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Aurora B Interacts with NIR-p53, Leading to p53 Phosphorylation in Its DNA-binding Domain and Subsequent Functional Suppression*[S]

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NIR (novel INHAT repressor) is a transcriptional co-repressor with inhibitor of histone acetyltransferase (INHAT) activity and has previously been shown to physically interact with and suppress p53 transcriptional activity and function. However, the mechanism by which NIR suppresses p53 is not completely understood. Using a proteomic approach, we have identified the Aurora kinase B as a novel binding partner of NIR. We show that Aurora B, NIR and p53 exist in a protein complex in which Aurora B binds to NIR, thus also indirectly associates with p53. Functionally, overexpression of Aurora B or NIR suppresses p53 transcriptional activity, and depletion of Aurora B or NIR causes p53-dependent apoptosis and cell growth arrest, due to the up-regulation of p21 and Bax. We then demonstrate that Aurora B phosphorylates multiple sites in the p53 DNA-binding domain *in vitro*, and this phosphorylation probably also occurs in cells. Importantly, the Aurora B-mediated phosphorylation on Ser^{269} or Thr^{284} significantly compromises p53 transcriptional activity. Taken together, these results provide novel insight into NIR-mediated p53 suppression and also suggest an additional way for p53 regulation.

The tumor suppressor p53 is a transcription factor that regulates various important biological processes, including apoptosis, cell cycle arrest, and senescence (1, 2), and p53 mutations have been identified in over 50% of human cancers (3, 4). The steady-state level of p53 in unstressed cells is low; in response to DNA damage, the stability and activity of p53 is modulated by various post-translational modifications, including phosphorylation and acetylation (5, 6). Activated p53 can induce the transcription of subsets of genes. Putative p53 target genes include the *p21* and some proapoptotic genes, such as *Bax*, *Puma*, and *NOXA* (2). Induction of the cyclin-dependent kinase inhibitor p21 leads to G_1 phase cell growth arrest (7), and the proapoptotic genes cause caspase activation and ultimately apoptotic cell death (4). Suppression of p53 function in the germinal center (GC) is important for high rate B cell proliferation (8). Physiologic DNA breaks occur when germinal center B cells undergo immunoglobulin class switch recombination (CSR)[2] and somatic hypermutation (9–11); in this situation, inhibition of p53 can protect B cells from p53-dependent apoptosis.

p53 is composed of an N-terminal transactivation domain, a central specific DNA-binding domain (DBD), and a C-terminal tetramerization domain followed by a regulatory domain (6). At least 20 phosphorylation sites have been reported for human p53; the majority of these sites are modified in response to DNA damage or stress, but some are phosphorylated under normal growth conditions. Most of the N-terminal-specific phosphorylation sites prevent MDM2-mediated ubiquitination and stabilize p53; in contrast, phosphorylation of p53 at its C-terminal and some N-terminal sites more often suppresses its function, in most cases by promoting its degradation, for example phosphorylation of Ser$^{362/366}$ by inhibitor of NF-κB kinase (12) and Thr^{55} by TAF1 (13). To date, little is known about phosphorylation in the p53 DBD.

NIR (novel INHAT repressor) has been identified as a potent transcriptional co-repressor with inhibitor of histone acetyltransferase (INHAT) activity (14). As a nuclear protein, NIR has been shown to directly bind to nucleosomes and core histones and prevent acetylation by histone acetyltransferases. Moreover, NIR also physically interacts with p53 and localizes to the promoter regions of some p53-targeted genes, thus suppressing p53 transcriptional activity and p53-dependent apoptosis (14). We previously found that the expression level of *NIR (DKFZp564C186)* increased in normal B cells upon stimulation with CD40L plus IL-4 (15). Using tandem affinity purification, we identified Aurora B as a novel NIR-interacting protein. Aurora B is a serine/threonine kinase that is highly expressed during mitosis (16, 17) and is the catalytic component of the chromosome passenger complex, the Aurora B-INCENP-Survivin-Borealin complex (18, 19). The kinase activity of Aurora B is regulated through autophosphorylation (20). It is well established that Aurora B is required for chromosome condensation, alignment, and cytokinesis (19). Here, we demonstrate that Aurora B, NIR, and p53 exist in a protein complex *in vivo*. NIR functions to mediate the indirect association between Aurora B and p53 within this complex, therefore facilitating Aurora B-mediated p53 phosphor-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

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[2] The abbreviations used are: CSR, class switch recombination; INHAT, inhibitor of histone acetyltransferase; NIR, novel INHAT repressor; DBD, DNA-binding domain; EDI, ectodermal dysplasia with immune deficiency; Edu, 5-ethynyl-2′-deoxyuridine.

ylation on multiple sites in the DBD and thus causes significantly impaired p53 transcriptional activity.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—U2OS, H1299 cells, and BL2 B cells were purchased from the ATCC and maintained in RPMI 1640 medium supplemented with 10% FBS. HEK293T cells were maintained in DMEM with 10% FBS. Nocodazole and AZD1152 were purchased from Sigma and Selleck Chemicals, respectively.

B Cell Stimulation and Microarray Analysis—CD40L and IL4 stimulation of CD19+ B cells and microarray analysis were performed as described previously (15).

Plasmids, Small Interfering RNAs, and Transfection—Human NIR, p53, and Aurora B cDNAs were cloned into gateway Donor vector pDONR201 (Invitrogen) and then transferred to N-terminal Myc-tagged or S/FLAG-tagged expression vector, as indicated. PathDetect p53-luciferase reporter (catalog no. 219085) was purchased from Stratagene (21). p21 promoter-containing reporter (catalog no. 16462), pcDNA3 FLAG-p53 (under immediate early CMV promoter; catalog no. 10838) and pcDNA3.1-p300 (catalog no. 23252) plasmids were purchased from Addgene. All deletions or point mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene) and verified by sequencing.

siRNAs were synthesized by Dharmacon as follows: nontargeting control siRNA (catalog no. D-001810-01); siRNAs specific for NIR 1, GAACUCGGCAUACAUCUGC and 2, GUACAGGCGUUCCAAGCAG; siRNA specific for Aurora B, CGCGGCACUUCACAAUUGA (22).

Transfections were performed using Lipofectamine 2000 or Oligofectamine (Invitrogen), following the manufacturer's instructions. Cells were used for assays 20–36 h after transfection, as indicated.

Co-immunoprecipitation Assay—For co-immunoprecipitation assays, cells were lysed with NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40) containing 50 mM β-glycerophosphate, 10 mM NaF, and protease inhibitor mixture on ice for 10 min. After removal of cell debris by centrifugation, the lysates were incubated with protein A-agarose coupled with antibodies (2 μg) as indicated, or with S protein-agarose beads (Novagen), for at least 2 h at 4 °C. The immunocomplexes were then washed with NETN buffer three times, boiled in 2× Laemmli buffer, and separated by SDS-PAGE.

