⌘ *Author's Choice*

Phosphorylation of p53 Is Regulated by TPX2-Aurora A in *Xenopus* Oocytes*⑤

Gaetan Pascreau[‡1], **Frank Eckerdt**[‡1], **Andrea L. Lewellyn**[‡], **Claude Prigent**[§], and **James L. Maller**[‡2]

From the ‡Howard Hughes Medical Institute and Department of Pharmacology, University of Colorado School of Medicine, Aurora, Colorado 80045 and §CNRS, University of Rennes I, 35043 Rennes, France

p53 is an important tumor suppressor regulating the cell cycle at multiple stages in higher vertebrates. The p53 gene is frequently deleted or mutated in human cancers, resulting in loss of p53 activity. This leads to centrosome amplification, aneuploidy, and tumorigenesis, three phenotypes also observed after overexpression of the oncogenic kinase Aurora A. Accordingly, recent studies have focused on the relationship between these two proteins. p53 and Aurora A have been reported to interact in mammalian cells, but the function of this interaction remains unclear. We recently reported that *Xenopus* p53 can inhibit Aurora A activity *in vitro* but only in the absence of TPX2. Here we investigate the interplay between *Xenopus* Aurora A, TPX2, and p53 and show that newly synthesized TPX2 is required for nearly all Aurora A activation and for full p53 synthesis and phosphorylation *in vivo* during oocyte maturation. *In vitro*, phosphorylation mediated by Aurora A targets serines 129 and 190 within the DNA binding domain of p53. Glutathione *S*-transferase pull-down studies indicate that the interaction occurs via the p53 transactivation domain and the Aurora A catalytic domain around the T-loop. Our studies suggest that targeting of TPX2 might be an effective strategy for specifically inhibiting the phosphorylation of Aurora A substrates, including p53.

Aurora A is an oncogenic protein kinase that is active in mitosis and plays important roles in spindle assembly and centrosome function (1). Overexpression of either human or *Xenopus* Aurora A transforms mammalian cells, but only when the p53 pathway is altered (2–4). Aurora A is localized on centrosomes during mitosis, and overexpression of the protein leads to centrosome amplification and aneuploidy (2, 3, 5, 6), two likely contributors to genomic instability (7, 8). Because of its oncogenic potential and amplification in human tumors, considerable attention has been focused on the mechanism of

Aurora A activation in mitosis. Evidence from several laboratories indicates that activation occurs as a result of phosphorylation of a threonine residue in the T-loop of the kinase (4, 9, 10). Purification of Aurora A-activating activity from M phase *Xenopus* egg extracts led to an apparent activation mechanism in which autophosphorylation at the T-loop is stimulated by binding of the targeting protein for Xklp2 (TPX2) (11–14). On the other hand, it has been shown that Aurora A activity can be inhibited by interaction with several proteins, including PP1 (protein phosphatase 1), AIP (Aurora A kinase-interacting protein), and, more recently, p53 (9, 15–17).

p53 is a well known tumor suppressor able to drive cell cycle arrest, apoptosis, or senescence when DNA is damaged or cell integrity is threatened (18, 19). In human cancers, the p53 gene is frequently deleted or mutated, leading to inactivation of p53 functions (20). p53 protein is almost undetectable in "normal cells," mainly due to its instability. Indeed, during a normal cell cycle, p53 associates with Mdm2 in the nucleus and thereafter undergoes nuclear exclusion, allowing its ubiquitination and subsequent degradation (21). In cells under stress, p53 is stabilized through the disruption of its interaction with Mdm2 (21), leading to p53 accumulation in the nucleus and triggering different responses, as described above.

Although p53 has mostly been characterized as a nuclear protein, it has also been shown to localize on centrosomes (22–24) and regulate centrosome duplication (23, 24). Centrosomes are believed to act as scaffolds that concentrate many regulatory molecules involved in signal transduction, including multiple protein kinases (25). Thus, centrosomal localization of p53 might be important for its own regulation by phosphorylation/dephosphorylation, and one of its regulators could be the mitotic kinase Aurora A. Indeed, phenotypes associated with the misexpression of these two proteins are very similar. For example, overexpression of Aurora A kinase leads to centrosome amplification, aneuploidy, and tumorigenesis, and the same effects are often observed after down-regulation of p53 transactivation activity or deletion/mutation of its gene (26, 27).

Several recent studies performed in mammalian models show interplay between p53 and Aurora A, with each protein having the ability to inhibit the other, depending on the stage of the cell cycle and the stress level of the cell (17, 28, 29). These studies reported that p53 is a substrate of Aurora A, and serines 215 and 315 were demonstrated to be the two major Aurora A phosphorylation sites in human p53 *in vitro* and *in vivo*. Phosphorylation of Ser-215 within the DNA binding domain of human p53 inhibited both p53 DNA binding and transactiva-

tion activities (29). Recently, our group showed that *Xenopus* p53 is able to inhibit Aurora A kinase activity *in vitro*, but this inhibitory effect can be suppressed by prior binding of Aurora A to TPX2 (9). Contrary to somatic cells, where p53 is nuclear, unstable, and expressed at a very low level, p53 is highly expressed in the cytoplasm of *Xenopus* oocytes and stable until later stages of development (30, 31). The high concentration of both p53 and Aurora A in the oocyte provided a suitable basis for investigating p53-Aurora A interaction and also evaluating *Xenopus* p53 as a substrate of Aurora A.

EXPERIMENTAL PROCEDURES

Antibodies—Anti-p53 mouse monoclonal (X77) antibody was from Novus (catalog number NB 200-566), anti-FLAG-M2/horseradish peroxidase and anti-actin (AC40) antibodies were from Sigma (catalog numbers A8592 and A3853), anti-Myc (9E10)/horseradish peroxidase antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (catalog number SC-40), and anti-phospho-Thr-288 Aurora A (equivalent to *Xenopus* phospho-Thr-295), -phospho-MAPK, and -phospho-Tyr-15 Cdc2 antibodies were from Cell Signaling (catalog numbers 3079, 9101, and 9111). Anti-Aurora A, -TPX2, and -Plx1 antibodies were previously described (65, 66).

Morpholino and Inhibitor Experiments—Morpholinos used to inhibit TPX2 expression during oocyte maturation were synthesized by Gene Tools, LLC (control morpholino, 5′-CCT-CTTACCTCAGTTACAATTTATA-3′; TPX2 morpholino, 5′-GTAGGTGTCCTGTGTATCTTCCATG-3′). Morpholinos were injected into resting Stage VI oocytes at a final concentration of 40 μM. Oocytes were incubated overnight and then stimulated or not with progesterone. Extracts of five oocytes were prepared in extraction buffer (80 mM β-glycerophosphate, 20 mM EGTA, 15 mM MgCl$_2$, 1 mM dithiothreitol, protease inhibitor mix (Roche Applied Science), 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 μM okadaic acid) either before progesterone stimulation or 2 h after germinal vesicle breakdown (GVBD),[3] when uninjected oocytes reached Meta II arrest. Proteins were resolved on Anderson gels and transferred to nitrocellulose membranes for Western blot analysis. The ATP-competitive Aurora A kinase inhibitor, cyclopropanecarboxylic acid {3-[4-(trifluoromethyl-phenylamino)-pyrimidin-2-ylamino]-phenyl}-amide, is from Sigma (C1368) and was injected into oocytes to a final concentration of 50 μg/ml. Progesterone (10 μg/ml) was added 30 min after injection, and extracts were prepared as described above.

