The study on regulatory mechanisms of Myt1 through its N-terminal domain in the cell cycle of Xenopus laevis

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Contents

SUMMARY

Immature animal oocytes are naturally arrested at the first meiotic prophase (Pro-I), which corresponds to the G2 phase of the cell cycle. In *Xenopus* oocytes, Myt1 kinase phosphorylates and inactivates cyclin-dependent kinase 1 (Cdk1) at Pro-I, thereby preventing oocytes from entering meiosis I (MI) prematurely. Previous studies have shown that, upon resuming MI, Cdk1 and p90rsk, which is a downstream kinase of the Mos-MAPK pathway, in turn phosphorylate the C-terminal region of Myt1, to suppress its activity, thereby ensuring high Cdk1 activity during M phase. However, the roles of the N-terminal region of Myt1 during meiosis and mitosis remain to be elucidated. In the present study, I show that the N-terminal region of Myt1 participates in the regulation of Myt1 activity in the *Xenopus* cell cycle. In particular, I found that a short, conserved sequence in the N-terminal region, termed here as the PAYF motif, is required for the normal activity of Myt1 in oocytes. Furthermore, multiple phosphorylations by Cdk1 at the Myt1 N-terminal region were found to be involved in the negative regulation of Myt1. In particular, phosphorylations at Thr11 and Thr16 of Myt1, which are adjacent to the PAYF motif, were found to be important for the inactivation of Myt1 in the M phase of the cell cycle. These results suggest that in addition to the regulation of Myt1 activity via the C-terminal region, the N-terminal region of Myt1 also plays an important role in the regulation of Myt1 activity.

INTRODUCTION

The eukaryotic cell cycle is composed of S (DNA synthesis) and M (mitosis) phases with intervening gap-phases, namely the G1 and G2 phases [1]. The transition through the M phase is driven by the activation of the Cdk1/Cyclin B complex (or M-phase promoting factor, called MPF) [2]. MPF promotes many mitotic events, such as nuclear envelope breakdown and chromosome condensation, through the regulatory phosphorylation of hundreds of divergent substrates [3, 4]. The activity of MPF is tightly regulated in multiple ways, including the oscillating levels of Cyclin B and phosphorylation of Cdk1 [5, 6]. In interphase, newly synthesized Cyclin B interacts with Cdk1, followed subsequently by Wee1 kinase family-mediated phosphorylation at Thr14 and Tyr15 of Cdk1 [7, 8], resulting in the suppression of Cdk1 activity [6]. In metazoans, the Wee1 kinase family comprises of Wee1 and Myt1. Wee1 is present in the cell nucleus and exclusively phosphorylates Tyr15 on Cdk1 [9, 10], whereas Myt1, a membrane-associated protein that is localized to both the endoplasmic reticulum and Golgi apparatus, phosphorylates both Thr14 and Tyr15 on Cdk1 [11, 12]. During mitotic entry, the Cdc25 phosphatase family antagonistically dephosphorylates these Cdk1 phosphorylation sites, thus activating the kinase [13, 14]. At the entry to mitosis and during M phase, Cdk1 phosphorylates both the Wee1 kinase and Cdc25 phosphatase families, causing both the downregulation of the Wee1 kinase family and upregulation of the Cdc25 phosphatase family [11, 15-17]. These double-negative and positive feedbacks during the M phase ensure a switch-like bi-stable Cdk1 activation system, which make the mutually exclusive cell cycle phase free of intermediate states [18].

During early animal development, the cell cycle changes drastically [1]. Regulation of inhibitory Cdk1 phosphorylation has important roles in cell cycle regulation during this period [19]. Fully grown immature *Xenopus* oocytes are arrested naturally at the prophase of the first meiotic cycle (Pro-I arrest) corresponding to the G2 phase of the cell cycle by the phosphorylated and inactivated Cdk1 [20-22]. Stimulation of oocytes with progesterone triggers meiotic resumption and progression through meiosis I (MI), following Cdk1 dephosphorylation and activation [20-22]. The interval

between MI and meiosis II (MII), called interkinesis, is very short and is not accompanied by DNA replication (or S phase) [20-22]. In vertebrates, oocytes are arrested again at the metaphase of MII (Meta-II arrest) by the dephosphorylated and highly activated Cdk1, until fertilization [20-22]. Meta-II arrest is released by fertilization to complete meiosis. The fertilized eggs then initiate a first mitosis with a short G2 phase accompanied by phosphorylated Cdk1, which is followed by 11 rapid cell cycles without appreciable G1 and G2 phases [23-25]. After the 12th cell cycle, zygotic genome activation of the embryos occurs, and the cell cycles elongate rather abruptly with phosphorylated Cdk1 at the mid-blastula transition (MBT) [24-26]. These dynamic changes in the cell cycle during *Xenopus* early development are precisely regulated by Cdk1-inactivating kinases such as Wee1A and Myt1 and Cdk1-activating phosphatases such as Cdc25A and Cdc25C [25, 27-29].

In *Xenopus*, Myt1, but not Wee1A, is present in immature oocytes, where it phosphorylates and inactivates Cdk1-Cyclin B complexes to maintain Pro-I arrest [27, 30, 31]. Upon progesterone-induced oocyte maturation, Myt1 activity is suppressed since Cdk1 activation results from the antagonistic regulation of Myt1 and Cdc25 activities while Cdc25 is activated [30, 31]. Inhibition of endogenous Myt1 induces Cdk1 activation and oocyte maturation without progesterone treatment [30, 31], indicating that Myt1 is pivotal for maintaining Pro-I arrest. However, there is little information on the suppression mechanism(s) of Myt1 during MI resumption. Myt1 consists of a short N-terminal regulatory domain (NRD), a central kinase domain (KD), and a long C-terminal regulatory domain (CRD) [12, 32]. The CRD of Myt1 is required for its localization to both the endoplasmic reticulum and Golgi apparatus [12]. In addition, Myt1 mutants lacking CRD cannot phosphorylate and inactivate Cdk1 efficiently *in vivo* [33]. In *Xenopus*, phosphorylation by Cdk1-Cyclin B, p90rsk, Cdk-RINGO, and Plk1 at multiple sites in the Myt1 CRD is thought to be involved in the inactivation of Myt1 [31, 33-35]. These phosphorylations weaken the interaction with Cdk1, resulting in the suppression of Myt1 kinase activity [33, 34]. These previous studies suggest that CRD plays an important role in the regulation of Myt1 kinase

activity. In addition to modification by these kinases, *Xenopus* Myt1 can autophosphorylate Ser66 and Ser76 in its NRD *in vitro* [36]. The autophosphorylation of Ser66 seems to be a prerequisite for further modifications, leading to the inactivation of Myt1 [36]. In starfish, Myt1 is also involved in Pro-I arrest in oocytes by inactivating Cdk1[37]; upon hormonal stimulation, serum- and glucocorticoid-regulated kinase (SGK) is activated and phosphorylates the N-terminal region of Myt1to resume MI [37]. However, the *Xenopus* Myt1 amino acid sequence corresponding to the starfish Myt1 sequence around the SGK phosphorylation site is not a typical SGK consensus sequence (RXRXXS/T, where X represents any amino acid; [38]). It is therefore unlikely that SGK phosphorylates and inactivates Myt1 during *Xenopus* oocyte maturation. Thus, compared to the role(s) of Myt1 CRD, those of most regions of the Myt1 NRD remain to be elucidated.

In this study, I investigated the role(s) of the NRD of Myt1 using *Xenopus* oocytes and embryos. We show that N-terminal truncation mutants of Myt1 display significantly reduced activities. Specifically, we found that a conserved sequence in the N-terminal region, termed the PAYF motif, is particularly important for its normal activity. In addition, I found that multiple phosphorylations by Cdk1 at the N-terminal region of Myt1 are involved in the negative regulation of Myt1. In particular, phosphorylations at Thr11 and Thr16 of Myt1, which are adjacent to the PAYF motif, are important for inactivation of Myt1 during oocyte maturation and cell cycle of the cleavage stage in *Xenopus*. These results indicate that not only the CRD of Myt1 but also the NRD is involved in the regulation of Myt1 activity during *Xenopus* development.

RESULTS

N-terminal truncations of Myt1 significantly reduced its activity

Myt1 consists of a highly conserved KD, a relatively divergent NRD, and a CRD (Fig. 1A) [12, 32]. Previous studies have revealed that the CRD is required for both the

subcellular localization and activity of Myt1 *in vivo* [12, 32]. In addition, multiple sites within the CRD of *Xenopus* Myt1 are phosphorylated by several kinases, such as MPF, p90rsk (a downstream kinase of MAPK), and the Cdk/XRINGO complex, for Myt1 inactivation during M phase in oocyte maturation [11, 33, 34]. Thus, many studies have been carried out to identify the important roles of the CRD in Myt1, but the NRD has not been studied in such detail. To elucidate the potential role(s) of the NRD, I constructed mRNAs encoding a series of mutants with truncations of N-terminal 10 to 100 amino acids (Δ10N- to Δ100N-Myt1), injected them (each tagged with three Myc epitopes) into *Xenopus* immature oocytes, and compared their activities in terms of inhibition of progesterone-induced GVBD, a hallmark of entry or progression into the meiotic M phase. In these experiments, each N-terminal-truncated Myt1 protein was expressed in the oocytes in amounts comparable to wild-type Myt1 (WT-Myt1) (upper panel in Fig. 1C). The GVBD inhibitory activity of Δ10N-Myt1 was very strong and similar to that of WT-Myt1 (Fig. 1B). Consistent with previous studies [33], the CRD-truncated Myt1 (ΔC-Myt1) had very weak GVBD-inhibiting activity (Fig. 1B), confirming the requirement of CRD for Myt1 activity *in vivo*. However, the activity of Δ25N-Myt1 was significantly weaker than that of WT-Myt1 (Fig. 1B). Moreover, Δ40N-, Δ50N-, Δ57N-, Δ67N-, and Δ90N-Myt1 had much weaker GVBD-inhibiting activities than WT-Myt1, while Δ100N-Myt1 had somewhat stronger GVBD-inhibiting activity than Δ90N-Myt1 (Fig. 1B). Interestingly, N-terminal truncations of more than 24 amino acids markedly reduced the biological activity of Myt1. I then attempted to compare the biochemical activity of WT-Myt1 and the N-terminal truncated Myt1 mutants that were expressed in oocytes. Ectopic expression of Myt1 does not completely impair progesterone-induced oocyte maturation [30]. Therefore, to evaluate the biochemical activities of WT-Myt1 and Myt1 mutants, oocytes injected with WT-Myt1 mRNA or each Myt1 mutant mRNA were treated with progesterone to induce maturation, collected immediately after GVBD, and subjected to immunoblotting with anti-phosphorylated Cdk1 antibody, as a measure of Myt1 activity. Before progesterone treatment, phosphorylated Cdk1 was appreciably detected at similar levels in control

immature oocytes and in oocytes ectopically expressing Myt1 (left side of middle panel in Fig. 1C: -PG). Just after GVBD, WT-Myt1 and a series of N-terminal truncated Myt1 mutants displayed reduced mobility in SDS-PAGE and this may be indicative of hyperphosphorylation in the oocytes (right side of upper panel in Fig. 1C: GVBD). In contrast, the mobility of ΔC-Myt1 was unaltered during maturation (middle panel in Fig. 1C), implying that the mobility shift of Myt1 at GVBD required the CRD. Consistent with the biological activity shown in Fig. 1B, phosphorylated-Cdk1 proteins were detected weakly (about 70% intensity compared to immature oocytes) in WT- or Δ10N-Myt1-expressing matured oocytes, slightly (about 25% intensity compared to immature oocytes) in Δ25N-Myt1-expressing matured oocytes, and hardly (about 10% intensity compared to immature oocytes) in Δ40N-, Δ50N-, Δ57N-, Δ67N-, Δ90N-, and Δ100N-Myt1-expressing oocytes and control matured oocytes. (Fig. 1C), suggesting that the N-terminal region of Myt1 is required for Myt1 to phosphorylate and inactivate Cdk1. Taken together, these results suggest that, similar to the CRD of Myt1, the NRD of Myt1 also has an important role in Myt1 activity in *Xenopus* oocytes.