Tandem Affinity Purification—293T cells stably expressing SFB-NIR were lysed in NETN buffer on ice. Cell debris was removed by centrifugation, and the supernatants were incubated with streptavidin-conjugated beads (Amersham Biosciences) for 1 h at 4° C. The immunocomplexes were washed three times with NETN buffer, and then bead-bound proteins were eluted with NETN buffer containing 2 mg/ml biotin (Sigma). The eluates were incubated with S protein beads (Novagen). After three washes, the immunocomplexes were analyzed by SDS-PAGE, and mass spectrometry analyses were performed by the Harvard Medical School Taplin Biological Mass Spectrometry facility.

Immunoblotting and Antibodies—Immunoblotting was performed following standard procedures. A polyclonal antibody against human NIR was raised by immunization of rabbits with the peptide CRDREIQLEISGKER. Other antibodies used in this study were as follows: anti-FLAG (M2, catalog no. F3165, Sigma), Aurora B (catalog no. 611082), Ser(P) antibody (catalog no. 612546) (BD Biosciences); Myc (9E10), p53 (DO-1), p53 (fl-393), and p21 (C19) (Santa Cruz Biotechnology); Bax (Ab-3, catalog no. AM32, Calbiochem); actin (Sigma); MDM2 (catalog no. 556353, BD Pharmingen); Thr(P) (catalog no. 9386, clone 42H4), Phospho-H3 (Ser^{10}) (Cell Signaling); and total acetyl-Lys (catalog no. 05-515, clone 4G12, Millipore).

Apoptosis Assay and EdU Incorporation Assay—For the apoptosis assay, siRNA-transfected cells were trypsinized, combined with floating cells, and washed sequentially with PBS and 1× staining buffer. Cells were stained with FITC-annexin V (BD Pharmingen) on ice for 30 min. After washing, cells were resuspended in PBS at $\sim 2 \times 10^5$ cells/100 μl and subjected to FACS analysis.

For the EdU (BrdU alternative) incorporation assay, cells were pulse-labeled with 10 μM EdU for 30 min at 37 °C and then stained following the manufacturer's protocol (Click-iT EdU kit, catalog no. C10337, Invitrogen).

Dual-Luciferase Assay—H1299 cells were seeded at 1×10^5 cells/well on 24-well plates. The next day, cells were transfected with 500 ng of p53 PathDetect reporter or p21 promoter reporter, 20 ng of p53 plasmid, and other plasmids as indicated. pRL-TK (5 ng) was included as an internal control. Each sample was triplicated. Luciferase assays were performed with the Dual-Luciferase reporter assay kit (Promega) following the manufacturer's instructions. Results were normalized to expression of pRL-TK as measured by *Renilla* luciferase activity.

RT-PCR—Total RNA was isolated from cells using the RNeasy mini kit (Qiagen). RT-PCR for p21, Bax, and GAPDH was performed using the two-step kit (Applied Biosystems). Primer sequences are available upon request.

In Vitro Kinase Assay—Aurora B kinase purified from insect cells was purchased from Cell Signaling Technology (catalog no. 7394). GST-p53 or mutant proteins were purified from BL21 bacteria following standard procedures. For the kinase reaction, protein substrate bound on GSH-Sepharose was incubated with 2 μg of Aurora B in 30 μl of kinase buffer (25 mM Tris-HCl, 2.5 mM β-glycerophosphate, 0.5 mM DTT, 5 mM $MgCl_2$, 0.1 mM $NaVO_3$, 20 μM ATP) at 30 °C for 30 min, in the presence of 3 μCi of [γ-^{32}P]ATP. Beads were washed twice, boiled with 2× Laemmli buffer, and applied to SDS-PAGE.

Mapping of p53 Phosphorylation Sites—GST-p53 (wild-type) protein was bound to GSH-Sepharose and phosphorylated by Aurora B in the presence of cold ATP. Phosphorylated GST-p53 was separated by SDS-PAGE, and the protein band was excised and subjected to mass spectrometry analysis.

RESULTS

Expression of NIR in B Cells and Its Role in B Cell Survival—We previously performed a microarray study to compare the gene expression profiles of B cells from patients

with ectodermal dysplasia with immune deficiency (EDI) and reference controls (15). EDI is a developmental disorder associated with severe immune deficiency and is caused by alterations in the gene encoding NEMO (NF-κB essential modulator). As a consequence of impaired induction of NF-κB signaling, the B cells from EDI patients are invariably naive and fail to undergo CSR *in vitro*. Supplemental Fig. S1*A* shows a subset of genes that are up-regulated upon stimulation in reference control B cells but not in B cells from EDI patients with the NEMO (C417R) mutation. Among these genes, many are known to have roles in immunoglobulin CSR and somatic hypermutation. Interestingly, one gene called *DKFZp564C186* was also identified in this analysis. This gene attracted our attention as later on it was functionally characterized and renamed as *NIR* (novel INHAT repressor) (14). To validate the microarray result, we treated CD19+ B cells with CD40L plus IL4 and harvested cell lysates at different time points for immunoblot analysis of NIR expression. Supplemental Fig. S1*B* shows that NIR expression level increased upon stimulation. Similar results were also observed in Burkitt lymphoma BL2 B cells (supplemental Fig. S1*C*).

Because NIR had previously been shown to negatively modulate p53 function (14), we examined whether NIR is required for B cell survival. To this end, we investigated the apoptosis induction caused by NIR depletion in B cells. As expected, NIR knockdown in BL2 cells (possessing wild-type p53) with siRNA enhanced the dose- and time-dependent induction of apoptosis by the DNA-damaging agent etoposide (supplemental Fig. S1, *D* and *E*). These data confirmed the results of previous studies with respect to the function of NIR and further indicate a role for NIR in B cell survival.

Aurora B Interacts with NIR to Form an Aurora B-NIR-p53 Complex—To better understand the function of NIR, we conducted studies to identify additional NIR-interacting protein(s). We established a 293T-derived cell line that stably expresses SFB (S protein, FLAG, and streptavidin-binding peptide)-tagged NIR. A two-step affinity purification using streptavidin-Sepharose beads and S protein-agarose beads followed by mass spectrometry analysis identified several NIR-interacting proteins. In addition to the previously reported p53, we repeatedly identified the Aurora B protein in the purified NIR complex (Fig. 1*A*). Aurora B is a serine/threonine mitotic kinase required for proper chromosome segregation and cytokinesis (18, 19). We initially confirmed the interaction between NIR and Aurora B by endogenous co-immunoprecipitation, showing that antibody specific for NIR, but not a control IgG, retrieved Aurora B from total cell lysate (Fig. 1*B*). This interaction was further confirmed by the reciprocal co-immunoprecipitation assay with ectopically expressed NIR and Aurora B (Fig. 1, *C* and 1*D*). In addition, immunostaining analysis showed that ectopically expressed FLAG-tagged Aurora B largely co-localizes with endogenous NIR in the cell nucleus (Fig. 1*E*).

