Greatwall is essential to prevent mitotic collapse after nuclear envelope breakdown in mammals

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Edited* by Tim Hunt, Cancer Research UK, South Mimms, United Kingdom, and approved September 19, 2013 (received for review June 6, 2013)

Greatwall is a protein kinase involved in the inhibition of protein phosphatase 2 (PP2A)-B55 complexes to maintain the mitotic state. Although its biochemical activity has been deeply characterized in *Xenopus,* its specific relevance during the progression of mitosis is not fully understood. By using a conditional knockout of the mouse ortholog, *Mastl*, we show here that mammalian Greatwall is essential for mouse embryonic development and cell cycle progression. Yet, Greatwall-null cells enter into mitosis with normal kinetics. However, these cells display mitotic collapse after nuclear envelope breakdown (NEB) characterized by defective chromosome condensation and prometaphase arrest. Intriguingly, Greatwall is exported from the nucleus to the cytoplasm in a CRM1-dependent manner before NEB. This export occurs after the nuclear import of cyclin B–Cdk1 complexes, requires the kinase activity of Greatwall, and is mediated by Cdk-, but not Polo-like kinase 1-dependent phosphorylation. The mitotic collapse observed in Greatwall-deficient cells is partially rescued after concomitant depletion of B55 regulatory subunits, which are mostly cytoplasmic before NEB. These data suggest that Greatwall is an essential protein in mammals required to prevent mitotic collapse after NEB.

cell cycle regulation | cell division | mitotic kinases | mitotic phosphatases | nuclear export

Greatwall was originally identified in *Drosophila* as a modulator of Polo activity and a protein required for DNA condensation and normal progression through mitosis (1–4). Biochemical assays in *Xenopus* extracts have demonstrated that Greatwall is able to inhibit PP2A–B55 phosphatase complexes by phosphorylating the cAMP-regulated phosphoprotein Arpp19 and α-endosulfine, thus participating in the maintenance of the mitotic state (5–8). The control of PP2A through the Greatwall-dependent phosphorylation of Arpp19/Ensa proteins has also been supported by genetic studies in *Drosophila* (9, 10). The mammalian ortholog of Greatwall, also known as microtubule-associated serine/threonine kinase-like protein (Mastl), also participates in the maintenance of the mitotic state by inhibiting PP2A phosphatases (11, 12). Inhibition of Greatwall is required for the activation of PP2A–B55α,δ complexes during mitotic exit (13), thus suggesting the relevance of this pathway in maintaining the mitotic state (4). How Greatwall activity and function is regulated is not well established. Several evidences support a role for cyclin-dependent kinase (Cdk)-dependent phosphorylation in the activation of Greatwall, and several phosphorylation sites for multiple kinases have been mapped (14, 15). In addition, although Greatwall is mostly nuclear in interphase (3, 11), the cellular and molecular basis of the control of its dynamic intracellular trafficking and its activity remains largely unknown.

We show in this work that the murine Greatwall ortholog, encoded by the *Mastl* gene, is essential for mouse development and cell cycle progression. Greatwall-null cultures, however, display normal kinetics during the G_2/M transition, suggesting that this protein is not required for mitotic entry. Greatwall is exported to the cytoplasm before nuclear envelope breakdown (NEB) but, interestingly, this export follows nuclear import of cyclin B–Cdk1. The lack of Greatwall activity results in defects in chromosome condensation after NEB, and these defects can be rescued by concomitant ablation of B55 proteins. Our results imply that cells are subjected to a mitotic stress resulting from NEB, a moment in which nuclear chromatin becomes exposed to cytoplasmic phosphatases. We therefore propose that Greatwall shuttles to the cytoplasm before NEB and prevents mitotic collapse by inhibiting the PP2A–B55-dependent dephosphorylation of Cdk substrates.

Results

Genetic Ablation of Mastl in the Mouse. *Mastl*(+/−) mice (Fig. 1 *A* and *B*) develop normally and are fertile. However, no viable homozygous mutants were found after intercrosses between these mice, suggesting embryonic lethality in the absence of Greatwall. *Mastl*(−/−) embryos developed normally in vitro during a few days and generate normal blastocysts (Fig. S1). However, these mutant embryos were not detected in the utero by embryonic day (E) 10.5, suggesting lethality at periimplantational stages (Fig. 1*C*).

Because the germ-line null mutation in Greatwall resulted in early embryonic lethality, we intercrossed *Mastl*(+/lox) mice harboring an additional allele that expresses the Cre-estrogen receptor (ERT2) recombinase in the ubiquitous RNA polymerase II locus (13). We treated pregnant females at E12.5 with tamoxifen to induce Cre activity, and embryos were analyzed at E14.5. Genetic ablation of Greatwall resulted in increased mitotic figures in the neuroepithelium of *Mastl*(Δ/Δ) embryos compared with *Mastl*(+/Δ) littermates (Fig. 1*D*). These mitotic figures displayed an accumulation of prometaphases/metaphases (PM) in the absence of Greatwall compared with more abundant anaphases/telophases (AT) in control sections (Fig. 1*D*), thus suggesting that Greatwall is essential for proper mitotic progression in vivo in mammals.

Significance

Nuclear envelope breakdown (NEB) leads to the exposure of nuclear structures to cytoplasmic activities. Greatwall is a kinase able to inhibit PP2A phosphatases that counteract Cdk-dependent phosphorylation required for mitosis. Here we show that Greatwall, an essential protein in mammals, is exported to the cytoplasm in a Cdk-dependent manner before NEB, thus protecting mitotic phosphates from phosphatase activity.

