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Phosphorylation by aurora kinase A induces Mdm2-mediated destabilization and inhibition of p53

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Aurora kinase A (also called STK15 and BTAK) is overexpressed in many human cancers. Ectopic overexpression of aurora kinase A in mammalian cells induces centrosome amplification, chromosome instability and oncogenic transformation, a phenotype characteristic of loss-of-function mutations of p53. Here we show that aurora kinase A phosphorylates p53 at Ser315, leading to its ubiquitination by Mdm2 and proteolysis. p53 is not degraded in the presence of inactive aurora kinase A or ubiquitination-defective Mdm2. Destabilization of p53 by aurora kinase A is abrogated in the presence of mutant Mdm2 that is unable to bind p53 and after repression of Mdm2 by RNA interference. Silencing of aurora kinase A results in less phosphorylation of p53 at Ser315, greater stability of p53 and cell-cycle arrest at G2-M. Cells depleted of aurora kinase A are more sensitive to cisplatin-induced apoptosis, and elevated expression of aurora kinase A abolishes this response. In a sample of bladder tumors with wild-type p53, elevated expression of aurora kinase A was correlated with low p53 concentration. We conclude that aurora kinase A is a key regulatory component of the p53 pathway and that overexpression of aurora kinase A leads to increased degradation of p53, causing downregulation of checkpoint-response pathways and facilitating oncogenic transformation of cells.

Aurora kinase A is a member of a new family of serine/threonine kinases that includes *Drosophila melanogaster* Aurora and *Saccharomyces cerevisiae* Ipl1 kinase, both of which are essential for controlling normal chromosome segregation and centrosome functions[1–3]. Aurora kinase A has been implicated in regulating centrosome function, spindle assembly, spindle maintenance and mitotic commitment in cells[4–7]. *AURKA*, encoding aurora kinase A, is a putative oncogene that is amplified and overexpressed in many human cancers[8–13]. The molecular targets of aurora kinase A have not been well characterized. We previously reported that phosphorylation-mediated feedback between aurora kinase A and protein phosphatase 1 operates through mitosis and that disruption of this interaction results in defects in chromosome segregation[14].

Overexpression of aurora kinase A[8] and loss of wild-type p53 function induce similar phenotypes of centrosome amplification and aneuploidy in cells[15,16]. These observations suggest that gain of aurora kinase A function and loss of wild-type p53 function may be interdependent in common pathways. The finding that human tumors with elevated expression of aurora kinase A have wild-type *TRP53* (encoding p53) also suggests that gain of aurora kinase A function may cause loss of wild-type p53 function, contributing to malignant transformation.

p53 induces growth arrest or apoptosis in cells exposed to stress and is frequently mutated or deleted in human cancers. Expression of p53 is controlled by Mdm2, which promotes ubiquitination by E3 ubiquitin ligase activity and degradation of p53 by the cytoplasmic 26S proteasome[17]. Stability and activity of p53 are also regulated by post-translational modifications[18–23] including phosphorylation, acetylation, glycosylation and attachment of a small ubiquitin-related modifier protein. Phosphorylation at multiple sites is the predominant mechanism known to stabilize and abrogate Mdm2-mediated ubiquitination and activates p53. In contrast, phosphorylation of the core domain at Thr155 by the COP9 signalosome has been reported to target p53 for degradation[24]. The present study investigated whether phosphorylation by aurora kinase A also regulates p53 activity.

RESULTS

Aurora kinase A phosphorylates and interacts with p53

We first investigated the ability of aurora kinase A to phosphorylate p53 in an *in vitro* kinase assay. We incubated bacterially expressed glutathione S-transferase (GST) and a GST-p53 fusion protein with aurora kinase A immunoprecipitated from mitotic HeLa cells and γ^{32}P ATP. The aurora kinase A immunocomplex clearly phosphorylated GST-p53 (**Fig. 1a**). To confirm the specificity of aurora kinase A in phosphorylating p53, we used immunoprecipitated wild-type and kinase-inactive aurora kinase A (K162R) in an *in vitro* kinase assay with GST-p53. Wild-type aurora kinase A phosphorylated p53 but the kinase-inactive mutant did not (**Fig. 1b**), confirming that aurora

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kinase A specifically phosphorylates p53 *in vitro*. We examined the pattern of phosphorylation of p53 by aurora kinase A *in vitro* by phosphorylated-amino-acid and two-dimensional phosphopetide analysis of ^{32}P-labeled GST-p53 after digestion with N-tosyl-L-phenylalanine-chloromethyl ketone–trypsin[25]. We detected radioactive signal at the phosphoserine marker, indicating that aurora kinase A phosphorylates p53 at a serine residue (**Fig. 1c**). Two-dimensional phosphopeptide analysis showed a phosphorylated peptide in the second dimension (**Fig. 1d**). Edman degradation[26] showed that the highest radioactivity was released after the ninth cycle (**Fig. 1e**), corresponding to the serine at position 315 of p53. An *in vitro* kinase assay using GST-p53 showed that wild-type p53, but not S315A mutant p53, was phosphorylated by aurora kinase A (**Fig. 1f**).

To investigate phosphorylation of Ser315 by aurora kinase A *in vivo*, we expressed wild-type and S315A mutant p53 with either constitutively activated (T288D) or kinase-inactive mutants of aurora kinase A in HEK293 cells. We detected a strong signal with an antibody to p53 phosphorylated at Ser315 in cells expressing the constitutively activated aurora kinase A and weaker signals in the cells expressing the kinase-inactive control, probably due to endogenous aurora kinase A in these cells. We detected no signal in cells expressing S315A mutant p53, confirming that aurora kinase A phosphorylates p53 at Ser315 *in vivo* (**Fig. 1g**).

