Control of mitotic exit by PP2A regulation of Cdc25C and Cdk1

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Inactivation of maturation-promoting factor [(MPF) Cdk1/Cyclin B] is a key event in the exit from mitosis. Although degradation of Cyclin B is important for MPF inactivation, recent studies indicate that Cdk1 phosphorylation and inactivation occur before Cyclin B degradation and, therefore, also may be important steps in the exit from mitosis. Cdk1 activity is controlled by the Cdc25C phosphatase, which is turned on at the G_2/M transition to catalyze Cdk1 activation. PP2A:B56δ is a negative regulator of Cdc25C during interphase. We show here that PP2A:B56δ also regulates Cdc25C at mitosis. Failure of PP2A:B56δ to dephosphorylate Cdc25C at mitosis results in prolonged hyperphosphorylation and activation of Cdc25C, causing persistent dephosphorylation and, hence, activation of Cdk1. This constitutive activation of Cdc25C and Cdk1 leads to a delayed exit from mitosis. Consistent with Cdk1 as a major biological target of B56δ, stable knockdown and germ-line mouse KO of B56δ leads to compensatory transcriptional up-regulation of Wee1 kinase to oppose the Cdc25C activity and permit cell survival. These observations place PP2A:B56δ as a key upstream regulator of Cdk1 activity upon exit from mitosis.

cell cycle | cyclins | protein phosphatase | knockout | Wee1

In the process of mitosis, cells divide their duplicated genetic material equally between two daughter cells in a complex and tightly orchestrated series of events. For cells to enter into mitosis, the cyclin-dependent kinase Cdk1 must be activated. During interphase, Cdk1(Cdc2) is repressed by inhibitory phosphorylation at Thr-14 and Tyr-15, catalyzed by the Myt1 and Wee1 kinases (1, 2). At the G_2/M transition, these phosphoryl groups are removed by the hyperphosphorylated, active form of the phosphatase Cdc25C, thus activating Cdk1/Cyclin B, also known as maturation-promoting factor (MPF) (3). The exit from mitosis requires the inactivation of Cdk1/Cyclin B by APC^{Cdc20}-targeted proteasomal degradation of Cyclin B (4, 5), which is completed during the metaphase–anaphase transition (6). Failure to inactivate MPF and degrade Cyclin B results in the maintenance of condensed mitotic chromosomes and a metaphase arrest (7–9).

Recent evidence suggests that the degradation of cyclin B may not be the only mechanism to inactivate MPF at the end of mitosis. Inhibitory phosphorylation of Cdk1 at Tyr-15 occurs transiently during the exit from mitosis and correlates with a decrease in Cdk1/Cyclin B kinase activity before Cyclin B disappearance (10). This mitotic phosphorylation of Cdk1 Tyr-15 may actually be required for cyclin B degradation (10). A nonphosphorylatable form of Cdk1 both shortens interphase and prolongs mitosis in *Xenopus* egg extracts (11). Rephosphorylation of Cdk1 is probably due to loss of Cdc25C activity because this phosphatase returns to its hypophosphorylated, inactive interphase form as Cdk1 associates with Wee1. This finding suggests that inactivation of Cdc25C is a key upstream event in MPF inactivation in the exit from mitosis. How Cdc25C is dephosphorylated in mitosis to return to its inactive, interphase form is an unanswered question (12).

A role for PP2A in mitosis has long been suspected because MPF (Cdk1/Cyclin B) inactivation depends on an okadaic acid-sensitive phosphatase in the metaphase–anaphase transition (13). We previously showed that heterotrimeric protein phosphatase 2A, containing a B56δ targeting subunit (PP2A:B56δ), is a negative regulator of Cdc25C during interphase. PP2A:B56δ dephosphorylates Cdc25C Thr-130, thereby allowing 14–3-3 binding to Cdc25C phosphorylated on Ser-216 and cytosolic sequestration of Cdc25C (14).

