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APC/Fizzy-Related targets Aurora-A kinase for proteolysis

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Aurora-A kinase is a mitotic spindle-pole-associated protein that has been implicated in duplication and separation of centrosomes and in spindle assembly. The proper timing and amplitude of Aurora-A expression seems to be important, as elevated levels of this protein have been associated with centrosome abnormalities and aneuploidy in mammalian cells. We show that Aurora-A increases at the G_2–M transistion and disappears completely at G_1 in XL2 cells. Using *Xenopus* oocyte extracts, we demonstrate that degradation of Aurora-A is mediated by the anaphase-promoting complex (APC) and is regulated by Fizzy-Related but not by Fizzy. Degradation of Aurora-A depends on a D-Box, but not on its KEN-Box motif, as mutation of its C-terminal D-Box sequence induces stabilization of the protein. Accordingly, addition into the extracts of a cyclin B-type D-Box-motif-containing peptide completely suppresses its degradation. Furthermore, APC/Fizzy-Related ubiquitylates the wild type but not a D-Box mutant form of Aurora-A *in vitro*. Consistent with these data, ectopic expression of Fizzy-Related in *Xenopus* oocytes induces complete degradation of endogenous Aurora-A. Aurora-A is thus the first protein, at least in our assay system, that undergoes a D-Box-dependent degradation mediated by APC/Fizzy-Related but not by APC/Fizzy.

INTRODUCTION

In vertebrates, the Aurora kinase family contains three different members, Aurora-A, -B and -C, that are associated with mitotic structures, such as spindle poles, centrosomes, chromosomes and the mid-body. Aurora-A is implicated in centrosome duplication/separation and spindle assembly. Interestingly, this kinase is amplified in human cancers, and its overexpression induces cell transformation (Bischoff *et al.*, 1998; Zhou *et al.*, 1998), indicating that the control of Aurora-A levels is important in modulating cell division. In HeLa cells, Aurora-A is degraded and re-synthesized during cell-cycle transit (Honda *et al.*, 2000). Despite the fact that Aurora-A proteolysis seems to be essential for preventing cell transformation, the pathway that mediates its degradation is not known.

The ubiquitin-dependent pathway is a precise and rapid mechanism used by the cell to induce proteolysis. At the metaphase–anaphase and M–G_1 transitions, degradation is initiated by the E3/ubiquitin-ligase known as the anaphase-promoting complex (APC) (for a review, see Zachariae and Nasmyth, 1999). This E3 is regulated by the Fizzy/Cdc20 and Fizzy-Related/Cdh1 proteins (Sigrist and Lehner, 1997; Fang *et al.*, 1998; Lorca *et al.*, 1998; Kramer *et al.*, 2000), two distinct activators that transiently interact with this ubiquitin ligase in a cell-cycle-specific manner. APC/Fizzy complex mediates securin and cyclin B degradation, a prerequisite for progression through mitosis (Cohen-Fix *et al.*, 1996; Lorca *et al.*, 1998). Fizzy-Related mainly controls progression through G_1 by ensuring the complete proteolysis of cyclin B and Fizzy (Visintin *et al.*, 1997; Pfleger and Kirschner, 2000).

Here, we establish a further role for APC/Fizzy-Related at the M–G_1 transition, namely the ubiquitylation and thus degradation of Aurora-A in a D-Box-dependent manner.

RESULTS AND DISCUSSION

Aurora-A proteolysis is not mediated by the APC/Fizzy complex

Similarly to human Aurora-A (Kimura *et al.*, 1997; Bischoff *et al.*, 1998), *Xenopus* Aurora-A increases at the G_2–M transition and then disappears at the M–G_1 transition of the cell cycle