As both p53 and Aurora B were identified as NIR binding partners, we next investigated the possibility that NIR, p53, and Aurora B may co-exist in a complex. To this end, we performed a co-immunoprecipitation assay for endogenous proteins. The result showed that NIR and Aurora B could be co-

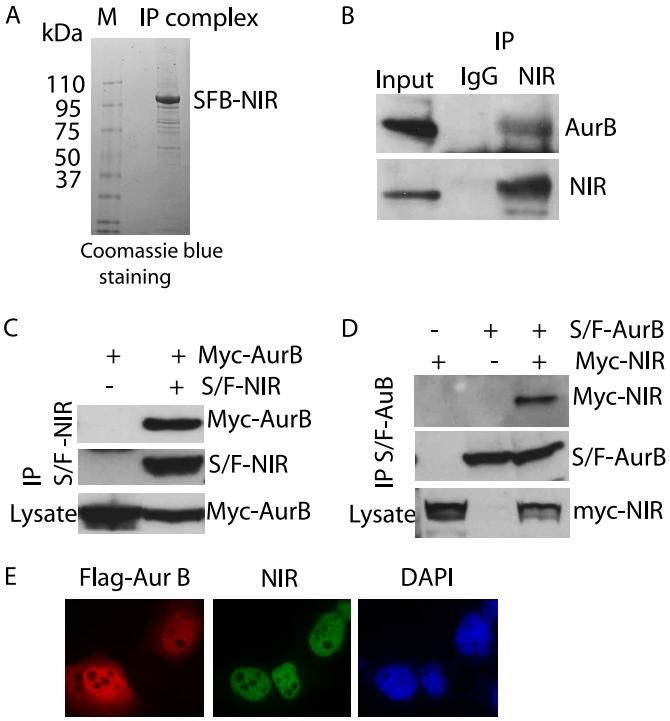

FIGURE 1. **Identification of Aurora B as a novel NIR-interacting protein.** *A*, the elution products of tandem affinity purification from 293T cells stably expressing S/FLAG/SBP (*SFB*)-tagged NIR were separated by SDS-PAGE and visualized by Coomassie Blue staining. The SFB-NIR band is shown on the *right*. *B*, association between endogenous NIR and Aurora B. Whole cell lysates from U2OS cells were immunoprecipitated (*IP*) with antibody specific for NIR or control IgG and immunoblotted with antibodies against NIR or Aurora B (*AurB*) as indicated. *C*, plasmid encoding Myc-tagged Aurora B was co-transfected with empty vector or plasmid encoding S/FLAG-tagged NIR. Cell lysates were then subjected to immunoprecipitation with S-Sepharose and blotted with antibodies against Myc (*AurB*) or FLAG (*NIR*). Input lysates were probed with anti-Myc antibody. *D*, plasmid encoding Myc-tagged NIR was co-transfected with or without plasmid encoding S/FLAG-tagged Aurora B (*S/F-AurB*). Immunoprecipitation and blotting were performed as described in *C*. *E*, U2OS cells were transfected with plasmid encoding FLAG-tagged Aurora B. After 24 h, cells were stained with anti-FLAG and NIR antibodies, counterstained with DAPI, and observed under a fluorescence microscope.

immunoprecipitated with each other (Fig. 2*A*, *lanes 1–4*) and, most strikingly, both proteins also associated with endogenous p53. In addition, only p53, but not NIR, retrieved endogenous MDM2, the p53-interacting E3 ubiquitin ligase (Fig. 2*A*, *lanes 5* and *6*). These results suggest that Aurora B, NIR, and p53 form an MDM2-independent protein complex *in vivo*.

In further studies to dissect the interactions within this complex, we transiently expressed these proteins and performed reciprocal co-immunoprecipitation. The result indicated that although NIR associates with both p53 and Aurora B, no strong interaction between Aurora B and p53 was observed (supplemental Fig. S2). To confirm this result, we performed a pulldown assay between GST-p53 protein and purified FLAG-tagged Aurora B or NIR. The result indicated that GST-p53 retrieved NIR, but not Aurora B alone, and the presence of NIR enabled the association between GST-p53 and Aurora B (Fig. 2*B*). These results suggest that NIR functions to mediate indirect association between p53 and Aurora B in a protein complex.

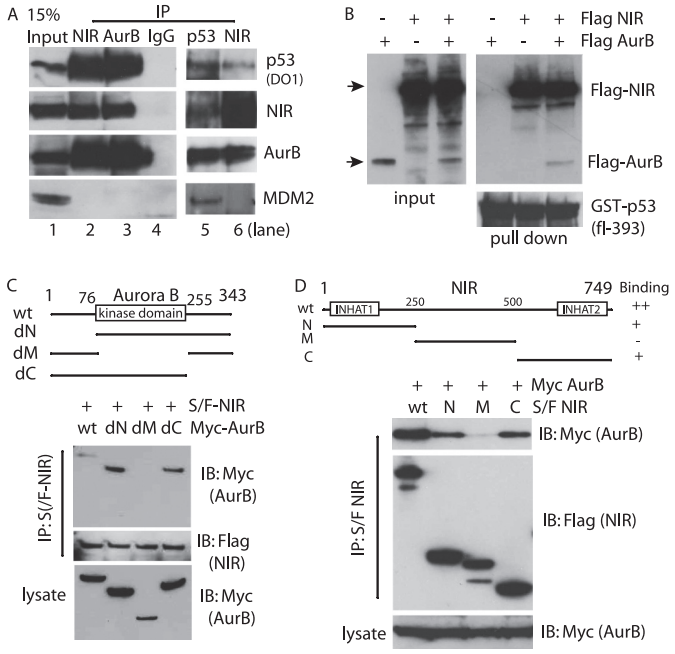

FIGURE 2. **Protein complex formation between p53, NIR, and Aurora B and mapping of the binding region between Aurora B and NIR.** *A*, U2OS cells were treated with irradiation (IR) (2 Gy) to increase the endogenous p53 level. Endogenous proteins were immunoprecipitated using antibodies as indicated, and then proteins bound to the beads, and input lysates were immunoblotted with antibodies to p53, NIR, Aurora B (*AurB*), and MDM2. Results in *lanes 5* and *6* are from a separate immunoprecipitation (*IP*) experiment. *B*, GST-p53 was incubated with purified FLAG-tagged Aurora B, NIR, or both. Bound Aurora B or NIR was analyzed by immunoblotting (*IB*) with anti-FLAG antibody. Input proteins were also blotted with antibodies as indicated. *C*, 293T cells were transfected with plasmid encoding SFB-NIR and wild-type or deletion mutants of Myc-tagged Aurora B plasmid. Cell lysates were subjected to immunoprecipitation with S-protein agarose. Bound Aurora B was analyzed by immunoblotting with anti-Myc antibody. *D*, 293T cells were transfected with plasmid encoding Myc-Aurora B and wild-type or truncation mutants of S/FLAG-tagged (*S/F*) NIR, as indicated. Cell lysates were subjected to immunoprecipitation with S-protein agarose. Bound Aurora B was analyzed by immunoblotting with anti-Myc antibody.