Identification of a key box, PAYF motif, which regulates Myt1 activity

Given the above results, the region between the residues 11 to 24 of Myt1 seemed to be important for its activities in phosphorylating and inhibiting Cdk1. Interestingly, this region contains a short sequence, $_{21}$(Pro)-Ala-Tyr-(Phe)$_{24}$, which is well conserved between *Xenopus* and mammalian Myt1 homologs (Fig. 1A and 2A). Therefore, I tested whether this sequence (hereafter referred to as the PAYF motif) had a role in Myt1 activity. To do this, I constructed alanine mutants of the PAYF motif, including Pro21 to Ala (P21A), Phe24 to Ala (F24A), or three Ala (replaced Pro21, Tyr23, and Phe24 with Ala; named PAYF3A), injected each of these mRNAs into oocytes, and examined their activities in the inhibition of GVBD. In these experiments, I confirmed that Myt1 proteins were expressed in the oocytes in amounts comparable to WT-Myt1 (upper panel in Fig. 2C). Compared to WT-Myt1, the P21A mutant was less efficient at inhibiting GVBD, and the F24A mutant was even lesser efficient than P21A (Fig. 2B). Moreover,

the PAYF3A mutant inhibited GVBD very weakly, to approximately the same level as the Δ25N mutant (Fig. 2B). To evaluate the biochemical activity of these mutants, I investigated the phosphorylation status of Cdk1 in mature oocytes expressing the PAYF motif mutants, as shown in Fig. 1C. As shown in the middle panel of Fig. 2C, the levels of phosphorylated Cdk1 in the oocytes expressing each PAYF motif-mutated Myt1 were as low as those in oocytes expressing Δ25N-Myt1(about 50% intensity compared to that in oocytes expressing WT-Myt1). These results indicate that the PAYF motif within the NRD of Myt1 is specifically required for Myt1 to phosphorylate and inhibit Cdk1.

The possibility that phosphorylation of Myt1 NRD by Cdk1 negatively regulates its activity

During *Xenopus* oocyte maturation, Myt1 is phosphorylated by Cdk1 and p90rsk and negatively regulated to maintain the mitotic state [33, 34]. Although previous studies have mainly focused on the negative regulation of Myt1 *via* CRD phosphorylation, the possibility of Myt1 regulation *via* NRD phosphorylation has not been fully verified. Therefore, I investigated whether NRD phosphorylation by Cdk1 would have any effect on Myt1 activity. *Xenopus* Myt1 harbors four Ser/Thr-Pro motifs (Thr11-Pro, Thr16-Pro, Ser79-Pro, and Thr84-Pro), a typical Cdk1 consensus sequence [43], within the NRD. According to a previous study, Cdk1 phosphorylates the Ser/Thr-X-$[Arg/Lys]_{2-5}$ sequence in addition to a canonical Ser/Thr-Pro motif [44]. The NRD contains a residue (Ser32-Leu-Arg-Arg-Lys) that matches this non-canonical Cdk1 consensus motif. To assess the influence of NRD phosphorylation by Cdk1, I constructed an alanine mutant containing all five potential Cdk1-phosphorylatable residues in the NRD (hereafter referred to as 5A-Myt1), expressed it in oocytes, and then examined its ability to inhibit GVBD. In this experiment, a kinase-dead (N231A) form of Myt1 (KD-Myt1) [35] was used as a negative control for mRNA injection. Injection of WT-Myt1 mRNA significantly inhibited the occurrence of GVBD induced by progesterone treatment in oocytes, whereas that of KD-Myt1 scarcely did (Fig. 3A). Using immunoblotting, I confirmed that the amounts of expressed Myt1 proteins were comparable in Myt1

mRNA-injected oocytes (Fig. 3B). Consistent with previous studies [11, 33, 34], at the time of GVBD, KD-Myt1 and WT-Myt1 showed prominent upward mobility shifts (Fig. 3B). This prominent mobility shift of Myt1 proteins at this time was almost abolished upon λ phosphatase treatment (Supplementary Fig. 1), indicating that the mobility shifts of Myt1 were caused by hyperphosphorylation. Interestingly, as shown in Fig. 3A, the expression of 5A-Myt1 in oocytes inhibited GVBD induced by progesterone treatment more efficiently than that of WT-Myt1. In addition, the expression of 5A-Myt1 in oocytes also inhibited GVBD occurrence induced by ectopic Cyclin B expression and progesterone treatment (Supplementary Fig. 2). Furthermore, 5A-Myt1 was less phosphorylated than WT-Myt1, as evidenced by smaller size shifts, post GVBD (Fig. 3B). In addition, the levels of phosphorylated Cdk1 in matured oocytes expressing 5A-Myt1 were higher than those in matured oocytes expressing WT-Myt1 (middle panel and the bar diagram in Fig. 3B). These results suggest the possibility that Myt1 NRD phosphorylation probably by Cdk1 could affects negatively its activity during oocyte maturation.

A previous study showed that autophosphorylation of *Xenopus* Myt1 at Ser66 is involved in the meiotic inactivation of Myt1 [37]. I next evaluated whether the phosphorylation of Ser66 is related to the phosphorylation of Cdk1. To verify this, I compared the activity of 5A-Myt1 with that of 5A-Myt1 mutated on Ser66 to alanine (6A-Myt1). As shown in Fig. 3A, 6A-Myt1 inhibited GVBD slightly more efficiently than 5A-Myt1 and exhibited mobility similar to 5A-Myt1 during SDS-PAGE (Fig. 3B). The phosphorylation levels of Cdk1 in matured oocytes expressing 6A-Myt1 were higher than those expressing WT-Myt1 and comparable to those expressing 5A-Myt1 (Fig. 3B). Thus, autophosphorylation of Ser66 probably additively impacts Myt1 activity with phosphorylation by Cdk1.

Next, I identified the Cdk1-phosphorylatable site(s) in the NRD of Myt1 that could be responsible for its inactivation. To achieve this, I constructed several non-phosphorylatable Myt1 mutants, that is, three single-Ala mutants (T11A, T16A, and S32A) and two double Ala mutants (T11A/T16A and S79A/T84A), expressed them (by

injection of mRNA) in oocytes, and then tested their GVBD inhibition activity. After hormonal stimulation, however, most of the mutants (T11A, T16A, S32A, and S79A/T84A) inhibited GVBD to a similar degree as WT-Myt1, but intriguingly, one mutant (T11A/T16A, hereafter referred to as 2A) inhibited GVBD more efficiently than WT-Myt1 (Supplementary Fig. 3 and Fig. 3A). 2A-Myt1 showed slightly higher mobility in SDS-PAGE than WT-Myt1, and the levels of phosphorylated Cdk1 in matured oocytes expressing 2A-Myt1 were higher than those in matured oocytes expressing WT-Myt1 (Fig. 3B). Conversely, a phosphomimetic double-Asp mutant (T11D/T16D, hereafter referred to as 2D) did not inhibit GVBD as efficiently as WT-Myt1, but single-Asp mutants (T11D and T16D) did (Supplementary Fig. 3 and Fig. 3A). Moreover, Cdk1 in oocytes expressing 2D-Myt1 was less phosphorylated than that in oocytes expressing WT-Myt1. The activity of the 2A-Myt1 mutant was not as high as the activities of the 5A- and 6A-Myt1 mutants, but was clearly higher than that of WT-Myt1 (Fig. 3A). Thus, among the candidate residues, phosphorylations at both Thr11 and Thr16 of Myt1 were found to be especially important for the inhibition of Myt1 during maturation. It is noteworthy that these sites are adjacent to the PAYF motif, suggesting that the phosphorylation of these sites may be involved in the functions of PAYF motif.

I next intend to test whether Cdk1 could directly phosphorylate the NRD on these Ser/Thr residues. For this, I performed *in vitro* Cdk1 kinase assay by using Myc-tagged NRD peptides (residues 1-102) synthesized by reticulocyte lysate, which contained either the WT or the mutated Cdk1 consensus motifs (2A: T11A/T16A or 5A: T11A/T16A/S32A/S79A/ T84A) as substrates. Active Cdk1 (Cdk1-WT), but not a kinase-dead (K33R) form of Cdk1 (Cdk1-KR) [39], is able to phosphorylate the NRD-WT, as judged by their higher mobility in phos-tag-containing SDS PAGE (Fig. 3C). In addition, roscovitine, a specific inhibitor for Cdk1, reduced the phosphorylation of the WT-NRD, confirming that these phosphorylations are dependent on the Cdk1 activity (Fig 3C). These results indicate that Cdk1 can directly phosphorylate WT-NRD. As expectedly, 2A-NRD exhibited slightly less phosphorylation by Cdk1 than WT-NRD,

whereas the 5A-NRD underwent little phosphorylation (Fig. 3C). Thus, these results suggest that Thr11, Thr16, Ser32, Ser79, and Thr84 in the NRD are direct phosphorylation sites by Cdk1 *in vitro*, consistent with it lying in Cdk1 consensus motif.

Next, I attempted to identify the kinase(s) responsible for phosphorylation of these sites in the NRD of Myt1 *in vivo*. Since both Cdk1 and MAPK can phosphorylate the Ser/Thr-Pro motif, I tested which kinase is involved in NRD phosphorylation. For this, I ectopically expressed WT-Myt1, 2A-Myt1, or 5A-Myt1 in oocytes treated with roscovitine, which is a specific inhibitor of Cdk1, and then expressed Mos, which is a MAP kinase kinase kinase [20, 21]. Under these conditions, MAPK was activated without Cdk1 activation in the oocytes, but none of the expressed Myt1 proteins induced appreciable mobility shifts (Fig. 4). In contrast, when the oocytes were treated with U0126, which is a specific inhibitor of MEK, and injected with the N-terminal-deleted non-degradable Cyclin B1 (ΔCycB) mRNA, Cdk1 was partially activated without MAPK activation (Fig. 4). Under these conditions, WT-Myt1 induced prominent mobility shifts (Fig. 4), while the mobility shift of 2A-Myt1 was smaller than that of WT-Myt1, and that of 5A-Myt1 was even smaller than that of 2A-Myt1 (Fig. 4). In addition, WT-Myt1 exhibited the mobility shift in the matured oocytes induced by progesterone in the presence of U0126 (Fig. 4). Taken together, these results suggest that Cdk1, but not MAPK, phosphorylates Myt1 at Thr11, Thr16, Ser32, Ser79, and Thr89, for negative regulation during oocyte maturation.

