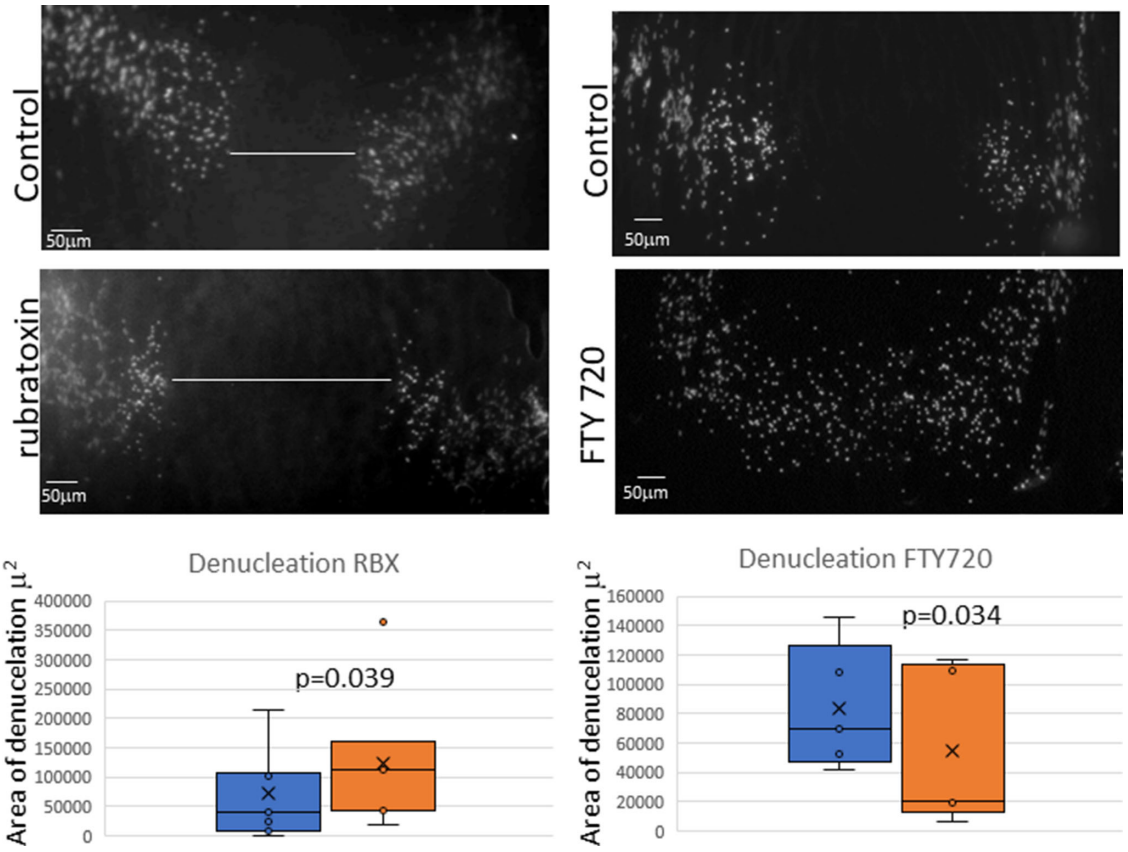

Figure 6. Inhibition of PP2A potentiates lens fiber cell denucleation, while activation of PP2A inhibits denucleation. (**A**, **B**) E12 chick lenses were placed into culture in the presence and absence of drugs for 3 days as noted. (**A**) 2 µM rubratoxin, $n = 7$. (**B**) 2.5 µM FTY720, $n = 5$. Full-size images of lens sections and data normalized to lens diameter are shown in Supplementary Fig. S6.

p27 transgene. Mice were treated with doxycycline at E15.5 or E18.5 of gestation, and offspring were examined at P1. Mice not exposed to doxycycline showed a clear organelle-free zone upon birth (Fig. 7A, left panels). In comparison, induction of p27 for 2 days from E18.5 to birth showed increased p27 levels and retained nuclei in the putative organelle-free zone (Fig. 7A, right panels). These findings indicate that increased expression of p27 impairs denucleation. Lenses in mice that were treated for 5 days (from day E15.5 to birth) showed even more p27 expression and also exhibited impaired LFCD (Supplementary Fig. S7A). Lenses from mice expressing p27 for 2 days, were ~35% smaller than WT lenses, and lenses from mice expressing p27 for 5 days were ~60% smaller. Confirming a p27-CDK1 block of cell proliferation lenses from these mice decreased staining of the proliferation marker Ki67 (Fig. 7B, 1Supplementary Fig. S7B).