We report here that PP2A:B56δ has a second role in addition to its maintenance of 14–3-3 binding to Cdc25C during interphase. Unexpectedly, given that PP2A:B56δ had a role in the response to DNA damage events in interphase, we found that B56δ strongly interacts with Cdc25C at the M phase. We also find that PP2A:B56δ regulates the activity of Cdc25C during mitosis, leading to the inactivation of MPF. Stable knockdown (KD) of B56δ in dividing cells is sufficient to cause a delay in the exit from mitosis. This failure to exit from mitosis is accompanied by prolonged hyperphosphorylation of Cdc25C and dephosphorylation of Cdk1 Tyr-15. Cyclin B1 degradation appears to be delayed in these cells as well. Although these events might appear incompatible with proliferation, remarkably B56δ KD cells and B56δ KO mice compensate well for the increased Cdc25C phosphatase activity that results from B56δ KD by transcriptional up-regulation of the Wee1 kinase. These findings support the importance of phosphorylation-regulated inhibition of Cdk1 during mitosis and identify B56δ-containing PP2A as a critical upstream regulator of Cdk1.

Results

KD of the Serine–Threonine Protein Phosphatase PP2A:B56δ Leads to Activation of Cdc25C in Human Cells. Prior work demonstrated that B56δ is the only member of the B56 family of PP2A-targeting subunits that binds to Cdc25C in human cells *in vivo* (14). This binding controls phosphorylation of a critical inhibitory site on Cdc25C, Threonine 130 (Thr-130). Thr-130 phosphorylation causes the release of 14–3-3, thereby allowing Cdc25C to increase specific activity and move to the nucleus (15, 16). To extend observations made in *Xenopus*, we examined whether B56δ regulates Cdc25C Thr-130 dephosphorylation in response to aphidicolin-induced checkpoint activation in mammalian cells. A short-hairpin-containing retrovirus, pMKO.1 B56δ (a gift from William Hahn, Dana–Farber Cancer Institute, Boston), was used to create HEK293 cells with stable KD of B56δ (B56δ KD) [see supporting information (SI) Figs. 5 and 6]. These cells, lacking ≈90% of B56δ protein, still proliferated and doubled only slightly slower than control cells,

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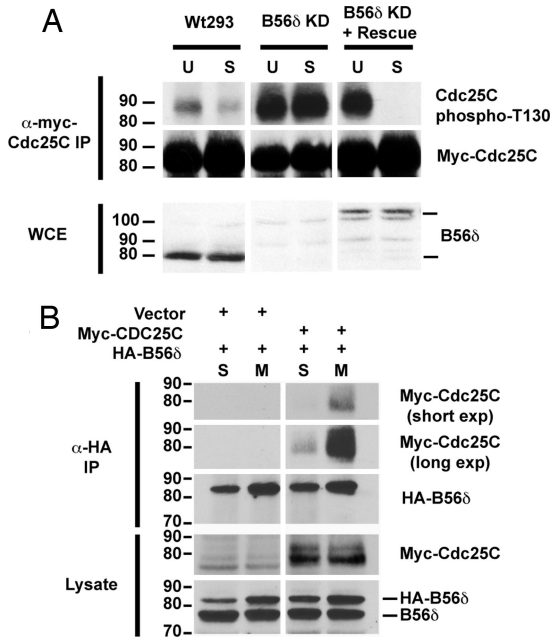

Fig. 1. PP2A:B56δ interacts physically and functionally with Cdc25C. (*A*) B56δ KD results in hyperphosphorylation of Cdc25C Thr-130. WT HEK293 and clonal B56δ KD cells were cotransfected with plasmids encoding 6XMyc-Cdc25C and short-hairpin-resistant GFP-B56δ (pEGFP-B56δ) or empty vector. Cells were synchronized to the S phase (4 μM aphidicolin for 16 h) or treated with vehicle alone (U, unsynchronized). Cdc25C was immunoprecipitated with 9E10 antibody. Immunoprecipitates were analyzed by SDS/PAGE and by immunoblotting with anti-phosphoT130 Cdc25C antibody, anti-myc (9E10), and anti-B56δ as indicated. WCE, whole cell extract. (*B*) B56δ interacts with Cdc25C at the M phase. Epitope-tagged B56δ and Cdc25C were coexpressed as indicated in HEK293 cells. The next day, cells were synchronized to the S phase (4 μM aphidicolin for 16 h) or to the M phase (100 ng/ml nocodazole for 18 h) and lysed. B56δ was immunoprecipitated with anti-HA antibody, and immunoprecipitates were analyzed by SDS/PAGE and immunoblotting with anti-myc and anti-B56δ antibodies.