Interaction between NIR and Aurora B—As the interaction between NIR and p53 has been characterized previously (14), we focused on the molecular interaction between NIR and Aurora B. For this purpose, we generated expression vectors encoding deletion or truncation mutants of Aurora B and NIR, as indicated in Fig. 2, *C* and *D*. Plasmids encoding WT or deletion mutants (dN, dM, dC) of Myc-tagged Aurora B were co-transfected with S/FLAG-tagged NIR vector into 293T cells, and their interactions were examined by co-immunoprecipitation. Although deletion of the N- or C-terminal regions of Aurora B did not affect its interaction with NIR, deletion of the middle region (dM) specifically abolished this interaction (Fig. 2*C*).

Next, vectors expressing wild-type and truncation mutants (N, M, C) of NIR were transfected into 293T cells together with full-length Aurora B and tested for interaction in a co-immunoprecipitation assay. Although both N terminus (N) and C terminus (C) truncations bound to Aurora B (as did WT NIR), the middle region of NIR (M) failed to do so (Fig. 2*D*). Taken together, these results suggest that the interaction between NIR and Aurora B requires the middle kinase domain of Aurora B and both the N and C termini of NIR.

Ectopic Expression of Either Aurora B or NIR Suppresses p53 Transcriptional Activity—It has been reported that NIR negatively regulates p53 function by suppressing its transcription activity, but the underlying mechanism remains elusive (14). In view of the binding assay results presented above, we propose that Aurora B might be involved in NIR-mediated suppression of p53 transcriptional activity. To test this hypothesis, we transiently transfected a constant amount of a luciferase gene reporter construct (PathDetect p53-luc) driven by p53-specific enhancer elements derived from p53-inducible genes (23) together with a plasmid expressing p53 under the CMV promoter (CMV-FLAG-p53), in the absence or presence of NIR or Aurora B expression vector. Plk1 was included as a control as it has been shown to suppress p53 transcription activity (24). Ectopic expression of p53 induced robust reporter activation (~100-fold) (supplemental Fig. S3*A*), and importantly, expression of either Aurora B or NIR efficiently suppressed p53 transcriptional activity to a similar extent in a dose-dependent manner (supplemental Fig. S3*A*). Furthermore, mutants of Aurora B (dM) or NIR (M) that failed to interact with each other lost their ability to suppress p53 transcriptional activity (supplemental Fig. S3*A*). To confirm these results, we transiently transfected p53-null H1299 lung cancer cells with increasing amounts of plasmids encoding Myc-NIR or Aurora B together with the p53 expression vector CMV-FLAG-p53. Expression of p53 clearly induced expression of its target genes *p21* and *Bax* (4, 7) (Fig. 3*A*, *lane 2*). Consistent with the luciferase reporter assay result, induction of p21 and Bax was suppressed by coexpression of NIR or Aurora B in a dose-dependent manner (Fig. 3*A*, *lanes 3–6*). Next, we asked whether NIR and Aurora B also suppress the transcriptional activity of endogenous p53. U2OS cells (p53 wild-type) were transfected with different expression vectors as indicated, and the induction of p21 and Bax protein was measured in the presence and absence of γ-irradiation. As shown in Fig. 3*B*, ectopic expression of Aurora B or NIR significantly inhibited the induction of p21 and Bax protein by irradiation (*lanes 4–9*). Collectively, these results support the hypothesis that, similar to NIR, increased expression of Aurora B suppresses p53 transcriptional activity and its ability to induce key target genes, such as *p21* and *Bax*.

Depletion of Aurora B or NIR Causes p53-dependent Apoptosis and Cell Growth Arrest—The above data show that NIR mediates the interaction between Aurora B and p53 and that expression of NIR or Aurora B suppresses p53 function. We therefore speculated that down-regulation of Aurora B would enhance p53-mediated apoptosis and/or G_1 cell cycle arrest in unstressed cells, similar to the effect of NIR depletion. To directly test this, siRNAs specific for Aurora B or NIR were transfected into U2OS cells and cells undergoing apoptosis were examined 48 h later by annexin V staining. Indeed, down-regulation of Aurora B or NIR dramatically increased the percentage of cells undergoing apoptosis from ~6 to ~18% (supplemental Fig. S3*B*). Furthermore, an EdU incorporation assay also showed that down-regulation of either NIR or Aurora B significantly decreased the percentage of EdU-positive cells, which are cells in S-phase, from ~50 to

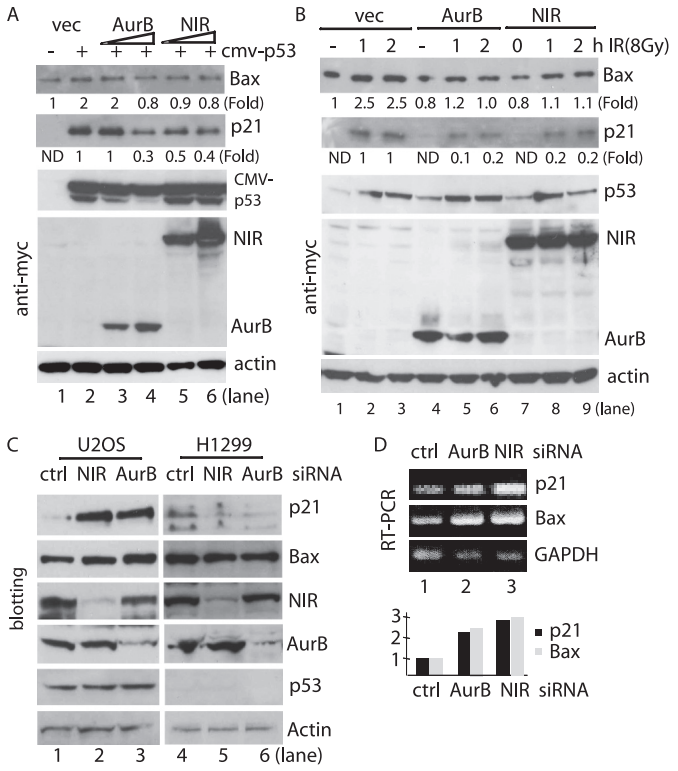

FIGURE 3. **Aurora B suppresses p53 transcription activity.** *A*, plasmid encoding p53 was transfected into H1299 cells together with empty vector or Aurora B (*AurB*) or NIR expression vector, as indicated. Lysates were harvested 20 h post-transfection and immunoblotted with antibodies as shown. *B*, empty vector (*vec*) or plasmid encoding Myc-tagged Aurora B or NIR was transfected into U2OS cells. 24 h post-transfection, cells were irradiated (8 Gy) or left untreated. Cell lysates were harvested at different time points and immunoblotted with antibodies specific for Bax, p21, p53, Myc, or actin. *ND*, not detected. *C*, U2OS or H1299 cells were transfected twice with siRNAs as indicated. 24 h post second transfection, cells were lysed and immunoblotted with antibodies as shown. *D*, Total RNA from transfected U2OS cells shown in *C* was subjected to RT-PCR to determine p21, Bax, and GAPDH expression. The *lower panel* shows the fold enhancement of mRNA expression relative to control cells. *ctrl*, control.