Myt1 NRD phosphorylation during oocyte maturation

I next examined the phosphorylation status of Myt1 NRD during *Xenopus* oocyte maturation. To do this, I injected immature oocytes with KD-Myt1 mRNA or Cdk1 non-phosphorylatable kinase-dead form of Myt1 (2A/KD- and 5A/KD-Myt1) mRNA, in order to reduce its effect on the progression of the cell cycle. Subsequently, the oocytes were treated with progesterone to induce maturation, and the activation of phosphorylation of MAPK, inhibition of phosphorylation of Cdk1, levels of Cyclin B1, and phosphorylation levels of ectopically expressed Myt1 were monitored using

immunoblotting. As shown in Fig. 5, progesterone treatment induced a slight mobility shift of KD-Myt1 prior to the activation of dephosphorylation of Cdk1, activation of MAPK phosphorylation, and GVBD occurrence (3 h after progesterone treatment in Fig. 5). Interestingly, both the mobility shift of 2A/KD- and that of 5A/KD-Myt1 were also observed to the same extent at this time, suggesting that the phosphorylation of the NRD on these sites by Cdk1 did not occur before GVBD. Upon GVBD, both Cdk1 and MAPK were abruptly activated in the oocytes (4 h after progesterone treatment in Fig. 5), and KD-Myt1 induced hyperphosphorylation, as determined using immunoblotting. As expected, just after GVBD, the phosphorylation of 2A/KD-Myt1 was slightly less than that of KD-Myt1, and the phosphorylation of 5A/KD-Myt1 was even lower than that of 2A/KD-Myt1, as judged by their smaller size shifts (4 h after progesterone treatment in Fig. 5). The mobilities of KD-, 2A/KD-, and 5A/KD-Myt1 were essentially unaltered 8 h after GVBD (Fig. 5). These results indicate that the phosphorylation of Myt1 NRD occurs from post GVBD stage to MII arrested stage, suggesting the possibility that multiple phosphorylations at the Myt1 NRD are involved in its inactivation throughout the M phase of meiosis.

Involvement of Myt1 NRD phosphorylation in the cell cycle of early Xenopus development

The above results suggest that NRD phosphorylation by Cdk1 negatively regulates Myt1 activity, to maintain high Cdk1 activity from Meta-I to Meta-II arrest during oocyte maturation. Next, I asked whether this regulation of Myt1 activity plays an important role post fertilization as well. In *Xenopus*, the first cell cycle, immediately after fertilization, takes approximately 90 min. Unlike the subsequent short (approximately 30 min) cell cycle up to MBT, there is a G2 phase of the cell cycle with phosphorylated Cdk1 in this cycle. According to a previous study, Myt1 is involved in the creation of the G2 phase in the first cell cycle [45]. Therefore, I addressed whether NRD phosphorylation by Cdk1 has any role in the first cell cycle. Since, due to the presence of a jelly layer, *Xenopus* eggs just post fertilization are very difficult to inject

with mRNAs for ectopic expression of Myt1, I utilized MII-arrested oocytes to analyze Myt1 phosphorylation during the first cell cycle. For this, I ectopically expressed either KD-Myt1, KD/2A-Myt1, or KD/5A-Myt1 in immature oocytes, treated them with progesterone to induce maturation, and then treated them with A23187, a calcium ionophore, to induce activation (which mimics fertilization), following which I monitored the phosphorylation status of Myt1 by means of immunoblotting (Fig. 6A). The inhibitory phosphorylation of Cdk1 in KD-Myt1-expressing oocytes increased 40 min after A23187 treatment and decreased 80 min after treatment (Fig. 6A), suggesting that the activated mature oocytes entered the M phase approximately 80 min post treatment. KD-Myt1 underwent dephosphorylation gradually from 20 min to 60 min post treatment and phosphorylated again from 80 min post treatment (Fig. 6A). In contrast, the change in the mobility shift of KD/2A-Myt1 in the matured oocytes treated with A23187 was smaller than that of KD-Myt1 (Fig. 6A). Interestingly, the size shift of KD/5A-Myt1 was scarcely observed before and after treatment. Collectively, these results suggest that Thr11, Thr16, Ser32, Ser79, and Thr84 on Myt1 are phosphorylated in Meta-II-arrested eggs, and these sites are preferentially dephosphorylated just after fertilization and phosphorylated again at the M phase in the cleavage stage, implying the possibility that negative regulation of Myt1 *via* NRD phosphorylation contributes to cell cycle progression during the early cleavage stage in *Xenopus*. To assess this possibility, I injected mRNA encoding either KD-Myt1, 2A/KD-Myt1, or 5A/KD-Myt1 into one-cell stage embryos and monitored their phosphorylation status using immunoblotting during the cleavage stage. Due to the difficulty in synchronization of the cell cycle in embryos and detection of Cdk1 phosphorylation during the cleavage stage, I was not able to discern the cell cycle stages of embryos precisely, but as shown in Fig. 6B, both KD/2A- and KD/5A-Myt1 in the embryos showed significantly higher mobility than KD-Myt1 during the cleavage stage, indicating that the phosphorylation of the NRD in Myt1 occurred during this period. At the early gastrula stage (600 min after fertilization), the inhibitory phosphorylation of Cdk1 was easily detected, and KD-Myt1 showed mobility similar to that of KD/2A- and KD/5A-Myt1 (Fig. 6B). These

results suggest that Cdk1-dependent phosphorylation of NRD also occurs during the *Xenopus* cleavage stage.

Finally, I investigated whether NRD phosphorylation is involved in the embryonic cell cycle. The expression of 2A-Myt1 in the one-cell stage embryos caused a more pronounced delay in cell division than that of WT-Myt1 and 2D-Myt1 (Fig. 7A). Moreover, the expression of 5A-Myt1 led to a more appreciable delay than that of 2A-Myt1 (Fig. 7A), as judged from the external morphology. Consistent with the external morphology, Cdk1 in 2A-Myt1-expressing embryos and 5A-Myt1-expressing embryos was more highly phosphorylated than that in WT-Myt1- or 2D-Myt1-expressing embryos (Fig. 7B). Thus, these results suggest that phosphorylation of the N-terminal region of Myt1 by Cdk1 plays a role, at least partially, in the progression of the cell cycle in early *Xenopus* embryos.

DISCUSSION

In eukaryotic cells, Wee1 and Myt1 kinases phosphorylate and inhibit Cdk1, to prevent cells from entering mitosis prematurely [8-10]. These kinases play a functionally redundant role in the inhibition of Cdk1 in somatic cells [11,12]. In contrast, Myt1, but not Wee1, is present in immature *Xenopus* oocytes [27,30,46]. Therefore, *Xenopus* oocytes are suitable for analyzing only the function of Myt1. To explore how Myt1 kinase activity is regulated, I expressed a series of Myt1 N-terminal truncation mutants in *Xenopus* oocytes. Myt1 mutants with truncations of the N-terminal 25 to 100 amino acids scarcely inhibited oocyte maturation and exhibited markedly reduced kinase activity (Fig. 1). These features were not observed in Myt1 mutants with truncation of the N-terminal 10 amino acids, as well as in WT-Myt1. Thus, removal of amino acids 11-25 from the N-terminus caused decreased activity of Myt1, suggesting that this N-terminal region is required for Myt1 kinase activity. I found that this region in Myt1 contains a conserved short sequence [$_{21}$(Pro)-Ala-Tyr-(Phe)$_{24}$], named the PAYF motif (Fig. 2). Substituting the PAYF motif in Myt1 with alanine resulted in decreased activity.

Interestingly, the activity of the PAYF-substituted alanine Myt1 (PAYF3A-Myt1) was comparable to that of the N-terminal 25 amino acids truncated Myt1 (Δ25N-Myt1). These results would imply that the N-terminal residues 11 to 25 amino acids affect normal Myt1 kinase activity positively because of, at least in part, the presence of the PAYF motif. Previous studies on the regulation of Myt1 have mainly focused on its C-terminal region, which is responsible for both localization in the cells and interaction with Cdk1 [12, 32-34]. To our knowledge, this is the first report that shows the involvement of the N-terminal region in Myt1 kinase activity *in vivo*.

In the M phase of the cell cycle, Myt1 undergoes hyperphosphorylation and inactivation by mitotic kinases, including Cdk1[12, 32-36]. A number of studies have shown that multiple phosphorylations in the C-terminal region of Myt1 during M phase negatively impact the biological activity of this kinase [32-35], but little research has been done on the phosphorylation of this kinase in the N-terminal region. To further address the role of the N-terminal region of Myt1, I expressed several mutants in oocytes that replaced the five potential phosphorylation sites of Cdk1 in the N-terminal region with alanine. I found that a Myt1 mutant in which all the five sites in the N-terminal region were replaced with alanine (5A-Myt1) significantly suppressed GVBD and strongly phosphorylated Cdk1 in oocytes, as compared to WT-Myt1 (Fig. 3). Intriguingly, a Myt1 mutant in which both Thr11 and Thr16 were replaced with alanine (2A-Myt1) inhibited GVBD induced by hormonal stimulation more efficiently than WT-Myt1, albeit less efficiently than 5A-Myt1 (Fig. 3). The PAYF motif (residues 21 to 24), which seems to have a positive effect on Myt1 activity, is adjacent to Thr11 and Thr16. Therefore, phosphorylation of these sites may have a negative effect on the PAYF motif. Since the PAYF motif contains a proline residue, there is a possibility that mutations to the PAYF motif can affect the structure of Myt1 regardless of proximity phosphorylation status. Compared to WT-Myt1, both 2A-Myt1 and 5A-Myt1 mutants showed less Cdk1-dependent mobility shifts in the M phase (Fig. 3B, Figs. 5-6), suggesting that the phosphorylation of these sites occurs in the M phase, thereby negatively regulating Myt1 activity. According to the previous paper [31], upregulation of Cyclin B synthesis

in response to progesterone causes the phosphorylation and the inactivation of Myt1, thereby causing in turn Cdk1 activation. Cdk1 activates Greatwall kinase (Gwl), which suppresses the protein phosphatase 2A-B55 (PP2A-B55) which antagonizes Cdk1 [47]. Therefore, it is thought that the phosphorylations of both the NRD and the CRD are maintained by Cdk1 activation and PP2A-B55 suppression by Gwl during M phase.

In this study, I showed that the NRD of Myt1 was phosphorylated post fertilization (Fig. 6A-B). In addition, expressing 2A- or 5A-Myt1 in fertilized eggs caused a significant delay in the cell cycle at the cleavage stage (Fig. 7A). These results suggest that the regulation of Myt1 *via* N-terminal phosphorylation is important for early *Xenopus* development. Notably, both the PAYF motif and Thr11 and Thr16 in Myt1 are conserved between mammals and *Xenopus* (Fig. 2A). In mammalian somatic cells, Myt1 is highly expressed in proliferating cells and is negatively regulated by phosphorylation during the M phase [33]. Therefore, a similar mechanism of Cdk1-mediated phosphorylation of the N-terminal region of Myt1 and negative regulation of its activity may operate in mammalian cells. Remarkably, the site corresponding to Thr16 in *Xenopus* Myt1 is also conserved in *C. elegans* Myt1. It is worth investigating whether the regulatory mechanism of Myt1 revealed in this study also operates in other animal species.