indicating that B56δ is not essential, that low levels are sufficient, or that there is efficient compensation for B56δ loss. Cells stably maintained KD of B56δ in the log, S, and M phases (SI Fig. 7). As predicted from studies using *Xenopus* egg extracts, induction of an S-phase checkpoint decreases Cdc25C Thr-130 phosphorylation, compared with unsynchronized cells in WT HEK293 cells (Fig. 1*A*). Stable KD of B56δ alone was sufficient to constitutively activate Cdc25C because myc-tagged-Cdc25C immunoprecipitated from both unsynchronized and S-phase checkpoint-induced B56δ KD cells show a marked increase of phosphorylation on Thr-130 (Fig. 1*A*). The checkpoint-induced hyperphosphorylation of Thr-130 is not an off-target effect of the short-hairpin expression because it can be rescued by the expression of a short-hairpin-resistant cDNA encoding a GFP-B56δ fusion protein (Fig. 1*A*). Therefore, B56δ is required to regulate Cdc25C activation in human cells. Because all other members of the B56 family are expressed in these cells (N. McAllister and D.M.V., unpublished data), we also conclude that the loss of B56δ cannot be compensated by endogenous levels of other B56 family members.

PP2A:B56δ Unexpectedly Interacts with Cdc25C at the M Phase. Regulation of Cdc25C activation occurs in interphase checkpoints, but also is crucial to both the entry into as well as the exit from mitosis (10, 17). Phosphorylation of Cdc25C on Thr-130 leads to the release of its interphase repressor, 14–3-3 (16). Therefore, the phosphatase that targets Thr-130 could regulate the catalytic activity of Cdc25C toward Cdk1. Protein phosphatase 2A is specifically targeted by its regulatory subunit B56δ to bind to Cdc25C in unsynchronized cells (14). If PP2A is targeted to Cdc25C to dephosphorylate Thr-130 solely to prevent precocious activation in interphase, we speculated that binding might be confined to the S phase. Unexpectedly, and in contrast to results seen in extracts from *Xenopus* eggs undergoing embryonic cell cycles (14), B56δ interacts with Cdc25C more robustly in the nocodazole-synchronized M-phase human HEK293 cells than in the aphidicolin-synchronized S-phase cells (Fig. 1*B*).

KD of B56δ Leads to a Delay in the Exit from Mitosis. Inactivation of Cdk1/Cyclin B is required to exit from mitosis. Because phosphorylation of Cdk1 precedes degradation of Cyclin B in the exit from mitosis, this finding suggests that the Cdk1 phosphatase, Cdc25C, may need to be inactivated during mitosis. B56δ-targeted PP2A has already been shown to inactivate Cdc25C during the S phase. Based on our finding that B56δ interacts with Cdc25C at the M phase, we asked whether PP2A:B56δ is important in transit through mitosis. Progression from mitosis into G_1 was examined by using a nocodazole release assay (18). WT HEK293 and B56δ KD cells were synchronized with nocodazole, and then nocodazole was removed and cells analyzed at various time points by flow cytometry. WT HEK293 cells rapidly exit from mitosis, whereas B56δ KD cells were significantly delayed in progression to the G_1 phase (Fig. 2*A*). To confirm that this result was indeed a delay in mitosis, in a separate experiment, cells were stained for phosphohistone H3 at various time points after release. Histone H3 is phosphorylated when Cdk1 is active during mitosis (19). The normal postmitotic decrease in phospho-Ser-10 histone H3 was significantly delayed in the B56δ KD cells (Fig. 2*B*), suggesting prolonged activation of Cdk1. This delay could not be attributed to clonal selection or off-target effects of the integrated short hairpin in the KD cells because the expression of a short-hairpin-resistant B56δ-GFP fusion protein rescued the defect in dephosphorylation of histone H3 (Fig. 2*B*). A similar delay in transit through mitosis was observed when cells were synchronized in the S phase with a double thymidine block (data not shown), indicating that the delay was not a unique feature of inducing a spindle assembly checkpoint with nocodazole. Thus, B56δ activity is required for timely exit from mitosis.

B56δ Is Required for the Inactivation of Cdc25C and Cdk1 at Mitotic Exit. To biochemically examine the signaling events involved in the delayed mitotic exit of B56δ KD cells, lysates were prepared from cells at the time points as in Fig. 2. Endogenous Cdc25C is hyperphosphorylated when active at the time of entry to mitosis (Fig. 3*A*) (20). This hyperphosphorylation of Cdc25C at the aminoterminus (including, but not limited to, phosphorylation of Thr-130) causes a marked electrophoretic mobility shift and is a well established marker of Cdc25C activation (21). WT HEK293 cells exit mitosis within 2–4 h, concurrent with the loss of hyperphosphorylation of Cdc25C and return to its 60-kDa interphase, inactive form (Fig. 3*A*). However, B56δ KD cells that exit mitosis slowly also show a slow dephosphorylation of Cdc25C, with persistent Cdc25C activation at 4 and 6 h after nocodazole release. These findings are consistent with the model that B56δ is required for mitotic inactivation of Cdc25C.