Recombinant Proteins—Full-length and truncated forms of Aurora A were subcloned into the pET-30 Ek/LIC vector (catalog number 69077-3; Novagen). [35S]Methionine-labeled p53 or Aurora A proteins were produced using the TNT Quick Coupled Transcription/Translation System (catalog number L1170; Promega) following the manufacturer's instructions. Full-length and truncated forms of GST-p53 were obtained by standard PCRs, adding a BamHI restriction site at the 5′-position and an EcoRI restriction site at the 3′-position. All PCR

products were subcloned into the pGEX-3X vector, and GST-tagged *Xenopus* p53 proteins were bacterially expressed and purified on glutathione-Sepharose beads.

In Vitro Pull-down Assays—4 μg of purified full-length or truncated GST-tagged *Xenopus* p53 was incubated for 2 h at 4 °C with 6 μl of 50% glutathione-Sepharose beads. Beads were then mixed with 5 μl of reticulocyte lysate containing [35S]methionine-labeled Aurora A for 2 h at 4 °C, washed, and then boiled in Laemmli sample buffer. Proteins were resolved by SDS-PAGE, and the gel was stained with Coomassie Blue to confirm that equal amounts of GST-protein were used in the pull-down. Association of the GST-protein with radiolabeled Aurora A was analyzed by autoradiography.

In Vitro Kinase Assays—500 ng of active recombinant WT Aurora A (11) was incubated with 5 μg of full-length p53 (WT or mutant) in kinase buffer (50 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1 mM dithiothreitol, 10 mM MgCl$_2$) and then assayed in the presence of 100 μM [γ-32P]ATP for 15 min at 30 °C. The reaction was stopped by the addition of Laemmli sample buffer, and proteins were resolved by SDS-PAGE. The Coomassie-stained bands containing p53 were excised from the gel, and radiolabel incorporation was analyzed by Cerenkov counting.

Immunoprecipitation Assays—The *Xenopus* p53 gene encoding full-length protein was subcloned into pOTV-3× FLAG-modified vector between XbaI and SalI, whereas the *Xenopus* Aurora A gene encoding full-length protein was subcloned into a pCS2–6× Myc-LIC-modified vector (Novagen). These constructs were then used for *in vitro* production of the corresponding mRNA with the mMessage mMachine T7 and SP6 systems, respectively (catalog numbers 1344 and 1340; Ambion). mRNA encoding Myc-Aurora A was injected into resting (prophase I (Pro I)-arrested) stage VI *Xenopus* oocytes and incubated overnight. Oocytes were then stimulated with progesterone, and extracts of five oocytes were prepared in extraction buffer 2 h after GVBD, a time when uninjected oocytes reached Meta II arrest. Extracts were precleared with 20 μl of protein A-agarose beads (catalog number 20334; Pierce) and 20 μl of normal anti-mouse IgG-agarose beads (catalog number SC-2343 AC; Santa Cruz Biotechnology) for 1 h at 4 °C on a wheel. Precleared supernatants were then incubated for 3 h at 4 °C on a wheel with 10 μl of anti-Myc (9E10) IgG-agarose beads (catalog number SC-40 AC; Santa Cruz Biotechnology). Beads were washed five times in extraction buffer and then boiled in Laemmli sample buffer. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes for Western blot analysis.

λ-Phosphatase Treatment—Oocytes were injected with mRNA encoding FLAG-p53 as described above and incubated overnight. Oocytes were then stimulated with progesterone or not, and extracts were prepared at Prophase I and Metaphase II. Extracts equivalent to one oocyte were then treated (+) or not (−) with 400 units of λ-phosphatase (catalog number P0753S; New England Biolabs) in λ-phosphatase buffer containing 2 mM MnCl$_2$ for 1 h at 37 °C. The reaction was stopped by the addition of Laemmli sample buffer, and samples were resolved by SDS-PAGE, followed by Western blot using anti-FLAG M2/horseradish peroxidase antibody.

---

[3] The abbreviations used are: GVBD, germinal vesicle breakdown; GST, glutathione *S*-transferase; Pro I, prophase I; Meta II, metaphase II; MAPK, mitogen-activated protein kinase.
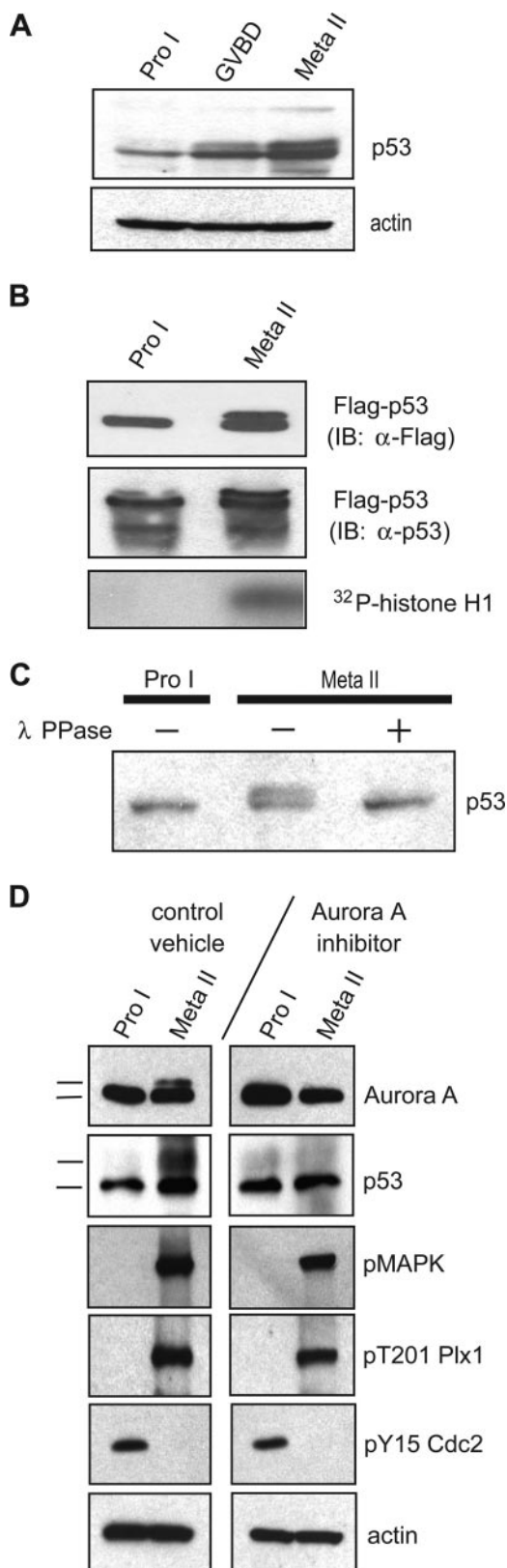
RESULTS

p53 Is Phosphorylated during Oocyte Maturation by TPX2-Aurora A—As mentioned above, *Xenopus* p53 is expressed at a significant level during development, from Stage VI of oogenesis (Prophase I) until the late tadpole stage (30). However, the behavior of p53 during oocyte maturation has not been investigated. Toward that aim, the abundance and electrophoretic mobility of endogenous *Xenopus* p53 was monitored in Pro I (G$_2$-arrested) oocytes, in oocytes undergoing GVBD after progesterone stimulation, and in oocytes arrested at metaphase II of meiosis (Meta II). As shown in Fig. 1*A*, the level of endogenous p53 increases markedly during oocyte maturation, and a substantial fraction undergoes an electrophoretic upshift between Pro I and GVBD that is maintained and even reinforced in MII oocytes. The same experiment conducted on oocytes injected with mRNA encoding FLAG-tagged full-length p53 shows that the upshift also occurs with the FLAG-p53 protein and can be observed either with anti-p53 antibody or with anti-FLAG antibody (Fig. 1*B*). To investigate whether this upshift is due to phosphorylation, we treated a metaphase II extract with λ-phosphatase and analyzed p53 electrophoretic mobility by Western blot. Fig. 1*C* shows that λ-phosphatase converts all p53 to the faster migrating form, indicating that the mobility shift is due to phosphorylation of p53.