An anticipated consequence of inhibition of CDK1 by increased levels of p27 is decreased phosphorylation of lamin A associated with retained nuclear membranes and impaired LFCD. As predicted, overexpression of p27 decreased the level of phosphorylated lamin A in the lens (Fig. 7C, upper panels). This was associated with retention of nuclei (Fig. 7C, lower panels). Staining of p27 was observed in the control epithelium (arrows in 7C) and in fiber cells when fluorescence intensity was increased (Supplementary Fig. S7C).

Degradation of DNA, among the final steps in LFCD, is accomplished by a lens-specific enzyme, DLAD/DNAse IIβ,[8] that digests genomic material following nuclear envelope breakdown. In control mice, DLAD/DNAse IIβ was allowed into the nucleus in the center of the lens (Fig. 7D, left panel, inset 2) but excluded from peripheral nuclei (Fig. 7D, left panel, inset 1). Consistent with retained nuclear membranes in lenses with elevated p27, DLAD/DNAse IIβ was excluded from central nuclei from p27 overexpressing mice as it was in outer zones of WT mice (Fig. 7D, right panel, see insets 1, 2).[16,33,34] This recapitulates observations when CDK1 is absent.[16]

p27 levels are regulated in part by ubiquitination with subsequent degradation by the proteasome. The ubiquitin ligase complex SCF^{Skp2} utilizes a substrate binding subunit called the F-box to ubiquitinate a number of cell cycle regulatory molecules, including p27.[46–49] We examined the lenses from embryos from which the F-box substrate targeting subunit *Skp2* was deleted. As expected, lenses from *Skp2*$^{-/-}$ showed increased levels of p27 (Supplementary Fig. S8). Lenses from *Skp2*$^{-/-}$ embryos were ~50% smaller than the WT lenses (Fig. 8 compare A1 to B1), consistent with a regulatory role in epithelial cell proliferation and the decreased growth phenotype of the mice.[36] Nevertheless, the overall lens structure was preserved. There was a single layer of epithelia, a discernable bow region and a posterior region free of epithelial cells. The equatorial (region 2) and anterior (region 3) epithelium regions appeared normal in these knockout mice. However, *Skp2*$^{-/-}$ embryos showed retained cell nuclei in the presumptive LFCD organelle-free zone (region 4). These findings indicate that *Skp2* deletion has a major regulatory effect on LFCD but perhaps a limited effect on overall lens formation.