~30% (supplemental Fig. S3*C*), suggesting that depletion of NIR or Aurora B causes a G_1 phase cell cycle arrest.

The increased apoptosis and G_1 cell cycle arrest induced by NIR or Aurora B depletion in U2OS cells presumably reflect the induction of key p53 target genes, such as *p21* and *Bax*. To verify this, U2OS and H1299 cells were transfected with NIR or Aurora B siRNA and lysates were analyzed for p21 and Bax protein levels. As expected, depletion of Aurora B or NIR increased the protein levels of endogenous Bax and p21 in U2OS cells (Fig. 3*C*, *lanes 1–3*), but not in H1299 cells (Fig. 3*C*, *lanes 4–6*). To further test whether the increased expression of p21 and Bax occurs at the transcriptional level, we isolated total RNA from NIR or Aurora B-depleted U2OS cells and performed a semiquantitative RT-PCR analysis. In support of our hypothesis, depletion of NIR or Aurora B increased mRNA levels of p21 and Bax (Fig. 3*D*). Taken together, these results suggest that depletion of NIR or Aurora B causes growth arrest and increases apoptosis due to p53-dependent p21 and Bax up-regulation.

p53 Phosphorylation by Aurora B—Our results indicate that ectopic expression of either Aurora B or NIR efficiently suppresses p53 function. Aurora B is a Ser/Thr protein kinase, raising the possibility that it may negatively regulate p53 though phosphorylation. To address this question, we first examined whether Aurora B kinase activity is required for its suppression on p53. As shown in Fig. 4*A*, whereas wild-type Aurora B efficiently suppressed p53 in a luciferase reporter assay, a kinase-inactive Aurora B mutant (K106R) (25, 26) had a minimal effect on p53 transcriptional activity. In line with this, treatment of U2OS cells, but not H1299 cells, with the Aurora B-specific inhibitor AZD1152 (27) markedly induced Bax expression (Fig. 4*B*). To further confirm that Bax induction by Aurora B inhibition occurs in G1 to early S-phase, we arrested U2OS cells in mitosis with nocodazole treatment and then released cells, waiting for ~2 h to allow cells to progress to G_1 phase; at that point, cells were either lysed (as control) or treated with or without AZD1152 for another 16 h. As shown in Fig. 4*C*, AZD1152 significantly induced Bax expression in the cells released from mitosis, suggesting that Aurora B is required for p53 suppression in G_1 to early S-phase. To determine whether Aurora B directly phosphorylates p53, we performed an *in vitro* kinase assay using Aurora B protein expressed in insect cells as the kinase source and GST-p53 purified from bacteria as the substrate and confirmed that Aurora B directly phosphorylates p53 (Fig. 4*D*).

Next, we set out to determine the site(s) of p53 phosphorylation by Aurora B. Following *in vitro* phosphorylation by Aurora B, full-length p53 was subjected to mass spectrometry analysis and three phosphorylation sites, Ser^{183}, Ser^{269}, and Thr^{284}, were identified (Fig. 4*E*). We noticed that these sites are all located in the p53 DBD domain (amino acids 100–300). These three sites were all converted to alanine (3A mutant) by site-directed mutagenesis. Results of a kinase assay indicated that phosphorylation of the 3A mutant by Aurora B was greatly impaired compared with wild-type p53 (Fig. 4*F*), suggesting that these three amino acid residues are major sites of phosphorylation by Aurora B. To explore whether these sites are also phosphorylated *in vivo*, we used antibody against both Thr(P) and Ser(P) to detect phosphoresidues in ectopically expressed p53 from H1299 cells. As shown in Fig. 4*G*, p53 was indeed phosphorylated at Thr and Ser residue(s), and importantly, this phosphorylation was markedly increased by Aurora B overexpression for the wild-type p53, but not for the 3A mutant. Taken together, these results support the idea that Aurora B phosphorylates p53 in the DBD domain and that this phosphorylation occurs both *in vitro* and *in vivo*.

Suppression of p53 Transcriptional Activity by Aurora B-mediated Phosphorylation—Sequence alignment revealed that the phosphorylation sites in the human p53 DBD domain identified above (Ser^{183}, Ser^{269}, Thr^{284}) are conserved across species (supplemental Fig. S4*A*). However, phosphorylation at these sites is unlikely to cause p53 degradation, as depletion of Aurora B did not increase endogenous p53 levels in U2OS cells (Fig. 3*C*). To test the alternative hypothesis that Aurora B-mediated p53 phosphorylation negatively regulates its transactivation activity and define which of these three sites play a major role in this process, we individually mutated the three phosphorylation sites of p53 (Ser^{183}, Ser^{269}, or Thr^{284}) to the phosphomimetic glutamic acid (Glu) and firstly exam-