To determine whether the interaction between aurora kinase A and p53 occurs *in vitro*, we incubated ^{35}S-labeled *in vitro*–translated p53 constructs expressing either GST-tagged full-length aurora kinase A or

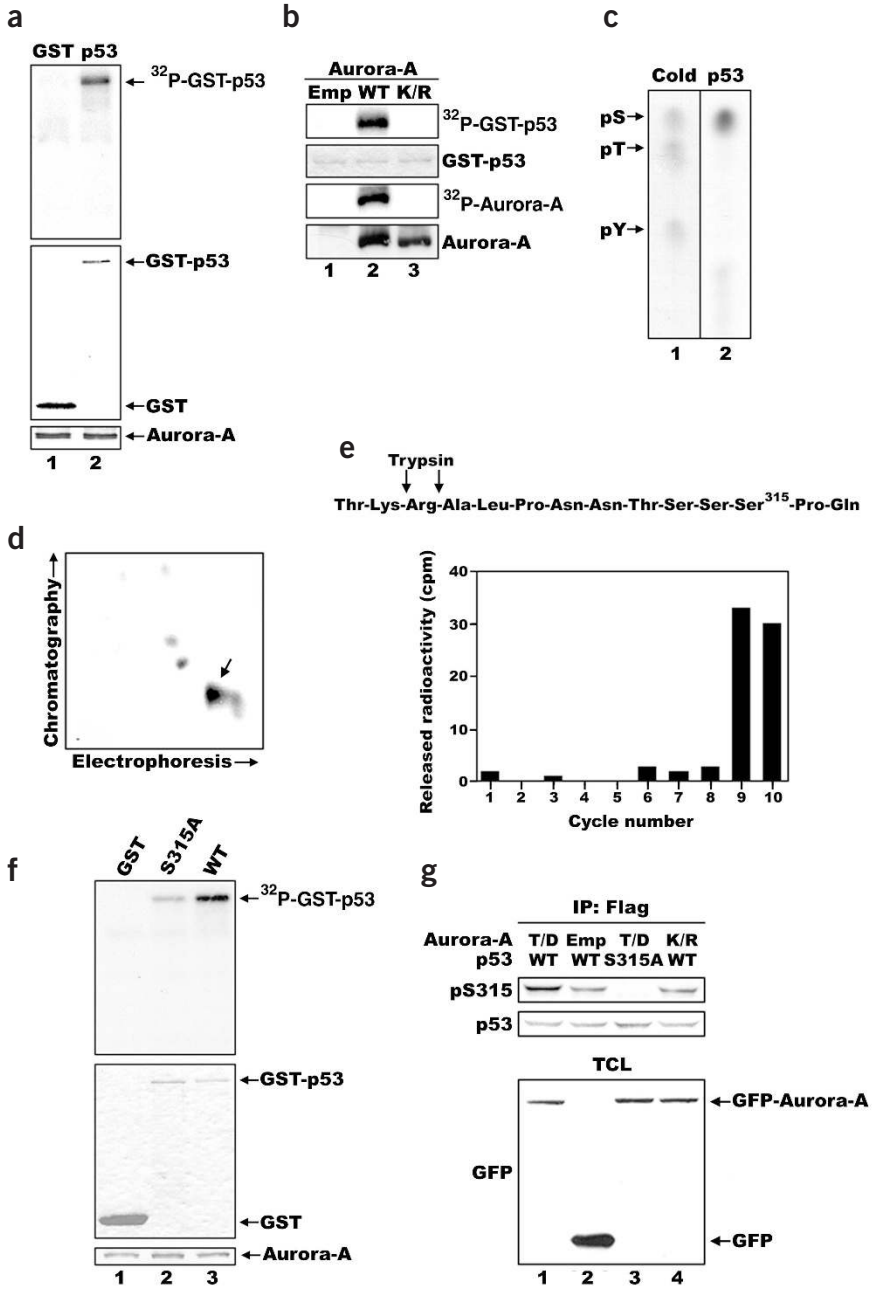

Figure 1 Aurora kinase A phosphorylates p53 at Ser315. (**a**) Immunoprecipitates with antibody to aurora kinase A from nocodazole-treated mitotic HeLa cells were incubated with either GST (lane 1) or GST-p53 (lane 2) in the presence of [γ^{32}P]ATP. GST proteins were resolved by SDS-PAGE and visualized by autoradiography (for kinase assay, top) or Coomassie blue staining (middle). Immunoprecipitates were detected with antibody to aurora kinase A (bottom). (**b**) Immunoprecipitates with antibody to Flag M2 from nocodazole-treated mitotic HeLa cells transfected with empty vector (Emp; lane 1), Flag–wild-type aurora kinase A (WT; lane 2) or Flag–aurora kinase A K162R (lane 3) were incubated with GST-p53 in the presence of [γ^{32}P]ATP. GST-p53 was resolved and visualized as in **a** (first and second rows). Immunoprecipitates were immunoblotted with antibody to Flag M2 (fourth row). Autophosphorylation of Flag–aurora kinase A was visualized by autoradiography (third row). (**c**) Trypsin-digested and HCl-treated ^{32}P-labeled GST-p53 purified by SDS-PAGE was resolved by thin layer chromatography together with phosphorylated amino acid marker. Cold indicates the position of phosphorylated amino acid markers (pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine). (**d**) Trypsin-digested ^{32}P-labeled peptides as in **c** were subjected to two-dimensional phosphopeptide mapping. Arrow indicates the major phosphopeptide. (**e**) Amino acid sequence including and surrounding Ser315 showing N-terminal trypsin cleavage sites (top). Phosphopeptide indicated by arrow was extracted from the thin-layer-chromatography plate and subjected to Edman degradation. Released radioactivity was monitored at each cycle (shown as bar graphs). (**f**) GST, GST–p53 S315A and GST–wild-type p53 (WT) were incubated with aurora kinase A immunoprecipitates and [γ^{32}P]ATP as in **a**. Proteins were resolved and visualized as in **a**. (**g**) Flag–wild-type p53 (WT; lanes 1, 2 and 4) or Flag–p53 S315A (lane 3) were cotransfected with GFP–aurora kinase A T288D (T/D; lanes 1 and 3), GFP-empty vector (Emp; lane 2) or GFP–aurora kinase A K162R (K/R; lane 4) into HEK293 cells. Twenty-four hours after transfection, p53 was immunoprecipitated (IP) with antibody to Flag M2 and immunoprecipitates were immunoblotted with antibody to p53 phosphorylated at Ser315 and antibody to p53 (top and middle). Aliquots of the same total cell lysate (TCL) were directly immunoblotted with antibody to GFP (bottom).

the control GST alone. Aurora kinase A showed specific binding to p53 (**Fig. 2a**). We also carried out a reverse experiment in which we incubated GST-tagged p53 constructs representing the N-terminal domain (amino acids 1–112), the central DNA binding domain (amino acids 93–290) and the C-terminal domain (amino acids 291–393) with ^{35}S-labeled *in vitro*–translated full-length aurora kinase A. Aurora kinase A bound to the C-terminal domain of p53 (residues 291–393; **Fig. 2b**). To map the aurora kinase A domains involved in this interaction, we tested the *in vitro* binding of GST-tagged deletion constructs of aurora kinase A with ^{35}S-labeled *in vitro*–translated full-length p53. The C-terminal end of aurora kinase A (residues 312–403) showed no binding, but the rest of the aurora kinase A peptide, including the N-terminal end, did bind p53 (**Supplementary Fig. 1** online). The aurora kinase A box, contrary to a previous report[27], was not essential for this interaction.