Since this phosphorylation occurs at GVBD, when many kinases, including Aurora A, are activated (32, 33), we considered whether Aurora A was involved in phosphorylation of p53. One approach to investigate this possibility is to inhibit Aurora A kinase activity during maturation. Accordingly, we used a commercially available Aurora A kinase inhibitor (catalog number C1368; Sigma). We first confirmed the ability of this inhibitor to suppress the *in vitro* kinase activity of purified *Xenopus* Aurora A (data not shown) and then tested its effect *in vivo*. To this aim, we microinjected the inhibitor into Stage VI (Pro I-arrested) oocytes at a final concentration of 50 μg/ml (Fig. 1*D*, *right*), whereas control oocytes were injected with the control vehicle (*left*). Oocyte extracts were then prepared at Pro I and Meta II stages. Fig. 1*D* (*top*) shows that the activation of Aurora A was completely blocked, as judged by the disappearance of the electrophoretic upshift characteristic of Aurora A kinase activation *in vitro* and during oocyte maturation (32, 33). In the same oocytes, the upshift of p53, indicative of phosphorylation (Fig. 1*C*), was blocked. It was also observed that less total p53 was present at Meta II in oocytes in which Aurora A was inhibited. Other major biochemical events of maturation,

FIGURE 1. **p53 phosphorylation during *Xenopus* oocyte maturation is regulated by Aurora A.** *A*, Western blot of endogenous p53 during oocyte maturation. *Pro I*, G$_2$-arrested oocytes; *GVBD*, progesterone-treated oocytes collected after germinal vesicle breakdown (meiosis I); *Meta II*, progesterone-treated oocytes collected at metaphase II (cytostatic factor arrest). The same Western blot was probed for actin as a loading control. *B*, immature oocytes were injected with mRNA encoding FLAG-tagged p53 and then induced to mature by the addition of progesterone. Expression of FLAG-p53 was checked with either anti-FLAG (*top*) or anti-p53 (*bottom*) antibodies, before (Pro I) or after (Meta II) maturation. Progression to Meta II was monitored by testing histone H1 kinase activity of the extract (*autoradiograph*; *bottom*). *C*, metaphase II-arrested oocyte extract was treated (+) or not (−) with λ-phosphatase, as described under "Experimental Procedures," and the electrophoretic mobility of endogenous p53 was checked by Western blot. *D*, resting prophase I-arrested oocytes were injected with the Aurora A inhibitor C1368 (*right*) or the control vehicle (DMSO) alone (*left*). After 30 min, half of the oocytes were treated with progesterone and incubated until reaching Meta II, whereas the other half was maintained in Pro I for the same period of time. Both sets of oocytes were homogenized and subjected to Western blotting analysis for the indicated proteins. *Hatch marks* on the *left* indicate phosphorylated and dephoshorylated forms of the proteins. Actin was monitored as a loading control.
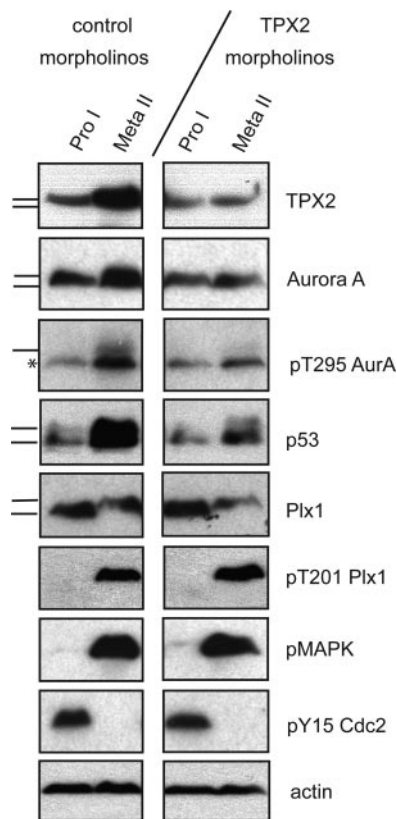
FIGURE 2. **Activation of Aurora A by TPX2 is required for full accumulation and phosphorylation of p53.** Immature oocytes were injected with control morpholinos (*left*) or morpholinos against TPX2 (*right*) and then stimulated to mature by the addition of progesterone. Expression and electrophoretic behavior of the indicated proteins were checked by Western blot with the indicated antibodies before (Pro I) or after (Meta II) maturation. Actin was monitored as a loading control. *Hatch marks* on the *left* indicate phosphorylated and dephoshorylated forms of the proteins. For the phospho-Thr-295 blot, the *asterisk* denotes a nonspecific band detected by the Cell Signaling antibody (35).

FIGURE 3. **p53 and Aurora A interact *in vivo* in *Xenopus* oocytes.** mRNA encoding $6\times$ Myc-Aurora A was injected into resting Stage VI *Xenopus* oocytes. Oocytes were stimulated by progesterone and extracts prepared when oocytes reached Meta II. Myc-Aurora A was then immunoprecipitated from the extract using anti-Myc antibody (*lanes 3* and *5*), and anti-mouse IgG was used as a control (*lane 4*). Immunoprecipitation of Myc-Aurora A was monitored by Western blot with anti-Myc/horseradish peroxidase antibody (*bottom panel*), whereas the co-immunoprecipitation of endogenous p53 was monitored by Western blot (*IB*) using anti-p53 antibody (*top*). *Lanes 1* and *2* display levels of endogenous p53 and Myc-Aurora A in 10% of extract input as judged by Western blot (*IB*). Actin was monitored as a loading control.

including activation of MAPK (phospho-MAPK) and Plx1 (phospho-Thr-201 Plx1), as judged by T-loop phosphorylation, and activation of cyclin B/Cdc2 (phospho-Tyr-15 Cdc2), as judged by removal of inhibitory phosphorylation at Tyr-15, were not affected by Aurora A inhibition. These results suggested that activated Aurora A was required for p53 phosphorylation during oocyte maturation.

