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BubR1 acetylation at prometaphase is required for modulating APC/C activity and timing of mitosis

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Regulation of BubR1 is central to the control of APC/C activity. We have found that BubR1 forms a complex with PCAF and is acetylated at lysine 250. Using mass spectrometry and acetylated BubR1-specific antibodies, we have confirmed that BubR1 acetylation occurs at prometaphase. Importantly, BubR1 acetylation was required for checkpoint function, through the inhibition of ubiquitin-dependent BubR1 degradation. BubR1 degradation began before the onset of anaphase. It was noted that the pre-anaphase degradation was regulated by BubR1 acetylation. Degradation of an acetylation-mimetic form, BubR1–K250Q, was inhibited and chromosome segregation in cells expressing BubR1–K250Q was markedly delayed. By contrast, the acetylation-deficient mutant, BubR1–K250R, was unstable, and mitosis was accelerated in BubR1–K250R-expressing cells. Furthermore, we found that APC/C–Cdc20 was responsible for BubR1 degradation during mitosis. On the basis of our collective results, we propose that the acetylation status of BubR1 is a molecular switch that converts BubR1 from an inhibitor to a substrate of the APC/C complex, thus providing an efficient way to modulate APC/C activity and mitotic timing.

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Introduction

Genetic integrity through accurate chromosome segregation requires the spindle-assembly checkpoint (SAC; Musacchio and Hardwick, 2002; Bharadwaj and Yu, 2004; Taylor *et al*, 2004). The activated SAC inhibits the anaphase-promoting complex (APC/C), a multicomplex E3 ligase that is responsible for destroying securin and Cyclin B (Yu, 2002). When this checkpoint fails, cells die or become aneuploid—a hallmark of cancer (Rieder and Maiato, 2004; Kops *et al*, 2005).

BubR1 is essential for the SAC and checkpoint signalling (Yu, 2002). Importantly, BubR1 binds directly to Cdc20, the WD domain-containing coactivator of the APC/C complex during mitosis (Tang *et al*, 2001; Yu, 2002; Chan and Yen, 2003). Cdh1 is another coactivator that is activated when it is no longer phosphorylated by Cdk1, and replaces Cdc20 in binding and activating the APC/C complex. Thus, Cdc20 serves as a coactivator of the APC/C E3 ligase until mitotic exit (Passmore and Barford, 2005; Peters, 2006; Thornton and Toczyski, 2006; Yu, 2007). The importance of BubR1 in the SAC is reflected by the observation that BubR1 forms the mitotic checkpoint complex (MCC) composed of Mad2, Bub3, BubR1, and Cdc20 and inactivates the APC/C–Cdc20 complex (Sudakin *et al*, 2001). A recent report suggested an alternative model for SAC activation, where Mad2 only loads Cdc20 onto BubR1 and then leaves the complex. Consequently, the Bub3–BubR1–Cdc20 complex is presented to the APC/C complex, followed by Cdc20 ubiquitination and degradation to maintain the SAC (Nilsson *et al*, 2008). In any model presented, BubR1 plays a central role in binding to and regulating the APC/C–Cdc20 complex.

It should be noted that BubR1 binds to APC as well as to Cdc20 (Chan *et al*, 1999; Sudakin *et al*, 2001; Morrow *et al*, 2005). BubR1, similar to its orthologue Mad3 in yeast (Burton and Solomon, 2007; Sczaniecka *et al*, 2008), has a D-box-like motif and two KEN boxes, which serve as destruction signals for the APC/C complex (Glotzer *et al*, 1991; King *et al*, 1996b; Pfleger and Kirschner, 2000). This suggests that BubR1 can be regulated by APC/C-mediated destruction. In this vein, it is noteworthy that BubR1 levels decrease late in mitosis (Chan *et al*, 1999). However, data from yeast Mad3, a homologue of mammalian BubR1, indicated that BubR1 is not degraded through APC/C-mediated polyubiquitination. Instead, BubR1 functions as a pseudosubstrate of the APC/C complex by competing with genuine substrates of the APC/C complex for the same Cdc20-binding sites (D-box and KEN box) (Burton and Solomon, 2007). Therefore, it remains to be elucidated whether BubR1 is regulated through destruction by the APC/C complex.

Regarding the regulation of BubR1 protein levels and its consequences in checkpoint activity, it has been shown that the levels of BubR1 during mitosis are crucial for mitotic timing. siRNA knockdown of BubR1 resulted in mitosis acceleration and checkpoint abrogation (Meraldi *et al*, 2004). In addition, BubR1 is required for the assembly of SAC components (Chen, 2002). Therefore, regulation of BubR1 levels might be an essential regulatory step in checkpoint activity.

BubR1 is a kinase, whereas its yeast homologue, Mad3, lacks a kinase domain. The kinase activity of BubR1 is

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stimulated by the microtubule motor protein, CENP-E, and is required for checkpoint maintenance and signalling in metazoans (Abrieu *et al*, 2000; Mao *et al*, 2003, 2005; Weaver *et al*, 2003). Phosphorylation of BubR1 in mitosis by kinases such as Cdk1, Aurora B, and Plk1 is essential for monitoring stable spindle attachments to kinetochores and tension. BubR1 phosphorylation is also required for checkpoint signalling (Ditchfield *et al*, 2003; Lampson and Kapoor, 2005; Elowe *et al*, 2007; Wong and Fang, 2007; Huang *et al*, 2008).

In this study, we show that BubR1 is acetylated exclusively at the prometaphase stage. We found that BubR1 is degraded during mitosis through APC/C–Cdc20-mediated ubiquitination. It is noteworthy that the acetylation of BubR1 inhibits its degradation by the APC/C–Cdc20 complex during mitosis, and this mechanism controls the timing of mitosis. Our results indicate that the acetylation status of BubR1 provides a molecular switch that controls the timely activation of the APC/C complex, thereby serving as a regulatory step for the SAC.

Results

BubR1 binds to and is acetylated by PCAF at prometaphase kinetochores

Protein modifications such as phosphorylation, SUMOylation, and acetylation contribute to the control of cell-cycle progression. While studying the regulation of BubR1 activity, we found that BubR1 might be controlled by acetylation. Asynchronously growing cells and nocodazole-arrested HeLa cells were compared and tested for the possibility of BubR1 acetylation. Immunoprecipitation (IP) followed by WB analysis showed that BubR1 might be acetylated in nocodazole-arrested cells (Figure 1A).

Next, we tested the binding ability of BubR1 to p300, CBP, and PCAF to verify whether BubR1 was capable of binding to acetyltransferase and being acetylated. In IP and WB, BubR1 interacted with PCAF but not with p300 or CBP acetyltransferases (Supplementary Figure 1). It is noteworthy that BubR1–PCAF complex formation was detected in nocodazole-arrested cells but was barely detectable in asynchronous cells (Figure 1B). This suggests the possibility that PCAF acetylates BubR1 and this acetylation might play a role in the control of BubR1 activity.

We then examined the location of the BubR1–PCAF complex formation because BubR1 localizes to kinetochores in mitosis. The localization of BubR1 to kinetochores is essential for SAC function; it monitors bipolar spindle attachment and chromosome alignment (Ditchfield *et al*, 2003), thereby regulating the APC/C complex and the metaphase–anaphase transition (Yu, 2002; Chan and Yen, 2003). Metaphase chromosomal spreads were prepared and subjected to an immunofluorescence assay with anti-PCAF and anti-BubR1 antibodies. The assays showed that BubR1 and PCAF colocalized at kinetochores (Figure 1C and D). Consistent with *in vivo* complex formation between BubR1 and PCAF at prometaphase kinetochores, we observed that BubR1 could be acetylated by PCAF. Recombinant BubR1, as well as immunoprecipitated BubR1, was acetylated by PCAF during *in vitro* acetylation assays (Figure 1E and F).

Next, we asked whether sustained BubR1 acetylation affected mitotic cell-cycle progression. As a first step towards answering this question, we treated cells in mitosis with the pan-HDAC inhibitor trichostatin A (TSA). HeLa cells were synchronized at prometaphase by nocodazole treatment followed by mitotic shake-off. Subsequently, synchronized cells were either released from the mitotic arrest by washing out nocodazole (Figure 2A, left panel), or kept in nocodazole to maintain mitotic arrest (Figure 2A, right panel). The effects of TSA treatment on BubR1 levels were analysed in both cases, with cycloheximide (CHX) treatment included to control for the effects of *de novo* protein synthesis. Cells were collected at the indicated time points and were analysed for BubR1 levels (Figure 2A) and cell-cycle profiles (Figure 2B).

Interestingly, BubR1 levels decreased markedly from the initial time point of analysis in cells progressing through mitosis (Figure 2A, left panel, none), and were further decreased by CHX treatment (Figure 2A, left panel, + CHX). When cells were arrested in mitosis by continuous nocodazole treatment, BubR1 levels were maintained (Figure 2A, right panel, none). However, when CHX was added, BubR1 levels decreased in the nocodazole-arrested condition (Figure 2A, right panel, + CHX). Taken together, these results suggest that, similar to Cdc20 (Nilsson *et al*, 2008), BubR1 is not only degraded but also continuously synthesized during mitosis.