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(see Supplementary figure 1 available at *EMBO reports* Online). Previously, Aurora-A was described to be degraded at metaphase II exit in *Xenopus* oocytes (Frank-Vaillant *et al.*, 2000), a transition regulated by APC/Fizzy (Lorca *et al.*, 1998). We investigated whether this reported decrease of Aurora-A is, in fact, mediated by this ubiquitin ligase. Using western blotting, we measured endogenous Aurora-A levels at different times following *in vitro* activation of APC/Fizzy by calcium addition to extracts prepared from *Xenopus* metaphase-II-arrested oocytes (CSF extracts). As expected, both Aurora-A and cyclin B2 were stable in the absence of calcium (Figure 1A). Surprisingly, the addition of Ca^{2+} did not trigger Aurora-A proteolysis, whereas cyclin B2 was completely degraded. To confirm these results, we investigated whether endogenous Aurora-A was degraded in metaphase-II-arrested oocytes activated by the calcium ionophore A32187. Oocytes were homogenized at different times following ionophore treatment, and the levels of endogenous Aurora-A and cyclin B2 were analysed by immunoblotting. In contradiction to Frank-Vaillant *et al.* (2000), endogenous Aurora-A levels were stable during the entire time course (Figure 1B). On the other hand, cyclin B2 was degraded 20 min post-activation and accumulated afterwards. Similar results were obtained when the oocytes were activated by electric shock or fertilization (data not shown). Taken together, these results demonstrate that Aurora-A is not degraded at metaphase II exit in *Xenopus* oocytes under conditions where APC/Fizzy is active. Thus, Aurora-A degradation observed at the M–G_1 transition in somatic XL2 cells may not be mediated by this ubiquitin ligase.

Aurora-A proteolysis requires the APC/Fizzy-Related complex

We next investigated whether the APC/Fizzy-Related complex was involved in the degradation of Aurora-A associated with mitotic exit in somatic cells. To reconstitute a functional APC/Fizzy-Related complex, interphase *Xenopus* egg extracts, which are devoid of Fizzy-Related (Lorca *et al.*, 1998), were supplied with an mRNA encoding this protein. One hour later, we added either Aurora-A or cyclin B, produced as [^{35}S]methionine labelled proteins in reticulocyte lysates. The stability of these proteins was monitored at 30 min intervals. Fizzy-Related expression was visualized principally by western blotting (Figure 1C). A minor proportion was also ^{35}S-labelled as a consequence of the free [^{35}S]methionine present in the reticulocyte lysate. As expected, both Aurora-A and cyclin B were stable in control interphase egg extracts that lack both APC/Fizzy and APC/Fizzy-Related activities (Figure 1C, lower panels). In contrast, cyclin B, a substrate of the APC/Fizzy-Related complex (Pfleger and Kirschner, 2000), was proteolysed within 60 min of