Author contributions: M.M. designed research; M.A.-F., R.S.-M., B.S.-C., P.P.G., M.S.-F., M.T., and M.R.-T. performed research; M.T., T.L., and A.C. contributed new reagents/analytic tools; M.A.-F., R.S.-M., B.S.-C., P.P.G., M.S.-F., M.T., M.R.-T., and M.M. analyzed data; and M.A.-F. and M.M. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1310745110/-/DCSupplemental.

serum, cells were monitored by using time-lapse microscopy during 2 d (Fig. 2*A*). No differences were observed in the kinetics of mitotic entry (determined by cell rounding and chromosome condensation) in these cultures (Fig. 2 *B* and *C*). In addition, no differences were detected in the accumulation of MPM2-positive cells by immunofluorescence (Fig. 2*D*) or in the accumulation of cyclin B1 or phospho-histone H3 (Fig. 2*E*). However, Greatwall-

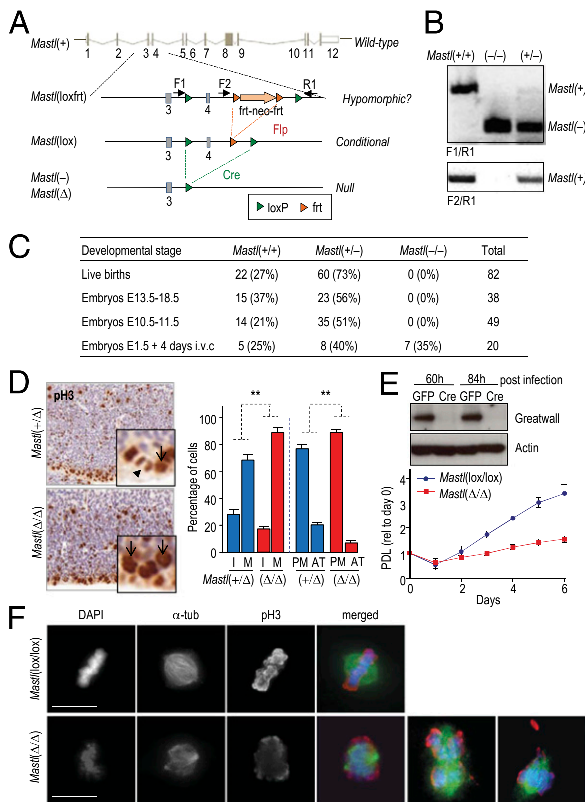

Fig. 1. Greatwall mutant mice and cells. (*A*) The exon 4 of the murine gene encoding Greatwall, *Mastl*, was flanked by loxP sites (green triangles) and a selection cassette [*Mastl*(loxfrt) allele]. Site-specific recombination by Flp results in the *Mastl*(lox) allele, which expresses normal levels of Greatwall. Cre-mediated recombination results in the germ-line [*Mastl*(–)] or conditionally induced [*Mastl*(Δ)] null alleles. (*B*) Representative PCR products showing the indicated alleles after amplification using oligonucleotides indicated in *A*. (*C*) The number (and percentage) of mice or embryos with the indicated genotype is shown, indicating that *Mastl*(–/–) embryos develop normally until blastocyte stage. (*D*) Immunohistochemical analysis of E14.5 embryos showing an increase in mitotic (M) figures (pH3, phosphohistone H3 signal, brown) versus interphasic (I) cells in the subventricular zone in *Mastl*(Δ/Δ) vs. *Mastl*(+/Δ). PM figures are indicated by arrows, whereas AT are indicated by arrowheads. $^{**}P < 0.01$. (*E*) Transduction with Cre-expressing adenoviruses (AdCre) results in the depletion of Greatwall in primary MEFs compared with control cells transduced with adenoviral vectors expressing GFP. β-actin was used as a loading control. Genetic ablation of Greatwall impairs proliferation of primary MEFs. Mean ± SD for each time point is shown in the plot. PDL, population doubling levels. (*F*) Representative immunofluorescence images of mitotic cells stained with DAPI (blue), α-tubulin (green), and pH3 (red). (Scale bars: 10 μm.)

We then intercrossed *Mastl*(+/lox) mice to generate *Mastl*(lox/lox) mouse embryonic fibroblasts (MEFs). These cells grew normally in vitro and displayed no differences in cell proliferation compared with wild-type cells. Acute expression of Cre, but not GFP or Flp, resulted in genetic ablation of Greatwall to generate the null [*Mastl*(Δ)] allele (Fig. 1*E*). Genetic ablation of *Mastl* in primary cells resulted in reduced proliferation in vitro accompanied by lack of Greatwall protein. These defects were accompanied by mitotic aberrations in prometaphase, including defective chromosome condensation and abnormal spindles (Fig. 1*F*).