The underlying molecular mechanism(s) of the N-terminal region, including the PAYF motif and its phosphorylation in M phase, which are involved in the regulation of Myt1 kinase activity, are, at present, uncertain. One possibility is that the multiple phosphorylations of the NRD negatively impact on the binding ability of Myt1 to Cdk1-Cyclin B complex. Therefore, I performed co-immunoprecipitation analysis to test this possibility. As a result, there was no significant difference in the binding ability between wild type and mutants (Supplementary Fig. 4). According to previous reports, Cdk1 binds the C-terminal region of Myt1, and the N-terminal region does not appear to be significantly involved in binding to Cdk1 [32].

In contrast, several studies have been conducted on the control mechanism of Myt1 activity *via* the C-terminal region of Myt1. According to previous studies, Myt1

binds to Cdk1 at the C-terminal region, thereby phosphorylating and inactivating Cdk1 during the interphase of the cell cycle [32]. In the M phase, the C-terminal region of Myt1 is phosphorylated by Cdk1/RINGO and p90rsk in *Xenopus* oocytes and by Cdk1 in mammalian somatic cells, and these phosphorylations change the conformation of Myt1, leading to full Cdk1 activation in cells [33, 34]. The cis/trans peptidyl-prolyl isomerase Pin1 binds to phosphorylated Ser/Thr-Pro sites of target proteins and changes their conformation *via* prolyl isomerization at the Ser/Thr-Pro bonds [48,49]. Pin1 binds to Myt1 at the phosphorylated C-terminal region, and Pin1 binding to Myt1 is thought to cause structural changes in Myt1 during the M phase [32,50]. As shown in Fig. 1C, the C-terminal-deleted Myt1 (ΔC-Myt1) exhibited little mobility shift at the time of GVBD. This result suggests that phosphorylation of NRD may be required for CRD phosphorylation. There is a possibility that Pin1-mediated structural changes in the phosphorylated CRD during the M phase may have some influence on the phosphorylation of NRD. In any case, structural analysis of both the NRD and CRD of Myt1 may reveal the mechanism of Myt1 kinase activation.

In summary, I found that the N-terminal region of Myt1, which has received little attention, plays an essential role in the inhibitory phosphorylation by Cdk1. In addition, the negative regulation of Myt1 *via* phosphorylation of the N-terminal region of Myt1 by Cdk1 is involved in normal cell cycle progression, during both meiosis and mitosis in *Xenopus*. Myt1 has essential developmental functions in male and female gametogenesis in *Caenorhabditis elegans*, *Drosophila melanogaster*, and the starfish *Asterina pectinifera* [38,51,52]. In the murine myeloid precursor cell line M1, suppression of p53-mediated Myt1 hyperphosphorylation is involved in the G2/M checkpoint [53]. The activity of Myt1 is regulated by its phosphorylation levels, and not by its protein levels. Elucidation of the regulatory mechanism of Myt1 would be useful not only for understanding cell cycle regulation during early development, but also in clinical applications.

MATERIALS AND METHODS

Preparation, microinjection, and treatment of Xenopus oocytes and embryos

Xenopus oocytes and embryos were prepared, cultured, and microinjected, as described previously [39, 40]. Stage VI oocytes were treated with progesterone (5 μg/mL) to induce maturation. Matured oocytes, 12 h after progesterone treatment, and at least 4 h after germinal vesicle breakdown (GVBD), were treated with the calcium ionophore A23187 (1 μg/mL; Sigma-Aldrich, St Louis, MO, USA) to induce egg activation. Animal studies were conducted according to the guidelines of the Animal Experiment Committee of Kyushu University.

Antibodies and immunoblot analysis

Frozen oocytes or embryos (usually five in number) were homogenized in 50 μL extraction buffer [80 mM β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 0.2 mM PMSF, 10 μg/mL aprotinin, 20 μM leupeptin, 20 μM pepstatin, 1 mM Na_3VO_4, 1 mM NaF, 1 mM DTT, and 0.01% Triton™ X-100] and centrifuged briefly at 2°C. The supernatant was diluted with an equal volume of 2× Laemmli's sample buffer, and a portion of it (equivalent to one oocyte or egg) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for immunoblotting. To determine the phosphorylation status of Cdk1, the blots were probed with anti-phospho-Thr14/Tyr15 Cdk1 antibody (BioSource, Camarillo, CA, USA; #44-686G), and then they were reprobed with anti-Cdk1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; SC-054). Other antibodies used in this study were anti-Myc antibody (Santa Cruz Biotechnology; A-14), anti-MAPK antibody (Santa Cruz Biotechnology; A-14), anti-phospho-p42/p44 MAPK antibody (Cell Signaling Technology, Danvers, MA, USA; 9106), and anti-Cyclin B1 serum (a gift from Dr. J.E. Maller). Antibodies were detected using chemiluminescence reagent. Digital images were acquired and analyzed using an Amersham Imager 680 (GE healthcare life sciences).

In vitro Cdk1 kinase assay

Myc-tagged Myt1-NRD (1-102) proteins for the substrates were synthesized in rabbit reticulocyte lysate (RRL) (Promega, L4960) in accordance with the manufacturer's protocol. Active Cdk1-Cyclin B complex was prepared as follows. Oocytes were injected with FLAG-tagged Cdk1 mRNA and Myc-tagged ΔN-Cyclin B mRNA. After 8 hours of incubation, they were homogenized in extraction buffer [10 mM HEPES-KOH (pH 7.5), 80 mM β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 0.2 mM PMSF, 10 μg/mL aprotinin, 20 μM leupeptin, 20 μM pepstatin, 1 mM Na_3VO_4, 1 mM NaF, 1 μM Okadaic acid, 1 mM DTT], and centrifuged briefly. The supernatants, equivalent to 10 oocytes, were incubated with 5 μL anti-FLAG antibody (Sigma-Aldrich, St Louis, MO, USA; F1840) and 10 μL Dynabeads Protein G (Invitrogen, Carlsbad, CA, USA) for 1 hour at 4°C with constant mixing for the immunopurificaton of Cdk1-Cyclin B complex. The beads were washed twice with extraction buffer, and then washed three times with kinase buffer [10 mM HEPES-KOH (pH 7.5), 80 mM β-glycerophosphate, 15 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM NaF, 1 μM Okadaic acid, 1 mM DTT]. For Cdk1 kinase assay, 3 μL of RRL expressing Myc-tagged Myt1-NRD and 2 μL of active Cdk1-bounded Dynabeads were mixed in kinase buffer supplemented with 20 mM ATP and then incubated 2 hours at 30°C. In some cases, roscovitine (Sigma-Aldrich, St Louis, MO, USA) was added to the reaction to a final concentration of 10 μM to inhibit the kinase reaction. The reactants were subjected to phos-tag (Fujifilm, Japan) immunoblotting analysis in accordance with the manufacturer's protocol with appropriate antibodies for evaluating Myt1-NRD phosphorylation.

cDNA and in vitro transcription

cDNA encoding *Xenopus* Myt1 was provided by RIKEN BRC through the National BioResource Project of the MEXT/AMED, Japan. N-terminal truncation mutants of Myt1 (Δ10N-, Δ25N-, Δ15N-, Δ40N-, Δ50N-, Δ57N-, Δ67N-, Δ90N-, and Δ100N-Myt1) were constructed using PCR of wild-type (WT) Myt1 with appropriate individual primers. The PCR products were subcloned into N-terminal Myc_3-tagged pT7G(UKII+)

transcription vector [41]. cDNA constructs of a non-degradable mutant *Xenopus* Cyclin B1 (ΔCyclin B1) and c-mos have been described in [39]. *In vitro* mutagenesis was performed as described previously [28]. *In vitro* transcription was performed using the MEGAscript™ T7 Transcription Kit (Thermo Fischer Scientific, Waltham, MA, USA), as described previously [28].

Immunoprecipitation

For immunoprecipitation assay, oocytes expressing Myc-tagged Myt1, hemagglutinin (HA)-tagged Cyclin B, and FLAG-tagged Cdk1-KR were homogenized in extraction buffer [10 mM HEPES-KOH (pH 7.5), 80 mM β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 0.2 mM PMSF, 10 μg/mL aprotinin, 20 μM leupeptin, 20 μM pepstatin, 1 mM Na_3VO_4, 1 mM NaF, 1 mM DTT, and 0.01% Triton™ X-100] and centrifuged briefly. The supernatants, equivalent to 10 oocytes, were incubated with 5 μL anti-FLAG antibody (Sigma-Aldrich, St Louis, MO, USA; F1840) for 1 hour at 4°C, and then incubated with 5 μL Dynabeads Protein G (Invitrogen, Carlsbad, CA, USA) for immunoprecipitation for 1 hour at 4°C with constant mixing. The beads were washed five times with the binding buffer, eluted with 2× Laemmli's sample buffer, and then subjected to immunoblotting with appropriate antibodies.

Phosphatase treatment

Frozen oocytes (usually five in number) were homogenized in λ phosphatase buffer [50 mM Tris-Cl (pH 7.5), 100 mM NaCl, 0.1 mM EGTA, 2 mM DTT, 0.01% Brij 35, 2 mM $MnCl_2$], and incubated with or without 200 U λ phosphatase/oocyte for 30 min at 30°C. The cell lysates were diluted with an equal volume of 2× Laemmli's sample buffer and subjected to immunoblot analysis.

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FIGURE LEGENDS

Fig. 1. The N-terminal region of Myt1 is required for its activity. (A) A schematic representation of Xenous Myt1 protein. Domain organization of Myt1, showing the N-terminal regulatory domain (NRD), kinase domain (KD), and C-terminal regulatory domain (CRD). See text for "PAYF" sequence. **(B)** GVBD inhibiting activity of the N-terminal- or C-terminal-truncated Myt1 mutants. Thirty immature stage VI oocytes were injected with water or 3-4 ng (depending on the size) of the indicated form of Myt1 mRNA (each tagged with three Myc epitopes). However, the C-terminal truncated Myt1 (ΔC) was injected with only 400 pg, because it expressed a large amount when injected with the same dose of mRNA as the other mutants. The oocytes were incubated overnight, treated with progesterone, and cultured. When water-injected oocytes fully underwent GVBD, the proportion of remaining oocytes that did not reach GVBD was scored. The score of wild-type (WT) Myt1-injected oocytes was set as 100%, following which the relative scores of the other mutant-injected oocytes were calculated. All values are mean ± SD of four independent experiments. **(C)** Immunoblotting analysis of oocytes injected with WT-Myt1 or truncated mutants. Five oocytes expressed either WT or the respective mutants were collected before progesterone treatment (-PG) or just after GVBD (GVBD), and then analyzed using immunoblotting with anti-Myc (for expressed Myt1), anti-phospho-Thr14/Tyr15 Cdk1, or anti-Cdk1 (used for as a loading control) antibodies. In the bar diagram, the relative value obtained for the pCdk1 level in WT-Myt1 injected immature oocytes was set at 1.0, and all values are means ± SD of four independent experiments.