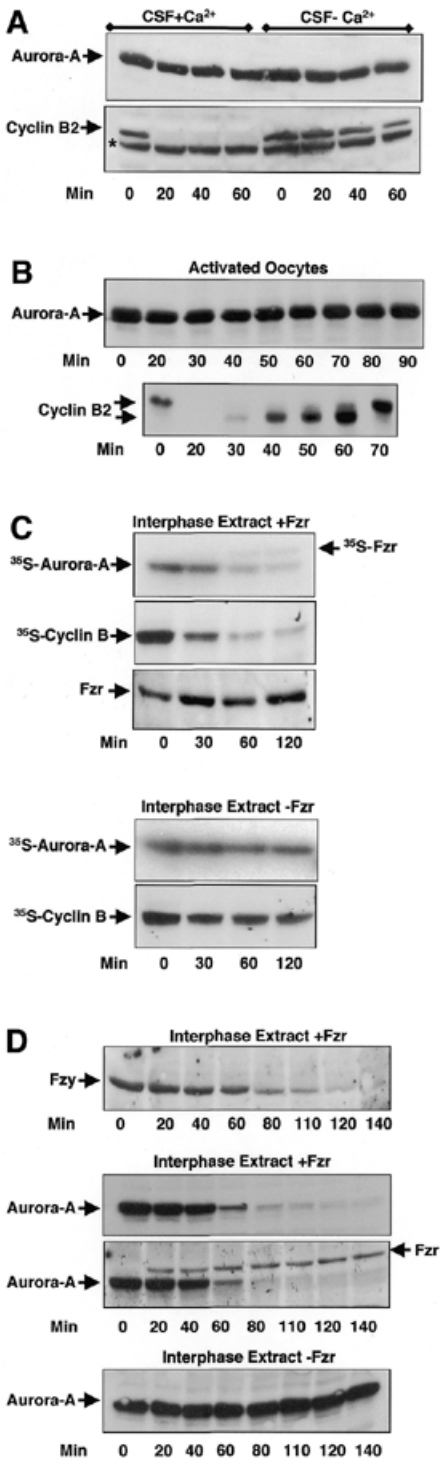

Fig. 1. Fizzy-Related but not Fizzy is required for Aurora-A degradation. (**A**) CSF extract (20 µl) was supplemented with 0.5 mM $CaCl_2$ where indicated (CSF+Ca^{2+}). Samples (2 µl) were taken at different times, and endogenous Aurora-A and cyclin B2 levels were analysed by western blotting. The asterisk represents an unspecific band recognized by the anti-cyclin B2 antibodies in the CSF extracts. (**B**) Metaphase-II-arrested oocytes were activated by the calcium ionophore A32187 (Activated Oocytes), homogenized individually and analysed at various times for the degradation of endogenous Aurora-A and cyclin B2. (**C**) Fizzy-Related mRNA was added to interphase extracts (50 µl) as indicated (Interphase Extract +Fzr). One hour later, 1 µl of either *in vitro* translated ^{35}S-labelled Aurora-A or the same amount of ^{35}S-labelled cyclin B was added. Samples (3 µl) were taken at different times and analysed by autoradiography. Fizzy-Related translation was verified by western blotting (Interphase Extract + Fzr and Fzr). (**D**) Interphase extracts (50 µl) were supplemented with Fizzy-Related mRNA as indicated (Interphase Extracts +Fzr). Endogenous Aurora-A and Fizzy degradation were analysed by western blotting. To verify Fizzy-Related translation, Aurora-A and Fizzy-Related were analysed on the same nitrocellulose membrane (Interphase Extract +Fzr, Aurora-A/Fzr).

addition to Fizzy-Related-containing extracts, indicating that the latter was functional. Similarly, [^{35}S]Aurora-A was degraded 60 min after its addition (Figure 1C, upper panels), suggesting a role for APC/Fizzy-Related in Aurora-A proteolysis. In a similar experiment, we examined the endogenous levels of this kinase by western blotting. Endogenous Aurora-A was completely degraded ~60 min after the addition of Fizzy-Related mRNA, which led to the synthesis of Fizzy-Related (Figure 1D). In its absence, endogenous Aurora-A levels did not vary (Figure 1D). Fizzy, another APC/Fizzy-Related substrate (Pfleger and Kirschner, 2000), also underwent complete degradation (Figure 1D).

To determine whether or not Aurora-A degradation depended upon APC, interphase extracts were immunodepleted with anti-CDC27 antibodies before the addition of the Fizzy-Related mRNA (Figure 2A, lower panel). This blocked degradation of endogenous Aurora-A, whereas this kinase was entirely proteolysed when non-specific antibodies were used for immunodepletion (Figure 2A). Thus, APC mediates degradation of Aurora-A induced by Fizzy-Related expression.

Phosphorylation of Fizzy-Related by cyclin/cdk complexes inhibits APC/Fizzy-Related activity (Jaspersen *et al.*, 1999; Blanco *et al.*, 2000; Kramer *et al.*, 2000). To gain a deeper insight into the requirement of APC/Fizzy-Related complex to induce Aurora-A degradation, we added recombinant cyclin A to produce H1 kinase activity similar to that present in mitotic egg extracts. This led to a delay in Aurora-A proteolysis (Figure 2B, middle panel), indicating that cyclin-A/cdk inhibited Aurora-A proteolysis, most likely through phosphorylation of Fizzy-Related.