Greatwall Is Not Required for Mitotic Entry in MEFs. To study the kinetics of mitotic entry and progression, *Mastl*(lox/lox) cells stably expressing histone H2B-mRFP were arrested in G_0, transduced with AdenoFlp or AdenoCre viruses, and stimulated with serum to reenter the cell cycle. Five hours after the addition of

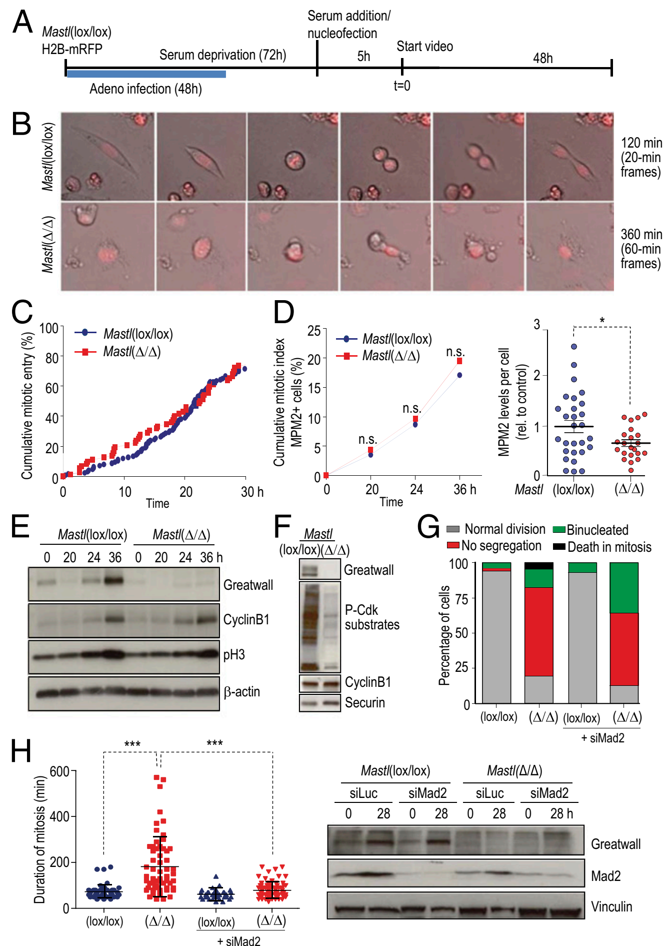

Fig. 2. Lack of Greatwall does not inhibit mitotic entry but prevents chromosome segregation. (*A*) Schematic representation of the protocol used for genetic ablation of Greatwall. Confluent cultures of immortalized *Mastl*(lox/lox) MEFs expressing a mRFP-tagged histone H2B (H2B-mRFP) were serum deprived and transduced with Flp (AdFlp)- or Cre (AdCre)-expressing adenoviruses, giving rise to *Mastl*(lox/lox) or *Mastl*(Δ/Δ) cells, respectively. These cells were stimulated with serum and monitored 5 h later by using time-lapse microscopy during an additional 48 h. (*B*) Representative time-lapse images of *Mastl*(lox/lox) and *Mastl*(Δ/Δ) cells. H2B-mRFP is in red. (*C*) Quantification of mitotic entry (as scored by cell rounding and chromosome condensation) in these cultures after visual analysis of these time-lapse images. (*D*) Quantification of the cumulative percentage of cells positive for the mitotic marker MPM2 (*Left*) or levels of MPM2 per mitotic cell (*Right*) in these cultures. (*E*) Level of the indicated proteins in these cultures after the addition of serum to quiescent cells ($t = 0$). (*F*) Levels of the indicated antigens in Greatwall-null or control mitotic cells after treatment with taxol and mitotic shake-off. (*G*) Quantification of mitotic aberrations in *Mastl*(lox/lox) and *Mastl*(Δ/Δ) cells in the absence or presence of short interfering RNAs against Mad2 (siMad2) or luciferase (siLuc). (*H*) Duration of mitosis (from NEB until mitotic exit, based on DNA decondensation and loss of rounded morphology) in Greatwall-deficient and control cells in the absence or presence of siMad2 or siLuc. Error bars indicate SD. The depletion of Greatwall or Mad2 in these cultures is shown after immunoblot. n.s., not significant differences ($P > 0.05$; Fisher's exact test); $^{*}P < 0.05$; $^{***}P < 0.001$ (Student *t* test).

deficient mitotic cells displayed reduced levels of phosphorylation of Cdk substrates as detected after quantifying the cellular levels of MPM2 antigens (many of which correspond to Cdk phospho-substrates; Fig. 2*D*) or after immunodetection of phospho-Cdk substrates in mitotic extracts (Fig. 2*F*). In addition, most *Mastl*(Δ/Δ) cells showed defects (defective chromosome condensation and prometaphase arrest) after entering into mitosis. Approximately 75% of *Mastl*(Δ/Δ) cells displayed mitotic arrest and lack of chromosome segregation (Movies S1 and S2), whereas 5% of the culture died during mitosis (Fig. 2*G*). Approximately 20% of the *Mastl*(Δ/Δ) culture displayed a normal mitosis likely as a consequence of defective transduction or lack of Cre activity, as observed in other models (13).

Whereas control cultures spent approximately 73 ± 5 min in mitosis, the duration of mitosis (DOM) in Greatwall-deficient cells reached 182 ± 16 min. This delay was due to the activation of the spindle assembly checkpoint (SAC) because knockdown of the SAC protein Mad2 significantly reduced DOM in Greatwall-deficient cells (Fig. 2*H*). In addition, lack of Greatwall did not significantly reduce the effect of taxol or nocodazole in mitotic arrest, indicating that this protein is not required for the SAC in these fibroblasts (Fig. S2). As a control, interference against Mad2 expression was able to overcome mitotic arrest in the presence of these microtubule poisons in parallel assays (Fig. S2).