TSA treatment resulted in sustained levels of BubR1 relative to the levels after CHX treatment in cells progressing through mitosis (Figure 2A, left panel, TSA and TSA + CHX) and in cells blocked for exit from mitosis (Figure 2A, right panel, TSA and TSA + CHX). Concomitantly, mitotic exit was delayed for at least 5 h, until the end of the time course, by treatment with TSA (Figure 2B, left panel, TSA and TSA + CHX), whereas control or CHX-treated cells exited from mitosis 1 h after nocodazole release (Figure 2B, left panel, none and CHX). In all cases of continuous nocodazole treatment, cells did not exit from mitosis (Figure 2A, right panel).

The fact that TSA treatment resulted in the stabilization of BubR1 levels suggests that BubR1 might be one of the targets of TSA during mitosis. Taken together with the results from Figure 1, BubR1 is likely to be acetylated in mitosis, which probably results in the inhibition of its degradation. In this regard, our observation that BubR1 interacted with HDAC 1–3 in transfected cells (Supplementary Figure 3) suggests the intriguing possibility that the BubR1 acetylation state might regulate BubR1 function and progression through mitosis.

BubR1 Lysine 250 (K250) is acetylated

To identify the acetylation site, we made a series of BubR1 deletion constructs, transfected them into 293T cells, and analysed the acetylation domain by IP and WB. The N-terminal region of BubR1 contained the acetylation domain (Supplementary Figure 4). Further *in vitro* acetylation assays showed that BubR1 amino acids 221–299 (ΔBR2–3) contained acetylated lysine residues (Figure 3A and B). In parallel, mass spectrometry analysis was carried out with full-length BubR1 purified from insect cells and K250 was identified as the site of acetylation (Supplementary Figure 5). By means of *in vitro* mutagenesis and acetylation assays, we confirmed that K250 was the acetylation site (Figure 3C and Supplementary Figure 6). To verify that K250 was acetylated *in vivo*, interphase and prometaphase HeLa cells prepared by nocodazole arrest and mitotic shake-off were subjected to IP using anti-BubR1 antibodies and to mass spectrometry analysis. Our findings showed that the K250 site was acetylated

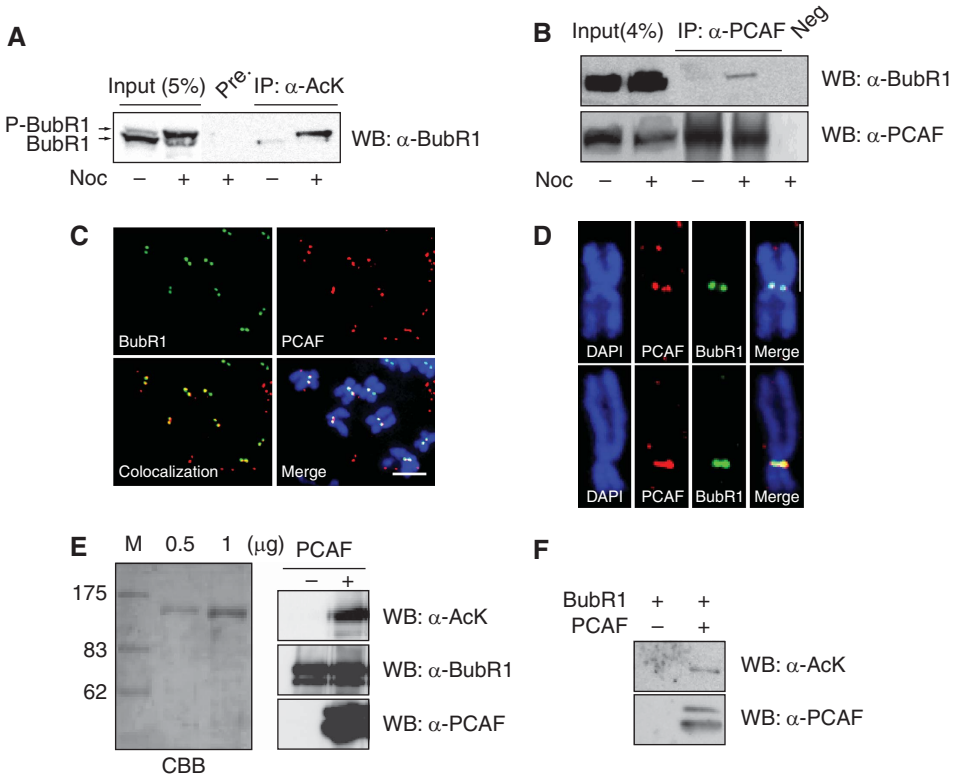

Figure 1 BubR1 interacts with PCAF and is acetylated at prometaphase kinetochores. (**A**) BubR1 acetylation was compared between asynchronously growing cells and prometaphase cells. HeLa cells were treated with nocodazole for 13 h (or left untreated) and subjected to mitotic shake-off. IP was carried out with anti-Ac-K antibodies and subsequent WB with anti-BubR1 antibodies. The slower migrating form of BubR1, phospho-BubR1 (as shown in WB of input control), was detected with the anti-Ac-K antibodies. (**B**) BubR1 and PCAF interact in nocodazole-treated cells. Cells were treated with nocodazole for 13 h (or left untreated), and lysates were analysed by IP with anti-PCAF, followed by WB using anti-BubR1. The blot was then re-probed with anti-PCAF to normalize the IP. (**C**) BubR1 interacts with PCAF at prometaphase kinetochores. Metaphase chromosome spreads were immunostained with anti-PCAF (red) and anti-BubR1 (green) antibodies. PCAF localization at prometaphase kinetochores was verified by siRNA experiments (Supplementary Figure 2). White scale bar, 5 μm. (**D**) Images in (C) were enlarged to visualize the localization of BubR1 and PCAF with the chromosome. (**E**) BubR1 purified from insect cells (left) was subjected to an *in vitro* acetylation assay in the presence of purified PCAF and acetyl-CoA. The reaction was analysed by WB with anti-Ac-K antibodies (right). One μg each of BubR1 (8.4 pmol) and PCAF (10.8 pmol) was employed for the assay, which corresponds to a molar ratio of 1:1.3. The same blot was re-probed with anti-BubR1 or anti-PCAF antibodies. (**F**) BubR1 IPs from 1 mg of HeLa cell lysates were subjected to acetylation assays with or without recombinant PCAF (1 μg), followed by WB with anti-Ac-K antibodies.

in prometaphase but not in interphase cells (Figure 3D). Interestingly, substrate recognition motifs of APC/C-dependent ubiquitination (Peters, 2006; Thornton and Toczyski, 2006; Yu, 2007), D-box (Glotzer *et al*, 1991; King *et al*, 1996a; Burton and Solomon, 2007) and KEN box motifs (Pfleger and Kirschner, 2000; Murray and Marks, 2001; Burton and Solomon, 2007; King *et al*, 2007) were found adjacent to K250. K250 and these degrons are conserved in vertebrates (Figure 3E), suggesting that BubR1 acetylation might regulate SAC activity by controlling either the binding affinity of BubR1 to the Cdc20 coactivator (Burton and Solomon, 2007) or the APC/C-dependent destruction of BubR1 (King *et al*, 2007).

To further confirm the *in vivo* acetylation of BubR1, we generated a polyclonal antibody that specifically recognizes K250-acetylated BubR1 (Figure 4A). To verify that the anti-Ac-K250 antibody specifically recognizes acetylated BubR1 in M phase, interphase and M phase HeLa cell extracts were subjected to IP with anti-BubR1 antibodies and to WB with the antibodies indicated. Similar to the anti-Ac-K antibody, the anti-Ac-K250 antibody recognized BubR1 only during M phase (Figure 4B). These results confirmed that the anti-Ac-K250 antibody recognizes acetylated BubR1 *in vivo*.

To further assess the time point when BubR1 is acetylated during the mitotic cell cycle, HeLa cells were synchronized in prometaphase and then released into the cell cycle in the presence of CHX. WB analysis with anti-acetyl-K250 serum at different time points after release indicated that acetylation occurred exclusively during prometaphase (time 0), but not during the other phases of the mitotic cell cycle (Figure 4C). This acetylation showed excellent correlation with BubR1–PCAF complex formation (Figure 1). Furthermore, TSA-treated cells showed an attenuated level of BubR1 and acetylation for more than 5 h (Figure 4D, + TSA, anti-Ac-K250), suggesting that one of the targets of TSA in mitosis might be acetylated BubR1, supporting our interpretation from Figure 2.

BubR1 acetylation participates in SAC activity

Substituting K250 with arginine (K250R) produces an acetylation-deficient mutant, whereas substituting lysine with glutamine (K250Q) generates a mutant that mimics the characteristics of acetylated BubR1 (Supplementary Figure 7B and C). Using these *in vitro* mutagenized constructs, we determined the effect of acetylation on various aspects of SAC activity.