Because the major function of Cdc25C is dephosphorylation and activation of Cdk1, we next asked whether the cells 4 and 6 h after nocodazole release had active Cdk1. In WT cells, Cdk1phosphorylation is present at 4 and 6 h after release and is followed by cyclin B degradation (Fig. 3*B*). In the B56δ KD cells, Cdk1 phosphorylation remains decreased by >50% at 4–6 h and cyclin B1 is still present, indicating persistent activity of MPF. This persistent Cdk1 activity is consistent with the persistence of histone H3 phosphorylation at similar time points (Fig. 2*B*).

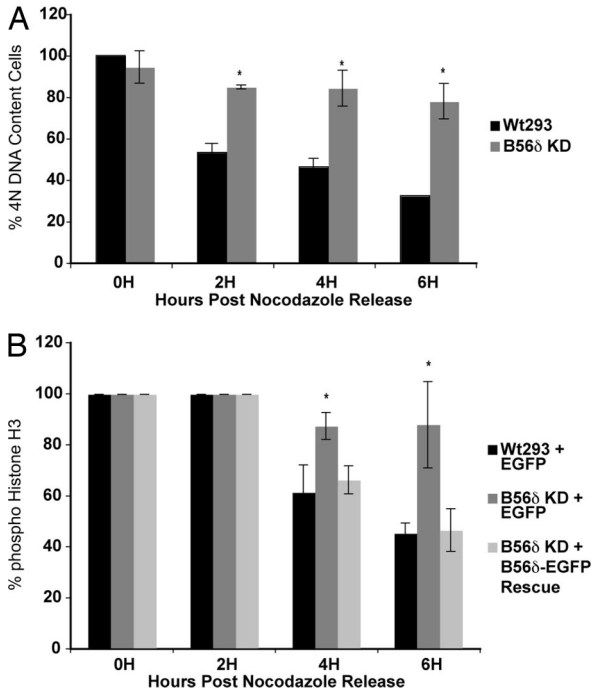

Fig. 2. KD of B56δ leads to delay in the exit from mitosis. (*A*) B56δ KD cells delay the transition from G_2/M to G_1 DNA content. WT HEK293 and stable B56δ KD cells were synchronized to the M phase with 18-h nocodazole incubation. Cells were washed three times with PBS, replated, and harvested every 2 h, and DNA content was analyzed by flow cytometry. Statistical significance was analyzed by using a χ^2 distribution test. The difference in 4N DNA population was statistically significant at 2 h ($P = 1.15 \times 10^{-6}$), 4 h ($P = 4.66 \times 10^{-9}$), and 6 h ($P = 2.60 \times 10^{-13}$) after nocodazole release, but not at 0 h ($P = 0.242$). (*B*) B56δ KD cells retain Cdk1-dependent histone H3 phosphorylation. To monitor for a delay in the exit from mitosis independent of 4N DNA content, phosphohistone H3 was assessed by flow cytometry in eGFP-expressing cells. Either short-hairpin-immune EGFP-B56δ or farnesylated EGFP (control) were expressed as indicated. B56δ KD cells lost phosphohistone H3 significantly slower than did WT HEK293 cells at 4 h ($P = 5.27 \times 10^{-5}$) and 6 h after nocodazole release ($P = 9.87 \times 10^{-11}$), and this effect was reversed by reexpression of EGFP-B56δ.