We verified *in vivo* binding of endogenous aurora kinase A and p53 in Cos1 cells by coimmunoprecipitation and immunoblotting experiments (**Fig. 2c**). We verified the specificity of this coimmunoprecipitation in p53-deficient Saos-2 cells, in which aurora kinase A did not precipitate with antibody to p53 (**Supplementary Fig. 1** online). Coimmunoprecipitation experiments in MCF7 and HEK293 cells with wild-type p53 and in SW480 cells with mutant p53 (R273H) showed that aurora kinase A interacts with both wild-type and mutant p53 under physiological conditions in human cells (**Fig. 2d**). We evaluated the role of conformational changes to p53 in influencing the interaction between p53 and aurora kinase A *in vivo* in human cells with different p53 mutants. Coimmunoprecipitation experiments showed that p53 conformational mutants R175H and R249S had substantially less or no interaction with aurora kinase A (**Supplementary Fig. 1** online), suggesting that conformation rather than functional inactivation is important for interaction of p53 with aurora kinase A. We carried out *in vivo* binding assays in p53-deficient H1299 cells cotransfected with wild-type or kinase-inactive aurora kinase A together with wild-type or S315A mutant p53. These assays showed that p53 could interact with both the wild-type and kinase-inactive forms of aurora kinase A with equal efficiency and that this interaction was not influenced by phosphorylation of p53 at Ser315 (**Fig. 2e**).

In vivo phosphorylation of p53 at Ser315 was previously mapped, but the biological relevance of this modification is controversial. Phosphorylation of p53 at Ser315 both enhances sequence-specific DNA-binding activity *in vitro*[28] and reverses the stabilizing and activating effects of Ser392 phosphorylation on tetramer formation[29]. Because p53-mediated regulation of checkpoint response is activated when p53 is stabilized at both G1-S and G2-M transitions of the cell cycle[30], we investigated whether interaction between p53 and aurora kinase A and phosphorylation of p53 at Ser315 also varies in a cell cycle–dependent manner. In U2-OS cells, with wild-type p53, the steady-state level of aurora kinase A progressively increased from G1-S

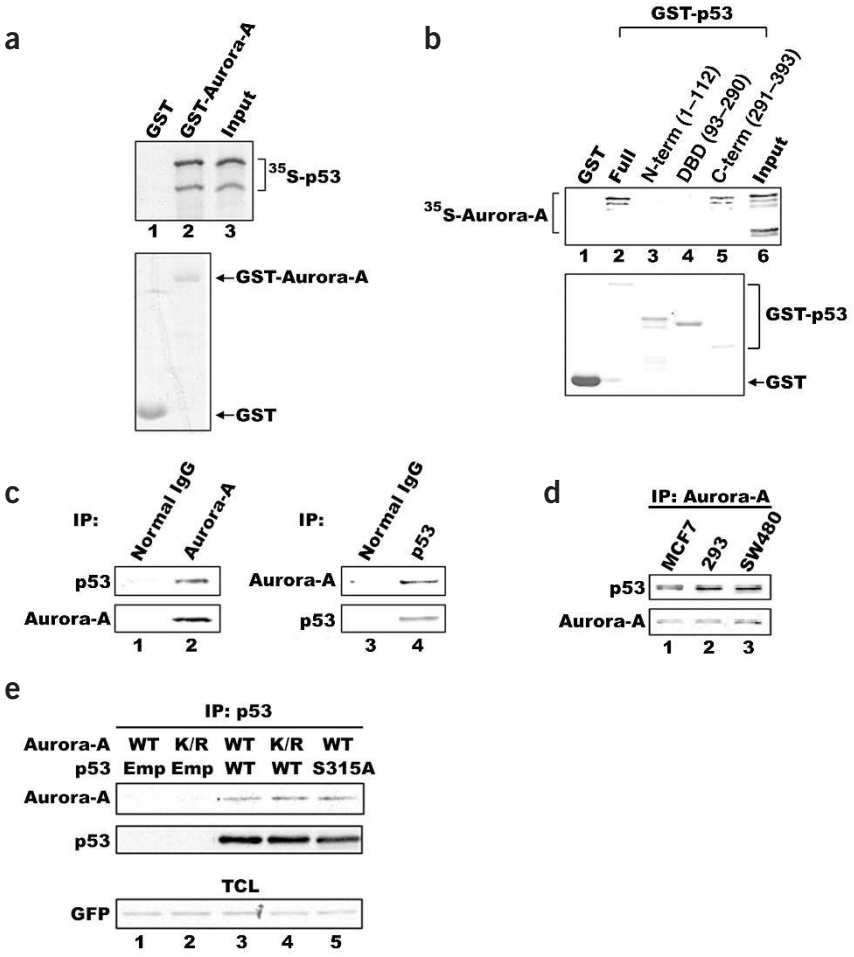

Figure 2 Aurora kinase A interacts with p53. (**a**) ^{35}S-labeled, *in vitro*–translated p53 was incubated with the beads bound either with GST or with GST–aurora kinase A. After binding, the beads were resolved by SDS-PAGE and visualized by autoradiography (for binding, top) or Coomassie blue staining (bottom). (**b**) ^{35}S-labeled, *in vitro*–translated aurora kinase A was incubated with the beads bound with GST, with GST–full length p53 (Full) or with a series of GST-p53 partial peptides (N-terminal (N-term), amino acids 1–112; DBD (DNA binding domain), amino acids 93–290; C-terminal (C-term), amino acids 291–393) followed by analysis as in **a**. (**c**) Cos1 cells were immunoprecipitated (IP) with normal IgG (lane 1) or with antibody to aurora kinase A (lane 2). Immunoprecipitates were immunoblotted with antibody to p53 and antibody to aurora kinase A (left). Right panel shows reciprocal experiment. Immunoprecipitates with normal IgG (lane 3) or with antibody to p53 (lane 4) were immunoblotted with antibody to aurora kinase A and antibody to p53. (**d**) MCF7 (lane 1), 293 (lane 2) and SW480 (lane 3) cells were immunoprecipitated (IP) with antibody to aurora kinase A and immunoprecipitates were immunoblotted with indicated antibodies. (**e**) H1299 cells were either transfected with GFP–wild-type aurora kinase A (WT; lane 1), with GFP–aurora kinase A K162R (K/R; lane 2) or with GFP–wild-type aurora kinase A and wild-type p53 (lane 3), with GFP–aurora kinase A K162R and wild-type p53 (lane 4) or with GFP–wild-type aurora kinase A and p53 S315A (lane 5). Twenty-four hours after transfection, p53 was immunoprecipitated with antibody to p53 and immunoprecipitates were immunoblotted with indicated antibodies (top and middle). Aliquots of the same total cell lysates (TCL) were directly immunoblotted with antibody to GFP (bottom). Emp, empty vector.

through G2-M phase, whereas the level of p53-bound aurora kinase A was comparable throughout the cell cycle. p53 phosphorylated at Ser315 was detectable throughout the cell cycle, but the relative amount of this species increased in S phase and during G2-M phase (**Supplementary Fig. 2** online).