However, both Aurora A and Aurora B are activated at GVBD (33–35), and it is likely that the Aurora A inhibitor C1368 targets both Aurora A and Aurora B, making it difficult to exclude an effect related to Aurora B inhibition (36–38). Thus, it was important to inhibit Aurora A kinase activity more specifically. Previous work has demonstrated that TPX2 is an activator of Aurora A present in Meta II oocytes that does not activate Aurora B (11). Therefore, we specifically impaired Aurora A activity by ablation of TPX2 with antisense oligonucleotides. First, we examined the level of TPX2 before and after maturation. TPX2 is present at a low level in Pro I-arrested oocytes and accumulates to a much higher level during maturation (Fig. 2, control morpholinos). Microinjection of oocytes with a morpholino specifically targeting TPX2 mRNA prior to progesterone stimulation inhibited accumulation of TPX2 protein at GVBD but did not eliminate the low level of protein
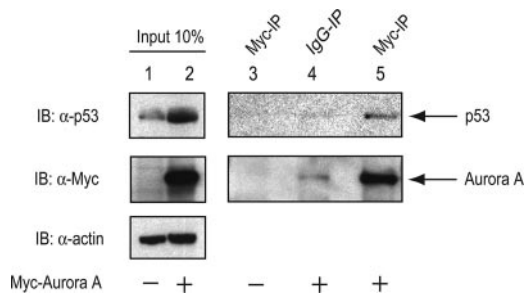
already present at Pro I (Fig. 2, *TPX2*). Importantly, inhibition of TPX2 accumulation blocked the Aurora A upshift in Meta II oocytes (Fig. 2, *Aurora A*). Furthermore, use of a phosphoantibody targeting the phosphorylated T-loop of Aurora A showed almost complete elimination of T-loop phosphorylation after ablation of TPX2 synthesis with morpholinos, suggesting that Aurora A kinase activity is strongly inhibited in these oocytes (Fig. 2, *pT295 Aur A*). Interestingly, this reduction in Aurora A activity was correlated with a partial downshift of p53 in the same extract, indicating that at least part of the p53 phosphorylation during maturation is due to Aurora A activity. To confirm that this effect on p53 phosphorylation is due to inhibition of Aurora A activity and not to secondary effects on other major kinases, we monitored the behavior of Polo-like kinase (Fig. 2, *Plx1* and *pT201 Plx1*), MAPK (*pMAPK*) and Cdc2 kinase (*pY15 Cdc2*). None of these kinases were affected by the TPX2 morpholino (Fig. 2). The reduced phosphorylation of p53 after TPX2 knockdown confirms that the effects on p53 seen with the Aurora A inhibitor (Fig. 1*D*) were indeed due to failure to activate Aurora A.

It was also noted that in addition to the effect on p53 phosphorylation, failure to activate Aurora A led to a decreased accumulation of p53. This could be explained by a decreased stability or synthesis of p53 in the absence of active Aurora A. However, p53 stability was not affected by Aurora A inhibition using either the Aurora A inhibitor or TPX2 morpholinos (Fig. S1). Therefore, the reduced p53 level observed in Figs. 1*D* and 2 may be due to a decrease in p53 synthesis. Altogether, these results provide strong evidence that p53 is synthesized and phosphorylated during oocyte maturation in a manner dependent, at least partially, on Aurora A activity.

Aurora A and p53 Interact in Vivo and in Vitro in Xenopus Oocytes—The dependence of p53 phosphorylation *in vivo* on Aurora A activation suggests both Aurora A and p53 might interact in *Xenopus* oocytes during maturation. To evaluate this hypothesis, we injected mRNA encoding Myc₆-tagged wild-type Aurora A (Myc-Aurora A) into resting Stage VI oocytes. Then meiotic maturation was induced with progesterone, and extracts were prepared when the oocytes reached metaphase II (Fig. 3). When Myc-Aurora A was immunoprecipitated from
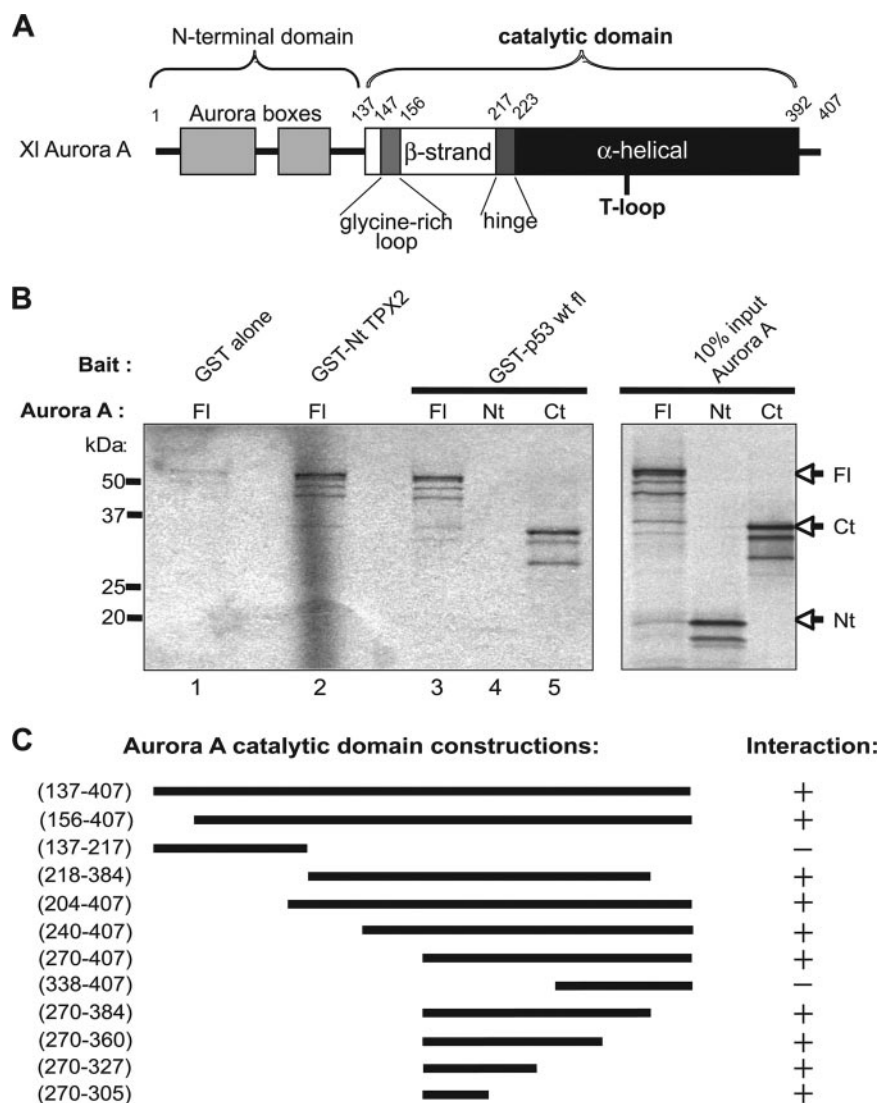
FIGURE 4. **p53 interacts with the catalytic domain of Aurora A.** *A*, Aurora A is composed of an N-terminal regulatory domain containing two Aurora boxes and a C-terminal kinase catalytic domain. DNA plasmids containing full-length Aurora A (*Fl*) as well as the N-terminal domain (*Nt*) or the catalytic domain (*Ct*) of Aurora A were transcribed and translated *in vitro* in the presence of [³⁵S]methionine as described under "Experimental Procedures." *B*, GST-tagged full-length p53 (*GST-p53 WT Fl*) was used as a bait in an *in vitro* pull-down assay to assess its interaction with the ³⁵S-labeled Aurora A constructs described in *A* (*lanes 3–5*). Radiolabeled Aurora A proteins pulled down by the GST-tagged proteins were resolved by SDS-PAGE and revealed by autoradiography. GST-Nt TPX2 was used as a positive control for the pull-down of Aurora A (*lane 2*), whereas GST alone served as a negative control (*lane 1*). *C*, smaller pieces of the Aurora catalytic domain expressed by *in vitro* transcription/translation in the presence of [³⁵S]methionine were assessed in a manner similar to that in *B*, using GST-p53 as bait. A *plus sign* indicates an interaction between GST-p53 WT Fl and the Aurora A piece equivalent to or stronger than the one observed with full-length Aurora A (*B*, *lane 3*), whereas a *minus sign* indicates no significant interaction (equivalent to or weaker than with GST alone; *B*, *lane 1*).