Greatwall Is a Nucleocytoplasmic Shuttling Protein. To investigate the cellular localization of Greatwall during the cell cycle, we stably expressed GFP-tagged human Greatwall (GFP-Gwl) or GFP alone (GFP) in U2OS cells and examined their intracellular localization. Whereas Greatwall is predominantly nuclear, overexpression of the exportin CRM1 resulted in a significant shift of Greatwall from the nucleus to the cytoplasm (Fig. S3). Conversely, inhibition of the CRM1-mediated nuclear export pathway by leptomycin B (LMB) resulted in accumulation of Greatwall in the nucleus (Fig. S3). Both GFP and Greatwall signals were found to colocalize in these stable clones after staining with Greatwall-specific antibodies (Fig. S4).

Two regions in the primary sequence of human Greatwall corresponded to potential nuclear localization signals (NLS; Fig. S3). Deletion of sequence 2 (ΔNLS2) resulted in cytoplasmic localization (Fig. S3), and a double ΔNLS1+ΔNLS2 (referred from now on as ΔNLS) mutant lacking both sequences did not further enhance the cytoplasmic accumulation observed in the ΔNLS2 mutant. Experiments in which the endogenous Greatwall protein from *Mastl* conditional-knockout cells was replaced by the ΔNLS mutant showed a significant rescue, although not complete, of the defects observed in Greatwall-deficient cells. In particular, expression of Gwl-ΔNLS was able to rescue the defects in chromosome segregation, although these cells displayed frequent lagging chromosomes (Fig. S3), indicating that nuclear accumulation of Greatwall is required for proper mitosis. The duration of mitosis was not completely rescued by this mutant or the wild-type protein, suggesting that overexpression of Greatwall itself may result in prolonged duration of mitosis. We also identified a few sequences that conform to the nuclear export signal (NES) consensus sequence (Figs. S3 and S5). Expression of mutant NES1-3 sequences resulted in a slight but significant decrease in cytoplasmic accumulation after coexpression of CRM1, and these domains displayed nuclear export activity in a well-established in vivo assay (Fig. S5). In general, these results show that Greatwall shuttles between the nucleus and cytoplasm in a CRM1-dependent manner and under the control of specific NLS and NES sequences.

Greatwall Is Exported from the Nucleus in a Cdk-Dependent Manner Before Nuclear Envelope Breakdown. We next monitored localization of Greatwall during the cell cycle by time-lapse microscopy. Greatwall displays a nuclear localization pattern during interphase, but it is exported to the cytoplasm before nuclear envelope breakdown (Fig. 3*A* and Movie S3). By using a CFP-laminA fusion protein, we observed that Greatwall is exported from the nucleus 7.9 ± 1.3 min before NEB (Fig. 3*A*). A crucial event required for mitosis is the nuclear import of cyclin B–Cdk1 complexes before NEB (16–18). We therefore tested the relative order of cyclin B–Cdk1 nuclear import and Greatwall export by combining cyclin B1-mCherry and GFP-Gwl reporters. As shown in Fig. 3*B*, cyclin B1 is internalized into the nucleus approximately 10.5 ± 1.2 min before NEB (see also Movie S4). Greatwall, on the other hand, is exported approximately 2.1 ± 1.4 min after cyclin B1 import. These data suggest a highly ordered process in which cyclin B1–Cdk1 complexes are imported into the nucleus during prophase, Greatwall is then exported, and NEB occurs few minutes later.

We next tested whether the activation of mitotic kinases influences Greatwall export. A pool of Polo-like kinase 1 (Plk1) is known to be active at the nucleus during mitotic entry and Polo kinases have been proposed to participate in Greatwall activation. However, Plk1 activity is not necessary for Greatwall export because treatment of cells with the Plk1 inhibitor BI2536 did not influence Greatwall shuttling before NEB (Fig. 3*C*). Plk1 was inhibited in these assays as treated cells displayed the typical morphologies found after Plk1 inhibition such as monopolar spindles and defective chromosome congression.