Phosphorylation by aurora kinase A destabilizes p53

Because gain of function of aurora kinase A through overexpression gives rise to cellular phenotypes similar to those seen with loss of function of p53, we hypothesized that phosphorylation of p53 at Ser315 by aurora kinase A inactivates p53 by enhancing its proteolytic degradation. To address the issue, we cotransfected H1299 cells with increasing ratios of aurora kinase A to p53 in independent experiments. Increasing expression of aurora kinase A led to declining steady-state levels of p53, coinciding with less cyclin-dependent kinase inhibitor p21 (**Fig. 3a**). Overexpression of aurora kinase A did not have a similar effect on the level of endogenous and ectopically expressed p21 in the absence of p53 (**Supplementary Fig. 3** online), suggesting that destabilization of p53 after overexpression of aurora kinase A affects downstream p53-transactivated effectors, such as p21. The levels of p27, also regulated by ubiquitin-proteasome degradation system[31], and a control GFP did not change in these experiments, indicating that aurora kinase A–mediated destabilization of p53 is not a nonspecific phenomenon. We assayed the effect of aurora kinase

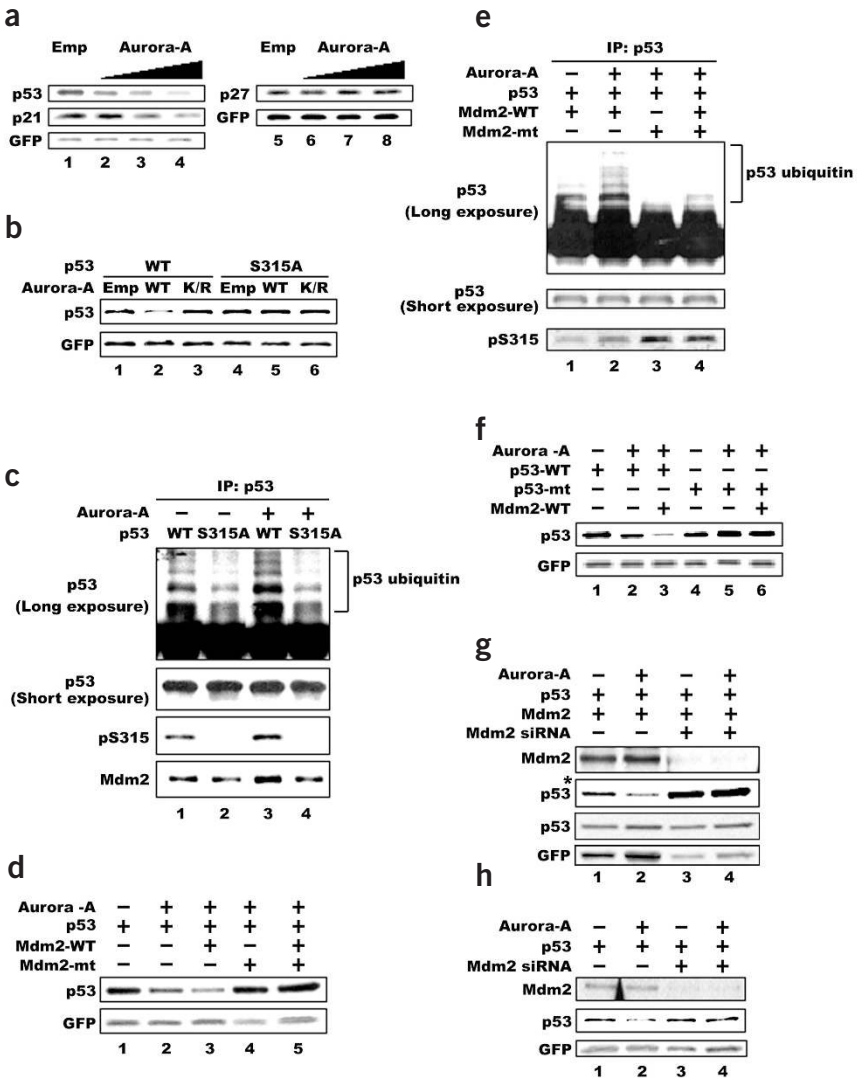

Figure 3 Phosphorylation by aurora kinase A destabilizes p53. (**a**) p53 or p27 was cotransfected with empty vector (Emp) and GFP (lane 1) or with different amounts of aurora kinase A and GFP into H1299 cells (lane 2–4). Twenty-four hours after transfection, aliquots of the same total cell lysates were immunoblotted with the indicated antibodies. Similar GFP expression indicates comparable transfection efficiency (bottom). (**b**) Wild-type p53 (WT; lanes 1–3) or p53 S315A (lane 4–6) was cotransfected with empty vector (Emp; lanes 1 and 4), with wild-type aurora kinase A (WT; lanes 2 and 5) or with aurora kinase A K162R (K/R; lanes 3 and 6) into H1299 cells and analyzed as in **a**. (**c**) Mdm2 was cotransfected with wild-type p53 (WT; lane 1), with p53 S315A (lane 2), with wild-type aurora kinase A and wild-type p53 (lane 3) or with wild-type aurora kinase A and p53 S315A (lane 4) into H1299 cells, and cells were treated with protease inhibitor N-acetyl-Leu-Leu-Norleucinal for 6 h before harvest. Twenty-four hours after transfection, p53 was immunoprecipitated (IP) with antibody to p53 and then immunoblotted with antibody to p53. The same membrane was stripped and reprobed with indicated antibodies. pS315, p53 phosphorylated at Ser315. Short exposure shows the unubiquitinated single band of p53. Long exposure of the same membrane shows multiple ubiquitinated bands of p53 in addition to the single unubiquitinated band seen after short exposure. (**d**) p53 was cotransfected with empty vector (lane 1), with aurora kinase A (lane 2), with aurora kinase A and wild-type Mdm2 (WT; lane 3), with aurora kinase A and a ubiquitination-defective Mdm2 mutant (mt; lane 4) or with aurora kinase A, wild-type Mdm2 and mutant Mdm2 (lane 5) into H1299 cells and analyzed as in **a**. (**e**) p53 was cotransfected with wild-type Mdm2 (WT; lane 1), with aurora kinase A and wild-type Mdm2 (lane 2), with aurora kinase A and mutant Mdm2 (mt; lane 3) or with aurora kinase A, wild-type Mdm2 and mutant Mdm2 (lane 4) into H1299 cells and analyzed as in **c**. IP, immunoprecipitation. Short exposure shows the unubiquitinated single band of p53. Long exposure of the same membrane shows multiple ubiquitinated bands of p53 in addition to the single unubiquitinated band seen after short exposure. (**f**) Wild-type p53 (WT; lanes 1–3) or a p53 mutant that does not bind Mdm2 (mt; lanes 4–6) was cotransfected with empty vector (lanes 1 and 4), with aurora kinase A (lanes 2 and 5) or with aurora kinase A and wild-type Mdm2 (lanes 3 and 6) and analyzed as in **a**. (**g**) H1299 cells transfected with control siRNA duplex (GL2; lanes 1 and 2) or siRNA duplex targeting Mdm2 (lanes 3 and 4). Twenty-four hours after transfection, p53 and Mdm2 were cotransfected with either empty vector (lanes 1 and 3) or with aurora kinase A (lanes 2 and 4) for 24 h and cells were immunoblotted with indicated antibodies. p53* (second row) shows the normalized amount of p53 in the four samples. Normalization was done after densitometric analyses of p53 (third row) and GFP (fourth row) band intensities in each lane, using Scion Image and NIH Image software. (**h**) H1299 cells transfected with control siRNA duplex (GL2; lanes 1 and 2) or Mdm2 siRNA duplex (lanes 3 and 4) for 24 h were subjected to cotransfection with p53 (lanes 1 and 3) or with p53 and aurora kinase A together (lanes 2 and 4). After 24 h, cells were analyzed as in **a**.

A–mediated phosphorylation on the stability of p53 by expressing wild-type or S315A variants of p53 with wild-type or kinase-inactive mutants of aurora kinase A in H1299 cells. Only wild-type p53 was destabilized in the cells expressing wild-type aurora kinase A, suggesting that Ser315 phosphorylation by aurora kinase A facilitates degradation of p53 (**Fig. 3b**).

Because degradation of p53 predominantly involves Mdm2-mediated ubiquitination, we assayed ubiquitination of p53 in H1299 cells cotransfected with Mdm2 and either empty vector or vector expressing aurora kinase A in combination with wild-type or S315A mutant p53. In cells expressing wild-type p53, we observed substantially more ubiquitination in the presence of aurora kinase A than in presence of the empty vector. We observed moderate ubiquitination in cells transfected with the empty vector, probably as a result of phosphorylation of p53 by endogenous aurora kinase A. We observed less ubiquitination of p53 in cells expressing the S315A mutant p53 (**Fig. 3c**). The difference in ubiquitination was not due to differences in amount of p53, as comparable levels of p53 were immunoprecipitated from all four transfections. We also noted greater binding of Mdm2 to highly ubiquitinated p53 in cells expressing aurora kinase A (**Fig. 3c**). Consistent with the levels of ubiquitinated p53, bound Mdm2 was more abundant in the cells transfected with empty vector and expressing wild-type p53 than in those expressing S315A mutant p53, possibly owing to phosphorylation of wild-type p53 by endogenous aurora kinase A.