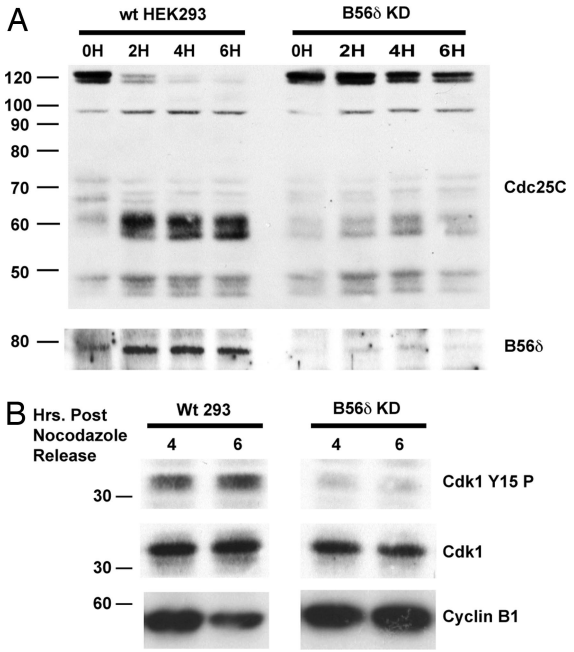

Fig. 3. Loss of B56δ leads to constitutive activation of Cdc25C after mitotic synchronization. (*A*) Mitotic hyperphosphorylation of Cdc25C persists in B56δ KD cells. WT HEK293 and B56δ KD cells were synchronized with nocodazole as in Fig. 2 and released. Lysates were prepared as described in Fig. 2*A* and analyzed by SDS/PAGE. Cdc25C and B56δ were probed by using anti-Cdc25C (TC-113/Ab2359; Abcam) and polyclonal rabbit anti-B56δ, respectively. (*B*) Delayed rephosphorylation of Cdk1 in B56δ KD cells. Lysates were analyzed as above and probed for Cdk1 Y15 phosphorylation (IMG-668; Imgenex), total Cdk1 (anti-PSTAIRE; Sigma–Aldrich), and Cyclin B1 (Ab7957–1; Abcam). All lanes were analyzed on the same blot and separated simply for clarity of presentation. Relative phosphorylation was quantitated by densitometry using ImageJ.

Thus, loss of B56δ activates Cdc25C to dephosphorylate and activate Cdk1.

Activation of Cdc25C by B56δ KD Is Compensated by Wee1 Up-Regulation. The first evidence for a role for B56δ in the regulation of Cdc25C and Cdk1 activity came from studies in *Xenopus* egg extracts, where biochemical changes cannot be compensated for by transcriptional changes (14). Stable KD of B56δ also results in activated Cdc25C in dividing mammalian cells (Fig. 1*B*). In the absence of secondary events, activated Cdc25C should cause constitutive activation of Cdk1. However, unregulated Cdk1 activity in interphase causes mitotic catastrophe and caspase-2-mediated apoptosis (22). How then do the B56δ KD cells continue to grow and divide? When we examined the phosphorylation state of Cdk1 in aphidicolin-arrested S-phase (Fig. 4*A*) and log-phase (Fig. 4*B*) B56δ KD cells, we unexpectedly found it to be highly phosphorylated, rather than dephosphorylated. Cdk1 is phosphorylated on Y15 by the Wee1 kinase, which coordinates cell-cycle timing and prevents inappropriate entry into mitosis (23). The difference in Cdk1 Y15 phosphorylation between WT and KD cells is greater in unsynchronized (Fig. 4*B*) than in aphidicolin-arrested (Fig. 4*A*) cells because of the described induction of Wee1 activity after aphidicolin-induced checkpoint activation (24). More important, the enhanced phosphorylation of Cdk1 Y15 in B56δ KD cells, despite activation of Cdc25C, appears to result from a compensatory increase in Wee1 protein abundance (Fig. 4*B*). The increase in Wee1 abundance appears to be an interphase-specific regulation because Wee1 is still degraded appropriately at the M phase in the B56δ KD cells (SI Fig. 8) (25, 26). The increase in Wee1 protein is due to the B56δ KD because it can be rescued by expression of the short-hairpin immune form of B56δ. Thus, cells appear to up-regulate Wee1 protein to compensate for B56δ KD.

Two potential mechanisms for this B56δ-associated increase in Wee1 protein abundance were considered: decreased Wee1 destruction or increased Wee1 production. The half-life of Wee1 was unchanged in B56δ KD cells (SI Fig. 9), whereas quantitative PCR showed an ≈10-fold increase in Wee1 mRNA (Fig. 4*C*). This up-regulation of Wee1 strongly supports the model that loss of B56δ acts through Cdc25C to increase Cdk1 activity, and that cells survive this activity only by an increase in Wee1 activity. As a final test of the importance of Wee1 in compensating for B56δ loss, we examined Wee1 abundance in mouse embryo fibroblasts (MEFs) from mice homozygous-null for B56δ. These mice have no overt phenotype and grow equally as well as WT littermates (E. Martens, J.V.L., and J.G., unpublished data). B56δ$^{-/-}$ MEFs have significantly elevated Wee1 protein and Wee1 mRNA (Fig. 4 *D* and *E*), compared with WT MEFs. This genetic evidence strongly supports the hypothesis that Cdk1 is a critical downstream target of B56δ in both HEK293 cells and murine fibroblasts, and that Wee1 transcriptional up-regulation compensates for loss of B56δ-dependent inactivation of Cdc25C.