Together, these results strongly suggest that Aurora-A proteolysis requires the APC complex and its activator Fizzy-Related.

The addition of a D-Box-motif-containing peptide blocks Aurora-A degradation

Substrates recognized by the APC contain a destruction signal sequence, often located near the N-terminus of target proteins. Two types of destruction sequences have been identified: the D-Box (RxxL, where 'x' is any amino acid) and the KEN-Box. D-Box-containing substrates typically appear to be recognized by both APC/Fizzy and APC/Fizzy-Related (Pfleger and Kirschner, 2000). The KEN-Box-containing substrates are believed to be recognized exclusively by APC/Fizzy-Related. Recently, a novel extended D-Box has been reported to confer APC/Fizzy- and APC/Fizzy-Related-specific degradation signals to human cyclin A and Nek2A. This motif contains the core cyclin-B-type D-Box, followed by an additional short sequence at its extreme C-terminus (Hames *et al.*, 2001).

N-terminal peptides of *Xenopus* and sea urchin cyclin B, containing the classic D-Box motif, inhibit the proteolytic activity of both APC/Fizzy and APC/Fizzy-Related by a competitive mechanism. Surprisingly, this works with substrates containing either the classic D-Box or KEN-Box motifs (Pfleger and Kirschner, 2000) but not those with the extended form of the D-Box motif (Hames *et al.*, 2001). Therefore, we added a cyclin B D-Box-containing peptide to interphase extracts supplemented previously with the Fizzy-Related mRNA and monitored endogenous Aurora-A levels. The peptide strongly delayed APC/Fizzy-Related-dependent proteolysis of Aurora-A, suggesting that this degradation is mediated by either D-Box or KEN-Box motifs (Figure 2B, lower panel).

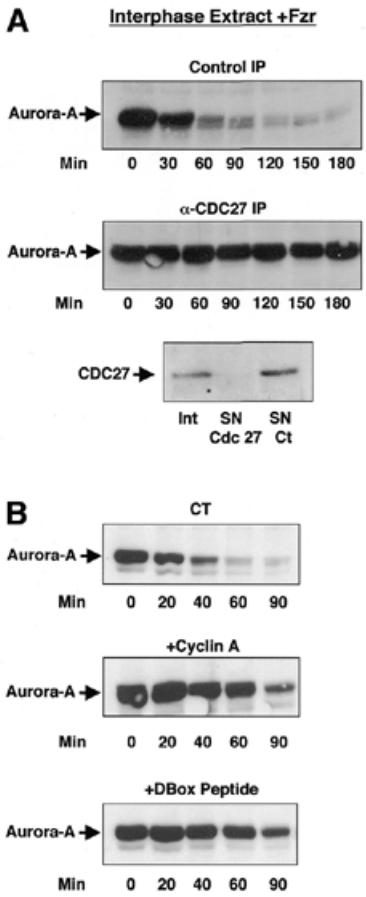

Fig. 2. The degradation of Aurora-A is mediated by the APC/Fizzy-Related complex and is blocked by the addition of a cyclin B D-Box-motif-containing peptide. (**A**) An interphase extract (50 µl) was first depleted with either α-CDC27 antibodies (α-CDC27 IP) or non-specific antibodies (Control IP) and then complemented with Fizzy-Related mRNA. A sample of 2 µl was then taken at different times, and endogenous Aurora-A levels were analysed by western blotting. The initial interphase extract (CDC27, Int) and the supernatants of α-CDC27 (CDC27, SN Cdc 27) and non-specific (CDC27, SN Ct) immunoprecipitations were analysed by western blotting with α-CDC27 antibodies to verify CDC27 depletion. (**B**) Fizzy-Related mRNA was added to interphase extracts (50 µl); 20 min later, we added a purified recombinant cyclin A protein (2 ng/µl, +Cyclin A), the cyclin B D-Box-containing peptide (7 µg/µl, +DBox Peptide) or buffer (CT). Endogenous levels of Aurora-A were then analysed by western blotting at different times.