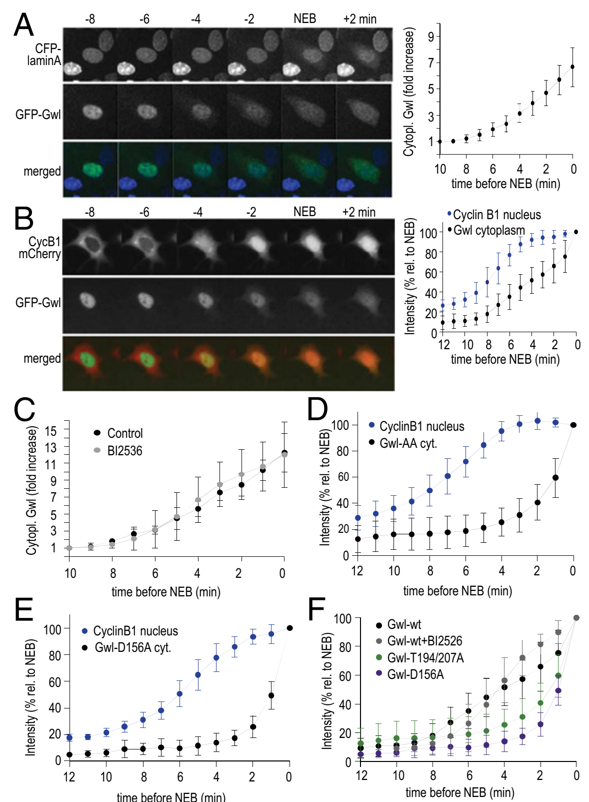

Fig. 3. Greatwall is exported to the cytoplasm before NEB in a Cdk-dependent manner. (*A*) Micrographs represent mitotic entry in U2OS cells stably expressing GFP-Gwl (green) and CFP-tagged lamin A (blue). Quantification of the cytoplasmic Greatwall signal before NEB. (*B*) Mitotic entry in cells stably expressing GFP-Gwl (green) and mCherry-tagged cyclin B1 (red). Quantification of cytoplasmic Greatwall and nuclear cyclin B1. Fluorescence mean intensity was set as 100% at NEB (time = 0). (*C*) Greatwall displays a normal export in the presence of the Plk1 inhibitor BI2536 (100 nM). (*D*) Quantification of cytoplasmic export (as indicated in *B*) of a Greatwall mutant harboring the T194A and T207A mutations (Cdk phosphorylation sites). (*E*) Quantification of cytoplasmic export of a Greatwall kinase-dead mutant harboring a mutation in the ATP-binding site (D156A). (*F*) Comparison of the dynamic changes in the subcellular localization of Greatwall in the different mutants or treatments. All graphs show means ± SD of at least 10 cells per condition.

We also tested the effect of interfering with the Cdk-dependent activation of Greatwall during mitotic entry. Because effective inhibition of Cdk1 prevents mitotic entry, we instead generated Greatwall isoforms with specific mutations in two residues known to be phosphorylated in a Cdk-dependent manner—T194 and T207—and proposed to be required for Greatwall activation (15). Mutation of these residues (T194A/T207A) resulted in a significant delay in nuclear export and that mutant stayed in the nucleus during prophase, being its cytoplasmic localization only observed concomitantly with NEB. Importantly, a Greatwall kinase-dead mutant harboring a mutation in the ATP-binding site (D156A) showed a similar impairment in nuclear export, suggesting that the activation of Greatwall, rather than its Cdk-dependent phosphorylation itself, is required for its cytoplasmic localization before NEB (Fig. 3 *E* and *F*).

Greatwall Protects Cells from a PP2A–B55-Dependent Mitotic Collapse. The fact that Gwl is exported to the cytoplasm in a kinase-dependent manner suggests that it is required to phosphorylate any cytoplasmic substrate. Greatwall is known to inhibit PP2A–B55α,δ complexes to avoid early dephosphorylation of Cdk substrates (5–8, 13). Indeed, B55α is mostly localized to the cytoplasm (Fig. S6) and we therefore hypothesized that the shuttling of Greatwall may be required for inhibiting cytoplasmic PP2A–B55 complexes, thus preventing a premature dephosphorylation of Cdk substrates upon NEB. Lack of Greatwall resulted in a significant decrease in phosphorylation of Cdk mitotic substrates (Figs. 4*A* and 2*F*). These defects were not due to delayed mitotic entry because *Mastl*(Δ/Δ) and control cultures displayed similar kinetics during mitotic entry (Fig. 2 *C*–*E*). In addition, knockdown of B55 isoforms rescued the defective phosphorylation of these substrates. These results are in agreement with previous data indicating that down-regulation of PP2A–B55 can suppress the effects of losing the Greatwall substrate Ensa (10), thus confirming that the inhibition of PP2A–B55, likely mediated by Ensa proteins, is one of the major functions of Greatwall during mitosis (Fig. 4 *A*–*C*). By analyzing metaphase spreads, we found that more than 75% of Greatwall-depleted cells displayed condensation defects, detected by the presence of longer mitotic chromosomes, which are indicative of hypocondensed DNA as shown in *Drosophila* (2). Importantly, these defects were almost completely prevented when we codepleted B55 isoforms (Fig. 4*D*). Greatwall-null cells showed a marked reduction in phosphorylation of S126 of Cyclin B1, which was also rescued by B55 codepletion (Fig. 4*E*). Interestingly, phosphorylation of CycB-pS126 by Cdk is absent in G_2 cells but accumulates specifically at mitotic chromosomes and is required for the stable association of cyclin B with mitotic chromosomes (18), which is in agreement with a requirement for Greatwall to maintain the mitotic state after NEB. All together, these data suggest that mammalian Greatwall is an essential protein in vivo, and its loss results in a mitotic collapse caused by cellular PP2A–B55 activity after NEB.

Subcellular Localization of Greatwall Is Required for Its Function. To specifically assess the relevance of the nucleocytoplasmic export of Greatwall, we first introduced a strong SV40 NLS sequence fused to the amino terminus of GFP-Greatwall sequence (NLS-GFP-Gwl). This mutant only displayed minor defects in nuclear export, and it was exported with a kinetics similar to wild-type Greatwall (approximately 60% of the signal was still cytoplasmic before NEB; Fig. S7). We then generated a fusion of Greatwall with histone H2B (H2B-GFP-Gwl). The cytoplasmic export was dramatically impaired in this mutant (Fig. 5*A* and Fig. S7), and we therefore used these constructs to reconstitute Greatwall in conditional-knockout fibroblasts. Reconstitution of *Mastl*(Δ/Δ) cells with the wild-type form of Greatwall and the NLS fusion resulted in a partial rescue of the prolonged duration of mitosis. The H2B fusion, however, was defective in this assay (Fig. 5*B*). More importantly, both wild-type and the NLS fusion significantly rescued the percentage of normal chromosome segregation in *Mastl*-null cells. However, complementation with the H2B

