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Transcriptional repression of *Aurora-A* gene by wild-type p53 through directly binding to its promoter with histone deacetylase 1 and mSin3a

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In this study, we firstly showed that p53 transcriptionally represses *Aurora-A* gene expression through directly binding to its promoter. DNA affinity precipitation assay and chromatin immunoprecipitation assay indicated that p53 physically bound to the *Aurora-A* promoter. Moreover, the *in vitro* and *in vivo* assays showed that p53 directly bound to the *Aurora-A* promoter together with histone deacetylase 1 (HDAC1) and mSin3a as corepressors. Furthermore, we identified that the nucleotides −360 to −354 (CCTGCCC), upstream of the *Aurora-A* transcriptional start site, was responsible for the p53-mediated repression. Mutation within this site disrupted its interaction with p53, mSin3a and HDAC1, as well as attenuated the repressive effect of p53 on *Aurora-A* promoter activity. Treatment with trichostatin A (TSA), a HDAC1 inhibitor, disrupted the interaction of p53-HDAC1-mSin3a complex with the nucleotides −365~-345 region, and enhanced the *Aurora-A* promoter activity and gene expression. Additionally, knockdown of p53 or mSin3a also drastically blocked the formation of p53-HDAC1-mSin3a repressive complex onto this promoter region and elevated the *Aurora-A* promoter activity and gene expression. Moreover, the p53-HDAC1-mSin3a repressive complex also involved in the inhibition of *Aurora-A* gene expression upon cisplatin treatment. Finally, the clinical investigation showed that *Aurora-A* and p53 exhibited an inverse correlation in both the expression level and prognostic status, and the low p53/high *Aurora-A* showed the poorest prognosis of NSCLC patients. Our findings showed novel regulatory mechanisms of p53 in regulating *Aurora-A* gene expression in NSCLC cells.

Introduction

The Aurora family is an evolutionally conserved serine/threonine kinase from yeast to *C. elegans, Drosophila, Xenopus* and mammals.[1] Three types of Aurora kinase are identified in human, named *Aurora-A*, *Aurora-B* and *Aurora-C*. The amplification and upregulation of *Aurora-A* are common characteristics in many human cancers including lung, breast, ovarian, colon, liver, head and neck, prostate cancer and leukemia; however, the overexpression of *Aurora-A* is more frequent than amplification.[2–7] Furthermore, the high expression of Aurora-A has been considered as a prognostic or diagnostic maker of many cancers such as bladder, breast, liver, colorectal and lung cancer.[8–10] Several studies demonstrate that overexpression of *Aurora-A* causes cellular transformation and tumorigenesis in nude mice, suggesting that *Aurora-A* is an oncogene.[11,12] These observations indicate that the deregulation of *Aurora-A* expressed levels might be one of the initial factors in priming cellular transformation. Regarding transcriptional activation, several transcription factors such as E2F3, E4TF1 and TRAP220/MED1 have been identified in the regulation of *Aurora-A* gene expression.[13–15] All these molecules are able to bind to *Aurora-A* promoter directly and stimulate its RNA expressed level. However, whether other factors contribute to the dysregulated high expression of *Aurora-A* in lung cancer cells remains to be explored.

The loss of function (including mutation and depletion) of tumor suppressor p53 was found in over half of all human

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What's new?
Many types of cancer feature a boost in production of the kinase Aurora-A, and several studies have implicated the protein in transformation and tumorigenesis. Some evidence suggests that p53 affects Aurora-A expression, and in this study, the authors set out to describe that relationship. They found that p53 binds directly to the Aurora-A promoter, repressing transcription. Next, they showed that treatment with trichostatin A thwarts this repression, allowing Aurora-A transcription to proceed. Clinical investigation revealed that lung cancer patients with low p53 and high Aurora-A expression had the worst prognosis.