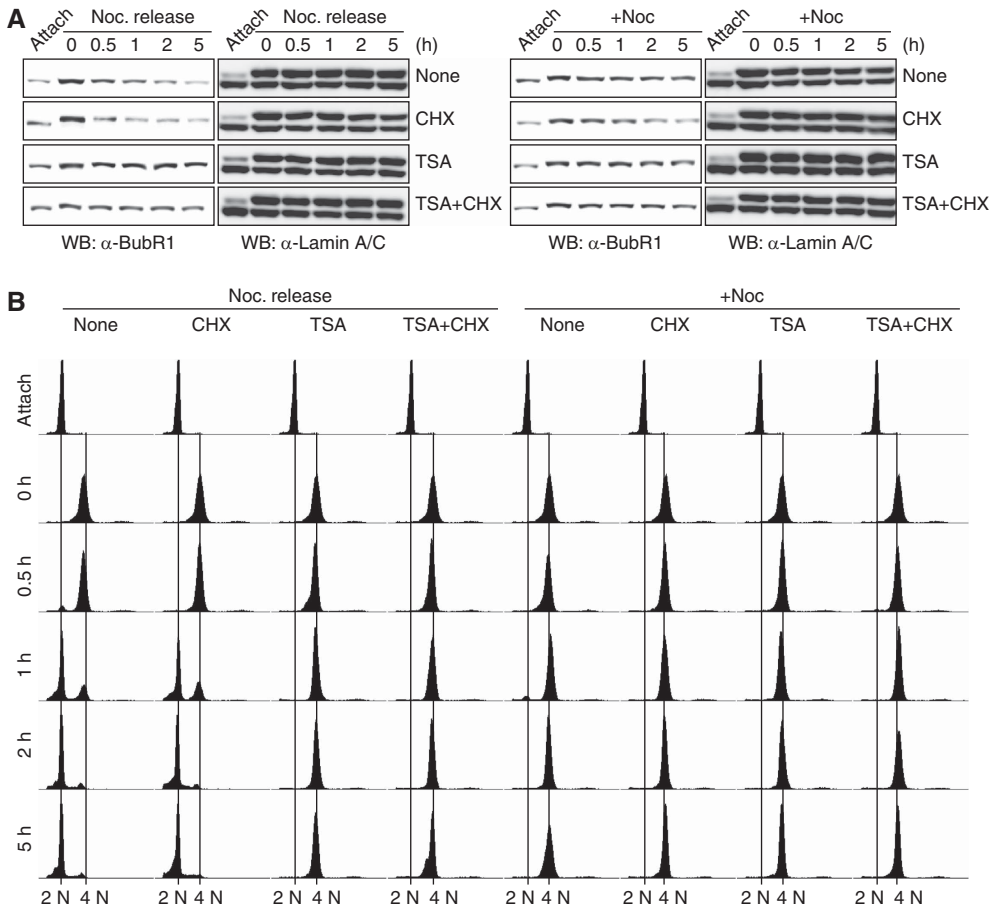

Figure 2 Treatment with TSA during mitosis inhibits the degradation of BubR1 and a delay in anaphase entry. (**A**) HeLa cells were synchronized at prometaphase by tyminidine–nocodazole arrest followed by mitotic shake-off, and then either released into the cell cycle (left panel) or maintained in nocodazole for 5 h (right panel). To assess the effect of HDAC inhibition on BubR1, cells were treated with 100 nM of TSA or left untreated for the indicated time points and subjected to WB; a total of 100 μg/ml of cycloheximide (CHX) was included as a control to suppress *de novo* protein synthesis. Blots were re-probed with an anti-Lamin A/C antibody to normalize protein levels. Attached cells (Attach) remaining after mitotic shake-off served as controls. (**B**) The cell-cycle stage was assessed by propidium iodide staining and flow cytometry at the same time points as in (A); 2 N and 4 N DNA contents are indicated.

To assess the checkpoint response to spindle damage, we compared MPM2 staining after nocodazole treatment in cells expressing three different BubR1 constructs (Figure 5A). Depletion of *BubR1* or *Mad2* by siRNA resulted in the abrogation of SAC function, as shown by a marked reduction in MPM2 staining (Figure 5C, square and cross, respectively). *Wild-type* and *K250Q*-expressing cells, depleted of endogenous BubR1, restored the MPM2 staining to a level comparable with control cells (Figure 5C, triangle and open circle, respectively). In contrast, *K250R*-expressing (Figure 5C, diamond) or *K250Q*-expressing cells depleted of *Mad2* expression could not recover the MPM2 staining (Figure 5C, closed circle). The importance of BubR1 acetylation for checkpoint function was confirmed by live-cell analysis. Control cells, cells expressing wild-type *BubR1*, and *K250Q*-expressing cells were blocked in mitosis by nocodazole treatment. By contrast, *BubR1*-depleted cells (siBubR1), *Mad2*-depleted cells (siMad2), cells expressing *K250R* (siBubR1 + K250R), and *Mad2*-depleted cells expressing *K250Q* (siMad2 + K250Q) exited from mitosis despite the presence of nocodazole (Table I). Taken together, these results confirm that BubR1 acetylation is required for SAC function.

We then determined whether BubR1 acetylation affected its phosphorylation in prometaphase. HeLa cells were transfected with *K250R*, *K250Q*, or wild-type (WT) *BubR1* plasmids, synchronized at prometaphase and analysed by IP and WB. To avoid proteolytic effects, MG132 treatments were included. The results showed that BubR1 acetylation did not affect the appearance of the characteristic slow-migrating band of BubR1 in prometaphase/metaphase cells after nocodazole treatment (Figure 5D).

Accurate chromosome segregation is guaranteed by the sensing of kinetochore–microtubule attachments and tension across sister kinetochores. This signal is transmitted and amplified by phosphorylation events in SAC components, resulting in inhibition of the APC/C complex even when only one chromosome is attached to spindle microtubules. Concerted BubR1 phosphorylations play a crucial role in spindle checkpoint signalling (Gillett and Sorger, 2001; Taylor *et al*, 2001; Chen, 2002; Mao *et al*, 2003, 2005; Morrow *et al*, 2005). It has been shown that Plk1 phosphorylates BubR1 in a tension-sensitive manner and that this event is crucial for stable kinetochore–microtubule attachments (Elowe *et al*, 2007; Wong and Fang, 2007). To investigate whether BubR1 phosphorylation by Plk1 was affected

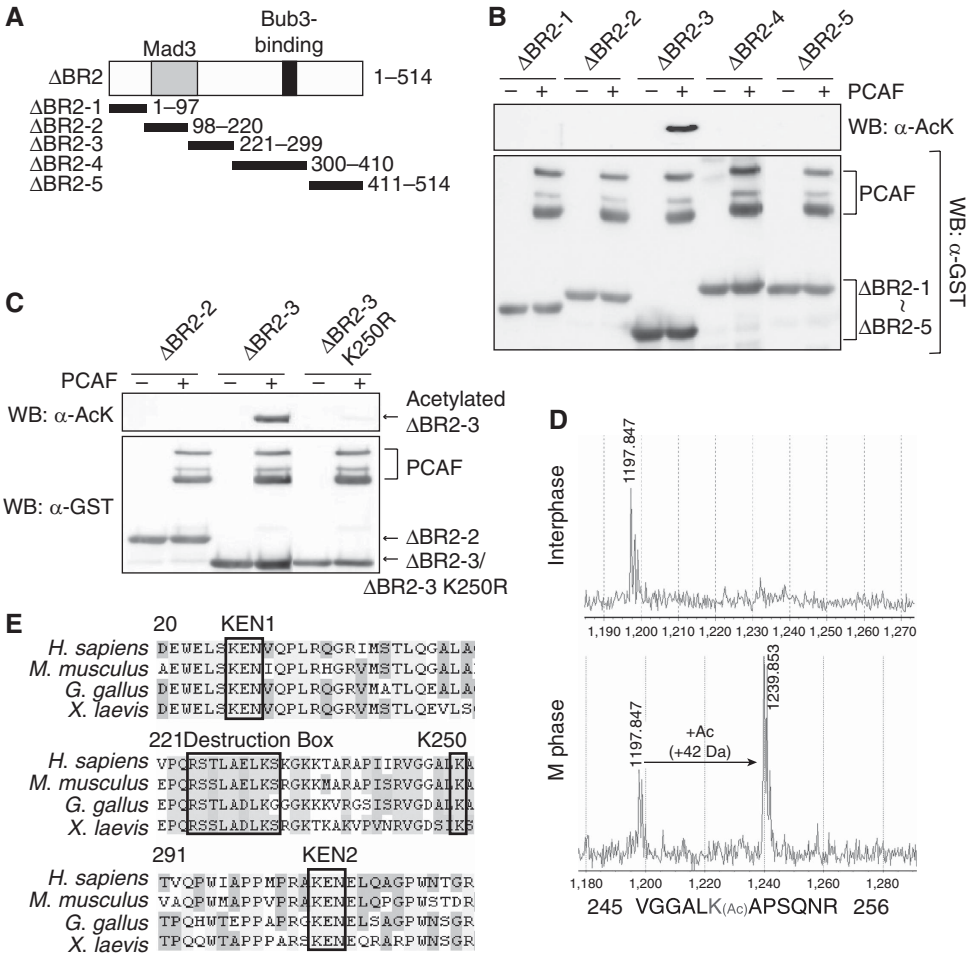

Figure 3 Identification of the BubR1 acetylation site. (**A**) The BubR1 N-terminal region (ΔBR2; amino acids 1–514) was subdivided into five regions to precisely map the acetylation site. (**B**) GST fusions of the recombinant proteins (ΔBR2-1–5) were purified from *Escherichia coli* and subjected to *in vitro* acetylation with or without PCAF in the reaction. (**C**) K250 in ΔBR2–3 was subjected to *in vitro* mutagenesis with Arg (ΔBR2–3 K250R), purified from *E. coli*, and subjected to an *in vitro* acetylation assay. Samples were analysed for acetylation by WB with anti-Ac-K antibodies. (**D**) Mass spectrometry analysis to detect *in vivo* acetylation of BubR1. HeLa cells were treated with nocodazole and prometaphase cells were collected by mitotic shake-off. Attached cells (interphase) and prometaphase cells (M phase) were immunoprecipitated with anti-BubR1 antibodies and SDS–PAGE. Excised BubR1 bands were analysed for the acetylated site by mass spectrometry. An additional peak at 1239.853, which was shifted by acetylation (42-Da) from 1197.847 at M phase, confirms that K250 of BubR1 is acetylated at prometaphase *in vivo*. (**E**) Comparison of BubR1 sequences surrounding K250 among vertebrates. Conserved Destruction Box (D-box)-like sequences and two KEN boxes are marked.

by acetylation, we utilized a BubR1 antibody that specifically recognizes Plk1-mediated BubR1 phosphorylation (Elowe *et al*, 2007). In a WB of the same blot that was used to assess BubR1 phosphorylation at prometaphase, we observed that BubR1 acetylation did not affect Plk1-mediated phosphorylation of BubR1 in prometaphase (Figure 5D, upper panel).