Discussion

To exit mitosis, cells must inactivate MPF. Although this process is primarily accomplished by proteolysis of Cyclin B, it also may

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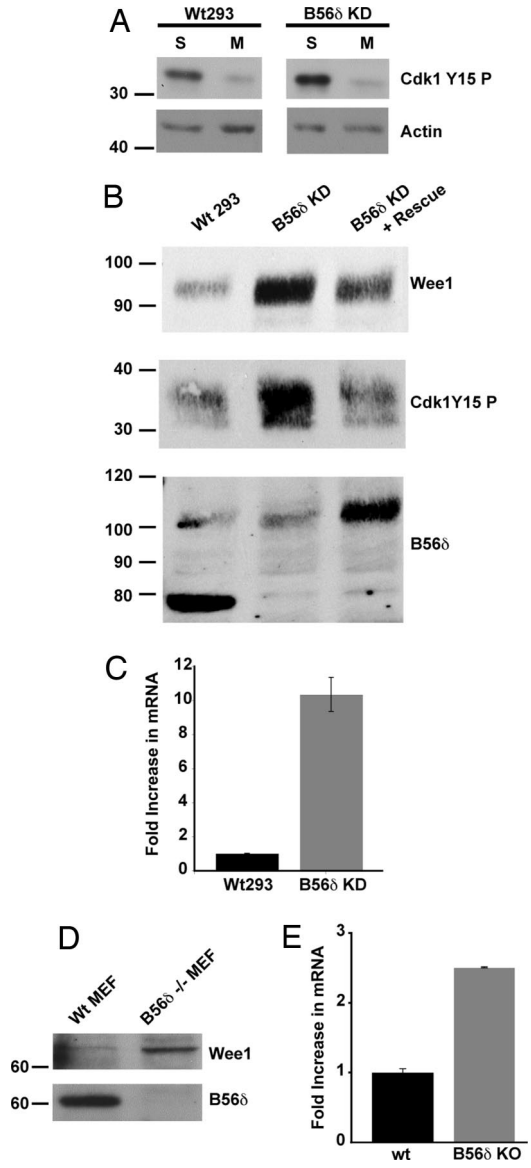

Fig. 4. Mammalian cells protect against constitutive Cdk1 activation in G_1/S by up-regulating Wee1 kinase. (*A*) Compensatory phosphorylation of Cdk1 Y15 in S-phase cells. Despite the KD of B56δ, Cdk1 is still phosphorylated in the S phase. Cells were synchronized to the S and M phases as in Fig. 1. The blot was probed with anti-Cdk1 phospho-Y15 and anti-Actin. (*B*) Up-regulation of Wee1 protein in B56δ KD cells. Interphase WT HEK293, B56δ KD, and B56δ KD + B56δ-GFP rescue cell lines were lysed and analyzed by SDS/PAGE and immunoblotting with the indicated antibodies. (*C*) Up-regulation of Wee1 mRNA in B56δ KD cells. Wee1 mRNA was quantitated in lysates from log-phase WT HEK293 and B56δ KD cells. Quantitative RT-PCR was used to determine the amount of reverse-transcribed mRNA. Wee1 message was normalized to GAPDH mRNA in cell lysates. (*D*) Up-regulation of Wee1 protein in B56$\delta^{-/-}$ MEFs. WT HEK293 MEF and B56$\delta^{-/-}$ MEF cells were lysed and analyzed by SDS/PAGE and immunoblotting with the indicated antibodies. B56δ was detected with anti-PR61δ that recognizes mouse B56δ (47). (*E*) Up-regulation of Wee1 mRNA in B56$\delta^{-/-}$ MEFs. Wee1 mRNA was quantitated as in *C*.

be accomplished by inhibitory rephosphorylation of Cdk1. The data here support a model in which PP2A:B56δ plays a key role in the exit from mitosis. After the onset of the M phase, B56δ binds to and inactivates Cdc25C by dephosphorylation of sites critical for its mitotic function. This step leads to increased phosphorylation and, hence, inhibition of Cdk1 before Cyclin B degradation. KD of B56δ has the opposite effect, causing increased activity of both Cdc25C and Cdk1, and resulting in prolonged histone H3 phosphorylation and a delay in the exit from mitosis. Cells and animals can compensate for this hyperactive Cdk1 by transcriptional up-regulation of Wee1, the kinase that inactivates Cdk1. Therefore, these data place PP2A:B56δ as an upstream regulator of mitosis that turns off Cdk1 during mitosis before degradation of Cyclin B1.