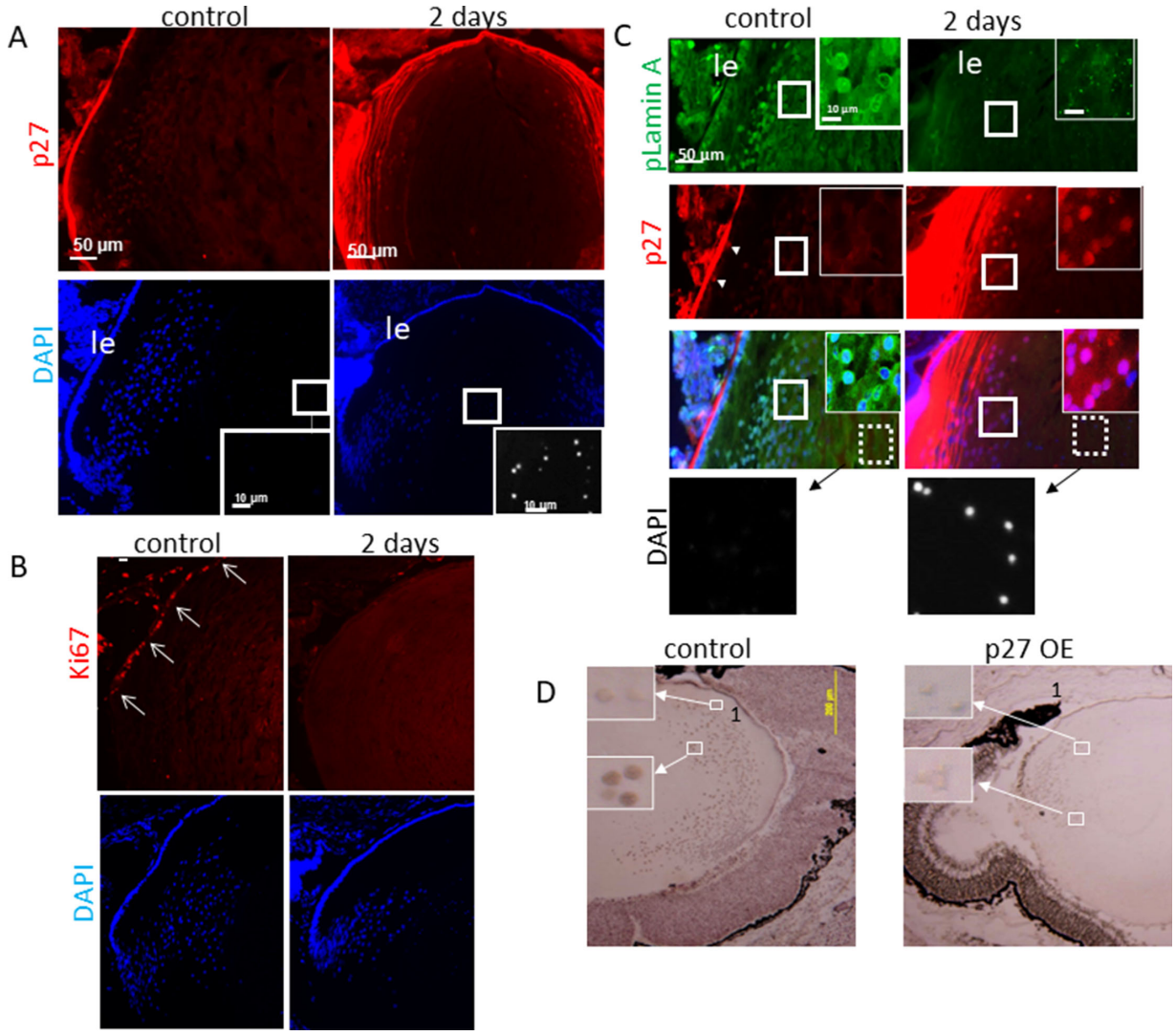

Figure 7. Expression of an inducible p27 transgene impairs lens fiber cell denucleation. Doxycycline-responsive p27 transgene was induced during gestation for 0, 2, or 5 days. Offspring were analyzed at P1. Le, lens epithelium; lc, lens cortex. (**A**) Lens sections were analyzed for p27 (*red*, *upper panels*) and DNA/DAPI (*lower panels*). (**B**) Staining of Ki67^{+} cells in lens epithelium in control (*left*) and 2-day induction (*right*). (**C**) P1 lenses from control (*left*) or 2-day p27 overexpresser mice (*right*) were stained for phospho–lamin A (*green*), p27 (*red*), and DNA/DAPI as indicated. *Arrowheads* show p27^{+} nuclei in control epithelium. *Lower panels* show DNA/DAPI staining in the center of the lens, broken line in panel above. (**D**) P1 lenses from control (*left*) and p27 two-day overexpresser mice (*right*) were stained for DLAD/DNase IIβ. *Insets* show peripheral (1) and central (2) nuclei.

A requirement for SCF^{Skp2}-catalyzed ubiquitination and degradation of p27 is phosphorylation at Thr187 of p27. We also examined lenses from embryos with a homozygous p27^{T187A} mutation that precludes T187 phosphorylation (T187AA[50]). Similar to *Skp2*$^{-/-}$, lenses from embryos expressing this degradation incompetent mutant p27 showed increased levels of p27 (Supplementary Fig. S8), and nuclei were retained in the center of the lens (Fig. 8C, region 4). Additionally, the lenses were ~50% smaller than the WT lenses. As with the *Skp2*$^{-/-}$ lenses, the equatorial (Fig. 8C, region 2) and anterior epithelium (region 3) of the T187AA lenses appeared morphologically normal, indicating that *Skp2*-dependent targeting of p27 does not affect the overall development of the lens. These results indicate that inhibiting the degradation of p27 inhibits the activity of the essential regulatory molecule CDK1 and impairs LFCD.

Discussion

The function of the lens is to focus light on the retina. Lens clarity is established and maintained by the expression and organization of high levels of crystallin proteins in the lens fibers along with the programmed removal of organelles in the central region. Failure to remove organelles is correlated with cataracts in animal models as well as in humans.[5,14,34,51] Previous work indicated that the mitotic kinase CDK1 has been adapted to initiate LFCD.[16] Here we asked if other processes that affect CDK1 activity in mitosis are also involved in LFCD in evolutionarily distant vertebrates. We demonstrate that a mitotic network including p27, WEE1, CDC25, and PP2A regulates CDK1 activity in the lens and tightly controls the process of LFCD in both the mouse and chick. This research establishes new biological roles for these widely expressed regulatory enzymes.