interact with the catalytic domain of Aurora A. This result contrasts with reports in human cells that p53-Aurora A interaction occurs within the N-terminal domain containing the Aurora boxes (17, 28). In order to determine directly which domain of *Xenopus* Aurora A interacts with p53, we performed *in vitro* pull-down assays. In these assays, full-length wild-type GST-p53 (GST-p53) was used as bait for several different [³⁵S]methionine-labeled Aurora A constructs: the full-length protein (*Fl*; residues 1–407), the N-terminal domain containing the Aurora boxes (*Nt*; residues 1–136), and the catalytic domain (*Ct*; residues 137–407) (Fig. 4*A*). The N-terminal domain of TPX2 fused to GST (GST-Nt TPX2) was used as a positive control for the pull-down assay (Fig. 4*B*, *lane 2*), whereas GST alone served as a negative control (*lane 1*). We first confirmed our *in vivo* result (Fig. 3) in oocytes that full-length Aurora A interacts with p53 (Fig. 4*B*, *lane 3*). Our results with Aurora A domains clearly demonstrate that *Xenopus* Aurora A interacts with p53 through its catalytic domain and not through its N-terminal domain (Fig. 4*B*, *lane 5 versus lane 4*). To determine more precisely which part of the catalytic domain mediates this interaction, we subjected even smaller pieces to pull-down analysis. Our results show that the site of interaction is a 35-amino acid domain (amino acids 270–305) in the α-helical region surrounding Thr-295 in the T-loop of Aurora A (Fig. 4*C*).

We also carried out reciprocal experiments to define which domain in p53 interacts with Aurora A. A set of constructs was generated that contain the p53 transactivation domain (*TA*), the DNA binding domain (*DNABD*), or the oligomerization domain (*OD*), alone or in combination together (*TADNABD*, *DNABDOD*), and with (+) or without the linker domains (Fig. 5*A*). These constructs were fused to GST and used as bait in pull-down assays with [³⁵S]methionine-labeled Aurora A WT. We found that Aurora A mainly interacts with the transactivation domain of p53 (*TA*; amino acids 1–29), and this interaction is even stronger when the construct includes the adjacent linker domain (TA+; amino acids 1–76) (Fig. 5*B*). Interaction of the p53 transactivation domain with Aurora A is particularly interesting, because

oocytes, endogenous p53 was co-immunoprecipitated (Fig. 3, *lane 5*). This was not the case when the immunoprecipitation was performed with a control anti-mouse IgG (*lane 4*) or in the absence of Myc-Aurora A (*lane 3*). These results indicate that Aurora A and p53 interact in *Xenopus* Meta II-arrested oocytes. We also noticed that when Aurora A was overexpressed, the amount of endogenous p53 was increased in the extract (*Input*, *lane 2 versus lane 1*), confirming that Aurora A may regulate p53 synthesis in *Xenopus* oocytes.

A previous study from our group showed that *Xenopus* p53 inhibits the kinase activity of full-length Aurora A as well as the Aurora A catalytic domain alone (9), suggesting that p53 may
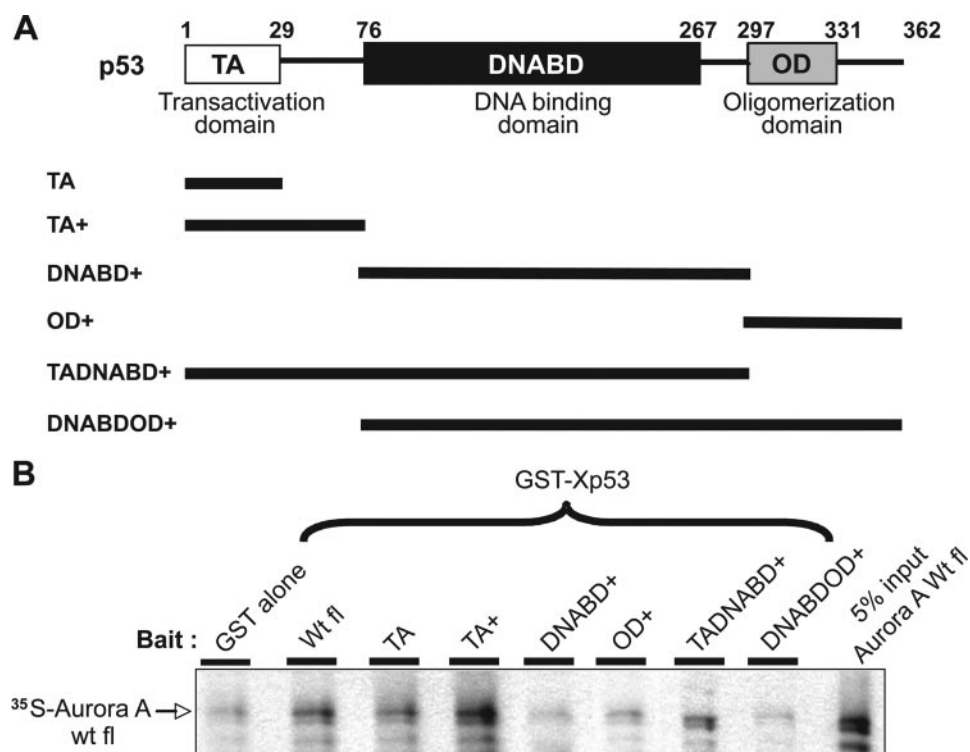
FIGURE 5. **Aurora A interacts with the transactivation domain of p53 *in vitro*.** *A*, GST-p53 constructs were expressed containing the transactivation domain (*TA*), the DNA binding domain (*DNABD*), the oligomerization domain (*OD*), or a combination of these domains (*TADNABD*, *DNABDOD*), with (+) or without the adjacent linker region, as indicated. *B*, these constructs were bacterially expressed, purified on glutathione-agarose beads, and used as bait in *in vitro* pull-down assays in the presence of ^{35}S-labeled Aurora A. ^{35}S-Labeled Aurora A pulled down by the GST-tagged proteins was resolved by SDS-PAGE and revealed by autoradiography.
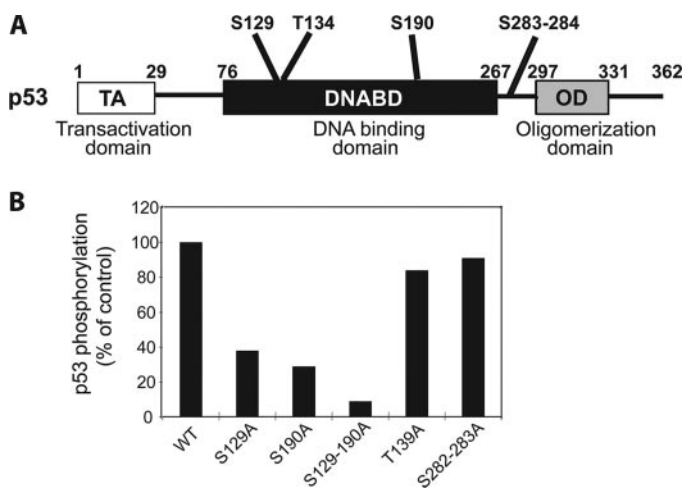

FIGURE 6. **Aurora A phosphorylates p53 on serines 129 and 190 *in vitro*.** *A*, three residues matching the minimum consensus site for Aurora A (R*X*(S/T)) were found in *Xenopus* p53: Ser-129, Thr-134, and Ser-190. Ser-283 and -284 is equivalent to human Ser-313, -314, and -315, where Ser-315 has been reported to be phosphorylated by human Aurora A *in vitro* (28, 29). Serine to alanine mutants were created for all of these putative Aurora A sites. *B*, phosphorylation by Aurora A of the p53 proteins mutated on the sites described in *A* was tested in an *in vitro* kinase assay, as described under "Experimental Procedures." The *graph* represents the percentage phosphorylation of these mutants compared with phosphorylation of wild-type p53 at the same concentration. *TA*, transactivation domain; *DNABD*, DNA binding domain; *OD*, oligomerization domain.

phosphorylation of numerous residues in this domain has been reported to be necessary for p53 transactivation in mammalian cells (39, 40).