Binding of BubR1 to Cdc20 is crucial in the inhibition of APC/C activity (Tang *et al*, 2001; Yu, 2007; Nilsson *et al*, 2008). Therefore, we tested whether BubR1 acetylation affected its binding to Cdc20. Our results showed that acetylation of BubR1 did not alter its direct binding to Cdc20 (Figure 5E). We then questioned whether BubR1 acetylation affected its binding to the Cdc20 complex in nocodazole-arrested cells. The 293T cells transfected with various BubR1 constructs were treated with nocodazole; some were subsequently treated with MG132 to inhibit protein degradation. Immunoprecipitation with 9E10 and subsequent WB showed that the formation of a complex between BubR1 and Cdc20 in prometaphase was not affected by BubR1 acetylation (Figure 5F).

BubR1 acetylation at K250 inhibits its ubiquitination-dependent proteolysis

Acetylation sometimes interferes with ubiquitination-mediated proteolysis by either competing for the same residue or altering protein structure (Minucci and Pelicci, 2006). This possibility is also supported by our observations that BubR1 levels decreased after nocodazole arrest in the presence of CHX (Figures 2A and 4C), TSA treatment produced higher protein levels (Figures 2A and 4D), and siRNA of PCAF resulted in a marked reduction of BubR1 staining at prometaphase kinetochores (Supplementary Figure 2). Therefore, we investigated whether BubR1 acetylation interfered with ubiquitination-mediated proteolysis.

HeLa cells were transfected with *Myc-K250R*, *-K250Q*, or wild-type *BubR1* expression constructs and their protein levels assessed in the presence or absence of the MG132 proteasome inhibitor. The results indicated that K250R was present at significantly lower levels than wild-type BubR1 or K250Q when *de novo* synthesis of proteins was inhibited (Figure 6A, CHX). When the same set of transfected cells was

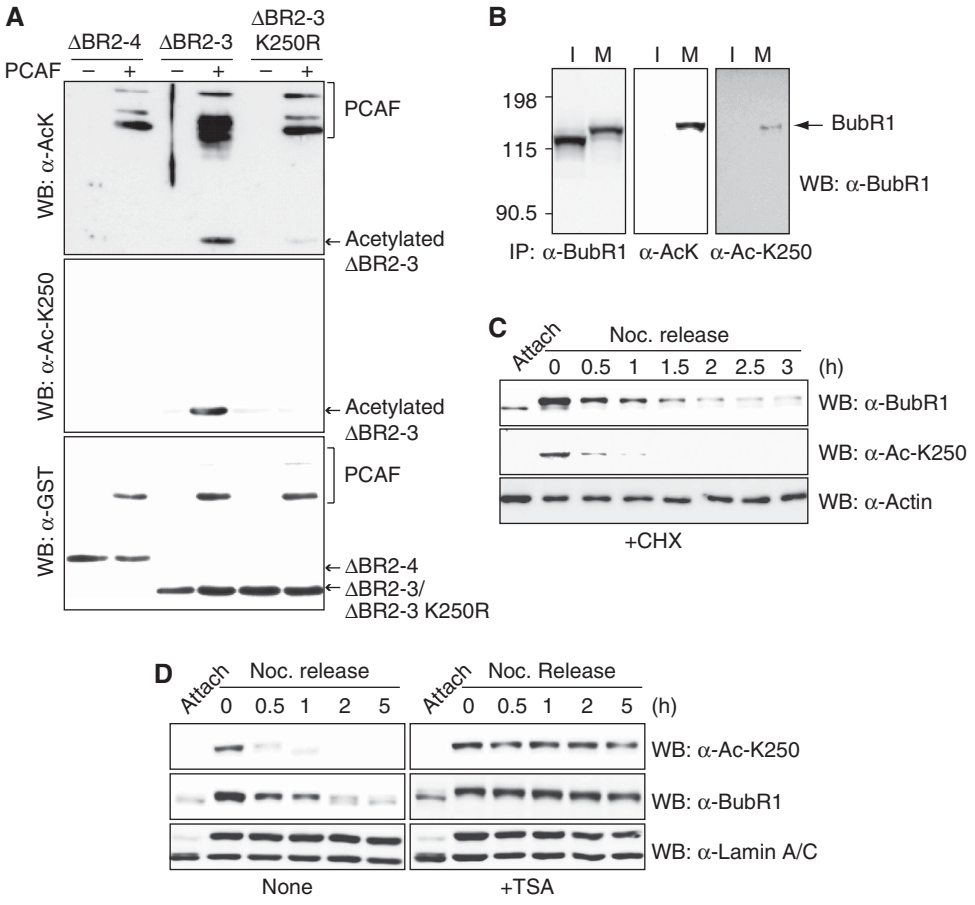

Figure 4 Antibodies raised against acetylated BubR1 detect BubR1 exclusively at prometaphase. The anti-Ac-K250 antibodies specifically recognize BubR1 acetylated at K250. (**A**) Recombinant proteins ΔBR2–3 and ΔBR2–3 K250R were subjected to *in vitro* acetylation and WB. Similar to the anti-Ac-K antibody, anti-Ac-K250 antibodies detect only acetylated BubR1 and not K250R. Antibodies raised against acetyl-K250 do not recognize autoacetylated PCAF, whereas the anti-Ac-K antibody does recognize it. The same blot was re-probed with anti-GST antibodies as loading controls. (**B**) HeLa cells in interphase (I) and M phase (M) were subjected to IP with anti-BubR1, anti-Ac-K, or anti-Ac-K250 antibodies and then immunoblotted with anti-BubR1 antibodies. BubR1 acetylation at K250 was detected in the M phase. (**C**) Levels of BubR1 and BubR1 acetylation after nocodazole release in the presence of CHX. HeLa cells were synchronized (time point 0), then washed and released into the cell cycle in the presence of 100 μg/ml of CHX. Lysates were prepared at the indicated time points after release and analysed by WB with anti-BubR1 and anti-Ac-K250 antibodies. The same blot was re-probed with anti-Actin antibodies. (**D**) BubR1 acetylation in mitosis is sustained by treating the cells with the pan-HDAC inhibitor TSA. WB with anti-Ac-K250 detects acetylated BubR1 exclusively at prometaphase-arrested cells (left panel). The right panel shows cells released from nocodazole arrest in the presence of 100 nM TSA, which resulted in the attenuation of BubR1 acetylation (Ac-K250). The same blot was re-probed with anti-BubR1 and anti-Lamin A/C.

treated with MG132, K250R protein levels were restored to wild-type levels (Figure 6A, MG132). Furthermore, ubiquitination of the acetylation-defective K250R mutant was consistently higher than that of the wild type, whereas ubiquitination of K250Q was markedly lower (Figure 6B).

Immunofluorescence assays substantiated the observation that BubR1 acetylation correlates with BubR1 levels. The intensity of K250Q staining was markedly higher than that of K250R at kinetochores when normalized to CREST staining (Figure 6C and D; Supplementary Figure 8). When the proteasome inhibitor, MG132, was included 3 h before fixation, K250R signals at kinetochores were restored (Figure 6C and D), suggesting that the checkpoint defects in K250R-expressing cells are because of insufficient amounts of BubR1 at kinetochores, resulting from acetylation-deficient BubR1 being susceptible to ubiquitin-mediated proteolysis. The localization of BubR1 at kinetochores was not altered by acetylation, which was corroborated by the finding that Bub3 binding was not affected by BubR1 acetylation (Supplementary Figure 9).

BubR1 acetylation/deacetylation regulates its degradation and timing in anaphase entry

Next, we questioned whether BubR1 acetylation and degradation affected the timing of mitosis. For this, we adopted live-cell analysis coupled with fluorescence measurements, which have earlier been used efficiently to measure the proteolysis of proteins in mitosis (Lindon and Pines, 2004; Pines and Lindon, 2005; Nilsson *et al*, 2008). Wild-type-, *K250R*-, or *K250Q*-expression plasmids tagged with DsRed at their N-termini were transfected into HeLa–H2B cells depleted of endogenous *BubR1* by siRNA. The transfected cells were synchronized and subjected to time-lapse imaging (Figure 7A).