Fig. 2. Identification of a key box, PAYF motif, which regulates Myt1 activity. (A) Conservation of the PAYF motifs in the NRDs of Myt1 kinase. The motif is underlined in red. **(B)** Thirty-five immature oocytes were injected with either water or 3 ng of the indicated Myt1 mRNAs (each tagged with three Myc epitopes), incubated overnight, and treated with progesterone. The oocytes were scored for the percentage of GVBD at the indicated times. **(C)** Immunoblot analysis of oocytes injected with RNA encoding

wild-type-Myt1, PAYF motif-mutated-Myt1, or Δ25N-Myt1. Five oocytes were collected just after GVBD, and then analyzed by means of immunoblotting for the indicated proteins. In the bar diagram, the relative value obtained for the pCdk1 level in WT-Myt1-injected immature oocytes was set at 1.0, and all values are means ± SD of four independent experiments. For both (B) and (C), essentially similar results were obtained in five other independent experiments, of which one representative result is shown.

Fig. 3. Phosphorylation of the Myt1 NRD involved in negative regulation of its activity. (A) Forty immature oocytes were injected with either water or 1 ng of the indicated Myt1 mRNAs (each tagged with three Myc epitopes), incubated overnight, and treated with progesterone. The oocytes were scored for the percentage of GVBD at the indicated times. **(B)** Immunoblotting analysis of oocytes injected with RNA encoding either wild-type-Myt1 or the indicated mutants. Five oocytes were collected either before progesterone treatment (-PG) or just after GVBD, and then analyzed by means of immunoblotting for the indicated proteins. In the bar diagram, the relative value obtained for the pCdk1 level in WT-injected immature oocytes was set at 1.0, and all values are means ± SD of four independent experiments. For both (A) and (B), essentially similar results were obtained in four other independent experiments, of which one representative result is shown. **(C)** Indicated the Myc-tagged NRD Myt1 peptides (with or without Ser/Ala mutations) were subjected to in vitro kinase assays with or without 30 mM roscovitine using Myc-tagged ΔN-Cyclin B and either FLAG-tagged Cdk1-WT or -KR complex, which is immunopurified from oocytes. The reactants were subjected to SDS-PAGE with or without phos-tag, followed by immunoblotting with indicated antibodies.

Fig. 4. Cdk1 is responsible for the phosphorylation of the Myt1 NRD. Immature oocytes were injected with 500 pg of the indicated mutant mRNA (each tagged with three Myc epitopes). After overnight incubation, oocytes were treated as follows:

DMSO only; injected with 10 ng of Myc-tagged Mos mRNA, immediately followed by 100 μM of roscovitine (Mos, roscovitine); injected with 10 ng of Myc-tagged ΔN-Cyclin B1, immediately followed by 100 μM of U0126 (ΔN-CycB, U0126); treated with 5 μg/mL of progesterone (PG) in the presence or absence of U0126. Five oocytes from the DMSO- and PG-treated groups were collected 12 h after each treatment. Five oocytes from the Mos plus roscovitine-treated group were collected 1 h after treatment. Five oocytes from the ΔN-Cyclin B plus U0126-treated group were collected 2 h after treatment. The samples were then subjected to immunoblot analysis for the indicated proteins. Anti-MAPK antibodies and anti-Cdk1 antibodies were used as loading controls. In the bar diagram, the relative value obtained for the pCdk1 level in immature oocytes (IMO) was set at 1.0. A representative result is shown.

Fig. 5. Phosphorylation status of the NRD of Myt1 during oocyte maturation. Immature oocytes were injected with either 500 pg of KD-, 2A/KD-, or 5A/KD-Myt1 mRNA (each tagged with three Myc epitopes). The oocytes were incubated overnight, treated with progesterone, and five of them were collected at the indicated time for immunoblot analysis. Immature oocytes (IMO) and mature oocytes (MO) were injected with dH_2O. GVBD indicates the time at which 50% of the injected oocytes underwent GVBD.

Fig. 6. The phosphorylation status of Myt1 NRD in the cleavage stage of Xenopus. **(A)** The phosphorylation status of Myt1 NRD after egg activation. Immature oocytes were injected with either 500 pg of KD-, 2A/KD- or 5A/KD-Myt1 mRNA (each tagged with three Myc epitopes), incubated overnight, treated with progesterone, and 12 h later, treated with a calcium ionophore to activate the eggs. Five of these activated eggs were collected at the indicated times and subjected to immunoblot analysis for the indicated proteins. Immature oocytes (IMO) were injected with KD-Myt1 mRNA before progesterone treatment. **(B)** One-cell stage embryos were injected with either dH_2O or 1 ng of the indicated form of kinase-dead mutant mRNA (each tagged with three Myc

epitopes) and cultured. Five of these embryos were collected at the indicated time and subjected to immunoblot analysis for the indicated proteins. Immature oocytes (IMO) and mature oocytes (MO) were injected with KD-Myt1 mRNA, and then collected before or 12 h after progesterone treatment, respectively.

Fig. 7. Involvement of Myt1 NRD phosphorylation in the cell cycle of early Xenopus development. (A) One-cell stage embryos were injected with either water or the indicated form of Myt1 mRNA (1 ng/embryo) and cultured until the water-injected embryos reached Stage 7. The embryos were subsequently fixed and photographed. **(B)** One-cell stage embryos were injected with either water or 1 ng of the indicated Myt1 mRNA (each tagged with three Myc epitopes) and cultured until the water-injected embryos reached Stage 5 (3 h post fertilization; 3 hpf). Subsequently, the embryos were collected and subjected to immunoblot analysis for the indicated proteins. IMO, immature oocytes injected with KD-Myt1 mRNA; MO, mature oocytes injected with KD-Myt1 mRNA and induced maturation by means of progesterone treatment. In the bar diagram, the relative value obtained for the pCdk1 level in immature oocytes (IMO) was set at 1.0, and all values are means ± SD of four independent experiments.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Fig. 1. Phosphorylation-dependent mobility shifts of Myt1-WT, 2A and 5A Myt1 after GVBD. Immature oocytes were injected with either 500 pg of WT, 2A, or 5A kinase-dead form mutant mRNA (each tagged with three Myc epitopes), incubated overnight, and then treated with progesterone. Five of these treated oocytes were collected before and 12 h after progesterone treatment and subjected to immunoblot analysis. Lysates of matured oocytes were incubated with (+λ) or without (-λ) lambda phosphatase before immunoblot analysis.

Supplementary Fig. 2. The expression of 5A-Myt1 in oocytes also inhibited GVBD occurrence induced by ectopic Cyclin B expression and progesterone treatment.

Forty immature oocytes were injected with either water (Ctrl) or 1 ng of either wild-type Myt1 mRNA (WT) or 5A-Myt1 mRNA (5A) (each tagged with three Myc epitopes), incubated overnight, and injected with or without 200 pg of Cyclin B2 (CycB) mRNA, and then treated with progesterone (PG). The oocytes were scored for the percentage of GVBD at the indicated times.

Supplementary Fig. 3. Biological activity of Myt1 mutants to inhibit GVBD. Fifty immature oocytes were injected with either dH_2O or 1 ng of Myt1 mRNA (each tagged with three Myc epitopes), incubated overnight, and then treated with progesterone. When all the dH_2O-injected oocytes underwent GVBD, the proportion of remaining oocytes that did not reach GVBD was scored. The score of the WT-injected oocytes was set at 100%, following which the relative scores of the other mutant-injected oocytes were calculated. All values represent mean ± SD of four independent experiments.

Supplementary Fig. 4. There was no significant difference in the binding ability to Cdk1 between wild-type and mutants. Immature oocytes were injected with indicated each Myc-tagged Myt1 mutant mRNA, FLAG-tagged Cdk1-KR mRNA, and HA-tagged Cyclin B2 mRNA. Twelve hours after injecting mRNA, FLAG-tagged Cdk1 proteins were immunoprecipitated with anti-FLAG antibody and then whole-cell extracts (WCE) and immunoprecipitates (FLAG-IP) were subjected to immunoblotting with either anti-Myc (for Myt1), anti-FLAG (for Cdk1), or anti-HA (for Cyclin B) antibodies.

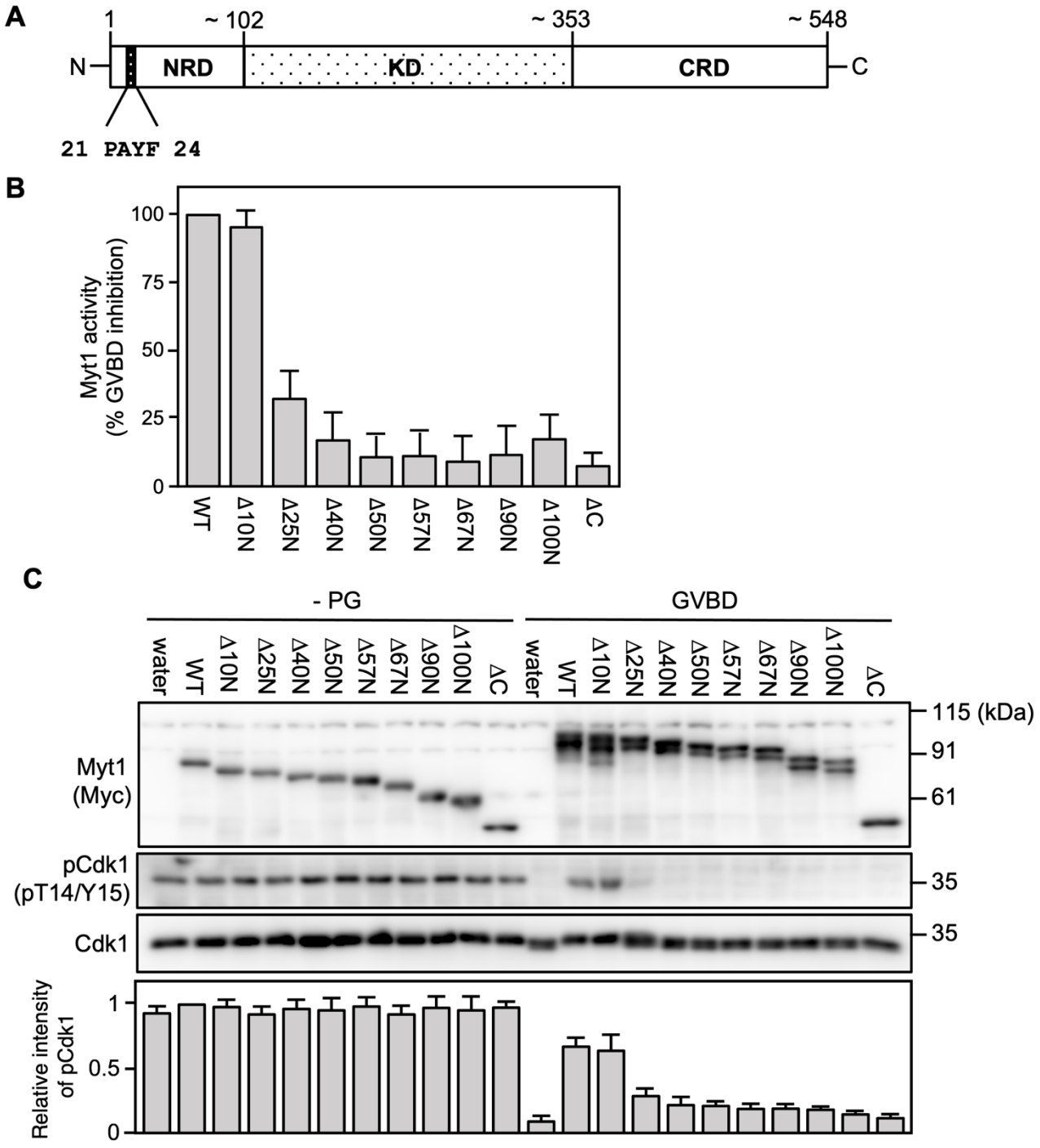
A
1
~ 102
~ 353
~ 548
N
NRD
KD
CRD
C
21 PAYF 24
B
Myt1 activity
(% GVBD inhibition)
100
75
50
25
0
WT
Δ10N
Δ25N
Δ40N
Δ50N
Δ57N
Δ67N
Δ90N
Δ100N
ΔC
C
- PG
GVBD
water
WT
Δ10N
Δ25N
Δ40N
Δ50N
Δ57N
Δ67N
Δ90N
Δ100N
ΔC
115 (kDa)
91
61
Myt1
(Myc)
pCdk1
(pT14/Y15)
35
Cdk1
35
Relative intensity
of pCdk1
1
0.5
0