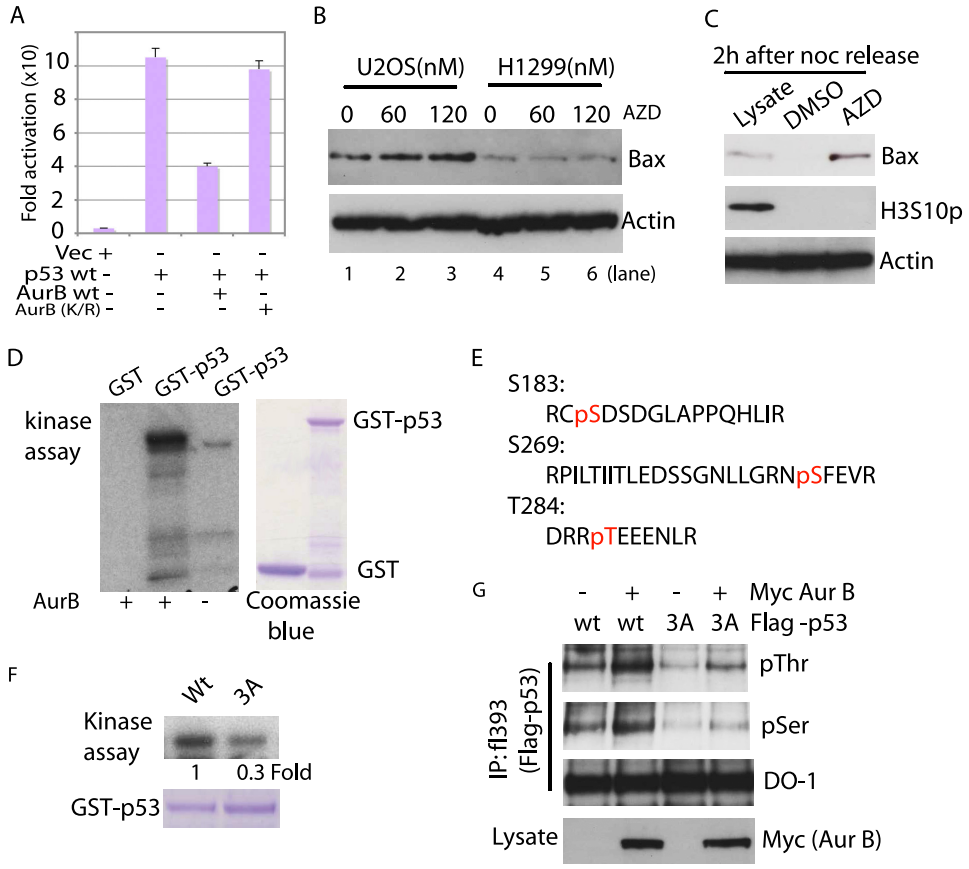

FIGURE 4. **p53 phosphorylation by Aurora B.** *A*, p53 reporter construct was co-transfected with the indicated plasmids into H1299 cells and reporter activation was determined as described under "Experimental Procedures." *B*, U2OS cells and H1299 cells were treated with AZD1152 (*AZD*) for ~12 h at the indicated doses. Cell lysates were harvested and immunoblotted with Bax and actin antibodies. *C*, U2OS cells were treated with 100 ng/ml nocodazole (*noc*) overnight, and then shake off cells were harvested, washed with PBS, and reseeded. Approximately 2 h later, cells were either lysated or treated with dimethyl sulfoxide (*DMSO*) or AZD1152 for another 16 h before harvesting. Cell lysates were immunoblotted with Bax, phospho-H3, and actin antibodies. *D*, GST-p53 or GST control proteins were incubated with Aurora B protein and analyzed for phosphate incorporation (*left panel*). Coomassie staining of GST-p53 and GST protein is also shown (*right panel*). *E*, *In vitro* phosphorylation sites of GST-p53 identified by mass spectrometry analysis. *F*, GST-p53 wild-type and 3A mutant proteins were analyzed in a kinase assay as in *B*. *G*, plasmids encoding wild-type or 3A mutant (CMV)-FLAG-p53 were transiently transfected into H1299 cells, with or without Myc-Aurora B (*AurB*) expression vector. 20 h post-transfection, cells were lysed and subjected to immunoprecipitation (*IP*) with p53 antibody (fl-393). Precipitates were immunoblotted with antibodies to p53 (DO-1), Thr(P) and Ser(P), as indicated. *Vec*, vector.

ined their effects on the p53 luciferase reporter assay in H1299 cells. As shown in Fig. 5*A*, WT p53 induced robust activation as expected. Although activation was only slightly affected in the S183E mutant, mutation of S269E or S284E almost completely abolished activation. Similar results were obtained for a p21 promoter reporter (Fig. 5*B*). Next, we examined the effect of these mutations on the induction of apoptosis. H1299 cells were transiently transfected with WT or mutant p53 constructs. 24 h later, cells undergoing apoptosis were evaluated by annexin V staining. Consistent with the luciferase reporter assay results, WT p53 caused robust apoptosis induction and the S183E mutant showed a modest change of induction. In contrast, S269E and T284E mutants showed minimal apoptosis induction (Fig. 5*C*). As the activation of p53 in these assays reflects its ability to induce expression of its target genes, we next examined the induction of p21 and Bax proteins. For this purpose, H1299 cells were transiently transfected with equal amounts of WT or mutant p53 expression vectors, and the cell lysates were analyzed by immunoblotting. We found that induction of p21 and Bax protein generally correlated with the results observed in Fig. 5, *A–C*, for transcription activity and apoptosis induction (Fig. 5*D*). As a control, the expression level of WT p53 and its phosphomimetic mutants were similar. Moreover, the individual alanine mutants (S183A, S269A, T284A) displayed similar capacity to induce p21 and Bax as wild-type p53. The K120R mutant was included as a control as it was previously shown to have impaired ability to induce Bax (supplemental Fig. S4*B*) (28, 29). Taken together, these self-consistent results support our hypothesis that Aurora B-mediated p53 phosphorylation negatively regulates its function. In addition, functional analysis of individual mutants indicated that phosphorylation on Ser^{269} and Thr^{284} plays an important role in this process.

NIR Affects p53 Acetylation Level—NIR is known to be able to prevent histone acetylation. Given that in many cases histone-modifying molecules also target p53 for modification, we explored whether NIR affects p53 acetylation. FLAG-tagged p53 was co-transfected with empty vector or a p300 expression vector, in the absence or presence of NIR. As expected, p300 enhanced total p53 acetylation (30) (Fig. S4*C*). Interestingly, NIR coexpression down-regulated this acetylation by ~50%, suggesting a role of NIR in p53 deacetylation.