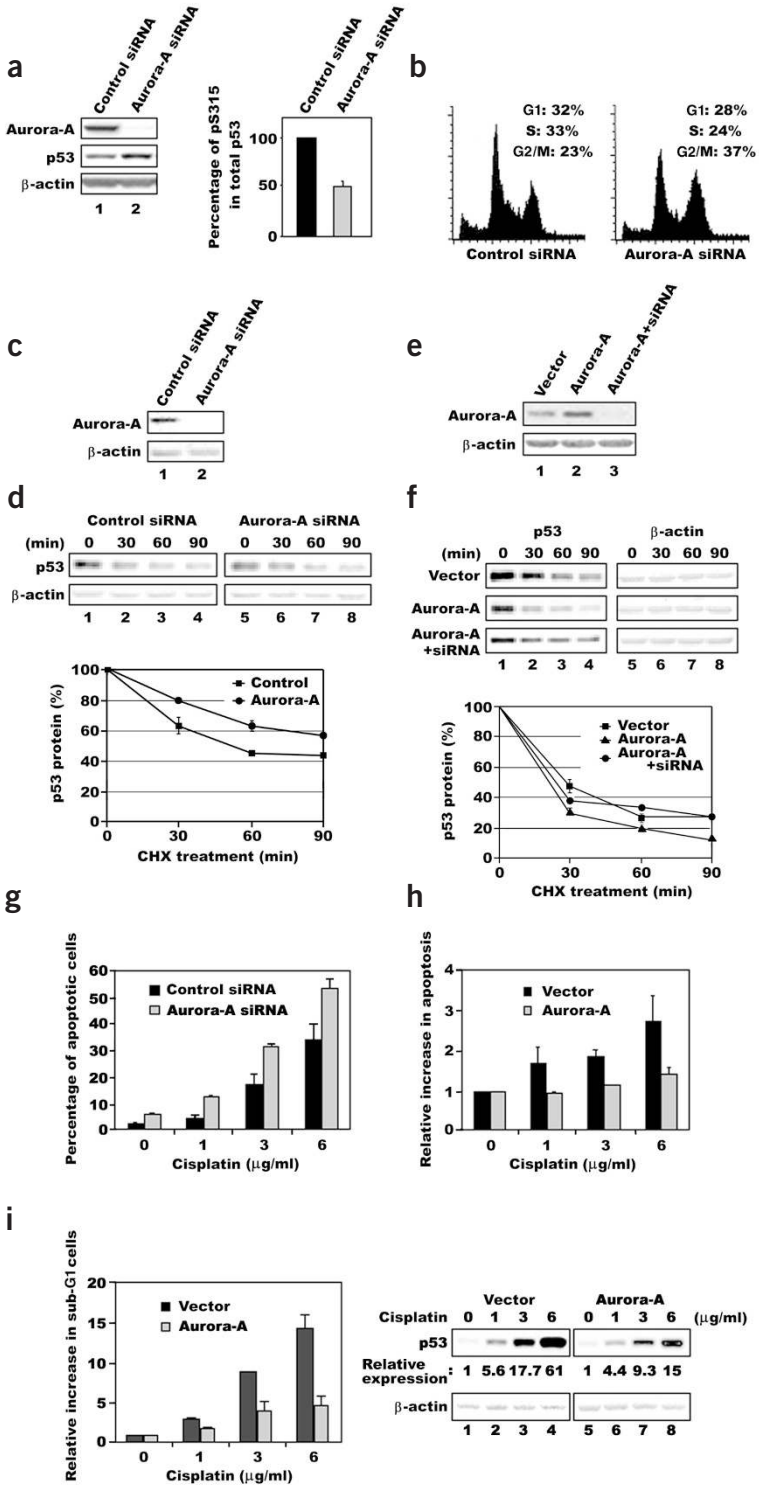

Figure 4 Effect of aurora kinase A expression on p53 stability and function *in vivo*. (**a**) Lysates from U2-OS cells transfected with control siRNA duplex (GL2; lane 1) or siRNA duplex targeting aurora kinase A (lane 2) for 48 h were immunoblotted with indicated antibodies. The same lysates were immunoprecipitated with antibody to p53 and immunoblotted with the indicated antibodies. The relative ratio of p53 phosphorylated at Ser315 (pS315) to total immunoprecipitated p53 was then estimated. (**b**) Cell cycle in **a** was analyzed by FACS. (**c**) Lysates from MCF7 cells transfected with control siRNA duplex (GL2; lane 1) or siRNA duplex targeting aurora kinase A (lane 2) for 72 h were analyzed as in **a**. (**d**) Lysates from MCF7 cells transfected with siRNA for 72 h as in **c** were collected at the indicated time points after addition of cycloheximide (CHX) and subjected to immunoblotting with the indicated antibodies. The amount of p53 was quantified by densitometry and is shown relative to the amount of p53 expressed in absence of cycloheximide. (**e**) Lysates from a stable clone expressing vector (lane 1), a stable clone expressing aurora kinase A (lane 2) and a stable clone expressing aurora kinase A treated with siRNA duplex (lane 3) were analyzed as in **a**. (**f**) Cells in **e** were treated with cycloheximide (CHX) and analyzed as in **d**. (**g**) MCF7 cells transfected with siRNA duplex for 48 h were treated with the indicated concentrations of cisplatin, and 24 h later the number of apoptotic cells was counted. (**h**) MCF7 cells were transfected with vector and GFP together or aurora kinase A and GFP together for 48 h, then treated with cisplatin and analyzed as in **g**. (**i**) Stable clones expressing vector and aurora kinase A were treated with cisplatin for 24 h and the sub-G1 cell population was analyzed by FACS. Expression levels of p53 in the same cells was analyzed as in **a**. The amount of p53 in the control and the cisplatin-treated cells was quantified to assess the relative p53 expression in the cisplatin-treated cells.

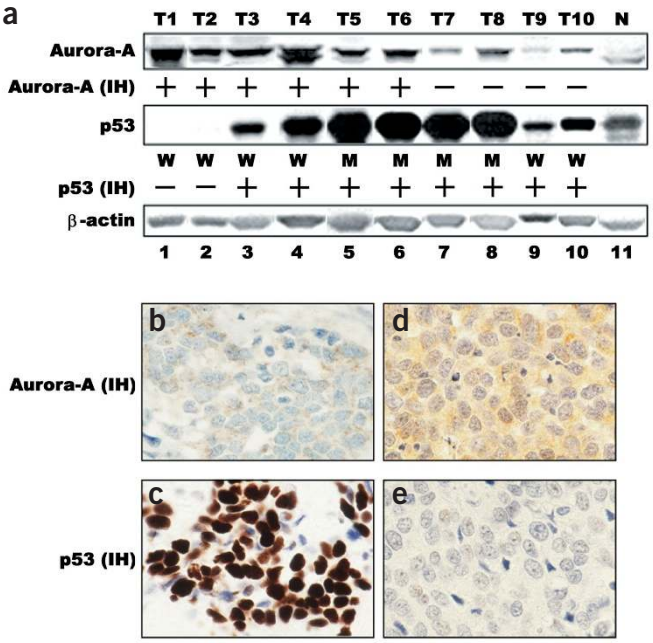