Aurora A Phosphorylates p53 in Vitro on Serines 129 and 190— Since the two proteins interact *in vitro* and *in vivo* and Aurora A is necessary for full p53 phosphorylation during oocyte maturation (Figs. 1*C* and 2), we next determined directly whether p53 was an *in vitro* substrate for Aurora A and which residues were targeted. Examination of p53 for any R*X*(S/T) sequences, reported to be the minimum consensus site for phosphorylation by Aurora A (41, 42), revealed three potential sites: Ser-129, Thr-134, and Ser-190 (Fig. 6*A*). We also examined Ser-283 and -284 as a potential site(s) because the equivalent residues in human p53 (Ser-313, -314, and -315) have been reported to be phosphorylated by Aurora A (28, 29). We prepared full-length constructs of p53 bearing a nonphosphorylatable amino acid (alanine) at each of these sites in fusion with GST. *In vitro* kinase assays with Aurora A showed that the T134A and the S283A/S284A mutants were phosphorylated as well as WT p53, indicating that these sites are not phosphorylated by Aurora A *in vitro*. However, phosphorylation of both the S129A and the S190A mutants by Aurora A was reduced by ~75%, and the double mutant S129A/S190A Aurora A was phosphorylated at less than 10% of the level of WT p53 (Fig. 6*B*). These results indicate that serine 129 and serine 190 are the primary sites of p53 phosphorylation by Aurora A *in vitro*, and both sites are in the DNA binding domain.

DISCUSSION

The results in this paper provide new evidence for p53-Aurora A interaction *in vivo* and *in vitro*. It is notable that the areas of interaction are important ones for both proteins: the kinase catalytic domain of Aurora A and the transactivation domain of p53. The fact that the phosphorylation sites for Aurora A on p53 are in the adjacent DNA binding domain of p53 suggests a model in which binding of Aurora A to the transactivation domain facilitates the phosphorylation of nearby residues in the DNA binding domain. It has been established from multiple studies that the ability of Aurora A to transform mammalian cells and to cause centrosome amplification is evident only in cells lacking a functional p53 pathway (2–4). Since p53 inhibits Aurora A activity, it is tempting to speculate that the absence of p53 enhances the transforming activity of Aurora A by removing an inhibitor of its activation.

The data presented here show a strong correlation between Aurora A activity stimulated by TPX2 and up-regulation of p53 levels and phosphorylation. Previous protein purification work

from this laboratory had identified TPX2 as an activator of Aurora A present in Meta II (cytostatic factor-arrested) *Xenopus* egg extracts (11), and the binding site for TPX2 on Aurora A has been well characterized both at the structural level and by mutagenesis (12–14, 43, 44). Our data here show that the level of TPX2 markedly increases during maturation (Fig. 2), and TPX2 undergoes an electrophoretic shift in Meta II oocytes, most likely due to phosphorylation by associated Aurora A, as shown previously (9, 11). The knockdown of TPX2 accumulation by morpholinos resulted in loss of virtually all Aurora A activation as judged both by the electrophoretic mobility of Aurora A and phosphorylation of the T-loop, which is required for Aurora A activity (4, 9, 10, 45). Although other activators of Aurora A have been reported in various cell types (42, 46–48), our data suggest that no other quantitatively important activator of Aurora A is present in Meta II *Xenopus* eggs. The dramatic effects of TPX2 knockdown on p53 accumulation and phosphorylation suggest that it is a specific consequence of a failure by TPX2 to activate Aurora A. This was further confirmed using a chemical inhibitor of Aurora A that did not affect TPX2 accumulation (not shown) but did block increased p53 level and phosphorylation (Figs. 1*D* and 2). On the other hand, the ectopic expression of Aurora A in oocytes resulted in an increased p53 level (Fig. 3). It is interesting that Aurora A affects not only the phosphorylation of p53 but also its accumulation during maturation. This increase seen upon ectopic expression of Aurora A is probably accounted for by increased synthesis of p53, because the half-life of microinjected radiolabeled p53 was unaffected by Aurora A inhibition (Fig. S1). The mechanism of increased synthesis is unclear, but other proteins (*e.g.* c-Mos and cyclin B1) that have been reported to be newly synthesized during maturation are translated only after complex phosphorylation and processing events occurring at the 3′-untranslated region of the mRNA (67). These events involve Maskin and the cytoplasmic element-binding protein, two known substrates of Aurora A (42, 68), and require the presence of a cytoplasmic polyadenylation element in the 3′-untranslated region of the target mRNA (67). Since examination of the p53 gene revealed the presence of a cytoplasmic polyadenylation element motif in its 3′-untranslated region, it seems likely that p53 translation may be regulated by Aurora A during maturation. Further work is necessary to evaluate whether the p53 cytoplasmic polyadenylation element is functional and the precise mechanism of its regulation. Preventing Aurora A activation with TPX2 morpholinos did not have any obvious effect on Meta II arrest or the activation of several other M phase kinases, including Plx1, MAPK, and Cdc2. The possibility cannot be excluded, however, that meiotic spindle assembly was affected by decreased Aurora A activity. Indeed, impairment of Aurora A function by injection of anti-Aurora A antibodies was previously reported to affect spindle rotation and polar body exclusion but not cytoplasmic cell cycle events, like cyclin B2 degradation following GVBD or parthenogenetic activation (49).

The analysis of p53 interaction with Aurora A indicates that p53 binds in a region near the T-loop containing Thr-295. Previous work has suggested that Thr-295 is readily accessible to PP1 in interphase. Upon binding to TPX2 at nuclear envelope breakdown, biochemical and structural studies indicate that the T-loop is shielded from dephosphorylation by PP1 (11, 14). TPX2 binding thus permits net autophosphorylation at Thr-295 and autoactivation of Aurora A, and TPX2 also targets the complex to polar microtubules (12, 13). As reported previously, p53 can inhibit the activity of full-length Aurora A or its catalytic domain *in vitro*, but inhibition is lost if the complex is activated by TPX2 (11). Since p53 binds near the T-loop, the shielding of this area by TPX2 may account for the resistance of the TPX2-Aurora A complex to inhibition by p53.