Aurora-A is degraded by a D-Box-dependent pathway

The Aurora-A protein sequence contains three potential D-Box motifs, D1 (^{212}RVYL), D2 (^{293}RTTL) and D3 (^{378}RLPL), and one potential KEN-Box motif near its N-terminus (^{6}KEN) (Figure 3A). The role of these destruction motifs on Aurora-A degradation has proved to be conflicting (Honda *et al.*, 2000; Arlot-Bonnemains *et al.*, 2001). By using deleted forms of *Xenopus* Aurora-A, Arlot-Bonnemains *et al.* (2001) concluded that this kinase contains a destruction motif required for its ubiquitylation and degradation. In contrast, Honda *et al.* (2000) showed that punctual mutations or deletions of the putative D-Box sequences did not alter human Aurora-A degradation. To clarify whether a functional D-Box sequence is present in Aurora-A, we developed a

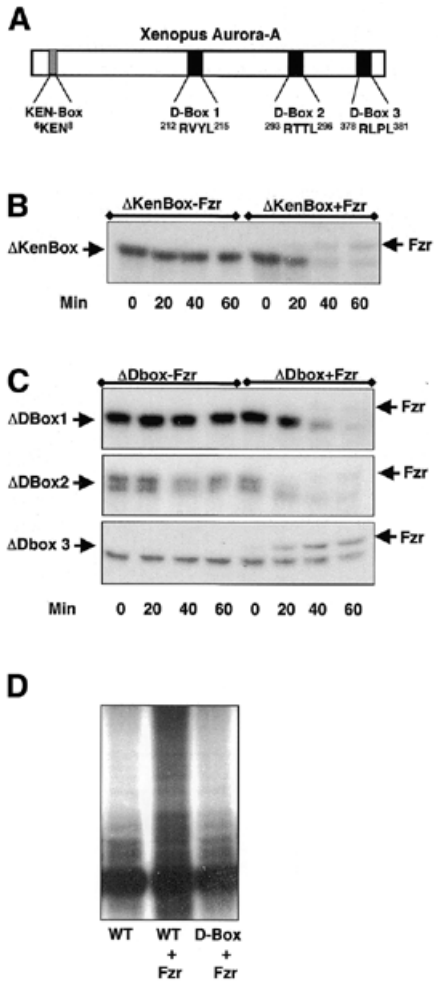

Fig. 3. Aurora-A targeting by the APC/Fizzy-Related complex requires a D-Box. (**A**) Schematic drawing of *Xenopus* Aurora-A depicting the presence of all the putative KEN-Box and D-Box motifs. (**B**) ^{35}S-radiolabelled KEN-Box mutant of Aurora-A was added to interphase extracts containing Fizzy-Related mRNA. The degradation of this protein was then analysed by autoradiography. (**C**) Similar to (B), except for the addition of D-Box 1 (ΔDBox1), 2 (ΔDBox2) or 3 (ΔDBox3) mutants instead of KEN-Box mutant. (**D**) ^{35}S-labelled wild type (WT and WT + Fzr) and D-Box 3 mutant (D-Box + Fzr) were added to an immunopurified APC in the presence (WT + Fzr) or absence (WT) of *in vitro* translated Fizzy-Related and assayed for ubiquitylation.