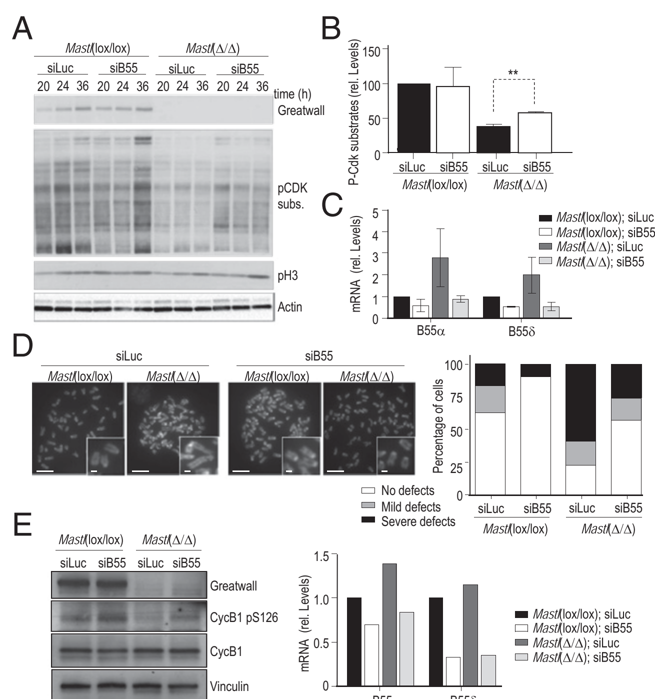

Fig. 4. Greatwall prevents PP2A–B55-dependent defects in chromosome condensation after nuclear envelope breakdown. (*A*) Immunodetection of the indicated antigens in *Mastl*(lox/lox) and *Mastl*(Δ/Δ) cells at the indicated time points after serum stimulation following the scheme represented in Fig. 2. Cells were treated with short interfering RNAs against the B55 subunits α, β, γ, and δ (siB55) or luciferase (siLuc) sequences as indicated. (*B*) Relative levels of phospho-Cdk substrates at 24 h, normalized versus *Mastl*(lox/lox) control cultures. (*C*) Quantification of the depletion of the most abundant B55 isoforms in MEFs (B55α and δ) by real-time RT-PCR after transfection with siLuc or siB55 oligonucleotides. (*D*) Representative images of metaphase spread from the indicated cultures. Condensation defects were determined based on the abnormal length of mitotic chromosomes and classified based on the number of affected chromosomes per metaphase, as mild (5–10 chromosomes) or severe (more than 10 chromosomes). At least 50 metaphases were counted per condition. (Scale bars: 10 μm; *Insets*, 1 μm.) (*E*) Mastl depletion and phosphorylation of cyclin B1 at S126 residue (pS126) was monitored by immunoblot. The level of depletion of the different B55 isoforms was analyzed by real-time RT-PCR 24 h after transfection with siLuc or siB55 oligonucleotides.

fusion did not improve chromosome segregation in the absence of the endogenous protein, although a few (approximately 15%) Mastl-null cells were able to divide in the presence of lagging chromosomes (Fig. 5*C*). These aberrations were not a consequence of additional problems or dominant-negative effects caused by the Gwl-H2B fusion because this construct induced no defects in chromosome segregation in wild-type cells (Fig. S7). These data suggest that cellular localization, in addition to kinase activity, is specifically required for the protective function of Greatwall during mitosis.

Discussion

Depletion of Greatwall from cycling extracts in *Xenopus* prevents cyclin B–Cdk1 activation and mitotic entry (19), and the concomitant inhibition or depletion of PP2A–B55 from these extracts rescues defective mitotic entry (5). However, genetic studies in *Drosophila* have shown that lack of Greatwall mostly leads to delays in mitosis accompanied by defective chromosome condensation (2, 20). These mitotic defects have also been reported in knockdown studies by using RNA interference in mammalian cells (11, 12). Because residual protein levels are frequently found after