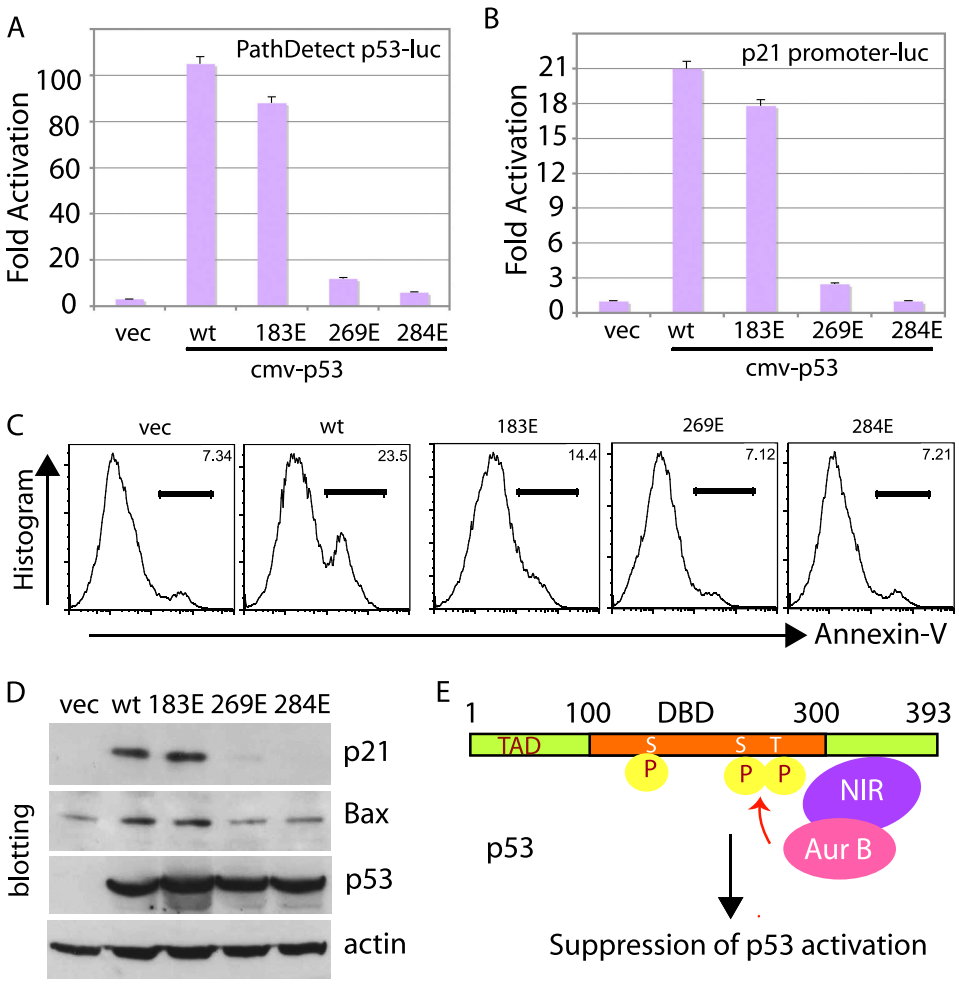

FIGURE 5. **Suppression of p53 transcriptional activity by Aurora B-mediated phosphorylation.** *A* and *B*, CMV constructs encoding P53 wild-type and E (Glu) mutants were co-transfected with the PathDetect p53-Luc reporter (*A*) or p21 promoter luciferase (*Luc*) reporter (*B*) into H1299 cells, and reporter activation was measured as described previously. *C*, H1299 cells were transfected with wild-type or mutant CMV-p53, as indicated. 20 h later, cells were trypsinized, combined with floating cells, and subjected to annexin V staining and FACS analysis. Experiments were repeated twice, and similar results were obtained. *D*, H1299 cells were transfected with wild-type or mutant CMV-p53, as indicated. Lysates were harvested 20 h post-transfection and immunoblotted with antibodies as indicated. *E*, a model of p53 phosphorylation and suppression by Aurora B (*Aur B*)-NIR complex. See text for details. *vec*, vector; *TAD*, transactivation domain.

DISCUSSION

In this study, we aimed to better understand the mechanism underlying NIR-mediated suppression of p53, a key protein already known to interact with NIR. With this in mind, we performed tandem affinity purification using a two-step protocol and identified Aurora B as a novel NIR-interacting protein. We then showed that Aurora B exists in a complex with NIR and p53, in which NIR might mediate the indirect association between Aurora B and p53 (Fig. 2). In further studies, we provide several lines of evidence that p53 is functionally suppressed in this protein complex. First, overexpression of wild-type Aurora B or NIR suppresses p53 transcriptional activity and the induction of p21 and Bax in a dose-dependent manner (Fig. 3, *A* and *B*). Second, depletion of Aurora B or NIR causes p53-dependent apoptosis and cell growth arrest through induction of p53 key target genes, such as p21 and Bax (Fig. 3, *C* and *D*). Finally, Aurora B-mediated p53 suppression requires its kinase activity (Fig. 4, *A* and *C*). Mechanistically, we demonstrated that Aurora B is able to directly phosphorylate p53 and that phosphorylation on Ser^{269} and Thr^{284} significantly compromised p53 activity (Figs. 4, *D*–*G*, and 5, *A*–*D*). Based on these results, we propose a model in which, under normal growth conditions and other specific conditions (*e.g.* in cells with spontaneous DNA damage or in B cells undergoing CSR), p53 is functionally suppressed within the Aurora B-NIR-p53 protein complex, mainly through constitutive phosphorylation within the DBD domain by Aurora B (Fig. 5*E*).

Previous studies indicated that NIR binds to two regions of p53 and suppresses p53 function (14). Our results showed that NIR binds to the kinase domain of Aurora B and that both the N terminus (amino acids 1–250) and C terminus (amino acids 500–749) of NIR are involved in the interaction with Aurora B. Under normal growth conditions, depletion of NIR causes p53-dependent apoptosis and cell growth arrest (supplemental Fig. S3, *B* and *C*, and Fig. 3). We speculate that NIR mainly functions to mediate the indirect association between Aurora B and p53, leading to constitutive p53 phosphorylation and functional suppression. At present, it is un-

clear whether NIR play a direct role in regulating Aurora B kinase activity, and due to the poor expression of NIR fusion protein in bacteria, we are currently unable to address this question in *in vitro* assays.

A functional connection between Aurora B and p53 has been documented previously. For example, overexpression of Aurora B kinase has been reported in colorectal (31) and prostate cancers (32), and treatment of cells with Aurora B inhibitors induces p53-dependent apoptosis in human leukemia cells (33). In this study, we demonstrate that Aurora B suppresses p53 through direct phosphorylation. Furthermore, we have identified the Aurora B-mediated p53 phosphorylation sites *in vitro* and shown that these sites are also most likely phosphorylated *in vivo* (Fig. 4*G*). Interestingly, all three phosphorylation sites (Ser^{183}, Ser^{269}, Thr^{284}) are located in the p53 DBD domain. Functional assays further defined that phosphorylation on Ser^{269} and Thr^{284} plays a major role in this p53-negative regulation (Fig. 5, *A–D*). Phosphorylation of these sites is unlikely to cause p53 degradation as depletion of Aurora B did not increase endogenous p53 protein levels (Fig. 3*C*) and the expression levels of WT p53 and its phosphomimetic mutants are similar (Fig. 5*D*). Previous structural studies indicated that Ser^{269} and Thr^{284} are located in the DBD core domain S10 strand and H2 helix region, respectively (34, 35). Thus, it is likely that phosphorylation at these sites affects p53 binding to some specific promoter regions of its target genes. In addition, although phosphorylation mimicking of either Ser^{269} or Thr^{284} is sufficient to abolish the induction of p21 and proapoptotic Bax protein (Fig. 5, *A–D*), it is unclear whether phosphorylation on Ser^{269} or Thr^{284} occurs simultaneously *in vivo* or exhibits some preference under certain conditions.

It is noteworthy that, although the expression level of Aurora B protein peaks at the late G_2 to M phase (16, 17, 36), Aurora B is expressed at a basal level throughout the cell cycle (36). Therefore, it is likely that in most of the cell population, Aurora B levels are comparable with the basal level of p53 and indispensable for p53 phosphorylation and suppression in the absence of DNA damage. Our results indicate that p53-NIR-Aurora B form a protein complex separate from MDM2 (Fig. 2*A*), and it is possible that upon DNA damage, p53 is stabilized and most "free" p53 can "escape" the suppression within this protein complex. Subsequently, the function of this free p53 is gradually down-regulated, partially through the phosphorylation by Aurora B. In favor of this notion, previous studies have reported slow kinetics of p53 phosphorylation after DNA damage, for example the phosphorylation of Ser^{366}, Ser^{378}, and Thr^{387} by Chk1/Chk2 (37).