PP2A is targeted to diverse substrates through its various B subunits (27, 28). The B56 family, in particular, has been implicated in the tumor-suppressor properties of PP2A, and SV40 small *t* antigen displaces B and B56 subunits to activate key signaling pathways, including the PI3K and RalA pathways and c-myc phosphorylation (29, 30, 48). The B56 family is required for cell survival because KD of both family members in *Drosophila* leads to apoptosis (31, 32). The yeast and mammalian B56 genes play a role in maintaining the stability of cohesin at centromeres and chiasmata (33, 34). Shugoshin protects cohesin from the cleavage by separase in both meiosis and mitosis. Recruitment of PP2A:B56 by shugoshin leads to the protection of cohesin degradation and prophase chromosome dissociation (34). In these studies, similar to studies on the involvement of B56 with adenomatous polyposis coli protein (35), all B56 subunits interacted equally well with shugoshin and appear to be functionally redundant. Thus, B56δ KD alone is not expected to have any effect on cohesin degradation and chromosome segregation nor on the Wnt/β-catenin signaling pathway.

Our study indicates that PP2A, through its B56δ subunit, regulates Cdc25C activity and, hence, the exit from mitosis (12). B56δ is subject to regulation by changes in both intracellular localization (36) and phosphorylation (14, 37, 38). B56δ localization could also play a role in regulating Cdc25C activity. Cdc25C is cytosolic in interphase because of 14–3-3 binding and nuclear at the M phase (15) after Cdk2/Cyclin A phosphorylates Thr-130 to release 14–3-3 (16). What regulates B56δ localization is unknown, but phosphorylation on specific sites has been proposed (37). It may be that the association of Cdc25C and PP2A is enhanced in mitosis because of nuclear envelope breakdown and physical accessibility of the proteins. The strong association of B56δ and Cdc25C at mitosis may not lead to the immediate dephosphorylation of T130 and the inactivation of Cdc25C, but rather may require an additional regulatory event. Given the number of phosphorylation events that regulate B56δ (14, 38), it is possible that dephosphorylation of T130 is further regulated by phosphorylation of the PP2A:B56δ bound to Cdc25C.

Unopposed activation of Cdc25C should lead to premature activation of Cdk1 and mitotic catastrophe. That cells and mice survive B56δ KD was therefore surprising. The finding that transcriptional up-regulation of Wee1 accompanies and is due to B56δ KD provides one potential solution to this problem because enhanced Cdc25C phosphatase activity can then be opposed by enhanced Wee1 kinase activity. A second solution is mitotic degradation of Cdc25C, a compensatory mechanism recently noted in the Ltzs1 KO mouse (39). The finding that transcriptional up-regulation of Wee1 accompanies and is because of B56δ KD provides additional strong genetic evidence that the normal function of B56δ is the regulation of Cdc25C and, hence, Cdk1 and illustrates how organisms can compensate for loss of what might otherwise be an essential gene. How cells sense loss of B56δ to increase transcription of Wee1 is currently unknown. *Wee1* is transcriptionally regulated, in part, by the transcriptional factors KLF-2 (40) and c-Fos/AP-1 (41, 42), and AP-1 activity may increase upon PP2A inhibition (43). The same mechanism in reverse might allow CDC25B and CDC25C KO mice to survive (44). Dissecting the pathways by which B56δ KO up-regulates Wee1 may provide further insights into the regulation of the cell cycle.

Materials and Methods

Cloning of Constructs. HA-B56 proteins were cloned into pCEP4-Lerner (36). Cdc25C was PCR cloned into HindIII/BamHI of pCS2 MT to produce a 6X myc-Cdc25C-tagged cDNA product. B56δ was BamHI/EcoRI cloned into pEGFP-C1 (Clontech) to create EGFP-B56δ. Two silent mutations were made by using QuikChange (Stratagene) to render the construct resistant (5′-AAACATAGCCAAGAAGTACAT-3′ changed to 5′-AAACAT*C*GC*G*AAGAATGACAT-3′) to the incorporated siRNA sequence of the stable B56δ KD cell line. pMKO.1-B56δ (gift of William C. Hahn) was used to create viral particles for transduction and stable KD of B56δ.