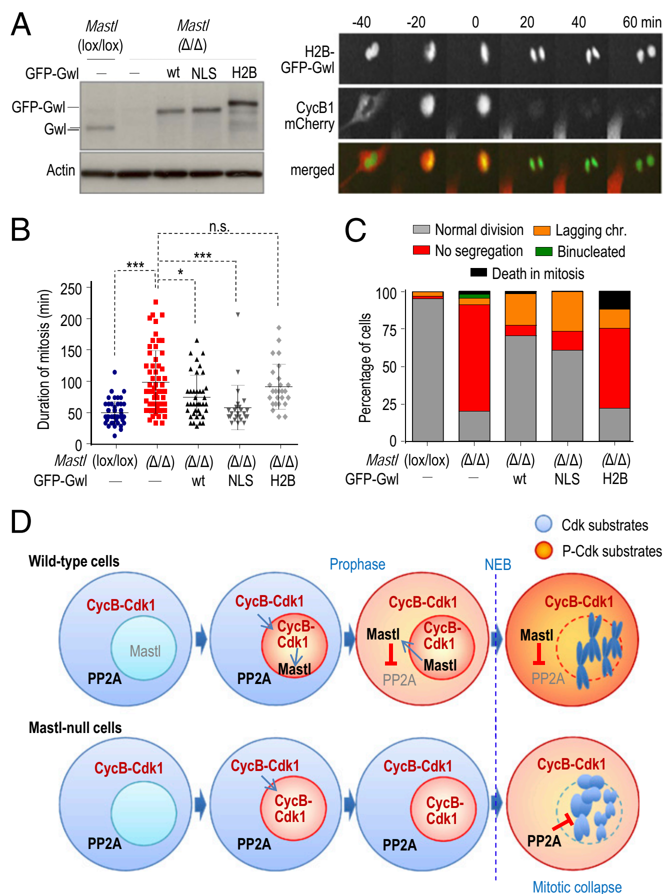

Fig. 5. Greatwall nuclear export prevents mitotic defects. (*A*) Greatwall-knockout cells were complemented with Greatwall variants (NLS or H2B-fusions) or the wild-type protein (wt). Cells were synchronized as indicated in Fig. 2*A*, and expression levels were monitored by immunoblot. Micrographs represent the localization of Gwl-H2B during mitosis (time 0 = metaphase). Analysis of duration of mitosis (*B*) and mitotic cell fate in those cultures showing a partial rescue by the wild-type (wt) and NLS fusion forms of Greatwall, but not in the H2B-fusion (*C*). $^{*}P < 0.05$; $^{***}P < 0.001$; n.s., not significant. (*D*) In normal cell cycles, activation and nuclear import of CycB/Cdk1 complexes leads to the phosphorylation of substrates (change from blue to reddish colors), thus triggering Greatwall export to the cytoplasm, where it inhibits PP2A–B55 complexes just before NEB (*Upper*). A defective inhibition of PP2A in early mitosis would cause a defective phosphorylation of Cdk substrates upon NEB, leading to the mitotic collapse observed in Greatwall-null cells (*Lower*).

RNAi knockdown studies, it has been suggested that mammalian Greatwall may also be required for mitotic entry.

We cannot rule out the possibility that residual Greatwall activity may be present after genetic ablation of the Mastl gene in our assays. However, *Mastl*(Δ/Δ) cells display homogenous mitotic defects despite no evidence for delayed mitotic entry in unperturbed cell cycles (Fig. 2). Moreover, we have extended the incubation of cells in the presence of Cre adenovirus and absence of serum in these assays to favor the degradation of the endogenous protein. In addition, the kinetics of mitotic entry is similar in *Mastl*(Δ/Δ) cultures irrespective of their mitotic fate, either strong prometaphase defects in chromosome condensation or normal chromosome segregation (Fig. 2), suggesting no correlation between mitotic phenotypes and a defect in mitotic entry.

An intriguing conclusion from these results is that Greatwall may be dispensable for proper activation of Cdk1 during mitotic entry. Our data and previous data in *Xenopus* and mammalian cells are in agreement with Greatwall being downstream of Cdk1 activation (4, 15, 19). The strong phenotype, not only in intrinsic kinase activity but also in subcellular localization, of Greatwall variants with mutations in the Cdk-phosphorylation sites (Figs. 3 and 5) confirms and extends the relevance of Cdk1 upstream of this kinase. Greatwall may be dispensable for the initial activation of Cdk1 at the cytoplasm, since it is almost exclusively nuclear before cyclin B–Cdk1 complexes enter into the nucleus. However, it is possible that Greatwall may contribute to further activation of Cdk1 just before NEB or after NEB, and we cannot exclude that Greatwall-deficient cells enter into mitosis with a deficient load of phosphorylated Cdk substrates (Fig. 2 *D* and *F*). It is also possible that Greatwall is not required for the levels of Cdk1 activity during a normal, unperturbed cell cycle, but for reaching more robust levels of Cdk1 activation, for instance, those levels required for checkpoint recovery (21). In fact, several synchronization protocols such as thymidine or aphidicolin blocks have been used in the previous RNAi studies in mammalian cells (11, 12), perhaps explaining defects in G_2/M transition as a consequence of defective checkpoint recovery. It is also possible that depletion assays in *Xenopus* extracts may partially eliminate other enzymatic activities required for mitotic entry such as Cdk1. Indeed, it is intriguing the recent proposal that Greatwall is a critical constituent of the M phase-promoting factor (MPF) originally thought to be formed exclusively by cyclin B–Cdk1 complexes (22). Altogether, the data from conditional knockout MEFs, along the accumulation of mitotic figures in Greatwall-null embryos (Fig. 1), suggest that Greatwall is essential for mitotic progression but largely dispensable for mitotic entry in unperturbed cell cycles in mammals.

Our data also suggest that control of the subcellular localization is critical for Greatwall function. Cyclin B–Cdk1 complexes are imported into the nucleus before NEB, and it is still a matter of debate whether this complex is activated before or after its nuclear import (18). We have observed that Greatwall export begins 2 min after cyclin B–Cdk1 nuclear import and is prevented by mutation of known Cdk-dependent phosphorylation sites in Greatwall. Thus, we propose that Cdk1 drives cytoplasmic export of Greatwall, in a CRM1-dependent manner, before NEB. Because B55 subunits are mostly cytoplasmic, we hypothesized that this export is critical to inhibit cytoplasmic PP2A–B55 complexes. NEB results in a stressful situation in which cytoplasmic components can access chromatin. In the absence of Greatwall, this combination of cytoplasmic and nuclear components would lead to a process originally described by Gorbsky and coworkers as mitotic collapse (23), in which uninhibited cytoplasmic PP2A–B55 complexes would counteract with the normal progression of mitosis (24). Based on these pioneer observations (23) and the results shown in this work, we therefore propose that nuclear import of cyclin B–Cdk1 complexes leads to the export of Greatwall, and these changes in subcellular localization may be important to properly inhibit PP2A–B55 complexes and prevent mitotic collapse after NEB (Fig. 5*D*).