cancers and strongly affected the sensitivity of tumor cells to chemotherapeutic and radio therapeutic agents (reviewed in[16,17]). The p53 is known to act biochemically as a nuclear transcription factor which can be activated in response to DNA damage, hypoxia, and abnormal expression of oncogenes.[18,19] The transcriptional targets induced by activated p53 include p21, GADD45, Fas, Puma and Bax, which are involved in arresting cell cycle or inducing apoptosis (reviewed in[20]). The consensus of the p53 DNA binding sequence of transactivated target genes is usually composed of two separated sites 5′-RRRCWWGYYY-3′ (R = purines; W = adenine or thymine; Y = pyrimidine) followed by a spacer of 0–21 bps between them.[21] These canonical p53 response elements were often arranged in a head to head (H-H) manner.[21] Previously, a set of p53 transcriptionally repressed genes, including, *stathmin,*[22] *Map4,*[23] heat shock protein *HSP90AB1,*[24] *MDR1* (Multiple Drug Resistance 1),[25] surviving,[26] *c-Myc*[27] and *TCTP,*[28] have been also identified. Unlike transcriptional activation, several studies demonstrate that the transcriptional repression by p53 is independent of the canonical consensus-binding motif.[22,29,30] The mechanisms of the p53-mediated transcriptional repression remain largely unclear. It has been reported that p53 can directly repress the expression of target genes such as stathmin and MAP4 via recruiting mSin3a and HDAC1 as corepressors;[22] however, the corresponding responsive elements were not identified yet.

Both p53 and Aurora-A are considered as prognostic and diagnostic factors in many cancers including lung cancer;[10,31–37] whether and how these two proteins functionally co-involved in the progress of lung cancer remained unclear. It has been shown that *Aurora-A* negatively regulates the p53 functionality *via* destabilizing p53 or blocking its binding ability to the target sequence.[38,39] Furthermore, *Aurora-A* exhibits reverse expressional relationships with p53 in human bladder cancer and hepatocellular carcinoma,[40,41] indicating that the inhibition of p53 by *Aurora-A* is a critical event in cellular transformation. On the contrary, Patrick *et. al.* have mentioned that formation of tetraploid induced by overexpression of *Aurora-A* could only be observed in $p53^{-/-}$ cells, suggesting an inhibitory role of p53 on *Aurora-A*-induced cellular transformation.[42] The mechanisms underlying how p53 regulates *Aurora-A* function and expression, especially in lung cancer, however, are not well characterized yet. Our previous report demonstrates that p53 may indirectly repress the expression level of *Aurora-A* via both p21-CDK-Rb-E2F3 and Fbw7-proteasome pathways.[43] In the present study, we identified *Aurora-A* as a new transcriptionally repressed gene of the p53 tumor suppressor in human lung cancer cells. Our observations provide evidence that p53 directly binds to the nucleotides −360∼-354 upstream region of the *Aurora-A* promoter *in vivo* and represses the promoter through a mechanism that involves recruiting the corepressor mSin3a and HDAC1.

Materials and Methods

Cell culture and reagents

The human lung cancer cell lines including A549, and p53-knockdown A549, p53-null H1299 and non-tumorous lung epithelial cell line BEAS-2B were maintained in RPMI 1640 medium (Gibco/BRL, MD, U.S.A.); the Hela cells and HEK 293 cells were maintained in DMEM medium (Gibco/BRL, MD, U.S.A.). Both media were supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Utah, U.S.A.), 2 mM glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin), at 37°C in a humidified atmosphere of 5% CO_2. PFT and cisplatin were purchased from Sigma (St. Louis, MO, USA) or Merck (NJ, USA).