Culture of Mammalian Cells. HEK293 cells were grown in complete media (DMEM, 10% FBS, Pen/Strep/Gln). B56δ$^{-/-}$ and WT MEF cells have been described (J.V.L. and J.G., unpublished data). MEF cells were grown in MEF media (DMEM, 12% FCS, 2.6X NEAA MEM, 4.8 mM L-glutamine, 8.6 × 10^{-4}% 2-β-mercaptoethanol, 46 mM sodium bicarbonate).

Lysate Analysis and Coprecipitation. To analyze human protein interactions, cell lysates were prepared as described (45). HA-tagged proteins were immunoprecipitated from 300 μg of total protein with anti-HA antibody 12CA5 (Santa Cruz Biotechnology) and collected with 20 μl of protein A agarose (Invitrogen). Beads were washed with lysis buffer [150 mM NaCl, 30 mM Hepes (pH 7.4), and 0.1% Tween 20] and immunoblotted. B56δ was detected by using a polyclonal antibody raised in rabbits against amino acids 1–80. Rabbit polyclonal anti-pT138 (a gift from S. Kornbluth, Duke University, Durham, NC) was raised against the peptide LPHLLCSpTPSFKKAC. Other antibodies used included total Cdk1 (sc-34; Santa Cruz Biotechnology), Cdk1 Y15P (IMG-668; Imgenex), Cdc25C (TC-113; Abcam), Actin (A2066; Sigma–Aldrich), and Cyclin B1 (ab7957–1; Abcam). Lysates from MEF cells were analyzed by using anti-Wee1 (ab2315–05; Abcam) and anti-B56δ (PR61δ).

Stable B56δ KD Cell Line. Viral particles were created by Fugene 6-mediated triple cotransfection of 293 EBNA cells with pMKO.1-shB56δ, along with constructs expressing GAG-Pol and VSV-G (a gift from S. Lessnick, University of Utah). Puromycin-resistant colonies were screened for KD of B56δ by SDS/PAGE and Western blotting by using the anti-B56δ antibody described earlier.

Nocodazole Release for Mitotic Progression. Cells were cultured in complete media to 70% confluency. Media were removed and replaced with 100 ng/ml complete media plus nocodazole (Sigma–Aldrich) for 18 h. Cells were then trypsinized, washed three times with PBS, and replated with complete media. Cells were harvested at indicated time points by trypsinization and washed three times with cold PBS, aliquoting half of cells for freezing at −80°C for lysate analysis and fixation of the remaining cells in 2:1 methanol/PBS for flow-cytometry analysis.

Flow-Cytometry Analysis. Methanol-fixed cells were spun at 400 × *g* for 5 min, rehydrated with PBS, and spun again at 400 × *g* for 5 min. Pellets were resuspended in DNA staining solution (PBS, 5% propidium iodide, 0.1% Triton X-100, 2 mg of RNase A) for 30 min at room temperature (RT) for DNA content analysis. For phosphor-histone H3 analysis, pellets were permeabilized in cold 0.1% Triton X-100, washed in FACS buffer (PBS, 2% FCS, 0.02% sodium azide, 0.5 mM EDTA), stained with Alexa Fluor 647 conjugated-phosphohistone H3 (Ser-10) antibody (no. 9716; Cell Signaling Technology) for 1 h at RT. Cells were washed with FACS buffer and resuspended in FACS buffer for analysis. Samples were gated for GFP-positive, Alexa 647-positive, compared with GFP-positive, Alexa 647-negative, for percentage of cells staining for phosphohistone H3. Samples were run on FACScan flow cytometers (Becton Dickinson), and data were analyzed by using CellQuest (Becton Dickinson).

Real-Time Quantitative RT-PCR assay. Real-time quantitative RT-PCR was performed by using previously described primers (46) to measure the level of *wee1* and *gapdh* mRNA. Total RNA was prepared by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RT-PCR was conducted by using First-Strand Supermix for quantative RT-PCR (Invitrogen) and BIORAD MJ Mini thermocycler. Reverse transcriptase-mediated cDNA was prepared by using 1 μg of total RNA. Quantification of the cDNA was performed by using the BIORAD iCycler with SYBR Green Supermix for iCycler (Invitrogen).

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