The level of wild-type BubR1 declined during mitosis; it began to decline before the onset of anaphase and declined further throughout mitosis (Figure 7B, blue line and 7C, upper panel). Mitosis took an average of 57 min from nuclear envelope breakdown (NEBD) to anaphase onset in wild-type BubR1-rescued cells (Figure 7B–D, top panel; Supplementary movie 1 and Supplementary Figure 11).

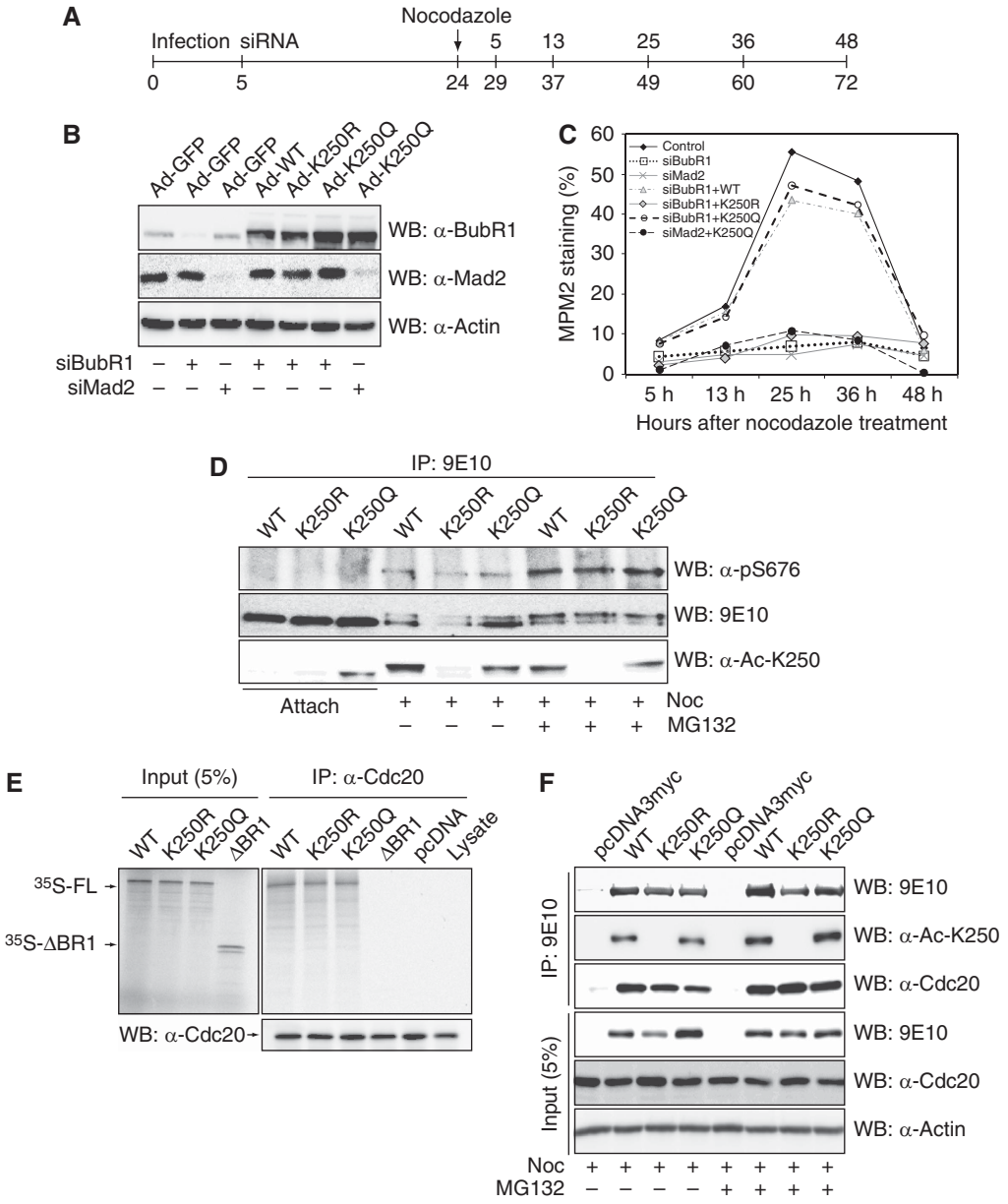

Figure 5 BubR1 acetylation is required for checkpoint activity. (**A**–**C**) BubR1 acetylation is required for checkpoint activity. (A) Schematic illustration of the experiment. HeLa cells were infected with 100 MOI of GFP-fused BubR1-*WT* (Ad-WT), *K250R* (Ad-K250R), or *K250Q* (Ad-K250Q)-expressing adenovirus. Adenovirus expressing *GFP* only was employed for control. Five hours later, cells were depleted of endogenous *BubR1* by synthetic siBubR1 targeting the 3′UTR (siBubR1) or siMad2 targeting *Mad2*, as marked. Nineteen hours post siRNA transfection, cells were treated with nocodazole (200 ng/ml) and aliquots of cells were collected at the indicated time points for WB (B) and measuring mitotic index in (C). Western analysis was used to determine the levels of BubR1 and Mad2 after siRNA transfection and adenovirus infection as indicated. (C) Comparison of the mitotic index of cells indicated after nocodazole treatment, measured by MPM2–FACS analysis (Davis *et al*, 1983). (**D**) Acetylation of BubR1 does not directly affect its phosphorylation. HeLa cells were transfected with the indicated plasmids and treated with nocodazole for 13 h. After mitotic shake-off, cells were washed with PBS five times and incubated in the presence or absence of 15 μM of MG132 for 1.5 h. Attached cells (Attach) were included for control. Lysates were prepared and subjected to IP (9E10), followed by WB with anti-p-S676 BubR1, which detects Plk-1-mediated phosphorylated BubR1. The same blot was re-probed with 9E10 (middle panel) and anti-Ac-K250 antibodies. (**E**) BubR1 acetylation at K250 does not alter Cdc20 binding. *Wild-type-*, *K250R-*, and *K250Q*-encoding constructs were translated *in vitro* in the presence of [^{35}S]-methionine and incubated with *in vitro*-translated Cdc20. Cdc20 complexes were immunoprecipitated using an anti-Cdc20 antibody and analysed by SDS–PAGE. ΔBR1 (amino acids 1–322), which lacks the Cdc20-interacting domain, a pcDNA3–myc empty vector, and reticulocyte lysate alone were included as negative controls. 5% of the *in vitro*-translated products were included as input controls. (**F**) 293T cells transfected with various BubR1 constructs were arrested with nocodazole, treated with MG132 for 3 h, and analysed for complex formation with Cdc20 by IP with 9E10 and by WB with anti-Cdc20 antibodies. In the lysis buffer, 10 μM of TSA was added. The same blot was re-probed with an anti-Ac-K250 antibody. Cells transfected with an empty vector (*pcDNA3–myc*) were included as a negative control.

Untransfected control cells took ~53 min (Figure 7D; Supplementary Figure 11) and *BubR1*-depleted cells took ~25 min (Figure 7D; Supplementary Figures 10 and 11) from NEBD to anaphase onset. Meanwhile, K250R intensities declined faster than wild-type BubR1-rescued cells (Figure 7B, red line). Furthermore, chromosomes segregated prematurely without proper alignment at the metaphase plane (Figure 7C, middle panel), and mitotic timing was

shortened to ~25–40 min (Figure 7B and D; Supplementary movie 2 and Supplementary Figure 11). In contrast, intensities for K250Q did not decline for more than 3 h ($n=40$), the chromosomes never segregated in those cells during the recording, and the cells subsequently died (Figure 7B, purple line and 7C, third row; Supplementary movie 3 and Supplementary Figure 11). Taken together, these results indicate that BubR1 destruction begins before the onset of anaphase and that BubR1 acetylation regulates its proteolysis. From these observations, we speculated that BubR1 acetylation and the timed degradation of BubR1 control the correct timing for anaphase entry.

Table I Frequency of exit from mitosis in 200 ng/ml nocodazole

Percentage of cells exiting from mitosis (total cell number analysed)	
Control	0% ($n=198$)
siBubR1	100% ($n=128$)
siMad2	100% ($n=97$)
siBubR1 + WT	6% ($n=18$)
siBubR1 + K250R	93% ($n=27$)
siBubR1 + K250Q	0% ($n=16$)
siMad2 + K250Q	94% ($n=17$)

Experimental schemes are similar to those described in Figure 5A. Nocodazole was continuously present and live-cell images were taken for 18 h after the start of nocodazole treatment. The number of cells exiting from mitosis during the time course was counted. Total cell numbers analysed (n) are indicated.

Acetylated BubR1 is an inhibitor of the APC/C complex, whereas unacetylated BubR1 becomes a substrate of APC/C-dependent proteolysis

We have shown that BubR1 acetylation occurs at prometaphase kinetochores and that BubR1 degradation began before the onset of anaphase. These results indicate that BubR1 might become a substrate of the APC/C complex when the SAC is satisfied. To test this hypothesis, we investigated whether BubR1 was ubiquitinated by APC/C–Cdc20 complex. HeLa cells were cotransfected with *Myc-BubR1*- and *HA-Cdc20*-encoding plasmids and subjected to IP with 9E10 and WB with anti-ubiquitin antibodies. The results showed that BubR1 was polyubiquitinated by ectopic expression of *Cdc20* in a dose-dependent manner (Figure 8A).