Tissue array

Two commercialized human lung cancer tissue arrays (LC1501, LC482t, Biomax, Rockville, MD, U.S.) were obtained. The tissue samples were double-stained with p53 and *Aurora-A* simultaneously by MaxDS M&R Double staining polymer detection kit (w/DAB&AP-red) (MaxVision Biosciences Inc. Washington, DC. USA). The immunohistochemistry (IHC) stain was performed according to the manufacture's instruction. The stained arrays were observed under phase-contrast microscopy, and the ratio of *Aurora-A* or p53 stained area in each tissue section was then estimated as elsewhere.[44]

Chromatin immunoprecipitation (ChIP) assay

This procedure was performed as described elsewhere.[45] Briefly, the cells were cross-linked and subjected to sonication followed by immunoprecipitation using 2 μg ChIP-grade anti-p53 (sc-126x), HDAC1 (sc-8410x) or mSin3a (sc-767x) antibody (Santa Cruz, Dallas, Texas), respectively. The IP-bead complex was then, washed and added to 150 μl SDS

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elution buffer (1% SDS, 0.1 M $NaHCO_3$) and incubated in a shaking water bath overnight at 67°C to reverse the crosslink. The supernatants were then purified by DNA-clean UP kit (promega) following the manufacturer's instructions. The quantitative Polymerase Chain Reaction (qPCR) was then performed on the ChIP-enriched DNA via an Applied Biosystems StepOnePlus^{TM} machine by using primer to amplify the region located at the *Aurora-A* or p21 promoter, respectively. The corresponding primers are as follows: For p21 promoter: (Forward: GTGGCTCTGATTGGCTTTCTG, Reverse: CTGAAAACA GGCAGCCCAAG). For Aurora-A promoter: (Forward: TC TCGCCATCTTACTTACTG, Reverse: TATCGGTGAAGCAACAGC). For Aurora-A promoter randomized primer: (Forward: GGTTCCCTATTCTCC CT, Reverse: TGCCCGTGAAGAATAGTGA)

Quantitative polymerase chain reaction (qPCR)

Total cellular RNA was extracted by a RNA-BeeTM RNA isolation kit (TEL-TEST, Friendswood, TX) in accordance with the manufacturer's instructions. One microgram of total RNA was reverse-transcribed using Advantage RT for PCR Kit (Clontech, Mountain View, CA) at 42°C for 1 hr. The qPCR primers for *Aurora-A* were forward (847): TCTTCCAGGA GGACCACTCTCT and reverse (917): TGCATCCGACCTTCAATCATT. The mRNA levels were also determined by real-time PCR with an ABI PRISM 7900 Sequence Detector system according to the manufacturer's instructions. The following quantitative PCR procedure was performed via a Applied Biosystems StepOnePlus^{TM} qPCR machine. β-actin was used as an endogenous control and the primer sequence were forward: 5′-TTCTACAATGAGCTGCGTGTG-3′ and reverse: 5′-GGGGTGTTGAAGGTCTCAAA-3′. The relative gene expression level (the amount of target, normalized to endogenous control gene) was calculated using the comparative Ct method formula E-ΔΔCt.

Cytosolic and nuclear fractionation

The cells were scraped with cold PBS and collected by centrifugation at 2,000 xg for 5 min followed by resuspension into hypotonic buffer (10 mM Hepes-KOH, 10 mM NaCl, 1 mM KH2PO4, 5 mM NaHCO3, 1 mM CaCal2 and 0.5 mM MgCl2). The cells were then homogenized with 10 strokes in a Dounce homogenizer after 5 min incubation on ice and repeated once. Cells were then spun at 2,000*g* for 15 min. The supernatant was collected as cytosol fractions. The pellet of the low-speed centrifugation was washed twice and resuspended in nuclear isolation buffer (10 mM Tris, pH 7.5, 300 mM sucrose, 0.1% Nonidet P-40) followed by stroking with a homogenizer and incubation on ice for 20 min. The mixture was then centrifuged at 14,000 xg for 30 min; the supernatant was then collected as a nuclear fraction.