Figure 1

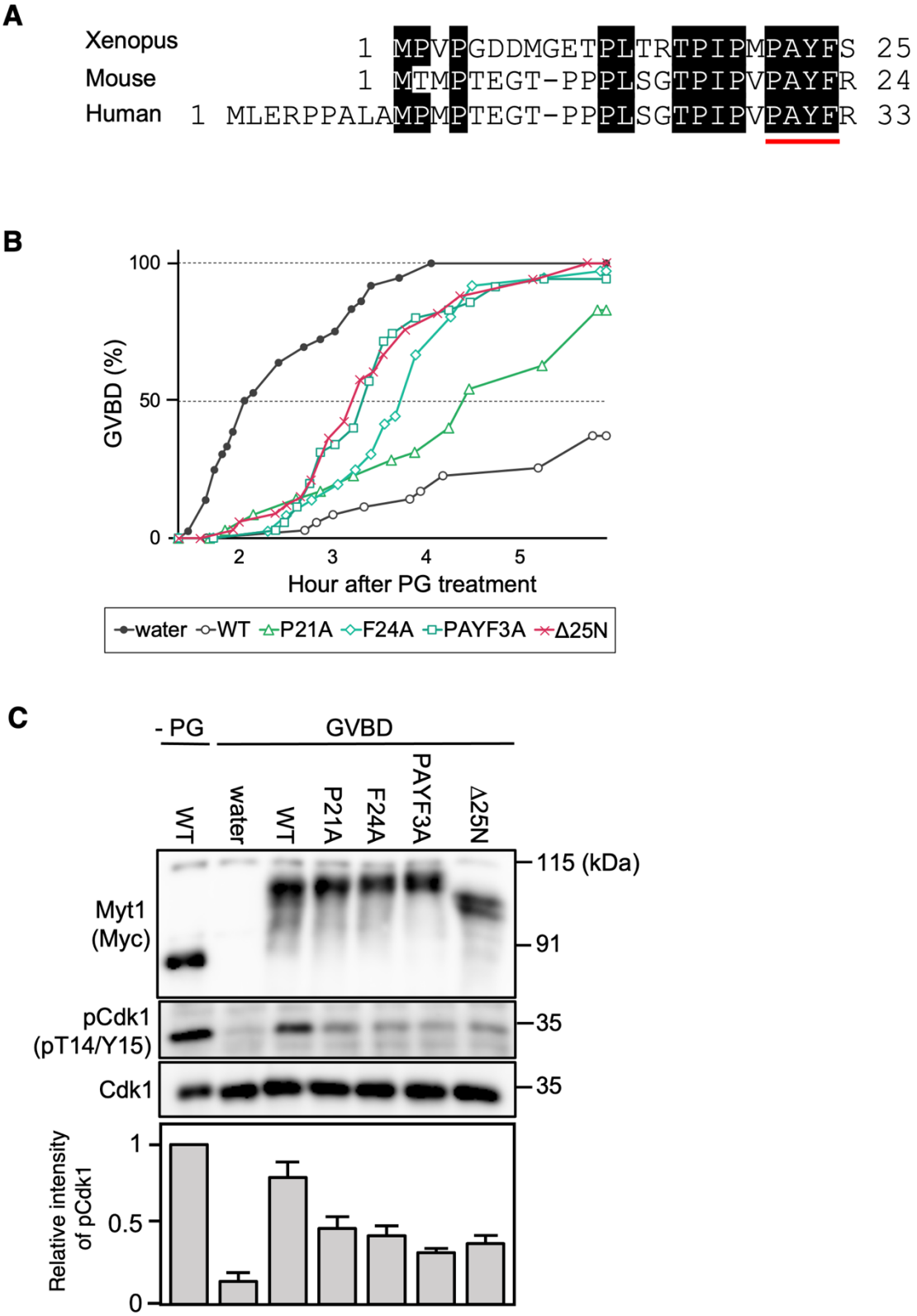
A
Xenopus 1 MPVPGDDMGETPLTRTPIPMPAYFS 25
Mouse 1 MTMPTEGT-PPPLSGTPIPVPAYFR 24
Human 1 MLERPPALAMPMPTEGT-PPPLSGTPIPVPAYFR 33
B
GVBD (%)
100
50
0
2
3
4
5
Hour after PG treatment
water WT P21A F24A PAYF3A Δ25N
C
- PG
GVBD
WT
water
WT
P21A
F24A
PAYF3A
Δ25N
115 (kDa)
91
Myt1 (Myc)
pCdk1 (pT14/Y15)
35
Cdk1
35
Relative intensity of pCdk1
1
0.5
0

Figure 2

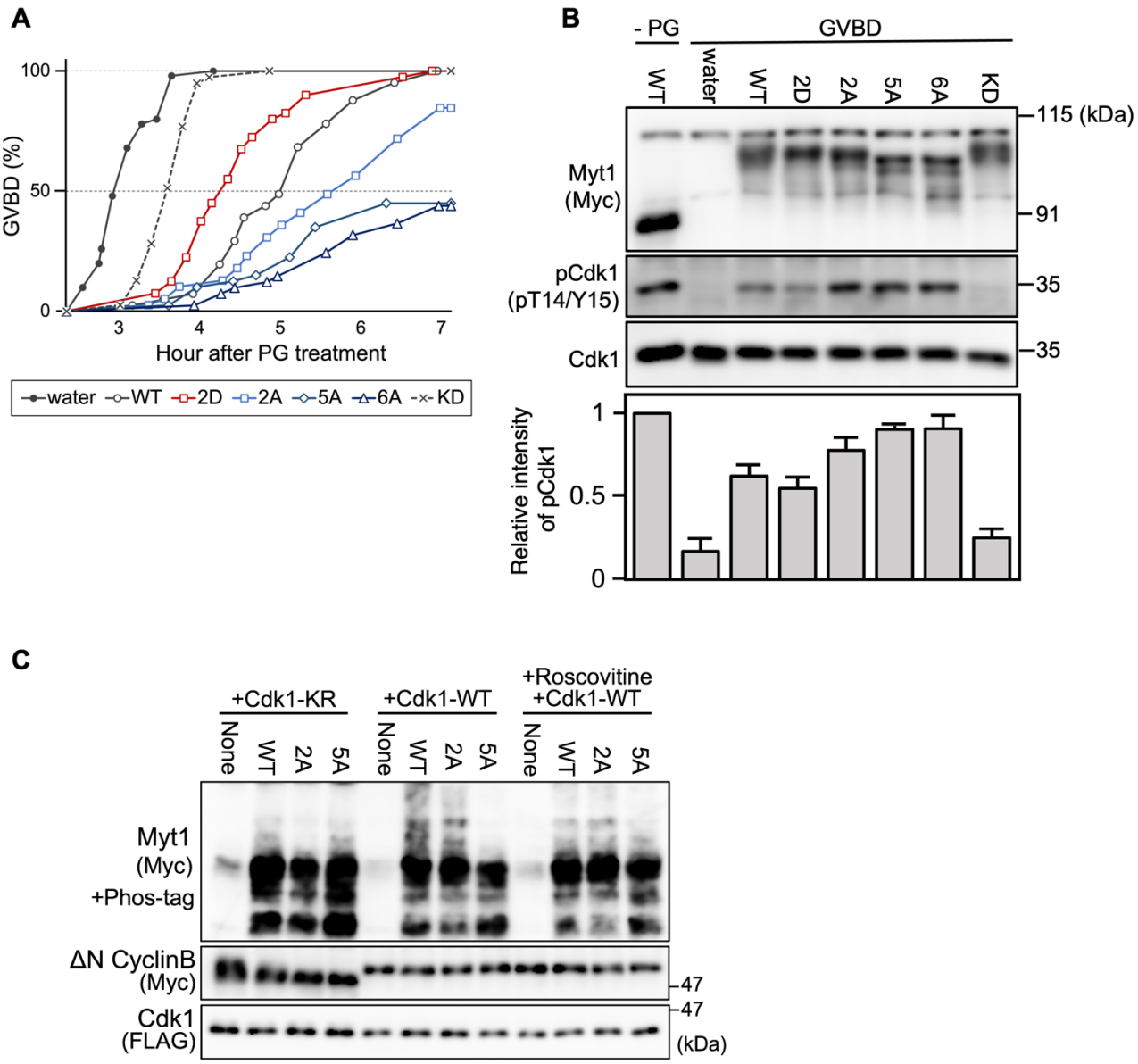
A
100
50
0
GVBD (%)
3
4
5
6
7
Hour after PG treatment
water
WT
2D
2A
5A
6A
KD
B
- PG
GVBD
WT
water
WT
2D
2A
5A
6A
KD
115 (kDa)
Myt1
(Myc)
91
pCdk1
(pT14/Y15)
35
Cdk1
35
Relative intensity
of pCdk1
1
0.5
0
C
+Cdk1-KR
+Cdk1-WT
+Roscovitine
+Cdk1-WT
None
WT
2A
5A
None
WT
2A
5A
None
WT
2A
5A
Myt1
(Myc)
+Phos-tag
ΔN CyclinB
(Myc)
47
Cdk1
(FLAG)
47
(kDa)