When the APC complex was inhibited by siRNA of *APC3*, the level of BubR1 remained constant for 60 min after nocodazole release in the presence of CHX, whereas it declined in control cells (Figure 8B). This result was corroborated by the finding from live-cell assay for proteolysis that depleting *APC3* expression abrogated the degradation of BubR1, and concomitantly cells did not enter anaphase for more than 5 h (Supplementary Figure 12 and Supplementary movie 7). When we compared the timing of BubR1 degradation with

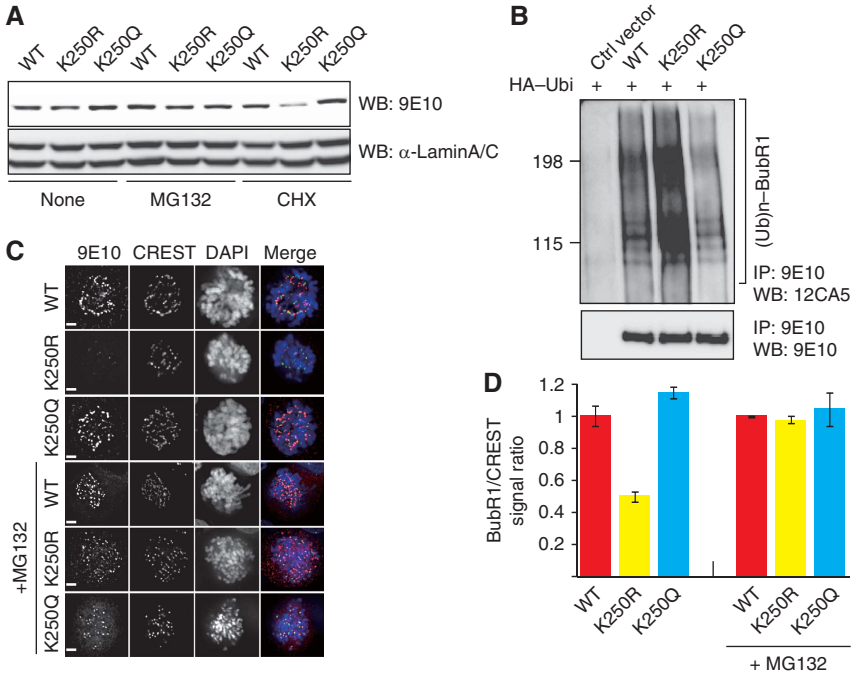

Figure 6 BubR1 acetylation inhibits its polyubiquitination-dependent proteolysis. (**A**) Two μg of wild-type *Myc-BubR1*-, *-K250R*-, or *-K250Q*-encoding expression plasmids was transfected into HeLa cells. The cells were then synchronized and arrested in mitosis by continuous nocodazole treatment. The cells were then treated with MG132 or CHX for 3 h in the presence of nocodazole before lysis. Total cell lysates were analysed by WB with 9E10 to detect the ectopically expressed BubR1. The same blot was re-probed with an anti-Lamin A/C antibody. (**B**) BubR1 acetylation results in the inhibition of polyubiquitination-dependent proteolysis. 293T cells were cotransfected with the plasmids encoding *wild type*, *K250R*, or *K250Q* with *HA–Ubiquitin*, and proteins were immunoprecipitated with 9E10 and analysed by WB using a 12CA5 anti-HA antibody to detect polyubiquitination of BubR1 [(Ub)n–BubR1]. The blot was re-probed with 9E10 to control for the amount of immunoprecipitation. (**C**) HeLa cells were transfected as in (A). At 16 h post transfection, the cells were treated with 100 μM of monastrol to arrest the cells in prometaphase without disturbing microtubule structures. After 5 h, cells were treated with MG132 (+MG132) or left untreated. Cells were then fixed and co-stained with 9E10 (red) and CREST (green). Other phases of mitosis are illustrated in Supplementary Fig 8. Bars, 5 μm. (**D**) Quantification of results in (C). Histograms summarize three different BubR1 intensities at kinetochores in the absence or presence of MG132, measured by staining with 9E10 and normalized for CREST staining. Results are from two independent experiments (mean ± s.e.m.; $n \geqslant 8$ prometaphase cells per experiment, $P<0.01$).

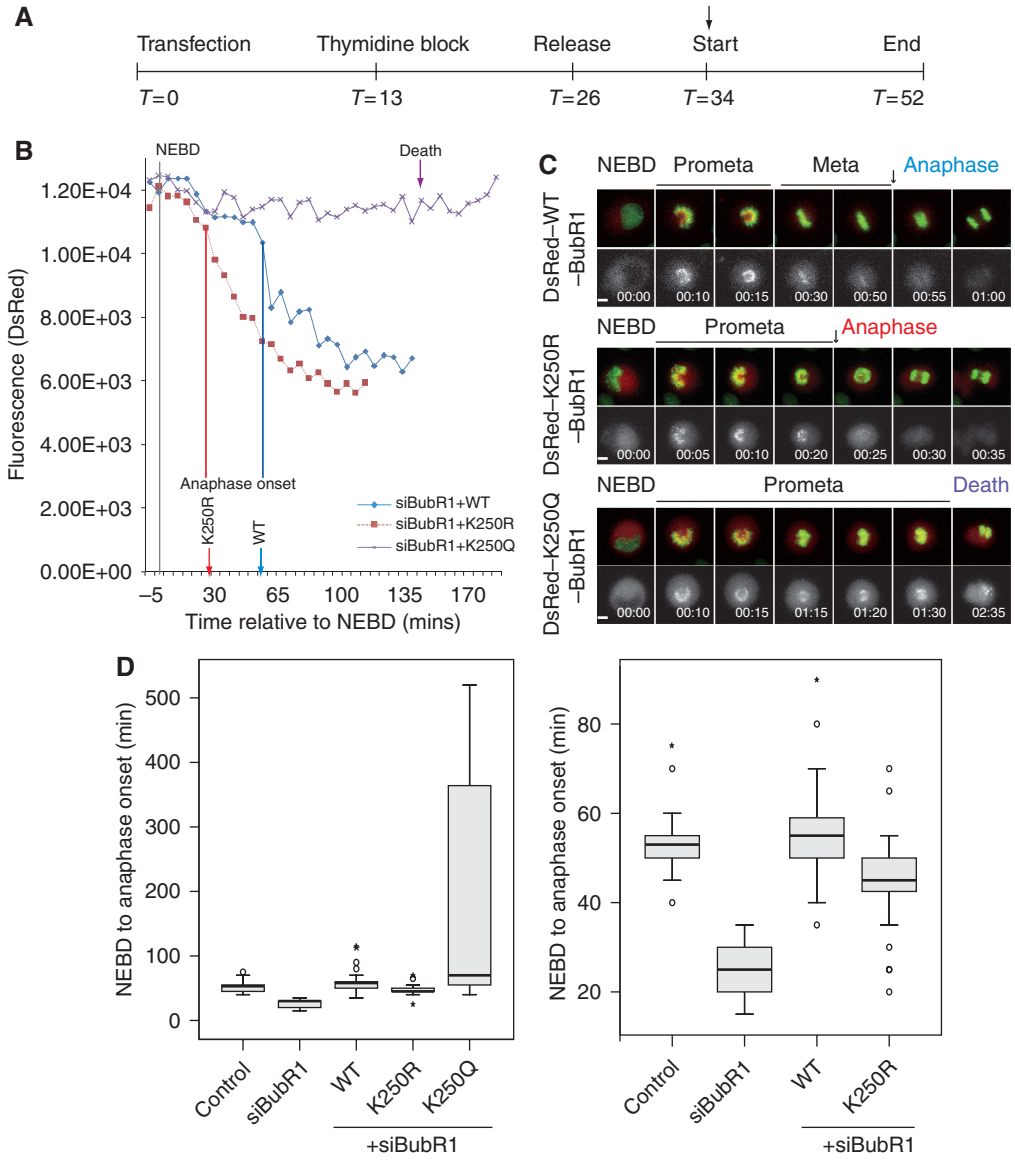

Figure 7 BubR1 acetylation inhibits its degradation and regulates the timing of anaphase onset. The HeLa cells stably expressing histone H2B–GFP (HeLa–H2B) were co-transfected with synthetic siBubR1 targeting the 3′UTR and *WT*, *K250R*, or *K250Q* expression plasmids, respectively, tagged with DsRed at the N-termini using lipofectamine™ 2000 and subjected to time-lapse microscopy. (**A**) Schematic illustration of the experiment. At 13 h after transfection, cells were synchronized at the S phase through the addition of 2 mM thymidine to the culture. After a further 13 h, cells were washed from the thymidine block and released into the cell cycle. Images were taken starting at 8 h after release and were processed for 18 h. (**B**) Fluorescence intensities (red) and mitotic timing (segregation of green) were measured and scored from NEBD. Mean red fluorescence was measured and plotted on the *Y*-axis as pixel values using Image J. The *X*-axis represents the time from NEBD in minutes. The curves shown are representative examples of several transfected cells from at least three separate experiments. Arrows indicate the timing of anaphase onset. (**C**) Captured images of (B) at indicated time points (marked at lower right). NEBD is marked as 00:00. BubR1 fluorescence (DsRed) images are shown in the bottom panels in grayscale to facilitate the measurement of fluorescence intensities for WT BubR1 (Supplementary movie 1), K250R (Supplementary movie 2), and K250Q (Supplementary movie 3), respectively. In the case of *K250Q*-rescued cells, cells were arrested in prometaphase for hours, then the cells frequently died. In the *K250R*-rescued cell, anaphase began without alignment of chromosomes in metaphase plane, and the mitotic timing was shortened to ~25 min. Stages in mitosis were determined from chromosome morphology. It should be noted that the fluorescence intensity around kinetochores declined before the onset of anaphase in *WT*- and *K250R*-expressing cells. Fluorescence intensity around kinetochores in *K250Q*-expressing cell did not decline for hours. (**D**) Box plot distributions of statistical mitotic timings from NEBD to anaphase onset for control HeLa cells ($n=60$); after depletion of *BubR1* (siBubR1) ($n=65$); WT *BubR1*-transfected cells rescued from *BubR1* depletion ($n=21$); *K250R* transfection after siRNA of *BubR1* ($n=43$); and *K250Q* transfection after siBubR1 ($n=40$). The bars in the box are the median values. Outliers (open circle) and suspected outliers (asterisk) as determined by statistical analysis are shown. Box plots were drawn again on a smaller *Y*-axis scale, without *K250Q*-rescued data, in the right panel to facilitate the comparisons of mitotic timing. Data are from three independent experiments analysing 20–65 transfected cells using SPSS software.