Figure 5 Steady-state level of aurora kinase A and p53 in human bladder tumor samples. (**a**) Expression levels of aurora kinase A (top), p53 (middle) and β-actin (bottom) in bladder tumor tissues (T1–T10; lanes 1–10) and in a normal bladder epithelial cells (N; lane 11). Intensity of immunohistochemical (IH) staining of each tumor specimen compared with normal tissue is represented as follows: –, no detectable expression or similar expression; +, higher expression. W, wild-type p53; M, mutant p53. (**b–e**) Immunohistochemical (IH) staining of tumor specimens with antibody to aurora kinase A (**b**,**d**) and antibody to p53 (**c**,**e**). **b** and **c** represent the same sample, and **d** and **e** represent the same sample.

To further investigate the role of Mdm2 in destabilization of p53 phosphorylated by aurora kinase A, we cotransfected H1299 cells with either mutant Mdm2 (C464A) lacking ubiquitination activity[32] or wild-type Mdm2 and aurora kinase A. The level of p53 was low in cells transfected with wild-type Mdm2 but unaltered in cells transfected with mutant Mdm2 compared with control cells not transfected with Mdm2 (**Fig. 3d**). Mutant Mdm2 also had a dominant negative effect of stabilizing p53 in the presence of aurora kinase A.

We further documented the role of aurora kinase A in Mdm2-mediated ubiquitination of p53 using ubiquitination assays on cells transfected with the same combinations of expression constructs described above (**Fig. 3e**). Ubiquitinated p53 in cells expressing aurora kinase A, p53 and wild-type Mdm2 was much more abundant than in cells expressing mutant Mdm2. As expected, ubiquitination of p53 was minimal in the absence of aurora kinase A and was undetectable in cells expressing mutant Mdm2. Mutant p53 (L22Q and W23S) that cannot interact with Mdm2 (ref. 33) was also not destabilized when expressed with aurora kinase A and Mdm2 (**Fig. 3f**). Silencing of Mdm2 with small interfering RNA (siRNA) in cells with transfected (**Fig. 3g**) and endogenous (**Fig. 3h**) Mdm2 inhibited destabilization of p53 in the presence of aurora kinase A. Absence of a perceptible increase in the amount of p53 after silencing of Mdm2 by siRNA (**Fig. 3h**), which seems to conflict with the existing model of p53 regulation, may reflect the fact that proliferating cells can tolerate only limited amount of p53. These results, taken together, indicate that Mdm2-mediated ubiquitination has a central role in the destabilization of p53 induced by aurora kinase A.