Figure 3

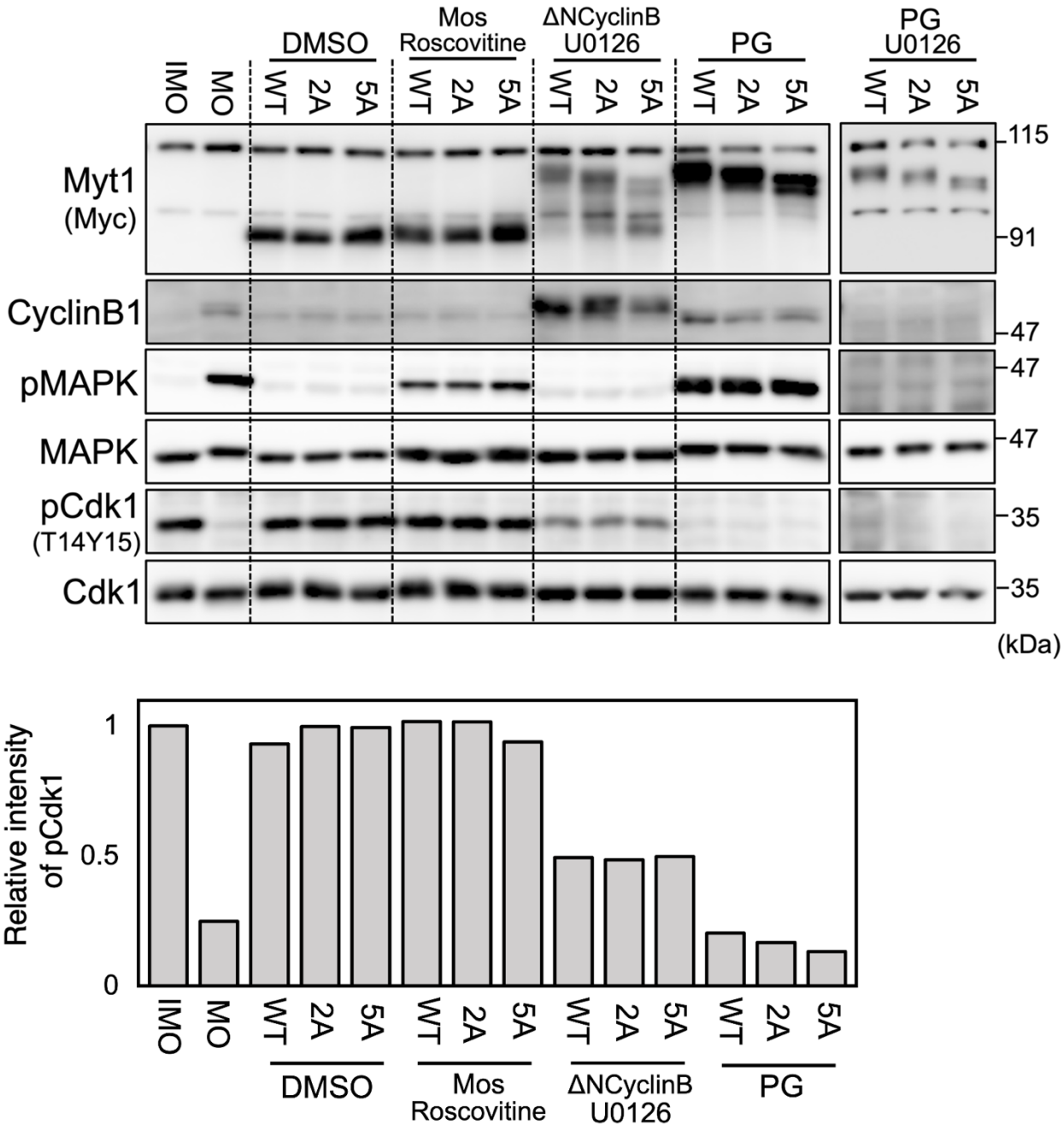
IMO
MO
DMSO
Mos Roscovitine
ΔNCyclinB U0126
PG
PG U0126
WT
2A
5A
Myt1 (Myc)
CyclinB1
pMAPK
MAPK
pCdk1 (T14Y15)
Cdk1
115
91
47
47
47
35
35
(kDa)
Relative intensity of pCdk1
1
0.5
0
DMSO
Mos Roscovitine
ΔNCyclinB U0126
PG

Figure 4

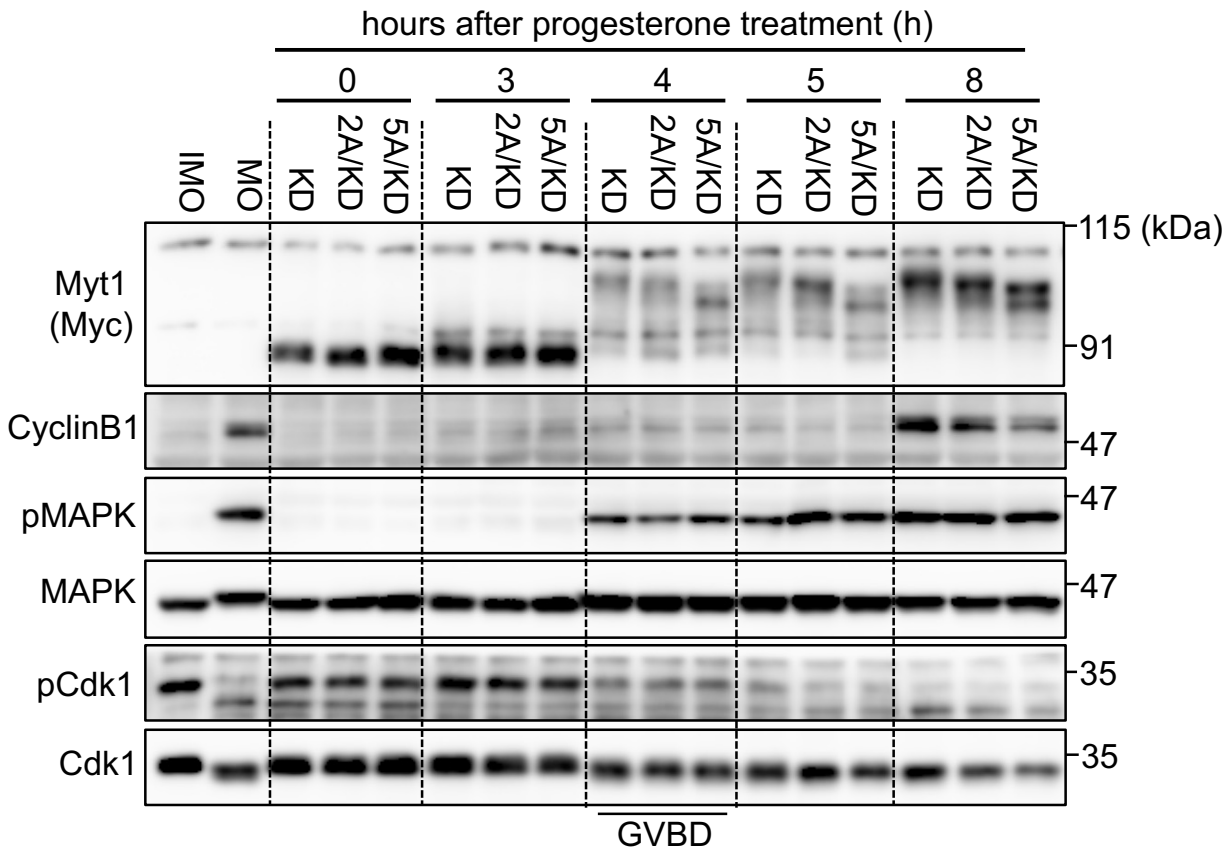
hours after progesterone treatment (h)
0
3
4
5
8
IMO
MO
KD
2A/KD
5A/KD
Myt1
(Myc)
CyclinB1
pMAPK
MAPK
pCdk1
Cdk1
115 (kDa)
91
47
47
47
35
35
GVBD

Figure 5

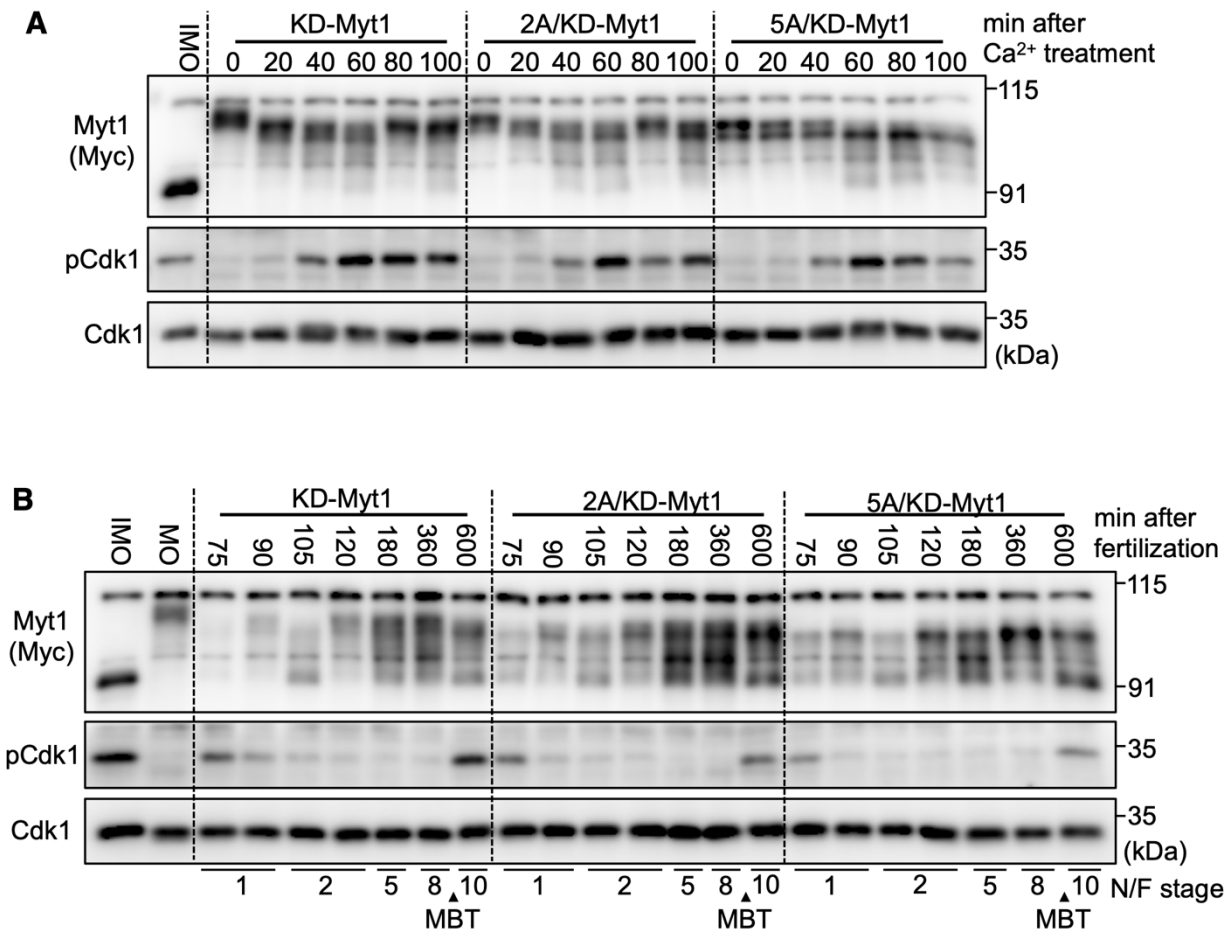
A
IMO
KD-Myt1
2A/KD-Myt1
5A/KD-Myt1
0 20 40 60 80 100
min after
Ca2+ treatment
Myt1
(Myc)
pCdk1
Cdk1
115
91
35
35
(kDa)
B
IMO
MO
KD-Myt1
2A/KD-Myt1
5A/KD-Myt1
75 90 105 120 180 360 600
min after
fertilization
Myt1
(Myc)
pCdk1
Cdk1
115
91
35
35
(kDa)
1 2 5 8 10
MBT
N/F stage