Immunoprecipitation

Experimental cells were collected and lyzed with immunoprecipitation-lysate buffer (20 mM Tris-Cl, pH 7.4, 135 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol and 1% Triton X-100) with protease inhibitor cocktail. 250 μg of lysates was then precleaned with protein A/protein G beads for 1 hrs at 4°C. The supernatants were then removed to a new eppendorf in the presence of either anti-p53 (sc-126) or anti-HADAC1 (sc-8410) and protein A/protein G beads o/n at 4°C. The immune complex was then resolved via SDS-PAGE and western blot. To avoid the shielding effect of antibody heavy chain, the 2nd antibody for this IP-western blot was AffiniPure Goat Anti-Mouse IgG, Light Chain specific or IgG Fraction Monoclonal Mouse Anti-Rabbit IgG, Light Chain Specific (Jackson), respectively.

DNA affinity purification assay

150 μg of the nuclear extracts was incubated with streptavidin for 1 hr at 4°C. The supernatant was then transferred to another new eppendorf tube and incubated with 1 μg of corresponding biotinylated DNA probes by PCR or chemical synthetic method for 1 hrs at 4°C followed by adding streptavidin for another 1 hr at 4°C. The complex was then washed with 1XTBST, added with SDS-loading dye and subjected to the SDS-PGAE and western blot analysis using anti-p53 antibody.

Transfection procedure and luciferase reporter assay

In the usual transfection procedure, the different forms of p53 plasmid or p53 specific siRNA were transfected into the tested cell lines by Lipofectamine 2000 (Invitrogen) for 24 hr. On the other hand, the full length, serial deletions or mutation form of *Aurora-A*–promoter luciferase vehicle were co-transfected with CMV-PRL control vehicle into A549, A549-shRNA or A549-p53shRNA cells by Lipofectamine 2000 (Invitrogen) for 24 hrs and then the transfection solution was replaced with fresh culture medium. After the desired experiments were conducted, the luciferase activity of each experimental set of cells was determined and normalized using the dual luciferase assay system according to the manufacturer's protocol (Promega, USA) by a luminometer (Minilumate LB 9506, Germany).

Meta-Analysis

In GSE50081, expression profiling platform is [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array, and was performed on RNA from frozen, resected tumor tissues corresponding to 181 Stage I and II NSCLC cases were collected at University Health Network (UHN181). In GSE19188, a genome-wide gene expression analysis was performed on a cohort of 91 patients that including 91 tumor and 65 adjacent normal lung tissue samples. Expression profiles in of 226 lung adenocarcinomas (127 with EGFR mutation, 20 with KRAS mutation, 11 with EML4-ALK fusion and 68 triple negative cases) were enrolled in GSE31210 dataset. In the TCGA cohort, the expression profile was performed by RNA sequencing using samples of 513 lung adenocarcinoma patients. Clinical outcome of lung adenocarcinoma cancer

patients with various *Aurora-A* and p53 expression status was analyzed by Kaplan-Meier plotter. On the Kaplan-Meier plotter website, gene symbol AURKA and TP53 were analyzed in the dataset GSE50081 and GSE19188, respectively. We split the patients enrolled in the datasets by "median" which subdivided the case number into half of high and low expression. Other parameters are according the default. Probe ID: 204092_s_at for *Aurora-A* and 201746_at for *p53* in GSE50081 were studied. In addition, probe ID: 204092_s_at for *Aurora-A* and 211300_s_at for *p53* were used to validate the results in GSE19188. In GSE31210 database, *Aurora-A* and p53 expression data were retrieved using 204092_s_at and 211300_s_at, respectively. For the study of relative *Aurora-A* and p53 expression, microarray data with 513 lung adenocarcinoma cases was downloaded from The Cancer Genome Atlas (TCGA) website. [genomic_TCGA_LUAD_exp_HiSeqV2_percentile_clinical]. Correlation coefficient was analyzed using Spearman's rho (2-tailed).

Statistical analysis

Figures were generated from at least 3 independent experiments with similar pattern. All data are presented as means ± S.D. of 9 replicates from 3 separate experiments. Statistical differences were evaluated using Student's *t test* (* denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$; these were considered significant) or by the calculation and grouped using the SAS program.