Phosphorylation of p53 at Ser315 affects cell cycle

Because p53 is a crucial regulator of cell cycle progression and DNA damage–induced checkpoint response in mammalian cells[30], we investigated whether inhibition of physiological levels of aurora kinase A with siRNA affects stability of p53 and, consequently, p53-regulated pathways in U2-OS and MCF7 cells with wild-type p53. Treatment of U2-OS cells for 48 h with aurora kinase A siRNA resulted in substantial depletion of aurora kinase A, which was directly correlated with an increase in the steady-state level of p53 and inversely correlated with the amount of p53 phosphorylated at Ser315 (**Fig. 4a**). These cells also had a larger G2-M population of 37%, compared with 23% in cells treated with control siRNA (**Fig. 4b**). This growth arrest or delay in the G2-M phase after depletion of aurora kinase A could be due to stabilization of p53. The lack of a similar growth-inhibitory effect in p53-deficient H1299 and Saos-2 cells depleted of aurora kinase A (data not shown) supports this conclusion.

We then assessed the stability of p53 in MCF7 parental cells and in MCF7 cells stably transfected with aurora kinase A with and without silencing of aurora kinase A by exposure to cycloheximide, a translational inhibitor, in time-course experiments. Treatment with aurora kinase A siRNA for 72 h almost completely inhibited expression of aurora kinase A (**Fig. 4c**,**e**). We estimated the relative amount of p53 in cells exposed to cycloheximide for 30, 60 and 90 min and found consistently higher levels of p53 in cells treated with aurora kinase A siRNA than in cells treated with a control siRNA. Treatment with cycloheximide for 90 min decreased p53 level by almost 60% in the control cells but only by ~40% in cells with silenced aurora kinase A (**Fig. 4d**), indicating that the degradation rate of p53 was greater in cells expressing aurora kinase A. We observed a similar phenomenon in cells overexpressing aurora kinase A: silencing of aurora kinase A in these cells stabilized p53 to almost the same extent as seen in control cells (**Fig. 4f**). These results suggest that expression of aurora kinase A at physiological levels *in vivo* mediates degradation of p53 and that this effect is enhanced in cells overexpressing aurora kinase A, possibly causing abrogation of p53-mediated checkpoint response pathways.

Aurora kinase A affects p53-mediated apoptosis

We investigated whether aurora kinase A affects p53-induced apoptosis in response to cisplatin-induced DNA damage in MCF7 cells. Silencing of aurora kinase A with siRNA substantially increased the incidence of apoptosis in these cells. Treatment with 6 μg ml^{-1} cisplatin increased the incidence of apoptosis by ~20% in cells with silenced aurora kinase A over that observed in cells transfected with a control siRNA (**Fig. 4g**). The lack of a similar increase in apoptotic response in p53-deficient H1299 cells treated with cisplatin (data not shown) indicated that the apoptotic response in MCF7 cells treated with cisplatin was a reflection of the effect of aurora kinase A on stability of p53. Consistent with its role as an inhibitor of p53 function, overexpression of aurora kinase A in transiently transfected cells inhibited p53-induced apoptotic response after treatment with cisplatin (**Fig. 4h**). Based on the number of sub-G1 DNA-harboring cells generated, cells stably transfected with aurora kinase A were also resistant to cisplatin-induced apoptosis. Substantially less induction of p53 in these cells corroborated the role of aurora kinase A as an inhibitor of p53-induced apoptotic response in DNA-damaged cells (**Fig. 4i**). The apoptotic response was not mediated through the two p53 homologous family members p63 and p73, as cotransfection with increasing amounts of aurora kinase A had no effect on the stability of p63γ and p73α isoforms, implicated in p53 like functions, in untreated (**Supplementary Fig. 3** online) and cisplatin-treated cells (data not shown).

Overexpression of aurora kinase A and low p53 levels

In agreement with the experimental data, we detected a trend between overexpression of aurora kinase A and reduced expression of p53 in human bladder tumors (**Fig. 5**), which frequently overexpress aurora kinase A, according to a recent report[13]. Of 23 tumors analyzed, 18 had no mutations of p53. Of these 18, 15 showed high expression of aurora kinase A, and 10 of these had reduced expression of p53.

DISCUSSION

Our findings indicate that aurora kinase A interacts with and phosphorylates p53 at Ser315, facilitating Mdm2-mediated ubiquitination and destabilization of p53. In view of this finding and the observed growth arrest of cells at G2-M phase after silencing of aurora kinase A, it is logical to suggest that degradation of p53 phosphorylated at Ser315 has physiological relevance related to allowing progression of cells through the normal cell proliferation cycle. Identification of aurora kinase A as an *in vivo* kinase for Ser315 does not rule out possible involvement of other S- and G2-phase cyclin-dependent kinases in the process, as precedents for multiple distinct kinases targeting the same phosphorylation site of p53 (ATM and ATR for Ser15; CK2 and PKR for Ser392) have been reported[34–36].

Phosphorylation of Ser315 has been implicated in enhancing sequence-specific DNA-binding affinity and transactivation function of p53. Total phosphorylation profile, rather than phosphorylation of a single residue, may determine the functional status of p53 at any given stage of the cell cycle. Indeed, the fact that phosphorylation of Ser315 along with Ser33 and Thr81 leads to binding of p53 to prolyl isomerase Pin 1 (refs. 37,38), which stimulates transactivation function and stabilization of p53, indicates that phosphorylation of Ser315 can direct the functional status of p53 to either stabilization or degradation. Selectivity towards a particular response may depend on the total profile of post-translational modifications on p53 and the resultant recruitment of additional proteins required for such a response.

The mechanism by which phosphorylation by aurora kinase A enhances Mdm2-mediated ubiquitination of p53 is not known at this time. Greater binding of Mdm2 to phosphorylated p53 undergoing ubiquitination suggests that p53 phosphorylated by aurora kinase A has greater binding affinity for Mdm2. Ser315 phosphorylation may help oligomerization of p53, which is required for Mdm2-mediated ubiquitination[39]. The ability of Mdm2 to degrade p53 requires shuttling between the nucleus and the cytoplasm[40,41], and inhibition of p53 nuclear export leads to accumulation of ubiquitinated p53 in the nucleus[32]. Localization of aurora kinase A in both nucleus and cytoplasm[42–44] makes it a credible catalyst for p53 ubiquitination.