While this manuscript was under review, similar changes in the localization of the *Drosophila* Greatwall have been reported (25), extending previous observations that suggested a cell cycle-regulated localization of this protein in flies (3). Whereas in *Drosophila*, Polo seems to play an important role in Greatwall localization (25), we have found no evidence of a similar control in mammalian cells, and the reasons for these differences remain unknown. However, these results indicate that the control of Greatwall localization adds a unique level of regulation that prevents stressful situations (mitotic collapse) due to the changes in the localization of proteins, such as phosphatases, after NEB.

Materials and Methods

Generation of Mastl Conditional Knockout Mice. *Mastl*(+/lox) mice (Fig. 1*A*) were crossed with knockin mice harboring a tamoxifen-inducible Cre recombinase [*RNAPII*(CreERT2)] expressed under the RNA polymerase locus (13). *Mastl*(+/lox); *RNAPII*(CreERT2/CreERT2) females were crossed with *Mastl*(lox/lox); *RNAPII*(CreERT2/CreERT2) males, injected with 4-hydroxytamoxifen (i.p., 5 mg; Sigma) by E12.5, and embryos were extracted 48 h later. For histological

observation, embryos were fixed in 10% (vol/vol) buffered formalin (Sigma) and embedded in paraffin wax. Immunohistochemical examination was performed by using specific antibodies against phospho-histone H3 (Millipore).

Plasmids and Mutagenesis. DNA mutagenesis was performed by using the QuikChange XL Site-Directed Mutagenesis kit (Stratagene) and sequence verified. The NLS and H2B-Greatwall fusions were generated by annealing oligos encoding the SV40 NLS signal (PKKKRKV), and subcloning the human histone H2B type 1 in frame at the N terminus of the GFP-Gwl plasmid, respectively. YFP-CRM1, CFP-lamin A, and CyclinB1-mCherry plasmids were kindly provided by M. Yoshida (The University of Tokyo, Tokyo, Japan), V. Andrés (Spanish National Cardiovascular Research Centre, Madrid, Spain), and G. Kops (University Medical Center, Utrecht, The Netherlands), respectively. siRNAs were from Dharmacon.

Cell Culture, Drugs, and Antibodies. Human cell lines and mouse embryonic fibroblasts were cultured and characterized by using routine protocols (13). Immortalization was performed by retroviral infection with a plasmid encoding the first 121 amino acids of the SV40 large T antigen (T121) followed by hygromycin selection. Plasmid transfection in U2OS cells was performed by using Lipofectamine 2000 (Invitrogen), whereas MEFs were transformed by nucleofection with Amaxa (Lonza) and Neon (Life Technologies) technology, respectively. Thymidine, leptomycin B (Sigma), and BI2536 (JS Research Chemicals) were used at 2.5 mM, 5 nM, and 100 nM, respectively. Immunodetection was performed with antibodies against Greatwall (11), α-tubulin (Sigma), β-actin (Sigma), phospho-histone H3 (Millipore), MPM2 (Millipore), Mad2 (BD Biosciences), vinculin (Sigma), phosphoSer-CDK substrates (Cell Signaling), cyclin B1 (BD Biosciences), and phosphor-S126 Cyclin B1 (Abcam).

Immunofluorescence and Videomicroscopy. For immunofluorescence, cells were fixed in 4% (vol/vol) buffered paraformaldehyde for 10 min at room temperature, permeabilized in 0.15% (vol/vol) Triton X-100 for 3 min at 37 °C, and stained with the indicated antibodies and/or with 4,6 diaminophenylindole (DAPI, Prolong Gold antifade; Invitrogen) to visualize nuclei. Images were captured by using a Nikon Eclipse 90i microscope or a Leica DMI 6000B microscope. For time-lapse studies, cells were plated on eight-well glass-bottom dishes (Ibidi) and imaged with a Leica DMI 6000B microscope equipped with a 20×/1.5 N.A. objective lens or DeltaVision RT imaging system (Applied Precision; IX70/71; Olympus) equipped with a Plan Apochromatic 20×/1.42 N.A. objective lens, and maintained at 37 °C in a humidified CO_2 chamber. Images were acquired every 10 min for mitotic analysis and 1 min for export assays. Quantitative analysis of immunofluorescence was performed by using Definiens or ImageJ software.

Statistical Analysis. Statistical analysis was carried out by using Prism 5 (Graphpad Software). All statistical tests of comparative data were done by using two-sided, unpaired Student *t* tests or Fisher's exact test for categorical data. Data with $P < 0.05$ were considered statistically significant (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

ACKNOWLEDGMENTS. We thank Diego Megias for help with microscopy and Drs. M. Yoshida, V. Andrés, and G. Kops for reagents. M.A-F. and P.P.G. were supported by the European Union-PEOPLE programme and Caja Navarra, respectively. B.S.-C. and M.T. were supported by Foundation La Caixa. R.S.-M. was supported by the Juan de la Cierva programme from the Spanish Ministry of Economy and Competitiveness (MINECO) and Asociación Española contra el Cáncer. M.S.-F. was supported by the Formación de Personal Universitario (FPU) programme and the Spanish Ministry of Education. This work was funded by a grant from Bayer Pharma AG, the Foundation Ramón Areces, MINECO Grant SAF2012-38215, OncoCycle Programme Grant S2010/BMD-2470 from the Comunidad de Madrid, and the European Union Seventh Framework Programme MitoSys (Systems Biology of Mitosis) project Grant HEALTH-F5-2010-241548.

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