the degradation of other players in mitosis, such as Cdc20, Cyclin B, Plk1, and Aurora A, we found that BubR1 degradation began before that of Cyclin B (Supplementary Figure 13).

Next, we tested whether Cdc20 was responsible for BubR1 degradation during mitosis. To prevent the cells from exiting mitosis before the analysis began, HeLa cells were transfected with an expression construct to force moderate expression of

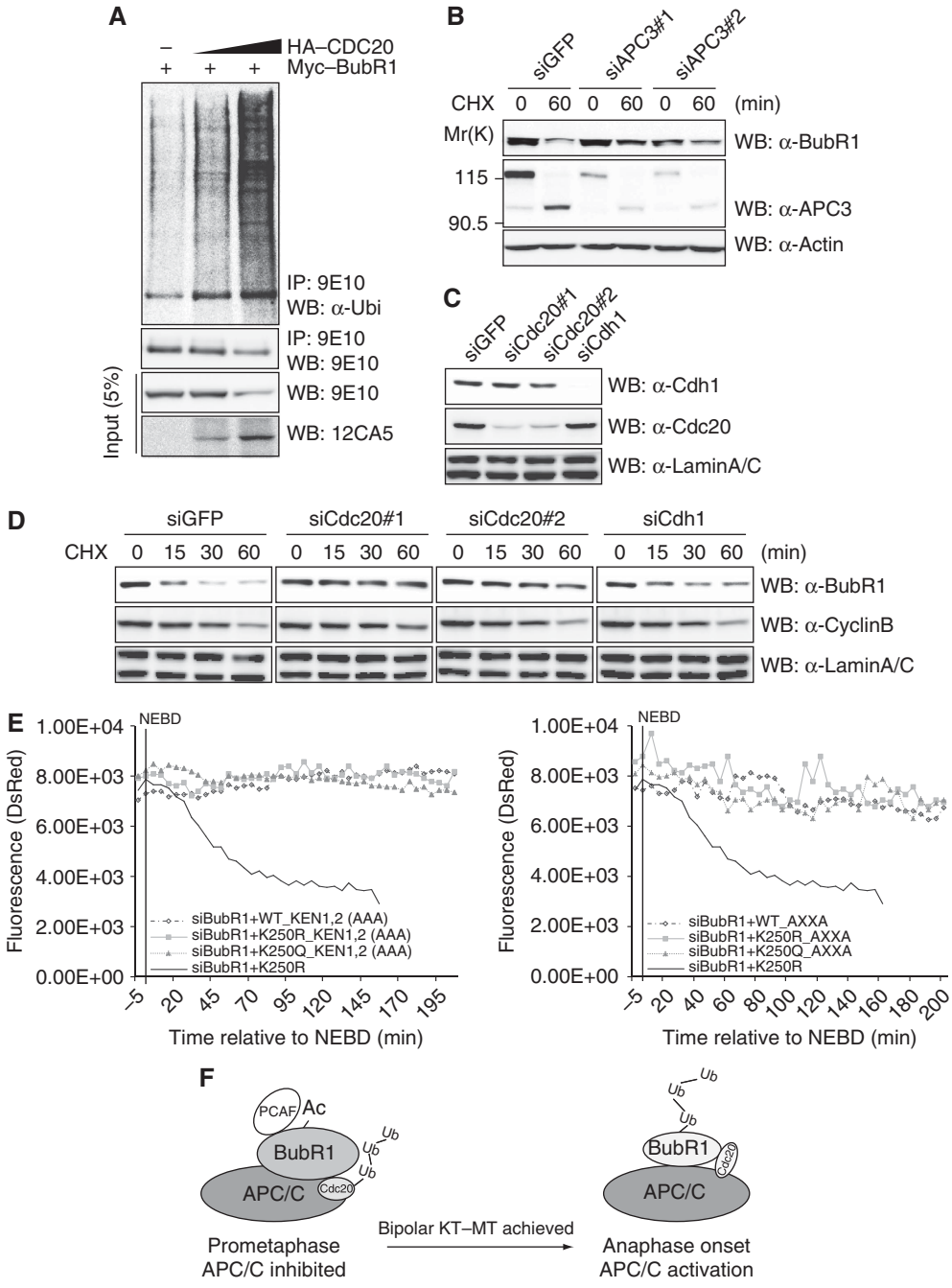

Figure 8 K250 acetylation functions as a switch between APC/C inhibition and APC/C-dependent BubR1 destruction. (**A**) 293T cells were co-transfected with Myc-tagged *BubR1* (1 μg each) and HA-tagged *CDC20* expression constructs in a dose-dependent manner (0, 1, and 2 μg). Lysates were then subjected to IP with 9E10 and to WB with anti-ubiquitin antibody. The blot was re-probed with 9E10 to control for the amount of IP. (**B**) BubR1 protein levels were compared after knockdown expressions of *APC3* to assess whether the APC/C complex was responsible for BubR1 degradation. HeLa cells were transfected with synthetic siRNAs. At 60 h later, cells were treated with 200 ng/ml nocodazole for 12 h, and the mitotic cells were collected by mitotic shake-off. Lysates were prepared before and after CHX addition for 1 h. Note that after nocodazole release for 60 min in the presence of CHX, APC3 appears as a faster migrating form, indicating that APC3 is highly modified in prometaphase. (**C**) WB analysis of Cdc20 and Cdh1 after transfection of siRNAs, as indicated. These cells were used to measure BubR1 levels in (D). (**D**) Experiments were carried out in a manner similar to (B), except that, to block the exit from mitosis, a *Cyclin B* expression construct was transfected simultaneously with siRNAs. The lysates for WB were collected at 0, 15, 30, and 60 min after nocodazole release and CHX treatment. (**E**) Intensities of fluorescence, which reflects the level of DsRed–BubR1 in mitotic cells, were recorded against time relative to NEBD. The D-box or the KEN boxes were destroyed by *in vitro* mutagenesis in *DsRed–K250R* by substituting AAA for KEN boxes (left panel) and RXXL to AXXA for the D-box (right panel). The curves shown are representative of at least 20 individual cells from at least two separate experiments. A fluorescence intensity curve for K250R was included in all experiments as a control. (**F**) Our working model for BubR1 acetylation and the regulation of APC/C activity. BubR1 is in a complex with Cdc20 and APC/C through its KEN1, KEN2, and D-box domains. BubR1 acetylation/deacetylation changes the surface structure of degrons in relation to APC/C–Cdc20 complex. When kinetochores are not yet attached to spindles (prometaphase), BubR1 is acetylated by PCAF. BubR1 acetylation prevents it from being a substrate of the APC/C complex, and acetylated BubR1 is a potent inhibitor of the APC/C–Cdc20 complex. When all kinetochores are stably attached to spindles, BubR1 is no longer acetylated because of a change in the stoichiometry of the BubR1 complex or its deacetylation. The conformational change allows the D-box and KEN box degrons of BubR1 to be recognized as a substrate of the APC/C-Cdc20 complex. In our model, the mode of Cdc20 binding to APC/C complex and BubR1 changes, depending on the status of BubR1 acetylation.

Cyclin B. siRNA for *GFP*, *Cdc20*, or *Cdh1* was simultaneously transfected. After 16 h of transfection, cells were synchronized at prometaphase, followed by release in the presence of CHX. Cell lysates were prepared at indicated time points after release and the levels of BubR1 and Cylin B were analysed. The results showed that BubR1 degradation was inhibited in cells depleted of *Cdc20*, whereas control cells (siGFP) showed significant decreases in BubR1 compared with initial analysis after nocodazole release. Cyclin B levels in the same blot showed that cells were in mitosis for at least 30 min after nocodazole release (Figure 8D). These results suggest that BubR1 is degraded through APC/C–Cdc20-dependent ubiquitination during mitosis.