A recent study using an ectopically expressed transactivation- and oligomerization-defective p53 deletion mutant found that oncogenic activity of aurora kinase A is suppressed by p53 in a transactivation-independent manner[27]. This observation, however, is inconsistent with our model of aurora kinase A function. Because the study cited used a transactivation- and oligomerization-defective p53 deletion mutant for functional assays, the physiological relevance of the reported finding needs to be investigated further.

The most important tumor-suppressor function of p53 involves its ability to induce apoptosis; thus, a negative regulator of p53, such as aurora kinase A, must have a crucial role in tumorigenic transformation of cells. This function of aurora kinase A may explain why overexpression causes oncogenic transformation in mammalian cells and why amplification or overexpression of aurora kinase A is commonly detected in human cancers[8–13]. Notably, our findings also provide a mechanistic explanation for the recently reported role of aurora kinase A as a tumor-susceptibility protein in mouse and human[45].

Overexpression of aurora kinase A resulting in loss of p53 function confers resistance to DNA damage–inducing agents, such as cisplatin, a drug commonly used in cancer chemotherapy. It is, therefore, expected that overexpression of aurora kinase A would be under positive selection in human tumors expressing wild-type p53. The function of aurora kinase A as a p53-destabilizing molecule makes it an important target for developing therapeutic strategies for those human cancers in which aurora kinase A is overexpressed.

METHODS

Mapping of phosphorylation site. We produced GST–aurora kinase A and GST-p53 proteins in BL21 pLys bacteria according to the manufacturer's protocol (Amersham Pharmacia Biotech). We carried out *in vitro* kinase assays as described[14]. We purified ^{32}P-labeled GST-p53 phosphorylated by aurora kinase A *in vitro* by SDS-PAGE and digested it with N-tosyl-L-pheylalanine chloromethyl ketone–trypsin (Sigma). We subjected digested phosphopeptides to phosphorylated amino acid analysis and two-dimensional analysis using the HTLE-7000 electrophoresis system (CBS Scientific) as described[25]. We carried out manual Edman degradation as described[26].

***In vitro* binding assay.** We produced *in vitro*–translated proteins in the presence of ^{35}S-methionine using the TNT coupled transcription/translation kit (Promega). We incubated GST–aurora kinase A or GST-p53 fusion proteins bound to glutathione-Sepharose beads with ^{35}S-labeled p53 or aurora kinase A, respectively, in binding buffer (20 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40) for 1 h at 4 °C and then washed them five times in wash buffer (20 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.25% Nonidet P-40, 10% glycerol). We analyzed the bound proteins by SDS-PAGE.

***In vivo* binding assay.** We transfected cells using LipofectAMINE according to the manufacturer's protocol (Invitrogen). We extracted cells with lysis buffer (50 mM Tris (pH 8.0), 20 mM sodium glycerophosphate, 140 mM NaCl, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 0.5 μM okadaic acid, 10 μM Microcystin LR, Protease inhibitor cocktail) 24 h after transfection. We incubated the lysate for 20 min on ice and then centrifuged it at 15,000*g* for 20 min. We used the supernatant for immunoblotting and immunoprecipitation. For immunoprecipitation, we incubated 0.3–2 mg of lysate for 3 h at 4 °C with 15 μl of polyclonal antibody to aurora kinase A conjugated to protein G-agarose or 5 μg of monoclonal antibody to p53 (Ab-6, Oncogene) conjugated to protein G-agarose, washed the immunocomplex four times with lysis buffer and then subjected it to SDS-PAGE.

p53 degradation and ubiquitination assay. We transfected p53-deficient H1299 cells with increasing amounts of either pcDNA3-Flag–tagged aurora kinase A or K162R mutant aurora kinase A together with 0.4 μg of pcDNA3-Flag–tagged p53, S315A mutant p53 or L22Q/W23S double mutant p53; pCMV-Mdm2 or C464A mutant Mdm2; pCEP4-p27, pcDNA3-empty vector and pEGFP-empty vector in separate transfections. We detected levels of p53, p21, p27 and GFP by immunoblotting with the respective antibodies. For this purpose, we used antibody to p21 F-5 (Santa Cruz), antibody to p27 F-8 (Santa Cruz) and antibody to GFP Living Colors A.v. Peptide (Clontech). To evaluate ubiquitination of p53, we treated transfected H1299 cells for 6 h with a proteasome inhibitor LLnL (50 μM; Sigma), collected them and extracted them with lysis buffer. We immunoprecipitated the lysate with monoclonal antibody to p53 (Ab-6) and then resolved it by SDS–PAGE (8% gel) and analyzed it by immunoblotting with antibody to p53 (Ab-6), antibody to p53 phosphorylated at Ser315 and monoclonal antibody to Mdm2 (Ab-1, Oncogene Science). To assess stability of p53, we treated MCF7 cells with 80 μg ml^{-1} of cycloheximide for indicated times.

siRNA, stable clone and cisplatin treatment. We carried out siRNA experiments for aurora kinase A and Mdm2 as previously described[46,47]. We obtained aurora kinase A stable transfectants by transfecting pcDNA3–wild-type aurora kinase A to MCF7 cells and then selecting the cells in the presence of G418 for 3 weeks. We added cisplatin to the culture medium for 24 h at indicated concentrations. For apoptosis analysis of siRNA-transfected cells, we fixed the cells in 3.7% formaldehyde, stained them with DAPI and counted the nuclear morphology of at least

500 cells under the fluorescence microscope. For FACS analysis of siRNA-transfected cells and stable transfectants, we stained the cells with propidium iodide and analyzed them by Becton Dickinson FACSCAN flow cytometer[48].

Bladder tissue samples. We analyzed the expression levels of aurora kinase A kinase and p53 in 23 bladder tumor tissues and 2 normal tissues by immunoblotting and immunohistochemistry as described[13]. We obtained frozen tissue samples from the interdepartmental tissue repository at the University of Texas M.D. Anderson Cancer Center. The tissue samples were collected under the approved institutional laboratory protocols and all individuals from whom samples were collected signed the informed. consent. We isolated exon 5–9 of *TRP53* (encoding p53) from all tumor samples, amplified the region and directly sequenced it by applying the cycle sequencing dye terminator protocol (PE Applied Biosystems).

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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