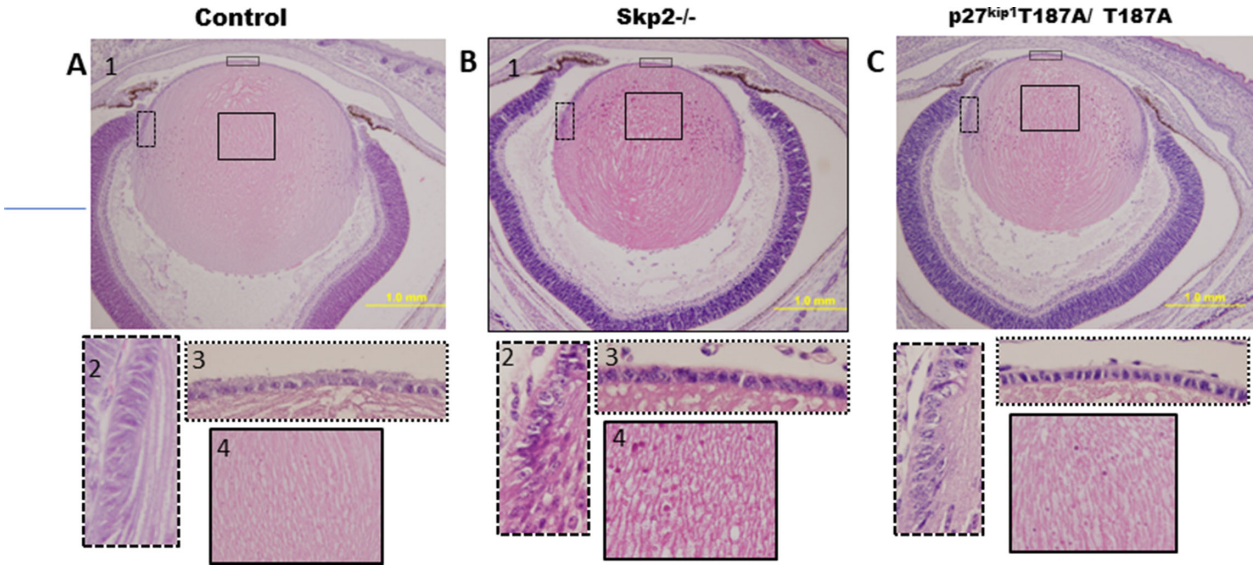

Figure 8. Impaired degradation of p27 leads to inefficient denucleation. E18.5 embryos from (**A**) WT, (**B**) *Skp2*$^{-/-}$, and (**C**) p27 T187AA knock-in mice were examined by hematoxylin and eosin. (1) Whole lens, (2) equatorial epithelium, (3) anterior epithelium, and (4) central fibers. Three individual embryos of each genotype were analyzed with similar results.

First, we observed that as in the mouse, CDK1 activity is upregulated in chick lens development. Furthermore, CDK1 was expressed in fiber cell nuclei in the chick until denucleation, and two inhibitors of CDK activity impaired LFCD in embryonic chick lenses. These data indicate that CDK1 activity is a master regulator of LFCD in both the mouse and chick.

CDK1 activity was controlled by hierarchical and counterbalanced phosphorylation and dephosphorylation activities of WEE1/MYT1 and CDC25 (Fig. 1). The colocalization of CDK1 with inhibitor WEE1 and activator CDC25B in nuclei just before nuclear envelope breakdown suggested that the activity of CDK1 was tightly regulated by these molecules in nuclei just before LFCD. We corroborated this pharmacologically: treatment of lens with drugs that inhibited the CDC25 family of phosphatases impaired LFCD, indicating that CDC25 activity is essential for activation of CDK1 during LFCD. While the inhibitors were active against all family members, the colocalization of CDC25B with CDK1 and prior literature indicating a role for CDC25B specifically in lens function suggest that CDC25B is the relevant phosphatase in LFCD.[25,26] It would be of interest to determine if there is a denucleation defect in the CDC25B$^{-/-}$ animals, although there are no reports of ocular defects. Inhibition of WEE1 activity with adavosertib potentiated LFCD, indicating that CDK1 activity is suppressed by WEE1 during lens development.