punctual mutation analysis of putative D-Box motifs in our assay, where the degradation of this kinase can selectively be induced by the addition of Fizzy-Related mRNA. Thus, the three arginine (^{212}R, ^{293}R and ^{378}R) and leucine (^{215}L, ^{296}L and ^{381}L) residues of each potential D-Box sequence were mutated to alanine (A). We then tested the degradation of the ^{35}S-labelled mutated forms translated in reticulocyte lysates when added to Fizzy-Related-containing interphase extracts. We also analysed proteolysis of an Aurora-A kinase mutant lacking the KEN-Box motif (^{6}KEN). The deletion of the KEN-Box motif did not affect Aurora-A stability (Figure 3B), nor did mutations of either D-Box 1 or D-Box 2 motifs (Figure 3C, upper and middle panels). However, a mutated D-Box 3 stabilized Aurora-A in this assay (Figure 3C, lower panel). We conclude that the D-Box 3 sequence of Aurora-A mediates its degradation. A Δ36-44 truncated form of *Xenopus* cyclin B has been reported to be ubiquitylated *in vitro* by APC/Fizzy-Related and not APC/Fizzy (Pfleger and Kirschner, 2000). This process is mediated by the recognition of the D-Box motif at position 7–15 of this protein. However, the authors found that a deleted form of cyclin B lacking this D-Box sequence was degraded to the same extent as wild-type protein by both APC/Fizzy and APC/Fizzy-Related. Therefore, the partial ubiquitylation dependent on APC/Fizzy-Related of cyclin B observed *in vitro* is not involved in physiological degradation of this protein. Thus, to our knowledge, this is the first example of a protein whose degradation is D-Box-dependent, at least in *Xenopus* egg extracts, and is exclusively mediated by APC/Fizzy-Related.

The APC/Fizzy-Related complex ubiquitylated wild type but not a D-Box 3 mutant of Aurora-A

To understand how mutation of the D-Box 3 motif blocks Aurora-A degradation, we tested whether this mutant is ubiquitylated *in vitro* by the APC/Fizzy-Related complex. Our *in vitro* ubiquitylation assay uses APC immunoprecipitated from interphase *Xenopus* egg extracts with α-CDC27 antibodies. This complex was incubated with ^{35}S-labelled wild type or a D-Box 3 mutated form of Aurora-A translated in reticulocyte lysates, along with E2, Ubch5B and bovine ubiquitin. Finally, the ubiquitylation assay was performed in the presence or absence of *in vitro* translated Fizzy-Related activator. As shown in Figure 3D, wild-type Aurora-A became strongly ubiquitylated only when both APC and recombinant Fizzy-Related were present in the reaction mixture (compare the first two lanes). Thus, Fizzy-Related is required for efficient APC-mediated ubiquitylation of Aurora-A. Furthermore, mutation of D-Box 3 blocked Aurora-A ubiquitylation, indicating that an intact D-Box 3 motif is essential, in our assay system, for its *in vitro* ubiquitylation (Figure 3D, right-hand lane).

Ectopic expression of Fizzy-Related by mRNA microinjection induces Aurora-A degradation in G_2-arrested oocytes

In quiescent *Xenopus* oocytes, Aurora-A is already present at stage VI oocytes, and no significant decrease in its levels has ever been observed during oocyte maturation. This is consistent with the lack of Fizzy-Related in *Xenopus* oocytes. We exploited this physiological situation to investigate whether the ectopic expression of Fizzy-Related could induce endogenous Aurora-A degradation *in vivo*. Stage VI oocytes were injected with Fizzy-Related mRNA. At different times, five oocytes were individually homogenized and analysed by western blotting for the presence or absence of Fizzy-Related and Aurora-A. We also followed the degradation of endogenous Fizzy protein as a positive control of APC/Fizzy-Related activation. Injection of Fizzy-Related mRNA induced the formation of a functional APC/Fizzy-Related complex, as endogenous Fizzy protein was clearly degraded (Figure 4, lower panel) in correlation with the appearance of Fizzy-Related (Figure 4, upper panel). Moreover, ectopic expression of Fizzy-Related in stage VI oocytes induced the degradation of endogenous Aurora-A protein, demonstrating that the proteolysis of this kinase is mediated *in vivo* by the

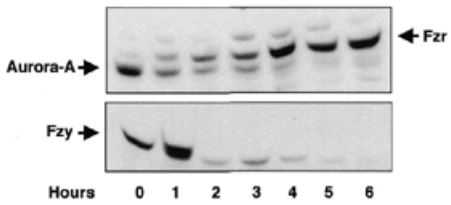

Fig. 4. APC/Fizzy-Related induces *in vivo* degradation of endogenous Aurora-A and Fizzy. Oocytes were microinjected with Fizzy-Related mRNA. Homogenates were prepared from individual oocytes hourly and analysed by coupled western blotting in the same nitrocellulose membrane for endogenous Aurora-A and Fizzy-Related. Fizzy degradation was followed by western blotting on a separate membrane.