Results

Negative regulation of *Aurora-A* by p53 in a transcriptional level

We first analyzed the effects of p53 on *Aurora-A* expression in parental and p53-knockdown A549 cells. As shown in Figure 1*a*, parental and vector control A549 cells expressed low levels of *Aurora-A* mRNA and protein. However, A549-p53shRNA stable clones showed a significant increase in *Aurora-A* mRNA and protein levels. Consistently, transient knockdown of p53 by specific siRNAs also increased both protein and mRNA levels of *Aurora-A* (Fig. 1*b*), ruled out the possibility of clonal effects of p53-knockdown sublines. In addition, the knockdown of p53 also induced the expression levels of Aurora-A in a non-tumorous lung epithelial cell line BEAS-2B (Supporting Information Fig. 1). Furthermore, ectopic expression of wild-type p53 in H1299 cells (p53-null) dose-dependently reduced both protein and mRNA levels of *Aurora-A* (Fig. 1*c*). These results indicated that the inversely expressional correlation between p53 and Aurora-A is ubiquitous in both normal and tumorous lung cells.

To define if the decrease of *Aurora-A* mRNA level following p53 expression is because of repression of *Aurora-A* promoter activity, a 1.4 kb *Aurora-A* promoter region (+15∼-1400 bp) was cloned into luciferase reporter vector pGL3-basic followed by transfecting into parental or A549-p53shRNA cells. As shown in Figure 2*a*, up to three-fold increase in luciferase activity in p53-knockdown A549 cells were observed in comparison with that of parental cells, indicating that p53 inhibited *Aurora-A* expression might be through a transcriptional repression manner. Next, the A549 cells were transiently transfected with p53 siRNAs followed by transfection of *Aurora-A* promoter luciferase vector. The luciferase activity of *Aurora-A* promoter increased (about 3–5 folds) when the p53 protein levels were reduced (Fig. 2*b*). Similar results were also observed in HEK293 cells (data not shown). However, ectopic expressed wild-type p53 decreased the *Aurora-A* promoter luciferase activity in H1299 cells (Fig. 2*c*), indicating that p53 inhibited *Aurora-A* expression in a promoter repression manner, and this effect was physiological and not clonal specific.

To further characterise the functional status of p53 in the regulation of *Aurora-A* promoter activity, the wild-type, R175H or R280K p53 (both are oncogenic forms of p53[46]) constructs were transfected into H1299 cells together with *Aurora-A* promoter luciferase vector. The result demonstrated that wild-type p53 downregulated the *Aurora-A* promoter activity to 50% (Fig. 2*d*), whereas p53-R175H showed no effects on the *Aurora-A* promoter. Interestingly, p53-R280K induced the *Aurora-A* promoter activity. Next, we investigated whether endogenous p53 induction could suppress *Aurora-A* expression, A549 cells transfecting with *Aurora-A* promoter-luciferase construct were treated with cisplatin (a p53 activator) and PFT (a p53 functional inhibitor). As shown in Figure 2*e*, the *Aurora-A* promoter activity was decreased when p53 activated while increased when p53 function was inhibited. These results demonstrate that the wild-type but not mutant p53, contributed to the transcriptional repression of the *Aurora-A* promoter.