In mitotic pathways, the activities of WEE1 and CDC25 are mechanistically linked via the PP2A phosphatase, which inhibits CDC25 and activates WEE1. As expected, inhibition of PP2A with rubratoxin A potentiated LFCD, while activation of PP2A with FTY720 inhibited LFCD. Although PP2A inhibits CDK1 activation and thus nuclear envelope breakdown, we cannot rule out whether other pathways regulated by PP2A are also contributing to the defect in LFCD we observe after FTY720 treatment.

In addition to regulating LFCD, CDK1 regulated lens epithelial cell division, which might also affect lens size.[16] However, we did not observe a difference in lens size between treatment and control during the lens culture. This may not be unexpected as we did not observe alterations in lens size in the lens-specific *CDK1* knockout mice.[16] Changes in the lens mass during the culture period are primarily due to postmitotic fiber cell differentiation and increases in cell size due to the significant upregulation of crystallin expression[52] and both intrinsic and extrinsic factors,[53] including compaction.[54] We cannot rule out whether inhibition of CDK1 activity in the epithelium is indirectly affecting fiber cell differentiation, and this would be interesting to explore.

Previously, by perturbing the ubiquitin proteasome system by expression of a mutant ubiquitin in the lens, we observed increased levels of the CDK inhibitor p27 correlated with impaired LFCD, and this was corroborated in multiple mouse models of cataract.[33,34] Here in addition to directly showing that increased levels of p27 impair LFCD, we demonstrate that retarding degradation of p27 also results in decreased LFCD. These data indicate that increased p27 levels directly inhibit LFCD. In confirmation of these findings, DLAD/DNAse IIβ, which usually enters the disassembled nuclei to degrade chromatin, is excluded from nuclei in lenses with increased levels of p27. Together, these observations suggest a CDK1-regulated LFCD process comparable to that which regulates the cell cycle (Fig. 1).

While organelle removal including the mitochondria, ER, and nucleus can be accelerated by stimulating autophagy,[2,3] mice deficient in autophagy proteins are not defective in lens organelle remova[6,7]; thus, the role of autophagy in these processes remains to be explored further. Elimination of ER, Golgi, and mitochondria can be separated from nuclear removal as mouse models with deletions of BNIP3L/NIX or SNFH2 show impaired removal of one organelle but not the other.[4,55] Our data indicate that mitotic regulators are controlling nuclear removal during lens differentiation, and we did not observe any alterations in the removal of ER and mitochondria upon CDK1 knockout, further confirming removal of these organelles are controlled by different pathways.[16]

Evidence indicates that lysosomal activities are required for organelle removal. Lysosomes are a source of DLAD/DNAse IIβ to digest DNA that remains after

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the nuclear membrane breakdown. Additionally, lysosomal phospholipases are critical for organelle removal in zebrafish and mouse models.[5] Whereas deletion of *Plaat1* in zebrafish impaired the degradation of nonnuclear organelles, deletion of the homologous phospholipase *PLAAT3* in the mouse impaired removal of organelles, including nuclei. The authors suggest that the activity of Plaat1/PLAAT3 is involved in lysosomal membrane breakdown, and the lack of sufficient DLAD/DNAse IIβ might be the cause of impaired LFCD in these mice.[5] However, initiation of LFCD requires that the nuclear membrane be permeabilized prior to access of nuclear material to DLAD/DNAse IIβ. Thus, there must be upstream regulators of nuclear membrane disassembly. We postulate that phosphorylation of nuclear lamins by CDK1 is responsible for permeabilization of the nuclear membrane, perhaps priming it for further destruction by phospholipases.

Nonmitotic nuclear envelope breakdown has been observed in other tissues. Keratinocytes and erythroid progenitor cells both remove their nuclei during differentiation.[56] However, the mechanisms by which they remove nuclei are distinct, relying on different kinases to direct nuclear disassembly, and thus they are examples of convergent evolution. Neutrophils use the process of NETosis, during which part of the nucleus breaks off and is extruded from the cell, as a means to destroy pathogens and to remodel vasculature.[57,58] NETosis is dependent on CDK4/6 and is regulated by the CDK inhibitor p21.[59] Furthermore, there is disassembly of cytoskeletal networks and remodeling of nuclear lamins in NETosis as in mitosis and LFCD.[58] Thus, our work provides further elucidation of nonmitotic nuclear envelope breakdown involving a mitotic regulatory network.

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