Cdh1, a homologue of Cdc20, serves as a coactivator of APC/C complex during mitotic exit. Therefore, we asked whether Cdh1 was also responsible for BubR1 degradation. When cells were depleted of *Cdh1* (siCdh1), BubR1 levels decreased during mitosis. However, in *Cdh1*-depleted cells, BubR1 levels remained similar between 30 and 60 min after nocodazole release, whereas control cells (siGFP) had a continuous decrease after release (Figure 8D). It should be noted that the level of Cyclin B declined between 30 and 60 min after release, indicating that cells are exiting from mitosis within 60 min after release. The result suggests that BubR1 is subjected to a second round of degradation during mitotic exit through APC/C–Cdh1-mediated ubiquitination.

If the destruction of BubR1 by the APC/C–Cdc20 complex is important for mitotic timing, the proteolysis of BubR1 might depend on its degrons, the D-box and KEN boxes. To test this hypothesis, we made BubR1 constructs in which the D-box or KEN boxes were destroyed in *DsRed-WT*, *-K250R*, and *-K250Q* expression vectors. HeLa cells depleted of endogenous BubR1 and transfected with various plasmids were subjected to live-cell analysis for proteolysis, as shown in Figure 7. The result showed that both KEN1 and KEN2-mutated (KEN1, 2 (AAA)) or D-box-mutated WT, K250Q, and K250R were stable during the recorded times, whereas K250R with intact degrons began to degrade $\sim$20 min after NEBD (Figure 8E). We also observed that when KEN boxes or the D-box was destroyed in BubR1, cells were not able to maintain the SAC, and the mitosis was accelerated, although BubR1 level remained constant (Supplementary movie 6).

To confirm that BubR1 degradation depended on the D-box and KEN boxes, we carried out WB analysis in HeLa cells transfected with various constructs. As it was impossible to obtain a similar mitotic index and synchronize the cells after BubR1 siRNA and *K250R*, *WT*, or *K250Q* transfection, we compared the level of ectopically expressed proteins in the presence of endogenous BubR1 (Supplementary Figure 14). The result corroborated the finding that the KEN box- or D-box-deleted K250R mutants were stable (Supplementary Figure 14B, middle and right panels). Collectively, these results suggest that the APC/C complex is responsible for BubR1 degradation, and BubR1 degradation requires the D-box and KEN boxes.

Discussion

In this study, we provide compelling evidence that BubR1 is acetylated at K250 by PCAF specifically at prometaphase. Acetylated BubR1 is a potent inhibitor of the APC/C–Cdc20 complex, whereas unacetylated (or deacetylated) BubR1 readily becomes a substrate of APC/C-dependent proteolysis. Thus, BubR1 acetylation/deacetylation serves as a molecular switch for the conversion of BubR1 from inhibiting the APC/C complex to becoming its substrate. This Janus-like mode of action provides an efficient way of controlling chromosome segregation after checking that everything is ready, which is a crucial requirement for accurate sister chromatid separation. A similar mode of switching from an inhibitor to a substrate of the APC/C complex has been reported in Emi1, an inhibitor of the APC/C–Cdh1 complex in interphase (Miller *et al*, 2006). Interestingly, a recent report suggested that Cdc20 serves as a substrate of APC/C complex when the SAC is activated and as a coactivator of APC/C complex during the metaphase–anaphase transition (Nilsson *et al*, 2008).

Our working hypothesis is as follows: BubR1 acetylation at K250 takes place at prometaphase, which inhibits the APC/C–Cdc20 complex until all the kinetochores are stably attached with bipolar spindles. This monitoring of microtubule attachment probably involves the motor protein CENP-E (Chan *et al*, 1999; Abrieu *et al*, 2000; Yao *et al*, 2000; Mao *et al*, 2003, 2005; Morrow *et al*, 2005; Zhang *et al*, 2008). When the spindle checkpoint is satisfied, BubR1 is no longer acetylated and the unacetylated form of BubR1 replaces acetylated BubR1. This process might involve the deacetylation of BubR1, because we observed that HDAC 1–3 could bind to BubR1. However, whether dynamic acetylation and deacetylation of BubR1 take place during mitosis has not yet been investigated. Another possibility is that PCAF detaches from BubR1 after all of the kinetochores are stably attached to spindles, because the BubR1 complex involving CENP-E and microtubules is very likely to change its stoichiometry. In support of the first view, a recent report showed that HDAC3 localizes to spindle microtubules and is required for kinetochore–microtubule attachments (Ishii *et al*, 2008). As for the latter view, we have shown that the BubR1–PCAF interaction takes place exclusively at prometaphase kinetochores (Figure 1).

Turning the APC/C–Cdc20 complex on and off

The timed proteolysis of key regulators of the cell cycle is vital for progression through mitosis. Regulated proteolysis in cell division is largely dependent on the APC/C E3 ligase activity. For APC/C activation, the WD40 domain-containing coactivator Cdc20 or its homologue Cdh1 is required (Peters, 2006; Thornton and Toczyski, 2006; Yu, 2007). It was suggested that coactivators recognize the destruction signals (D-box or KEN box) and transfer them to the APC/C complex for polyubiquitination. However, accumulating evidence indicates that both the D-box and the KEN box degrons are recognized by the APC/C complex as well. Furthermore, it was convincingly shown in *Xenopus* extracts that destruction signals are recognized and bound by the APC but not the Cdc20 coactivator (Yamano *et al*, 2004). Therefore, it is possible that BubR1 binds to Cdc20 and the APC simultaneously through its degrons. It is noteworthy that a recent structural analysis using single-particle electron microscopy showed that the APC/C interaction domain partially overlaps with the Cdc20 binding site on BubR1 (Herzog *et al*, 2009).

How, then, can BubR1 avoid degradation while binding to and inhibiting the APC/C–Cdc20 complex? When two KEN boxes or a D-box was destroyed in WT BubR1, K250Q, or

K250R, the checkpoint was compromised even though the levels of BubR1 did not decrease. We believe this was because of the inability of BubR1 to bind to the APC/C complex and Cdc20 in the absence of its degron domains. The acetylation status of BubR1 does not affect Cdc20 binding (Figure 5). Therefore, the availability and the binding mode of individual KEN1, KEN2, and D-box degrons for binding to APC/C–Cdc20 complex might depend on BubR1 acetylation and bipolar spindle attachment. Notably, a recent report showed that BubR1 binds and presents Cdc20 to promote APC/C-mediated Cdc20 degradation when SAC is active (Nilsson *et al*, 2008). Taken together with our findings, we postulate that in SAC-on conditions, BubR1 is acetylated and Cdc20 degradation is promoted. When all the chromosomes are attached to spindle microtubules, BubR1 is deacetylated (or no longer acetylated) because of conformational changes after spindle attachment. The structure of BubR1-containing complex changes, and the degrons of BubR1 are exposed to APC/C complex and Cdc20 and serve as substrate recognition motifs for the APC/C–Cdc20 complex. Thereafter, destruction of BubR1 might result in additional activation of the APC/C–Cdc20 complex (Figure 8F). Validation of this hypothesis will require further study.

Studies from yeast have shown that Mad3 is capable of binding to Cdc20 but is not degraded by the APC/C complex (Burton and Solomon, 2007). We wish to point out that there is a marked difference in BubR1/Mad3 acetylation and proteolysis between yeast and mammals. The acetylation site K250 and its neighbouring degrons are not really conserved between yeast Mad3 and hBubR1. Yeast Mad3 was not acetylated in our *in vitro* acetylation assays (Supplementary Figure 15). Thus, vertebrate BubR1 has acquired additional multilayered regulatory functions in evolution.

BubR1 is ubiquitinated and degraded in two steps: first through APC/C–Cdc20 complex and then through APC/C–Cdh1 complex

With compelling lines of evidence, we have shown that the APC/C–Cdc20 complex is responsible for BubR1 destruction in mitosis, although we were not able to detect polyubiquitinated chains on BubR1 using *in vitro* ubiquitination assays. As with Cyclin B, this could be because of the as-yet-unidentified biochemical nature of *in vitro*-translated BubR1. Owing to aggregation, full-length Cyclin B is not an efficient substrate of the APC/C complex, *in vitro* (Pfleger and Kirschner, 2000; Yamano *et al*, 2004). Interestingly, we found that APC/C–Cdh1 complex was responsible for BubR1 degradation after exit from mitosis, similar to the yeast Mad3 (King *et al*, 2007) and Bub1 (Qi and Yu, 2007). Our results suggest that BubR1 is degraded in two steps: first during mitosis and then during mitotic exit.

We have shown earlier that BubR1 is activated when mitotic cells encounter DNA damage (Choi and Lee, 2008). As the DNA damage response is central to cancer development, forced activation of BubR1 in mitosis might be helpful in the treatment of cancer. Furthermore, inhibitors of histone deacetylases have proven to be successful in many preclinical cancer models and have shown encouraging results in clinical trials (Minucci and Pelicci, 2006). Therefore, our results imply that BubR1-specific inhibition of deacetylation might be a promising target for future cancer therapeutics.

Materials and methods

Mouse and rabbit polyclonal antibodies generated against acetylated BubR1

Antibodies specific to BubR1 acetylated at K250 were generated by injecting mice or rabbits with the synthetic peptide chemically acetylated at K250 (-CGGAL(AcK)APSQ-C), corresponding to human BubR1 245–254 amino acids (Peptron, Korea). Anti-Ac-K250 antiserum was raised against the peptide.

Note: Additional Materials and methods are provided in the Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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