Aurora-A is downregulated by direct binding of p53 to its promoter

Next, the DNA Affinity Precipitation Assay (DAPA) was performed by using a 1.4 kb *Aurora-A* promoter to test whether p53 is physically binding onto the *Aurora-A* promoter. As indicated in Figure 3*a*, the *Aurora-A* promoter formed complex with p53 in A549 cells; however, such interaction was limited in A549-p53shRNA cells. To functionally identify the region upstream *Aurora-A* gene promoter responsible for p53-mediated repression, a series of 5′-end deletion mutants (Fig. 3*b*) were constructed into pGL3-luciferase vector followed by transfecting into HEK293 cells. Deletion analysis of this promoter showed that the truncated versions (from the −1.4 kb to −0.4 kb) were negatively regulated by p53. However, the luciferase activity drastically increased when deletion of 200 base pairs from −0.4 kb to −0.2 kb (Fig. 3*b*), indicating that −400 to −200 region of the *Aurora-A* promoter contained an element responsible for p53-mediated repression. No typical "head-to-head" or "head-to-tail" canonical binding sequence for p53[21] was found in this region. However, nucleotide sequence analysis via website ALGGEN (http://alggen.lsi.upc.es/) indicated that six predicted p53

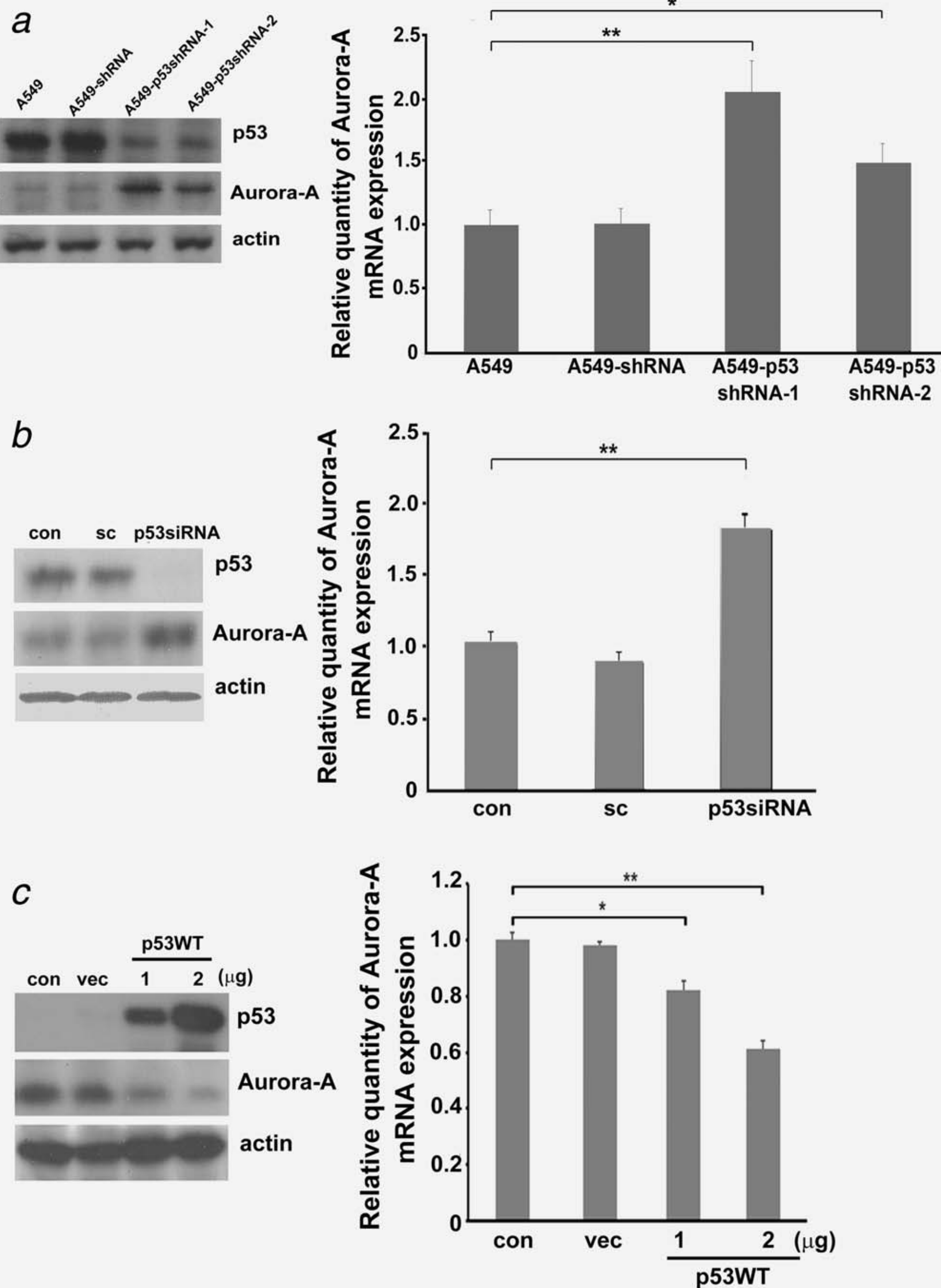