At this point, the consequences of TPX2-Aurora A-dependent phosphorylation of p53 are unclear. In oocytes, all p53 appears to be cytoplasmic and therefore unlikely to be involved in transcription (30). During the postfertilization cleavage divisions, a small fraction of p53 translocates into the nucleus at each division (31), but since transcription is largely absent from embryos until the midblastula transition (12th cleavage), it is unlikely that p53 is involved in transcription-dependent processes. Instead, in early embryos, it has been suggested to play a role in S phase regulation and in DNA repair, to prevent reinitiation of DNA replication during S phase, and to block premature entry from S phase into mitosis (50). During mitosis or M phase, many transcription factors are inactivated by phosphorylation (51). Since the Aurora A phosphorylation sites are in the DNA binding domain, one could anticipate that p53-dependent transcription would be inhibited during M phase. Indeed, it is interesting to note that Aurora A-mediated phosphorylation of human p53 on Ser-215, the equivalent of *Xenopus* Ser-190, leads to abrogation of both DNA binding and transactivation activity of p53 (29). Thus, a potential function for these phosphorylations during oocyte maturation could be to silence p53 transactivation activity. The conservation of this site and its phosphorylation by Aurora A suggest that it plays an important regulatory role. A major function of p53 in somatic cells is to participate in the DNA damage response, inducing transcription of either cell cycle arrest genes or those promoting apoptosis (19). However, in oocytes or embryos before midblastula transition, the DNA damage response is not evident in response to X-irradiation or DNA double-stranded breaks (52, 53). Therefore, at this stage of development, Aurora A-dependent phosphorylation of p53 is unlikely to regulate the DNA damage response.

Another potential function of Aurora A-p53 interaction is on the centrosome. Both Aurora A and a small fraction of p53 are localized on the centrosome in mammalian cells (22, 54–56). Studies in *Xenopus* XL2 cells have also shown that they are co-localized on the centrosome.[4] There is considerable indirect evidence that p53 and Aurora A are important for centrosome duplication. Overexpression of either human or *Xenopus* Aurora A can transform mammalian cells, but only if p53 function is deficient. In tumors in which Aurora A is overexpressed, centrosome amplification is common and is thought to contribute to chromosomal instability (2, 3, 6, 57). On the other hand, studies of mouse embryo fibroblasts from mice lacking p53 show that even after early passages, centrosome amplification occurs (26, 27). These results suggest that p53 exerts a

---

[4] G. Pascreau and J. Maller, unpublished data.

negative impact on centrosome duplication, although details are lacking. More work is needed to evaluate whether a negative role might be exerted via inhibition of Aurora A. In early embryos, centrosomes duplicate once and only once in each cell cycle and are initially involved in organizing a bipolar spindle at each mitosis. However, in oocytes, where Aurora A-p53 interaction was defined in this paper, spindles form in the absence of centrosomes by fusion of two half-spindles (58, 59). Therefore, the Aurora A-p53 interactions that form during oocyte maturation may be established to support the rapid cell divisions that occur after Meta II oocytes are fertilized and spindle poles become organized by centrosomes. This concept is consistent with evidence that enzymes and proteins needed for DNA synthesis and centrosome duplication after fertilization appear in Meta II, although no DNA synthesis occurs during oocyte maturation (60).

By using antisense morpholinos, we were able to show that new synthesis of TPX2 is required for full synthesis and phosphorylation of p53 during oocyte maturation. Thus, this study provides the first evidence that TPX2 plays an important role in the regulation of p53 via Aurora A. The fact that Aurora A-mediated p53 phosphorylation is blocked efficiently by TPX2 inhibition, whereas other mitotic regulators (*e.g.* Polo-like kinase 1, MAPK, and Cdc2) are not affected is important for the following reasons. First, both Aurora A and TPX2 (initially identified as REPP86 (restrictedly expressed proliferation-associated protein of 86 kDa) are overexpressed in some tumors (61–63). Furthermore, knockdown of TPX2 significantly reduces the survival of multiple human cancer cell lines (64). Second, pharmacological inhibitors targeting the oncogenic kinase Aurora A also target Aurora B in most cases. Therefore, specifically targeting Aurora A by inhibiting TPX2 might be a promising strategy for cancer therapy.