APC/Fizzy-Related pathway (Figure 4, upper panel). This confirms our *in vitro* results in interphase *Xenopus* egg extracts. Thus, *Xenopus* oocytes contain the machinery required to induce Fizzy and Aurora-A degradation upon the expression of Fizzy-Related, confirming that this APC activator is a key regulator of the proteolysis of these proteins. Taken together, these results clearly show that degradation of *Xenopus* Aurora-A is mediated by the APC/Fizzy-Related complex in a D-Box-dependent way, where this motif acts by modulating Aurora-A ubiquitylation.

METHODS

Immunization procedure, antibodies and immunofluorescence microscopy. A wild-type *Xenopus* His_6–Aurora-A fusion protein, produced in *Escherichia coli*, was used to immunize rabbits. Immune serum was affinity-purified on immobilized His_6–Aurora-A. The *Xenopus* anti-cyclin B2, anti-Fizzy, anti-CDC27 and anti-Fizzy-Related antibodies have been described previously (Lorca *et al.*, 1998; Castro *et al.*, 2001). Immunofluorescence assays were developed as described previously (Roghi *et al.*, 1998).

Translation and degradation in *Xenopus* egg extracts. Interphase and CSF extracts, as well as extracts competent in translation of *Xenopus* Fizzy-Related mRNA, were prepared as described previously (Fesquet *et al.*, 1997; Lorca *et al.*, 1998). For protein degradation assays, 1 µl of either ^{35}S-labelled cyclin B or Aurora-A was incubated at room temperature with 20 µl interphase extracts supplemented (1 h before) or not with Fizzy-Related mRNA.

***In vitro* ubiquitylation assay.** Wild-type and mutated (R398A–L401A) Aurora-A proteins were translated *in vitro* in rabbit reticulocyte lysates in the presence of [^{35}S]methionine. To obtain APC/Fizzy-Related complexes, APC was immunopurified from interphase *Xenopus* egg extracts with anti-CDC27 antibodies and activated by reticulocyte-lysate-expressed *Xenopus* Fizzy-Related. The *in vitro* ubiquitylation reaction was performed as reported previously (Bembenek and Yu, 2001).

mRNA microinjection in *Xenopus* oocytes. Fifty oocytes were microinjected with 20 ng Fizzy-Related mRNA. Each hour, five oocytes were homogenized individually in 10 µl homogenization buffer (20 mM Tris pH 7.5, 50 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 5 mM $Na_4P_2O_7$, 1 mM EDTA). After extract centrifugation (13 000 r.p.m. for 3 min at 4°C), the clear supernatant was recovered and used for western blotting.

Site-directed mutagenesis. Oligonucleotides D1 (5′-GGCTATTTC-CACGATGCTTCCGCAGTCTACGCAATCCTGGATTATGCCC-3′) (5′-GGGCATAATCCAGGATTGCGTAGACTGCGGAAGCAT-CGTGGAAATAGCC-3′), D2 (5′-CATGCTCCATCCTCCAGGGC-GACCACTGCGTGTGGAACACTGGAC-3′) (5′-GTCCAGTGTTC-CACACGCAGTGGTCGCCCTGGAGGATGGAGCATG-3′) and D3 (5′-CACAACCCAAACCACGCGCTGCCAGCGAAAGGGGT-TCTCGAAC-3′) (5′-GTTCGAGAACCCCTTTCGCTGGCAGCG-CGTGGTTTGGGTTGTG-3′) were used for site-directed mutagenesis of D-Box 1, 2 and 3, respectively, according to the manufacturer's recommendations (Stratagene). Deletion of the KEN-Box has been described previously (Arlot-Bonnemains *et al.*, 2001). All constructs were verified by sequencing.

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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