Figure 1. The inverse correlation between p53 and *Aurora-A* gene expression in lung cancer cells. (*a*) Knockdown of p53 increases Aurora-A expression in both protein and mRNA levels. The cell lysates of wild-type p53 and p53 shRNA knockdown A549 cells were collected, the mRNA and protein levels of p53 and Aurora-A were examined by Western and real-time PCR (RT-PCR), respectively. (*b*) Transient knockdown of p53 by p53-specific siRNA induces Aurora-A expression. The parental (con), p53-siRNA or scramble RNA (sc)-transfected A549 cells were collected and the mRNA and protein levels of p53 and Aurora-A were then determined (*c*) Overexpression of wild-type p53 decreases Aurora-A expression. The parental or p53-transfected H1299 cells were isolated 48 hrs after transfection, and then the mRNA and protein levels of p53 and Aurora-A were analyzed, respectively.

response elements located at the *Aurora-A* promoter region (Fig. 3*c*, upper panel). The DAPA were then performed to test the possibility of these putative-binding sites for p53-binding. As expected, the −365∼–345 (containing −360∼–354 core sequence) of the *Aurora-A* promoter exhibited the highest binding affinity for p53 in comparison with other five regions (Fig. 3*c*). That binding of the *Aurora-A* promoter by p53 was sequence-specific supported by the finding that the interaction of this region to p53 was dose dependently and it could be eliminated by incubation with a 5-fold excess of unlabeled same