REFERENCES

1. Fu, J., Bian, M., Jiang, Q., and Zhang, C. (2007) *Mol. Cancer Res.* **5,** 1–10
2. Bischoff, J. R., Anderson, L., Zhu, Y., Mossie, K., Ng, L., Souza, B., Schryver, B., Flanagan, P., Clairvoyant, F., Ginther, C., Chan, C. S., Novotny, M., Slamon, D. J., and Plowman, G. D. (1998) *EMBO J.* **17,** 3052–3065
3. Zhou, H., Kuang, J., Zhong, L., Kuo, W. L., Gray, J. W., Sahin, A., Brinkley, B. R., and Sen, S. (1998) *Nat. Genet.* **20,** 189–193
4. Littlepage, L. E., Wu, H., Andresson, T., Deanehan, J. K., Amundadottir, L. T., and Ruderman, J. V. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 15440–15445
5. Meraldi, P., Honda, R., and Nigg, E. A. (2002) *EMBO J.* **21,** 483–492
6. Tatsuka, M., Katayama, H., Ota, T., Tanaka, T., Odashima, S., Suzuki, F., and Terada, Y. (1998) *Cancer Res.* **58,** 4811–4816
7. Ghadimi, B. M., Sackett, D. L., Difilippantonio, M. J., Schrock, E., Neumann, T., Jauho, A., Auer, G., and Ried, T. (2000) *Genes Chromosomes Cancer* **27,** 183–190
8. Pihan, G. A., Purohit, A., Wallace, J., Knecht, H., Woda, B., Quesenberry, P., and Doxsey, S. J. (1998) *Cancer Res.* **58,** 3974–3985
9. Eyers, P. A., and Maller, J. L. (2004) *J. Biol. Chem.* **279,** 9008–9015
10. Ohashi, S., Sakashita, G., Ban, R., Nagasawa, M., Matsuzaki, H., Murata, Y., Taniguchi, H., Shima, H., Furukawa, K., and Urano, T. (2006) *Oncogene* **25,** 7691–7702
11. Eyers, P. A., Erikson, E., Chen, L. G., and Maller, J. L. (2003) *Curr. Biol.* **13,** 691–697
12. Kufer, T. A., Sillje, H. H., Korner, R., Gruss, O. J., Meraldi, P., and Nigg, E. A. (2002) *J. Cell Biol.* **158,** 617–623
13. Tsai, M. Y., Wiese, C., Cao, K., Martin, O., Donovan, P., Ruderman, J., Prigent, C., and Zheng, Y. (2003) *Nat. Cell Biol.* **5,** 242–248
14. Bayliss, R., Sardon, T., Vernos, I., and Conti, E. (2003) *Mol. Cell* **12,** 851–862
15. Katayama, H., Zhou, H., Li, Q., Tatsuka, M., and Sen, S. (2001) *J. Biol. Chem.* **276,** 46219–46224
16. Kiat, L. S., Hui, K. M., and Gopalan, G. (2002) *J. Biol. Chem.* **277,** 45558–45565
17. Chen, S. S., Chang, P. C., Cheng, Y. W., Tang, F. M., and Lin, Y. S. (2002) *EMBO J.* **21,** 4491–4499
18. Lane, D. P. (1992) *Nature* **358,** 15–16
19. Vousden, K. H., and Lu, X. (2002) *Nat. Rev. Cancer* **2,** 594–604
20. Xu, Y. (2008) *Oncogene* **27,** 3501–3507
21. Yang, Y., Li, C. C., and Weissman, A. M. (2004) *Oncogene* **23,** 2096–2106
22. Brown, C. R., Doxsey, S. J., White, E., and Welch, W. J. (1994) *J. Cell. Physiol.* **160,** 47–60
23. Shinmura, K., Bennett, R. A., Tarapore, P., and Fukasawa, K. (2007) *Oncogene* **26,** 2939–2944
24. Tarapore, P., Horn, H. F., Tokuyama, Y., and Fukasawa, K. (2001) *Oncogene* **20,** 3173–3184
25. Jackman, M., Lindon, C., Nigg, E. A., and Pines, J. (2003) *Nat. Cell Biol.* **5,** 143–148
26. Tarapore, P., and Fukasawa, K. (2002) *Oncogene* **21,** 6234–6240
27. Fukasawa, K., Choi, T., Kuriyama, R., Rulong, S., and Vande Woude, G. F. (1996) *Science* **271,** 1744–1747
28. Katayama, H., Sasai, K., Kawai, H., Yuan, Z. M., Bondaruk, J., Suzuki, F., Fujii, S., Arlinghaus, R. B., Czerniak, B. A., and Sen, S. (2004) *Nat. Genet.* **36,** 55–62
29. Liu, Q., Kaneko, S., Yang, L., Feldman, R. I., Nicosia, S. V., Chen, J., and Cheng, J. Q. (2004) *J. Biol. Chem.* **279,** 52175–52182
30. Tchang, F., Gusse, M., Soussi, T., and Mechali, M. (1993) *Dev. Biol.* **159,** 163–172
31. Amariglio, F., Tchang, F., Prioleau, M. N., Soussi, T., Cibert, C., and Mechali, M. (1997) *Oncogene* **15,** 2191–2199
32. Andresson, T., and Ruderman, J. V. (1998) *EMBO J.* **17,** 5627–5637
33. Frank-Vaillant, M., Haccard, O., Thibier, C., Ozon, R., Arlot-Bonnemains, Y., Prigent, C., and Jessus, C. (2000) *J. Cell Sci.* **113,** 1127–1138
34. Yamamoto, T. M., Lewellyn, A. L., and Maller, J. L. (2008) *Mol. Cell. Biol.* **28,** 4196–4203
35. Keady, B. T., Kuo, P., Martinez, S. E., Yuan, L., and Hake, L. E. (2007) *J. Cell Sci.* **120,** 1093–1103
36. Gadea, B. B., and Ruderman, J. V. (2005) *Mol. Biol. Cell* **16,** 1305–1318
37. Harrington, E. A., Bebbington, D., Moore, J., Rasmussen, R. K., Ajose-Adeogun, A. O., Nakayama, T., Graham, J. A., Demur, C., Hercend, T., Diu-Hercend, A., Su, M., Golec, J. M., and Miller, K. M. (2004) *Nat. Med.* **10,** 262–267
38. Hauf, S., Cole, R. W., LaTerra, S., Zimmer, C., Schnapp, G., Walter, R., Heckel, A., van Meel, J., Rieder, C. L., and Peters, J. M. (2003) *J. Cell Biol.* **161,** 281–294
39. Scoumanne, A., Harms, K. L., and Chen, X. (2005) *Cancer Biol. Ther.* **4,** 1178–1185
40. Kaustov, L., Yi, G. S., Ayed, A., Bochkareva, E., Bochkarev, A., and Arrowsmith, C. H. (2006) *Cell Cycle* **5,** 489–494
41. Giet, R., and Prigent, C. (1999) *J. Cell Sci.* **112,** 3591–3601
42. Pascreau, G., Delcros, J. G., Cremet, J. Y., Prigent, C., and Arlot-Bonnemains, Y. (2005) *J. Biol. Chem.* **280,** 13415–13423
43. Anderson, K., Yang, J., Koretke, K., Nurse, K., Calamari, A., Kirkpatrick, R. B., Patrick, D., Silva, D., Tummino, P. J., Copeland, R. A., and Lai, Z. (2007) *Biochemistry* **46,** 10287–10295
44. Bayliss, R., Sardon, T., Ebert, J., Lindner, D., Vernos, I., and Conti, E. (2004) *Cell Cycle* **3,** 404–407
45. Pascreau, G., Delcros, J. G., Morin, N., Prigent, C., and Arlot-Bonnemains, Y. (2008) *Dev. Biol.* **317,** 523–530
46. Hirota, T., Kunitoku, N., Sasayama, T., Marumoto, T., Zhang, D., Nitta, M., Hatakeyama, K., and Saya, H. (2003) *Cell* **114,** 585–598
47. Pugacheva, E. N., Jablonski, S. A., Hartman, T. R., Henske, E. P., and Golemis, E. A. (2007) *Cell* **129,** 1351–1363
48. Satinover, D. L., Leach, C. A., Stukenberg, P. T., and Brautigan, D. L. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 8625–8630
49. Castro, A., Mandart, E., Lorca, T., and Galas, S. (2003) *J. Biol. Chem.* **278,** 2236–2241

50. Tchang, F., and Mechali, M. (1999) *Exp. Cell Res.* **251,** 46–56
51. Holmberg, C. I., Tran, S. E., Eriksson, J. E., and Sistonen, L. (2002) *Trends Biochem. Sci.* **27,** 619–627
52. Anderson, J. A., Lewellyn, A. L., and Maller, J. L. (1997) *Mol. Biol. Cell* **8,** 1195–1206
53. Hensey, C., and Gautier, J. (1997) *Mech. Dev.* **69,** 183–195
54. Blair Zajdel, M. E., and Blair, G. E. (1988) *Oncogene* **2,** 579–584
55. Morris, V. B., Brammall, J., Noble, J., and Reddel, R. (2000) *Exp. Cell Res.* **256,** 122–130
56. Tritarelli, A., Oricchio, E., Ciciarello, M., Mangiacasale, R., Palena, A., Lavia, P., Soddu, S., and Cundari, E. (2004) *Mol. Biol. Cell* **15,** 3751–3757
57. Terada, Y., Tatsuka, M., Suzuki, F., Yasuda, Y., Fujita, S., and Otsu, M. (1998) *EMBO J.* **17,** 667–676
58. Sawin, K. E., and Mitchison, T. J. (1991) *J. Cell Biol.* **112,** 925–940
59. Vernos, I., and Karsenti, E. (1995) *Trends Cell Biol.* **5,** 297–301
60. Rempel, R. E., Sleight, S. B., and Maller, J. L. (1995) *J. Biol. Chem.* **270,** 6843–6855
61. Krams, M., Heidebrecht, H. J., Hero, B., Berthold, F., Harms, D., Parwaresch, R., and Rudolph, P. (2003) *J. Clin. Oncol.* **21,** 1810–1818
62. Ma, Y., Lin, D., Sun, W., Xiao, T., Yuan, J., Han, N., Guo, S., Feng, X., Su, K., Mao, Y., Cheng, S., and Gao, Y. (2006) *Clin. Cancer Res.* **12,** 1121–1127
63. Gautschi, O., Heighway, J., Mack, P. C., Purnell, P. R., Lara, P. N., Jr., and Gandara, D. R. (2008) *Clin. Cancer Res.* **14,** 1639–1648
64. Morgan-Lappe, S. E., Tucker, L. A., Huang, X., Zhang, Q., Sarthy, A. V., Zakula, D., Vernetti, L., Schurdak, M., Wang, J., and Fesik, S. W. (2007) *Cancer Res.* **67,** 4390–4398
65. Eckerdt, F., Eyers, P. A., Lewellyn, A. L., Prigent, C., and Maller, J. L. (2008) *Curr. Biol.* **18,** 519–525
66. Qian, Y. W., Erikson, E., Li, C., and Maller, J. L. (1998) *Mol. Cell. Biol.* **18,** 4262–4271
67. Radford, H. E., Meijer, H. A., and de Moor, C. H. (2008) *Biochim. Biophys. Acta* **1779,** 217–229
68. Mendez, R., Hake, L. E., Andresson, T., Littlepage, L. E., Ruderman, J. V., and Richter, J. D. (2000) *Nature* **404,** 302–307