Double-stranded DNA breaks trigger p53-mediated cell cycle arrest or apoptosis pathways. B cells undergoing CSR and somatic hypermutation have physiologic double-stranded DNA breaks, and suppression of p53 function is necessary to allow efficient rearrangement of antigen receptors and generation of neutralizing antibody responses (9–11). The oncogene BCL6 has been shown to inhibit p53 function by suppressing its expression in germinal center B cells (38). However, significant p53 protein levels can be observed in germinal center B cells (38), suggesting that additional factors independent of BCL6 may be required for further inhibition of p53 function and B cell terminal differentiation. Because NIR functions to suppress p53 and shows increased expression upon CD40L and IL-4 stimulation in normal B cells (supplemental Fig. S1, *A* and B), NIR may be such a factor. In support of this, we have shown that NIR forms a complex with Aurora B and facilitates p53 protein phosphorylation and suppression. Additionally, B cells from EDI patients with defects in CSR fail to express NIR upon stimulation (15). Because NIR-deficient mice are embryonic lethal,[3] it will be important to make NIR-conditional knock-out mice and examine whether specific depletion of NIR expression in the B cell lineage impairs germinal center formation and the development of neutralizing antibody responses.

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REFERENCES

1. Kruse, J. P., and Gu, W. (2009) *Cell* **137,** 609–622
2. Vousden, K. H., and Prives, C. (2009) *Cell* **137,** 413–431
3. Vogelstein, B., Lane, D., and Levine, A. J. (2000) *Nature* **408,** 307–310
4. Vousden, K. H., and Lu, X. (2002) *Nat. Rev. Cancer.* **2,** 594–604
5. Brooks, C. L., and Gu, W. (2003) *Curr. Opin. Cell. Biol.* **15,** 164–171
6. Toledo, F., and Wahl, G. M. (2006) *Nat. Rev. Cancer.* **6,** 909–923
7. el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) *Cell* **75,** 817–825
8. MacLennan, I. C. (1994) *Annu. Rev. Immunol.* **12,** 117–139
9. Sale, J. E., and Neuberger, M. S. (1998) *Immunity* **9,** 859–869
10. Papavasiliou, F. N., and Schatz, D. G. (2000) *Nature* **408,** 216–221
11. Bross, L., Fukita, Y., McBlane, F., Démollière, C., Rajewsky, K., and Jacobs, H. (2000) *Immunity* **13,** 589–597
12. Xia, Y., Padre, R. C., De Mendoza, T. H., Bottero, V., Tergaonkar, V. B., and Verma, I. M. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 2629–2634
13. Li, H. H., Li, A. G., Sheppard, H. M., and Liu, X. (2004) *Mol. Cell.* **13,** 867–878
14. Hublitz, P., Kunowska, N., Mayer, U. P., Müller, J. M., Heyne, K., Yin, N., Fritzsche, C., Poli, C., Miguet, L., Schupp, I. W., van Grunsven, L. A., Potiers, N., van Dorsselaer, A., Metzger, E., Roemer, K., and Schüle, R. (2005) *Genes Dev.* **19,** 2912–2924
15. Jain, A., Ma, C. A., Lopez-Granados, E., Means, G., Brady, W., Orange, J. S., Liu, S., Holland, S., and Derry, J. M. (2004) *J. Clin. Invest.* **114,** 1593–1602
16. 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
17. Terada, Y., Tatsuka, M., Suzuki, F., Yasuda, Y., Fujita, S., and Otsu, M. (1998) *EMBO J.* **17,** 667–676
18. Vagnarelli, P., and Earnshaw, W. C. (2004) *Chromosoma* **113,** 211–222
19. Ruchaud, S., Carmena, M., and Earnshaw, W. C. (2007) *Nat. Rev. Mol. Cell. Biol.* **8,** 798–812
20. Yasui, Y., Urano, T., Kawajiri, A., Nagata, K., Tatsuka, M., Saya, H., Furukawa, K., Takahashi, T., Izawa, I., and Inagaki, M. (2004) *J. Biol. Chem.* **279,** 12997–13003
21. Rui, Y., Xu, Z., Lin, S., Li, Q., Rui, H., Luo, W., Zhou, H. M., Cheung, P. Y., Wu, Z., Ye, Z., Li, P., Han, J., and Lin, S. C. (2004) *EMBO J.* **23,** 4583–4594

[3] L. Wu, C. A. Ma, and A. Jain, unpublished observations.

22. Fuller, B. G., Lampson, M. A., Foley, E. A., Rosasco-Nitcher, S., Le, K. V., Tobelmann, P., Brautigan, D. L., Stukenberg, P. T., and Kapoor, T. M. (2008) *Nature* **453,** 1132–1136
23. el-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W., and Vogelstein, B. (1992) *Nat. Genet.* **1,** 45–49
24. Ando, K., Ozaki, T., Yamamoto, H., Furuya, K., Hosoda, M., Hayashi, S., Fukuzawa, M., and Nakagawara, A. (2004) *J. Biol. Chem.* **279,** 25549–25561
25. Honda, R., Körner, R., and Nigg, E. A. (2003) *Mol. Biol. Cell.* **14,** 3325–3341
26. Wheatley, S. P., Henzing, A. J., Dodson, H., Khaled, W., and Earnshaw, W. C. (2004) *J. Biol. Chem.* **279,** 5655–5660
27. Wilkinson, R. W., Odedra, R., Heaton, S. P., Wedge, S. R., Keen, N. J., Crafter, C., Foster, J. R., Brady, M. C., Bigley, A., Brown, E., Byth, K. F., Barrass, N. C., Mundt, K. E., Foote, K. M., Heron, N. M., Jung, F. H., Mortlock, A. A., Boyle, F. T., and Green, S. (2007) *Clin. Cancer Res.* **13,** 3682–3688
28. Sykes, S. M., Mellert, H. S., Holbert, M. A., Li, K., Marmorstein, R., Lane, W. S., and McMahon, S. B. (2006) *Mol. Cell.* **24,** 841–851
29. Tang, Y., Luo, J., Zhang, W., and Gu, W. (2006) *Mol. Cell.* **24,** 827–839
30. Gu, W., and Roeder, R. G. (1997) *Cell* **90,** 595–606
31. Katayama, H., Ota, T., Jisaki, F., Ueda, Y., Tanaka, T., Odashima, S., Suzuki, F., Terada, Y., and Tatsuka, M. (1999) *J. Natl. Cancer Inst.* **91,** 1160–1162
32. Lee, E. C., Frolov, A., Li, R., Ayala, G., and Greenberg, N. M. (2006) *Cancer Res.* **66,** 4996–5002
33. Ikezoe, T., Yang, J., Nishioka, C., and Yokoyama, A. (2010) *Int. J. Hematol.* **91,** 69–77
34. Cho, Y., Gorina, S., Jeffrey, P. D., and Pavletich, N. P. (1994) *Science* **265,** 346–355
35. Kitayner, M., Rozenberg, H., Kessler, N., Rabinovich, D., Shaulov, L., Haran, T. E., and Shakked, Z. (2006) *Mol. Cell.* **22,** 741–753
36. Stewart, S., and Fang, G. (2005) *Cancer Res.* **65,** 8730–8735
37. Ou, Y. H., Chung, P. H., Sun, T. P., and Shieh, S. Y. (2005) *Mol. Biol. Cell.* **16,** 1684–1695
38. Phan, R. T., and Dalla-Favera, R. (2004) *Nature* **432,** 635–639