Figure 6

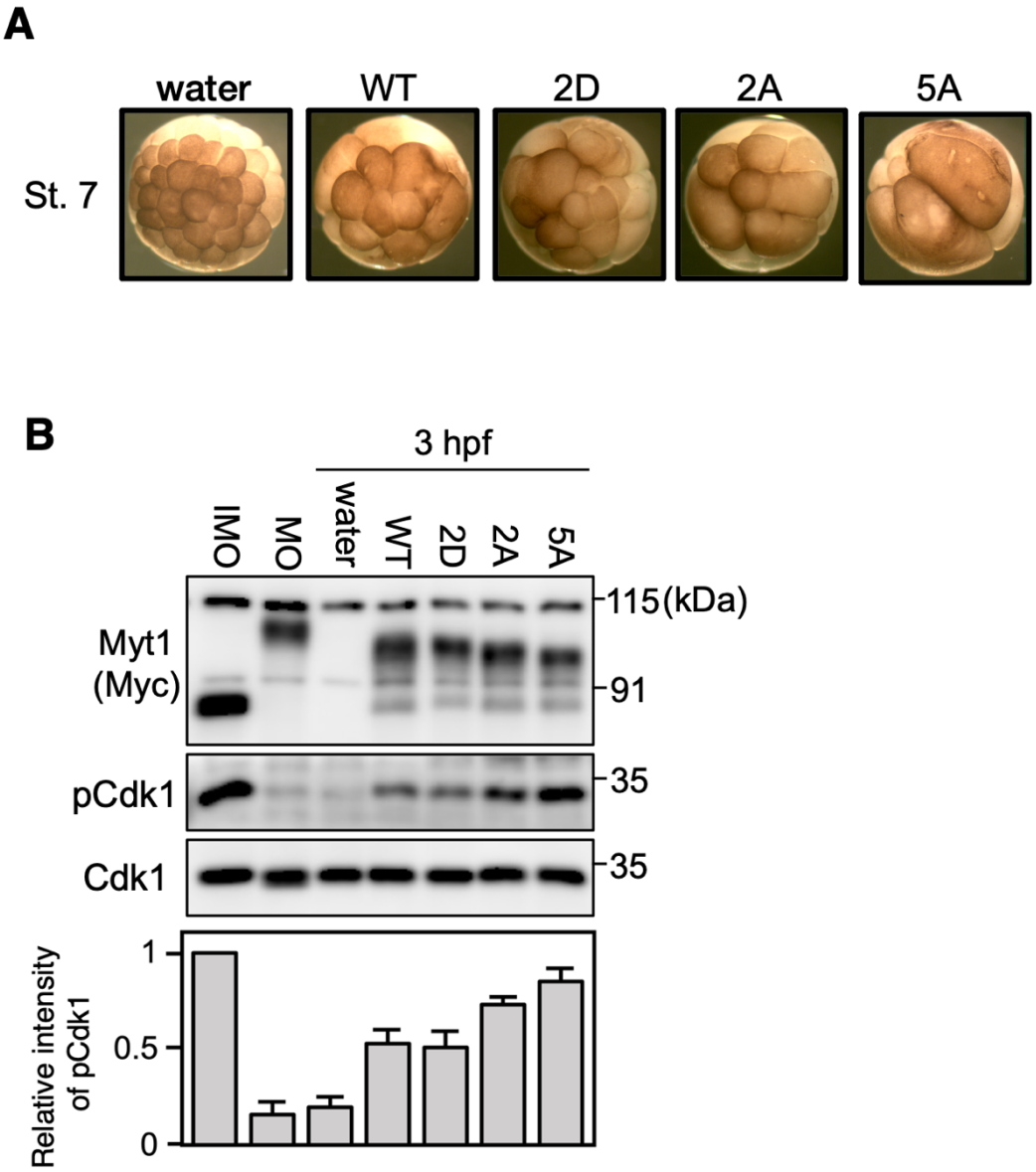
A
water
WT
2D
2A
5A
St. 7
B
3 hpf
IMO
MO
water
WT
2D
2A
5A
115(kDa)
Myt1
(Myc)
91
pCdk1
35
Cdk1
35
Relative intensity
of pCdk1
1
0.5
0

Figure 7

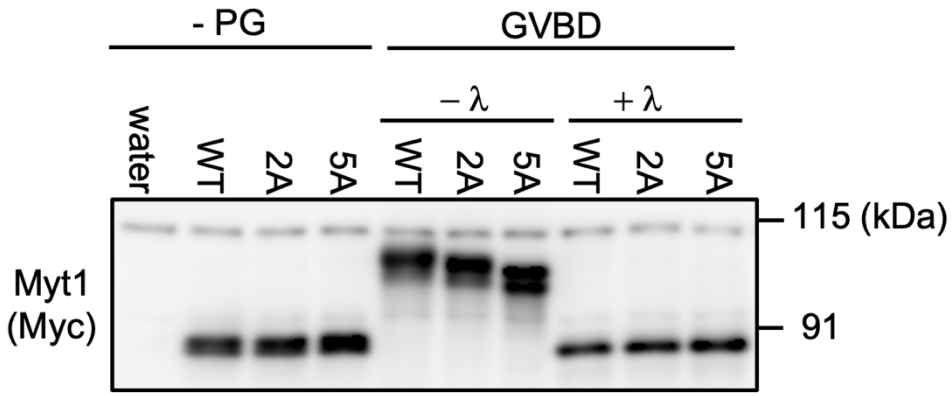

Figure S1

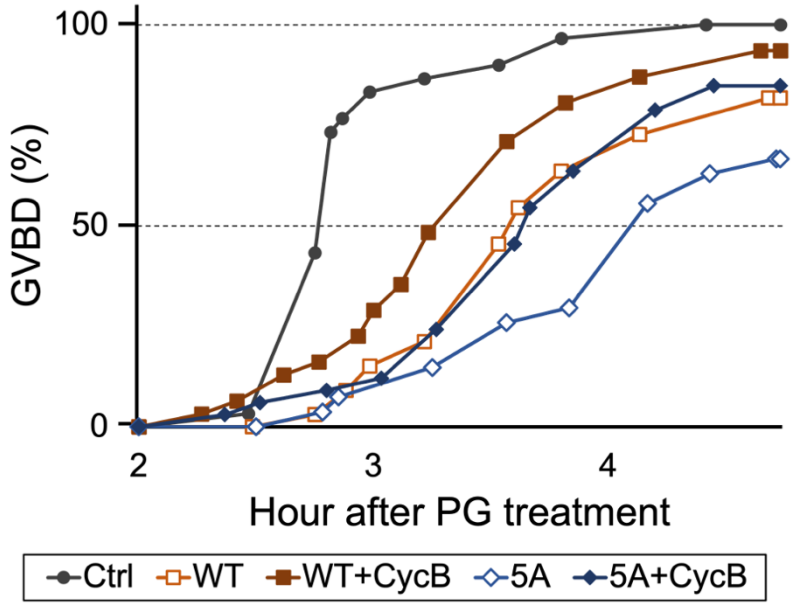
100
50
0
GVBD (%)
2
3
4
Hour after PG treatment
Ctrl
WT
WT+CycB
5A
5A+CycB

Figure S2

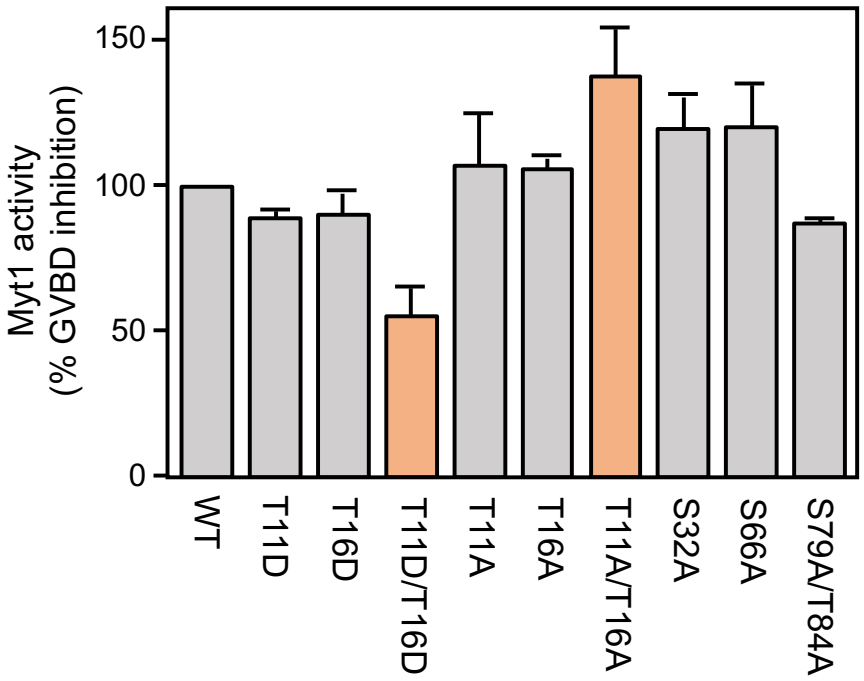
Myt1 activity
(% GVBD inhibition)
150
100
50
0
WT
T11D
T16D
T11D/T16D
T11A
T16A
T11A/T16A
S32A
S66A
S79A/T84A

Figure S3

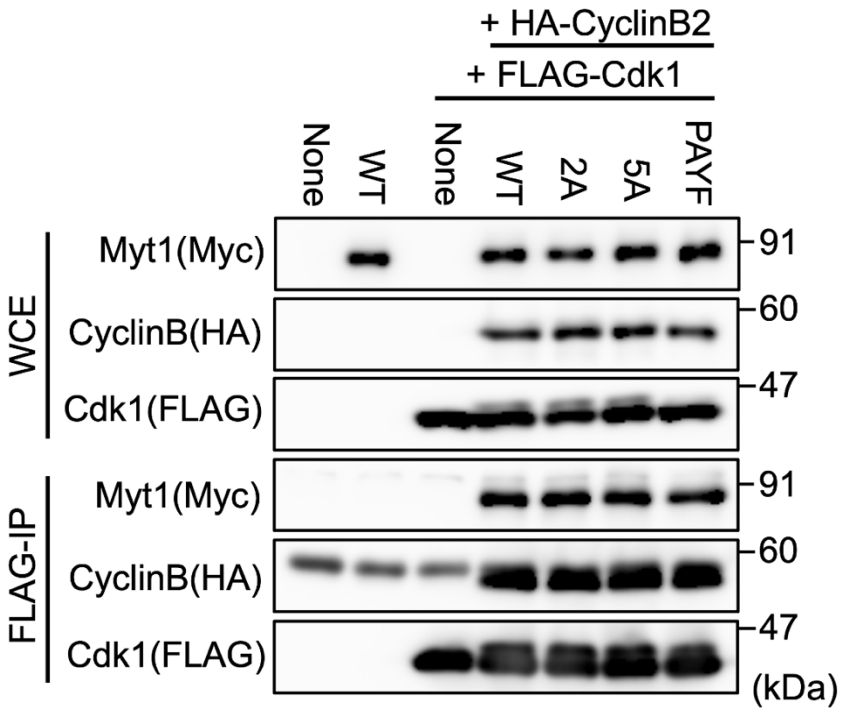
+ HA-CyclinB2
+ FLAG-Cdk1
None
WT
None
WT
2A
5A
PAYF
WCE
Myt1(Myc)
CyclinB(HA)
Cdk1(FLAG)
FLAG-IP
Myt1(Myc)
CyclinB(HA)
Cdk1(FLAG)
91
60
47
91
60
47
(kDa)

Figure S4