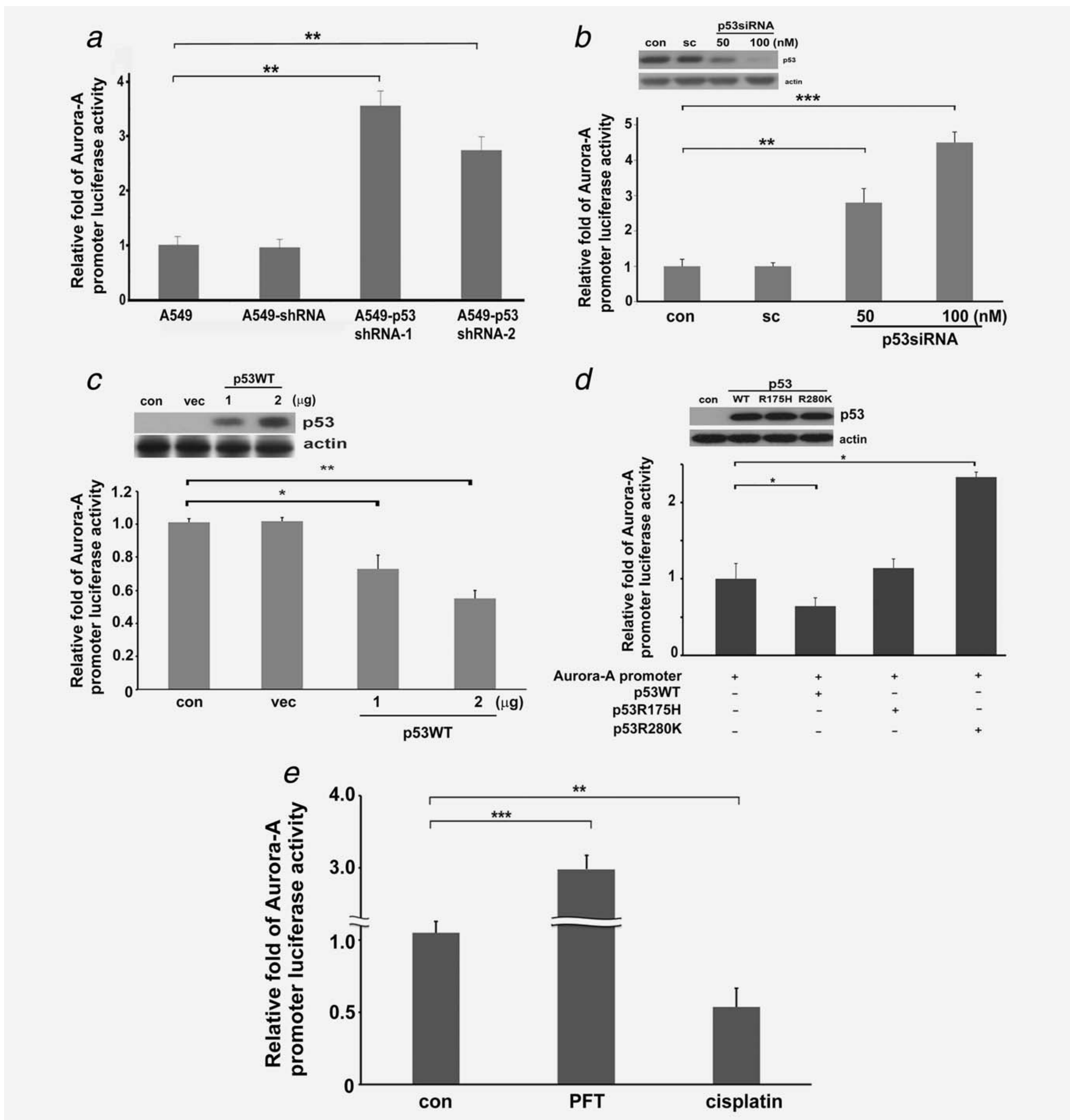

Figure 2. **Wild-type but not mutated p53 represses *Aurora-A* promoter activity.** (*a*) p53 represses *Aurora-A* promoter activity. The pGL3-*Aurora-A* promoter (–1400∼ +1 bp) transfected wild-type, shRNA and p53-knockdown A549 cells were subjected to a luciferase activity assay. The luciferase activity of each experimental set was normalized with parental A549 cells and presented as fold change. (*b*) The parental or p53-siRNA transfected A549 cells were transiently transfected with pGL-3-*Aurora-A*-promoter reporter. The cells were then collected and subjected to Western blot (upper panel) or luciferase activity assay (lower panel). (*c*) Overexpression of wild-type p53 inhibits *Aurora-A* promoter activity. The pGL3-*Aurora-A* promoter-transfected H1299 cells were transfected without or with wild type p53 (p53WT) for indicated time periods. The cells were collected and subjected into Western blot or luciferase assay. (*d*) Wild-type p53 but not mutant p53 represses *Aurora-A* promoter activity and gene expression. The pGL3-*Aurora-A* promoter vector-transfected H1299 cells were transfected without or with equal amount of wild type or mutant p53 (R175H or R280K) vector. The cell lysates were then collected and subjected to Western blot or luciferase assay. (*e*) Modulation of p53 function or expression by p53 inhibitor PFT and DNA damage agent cisplatin affects *Aurora-A* promoter activity. The pGL-3-*Aurora-A*-promoter-tranfected A549 cells were subcultured into three 6-cm petri-dishes equally followed by treatment with PFT (50 μM) or cisplatin (10 μM) for indicated time periods. The cells were then collected and subjected to a luciferase activity assay. For all experiments, aata are representative or expressed as the mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$ or ***$p < 0.01$, compared with control group.

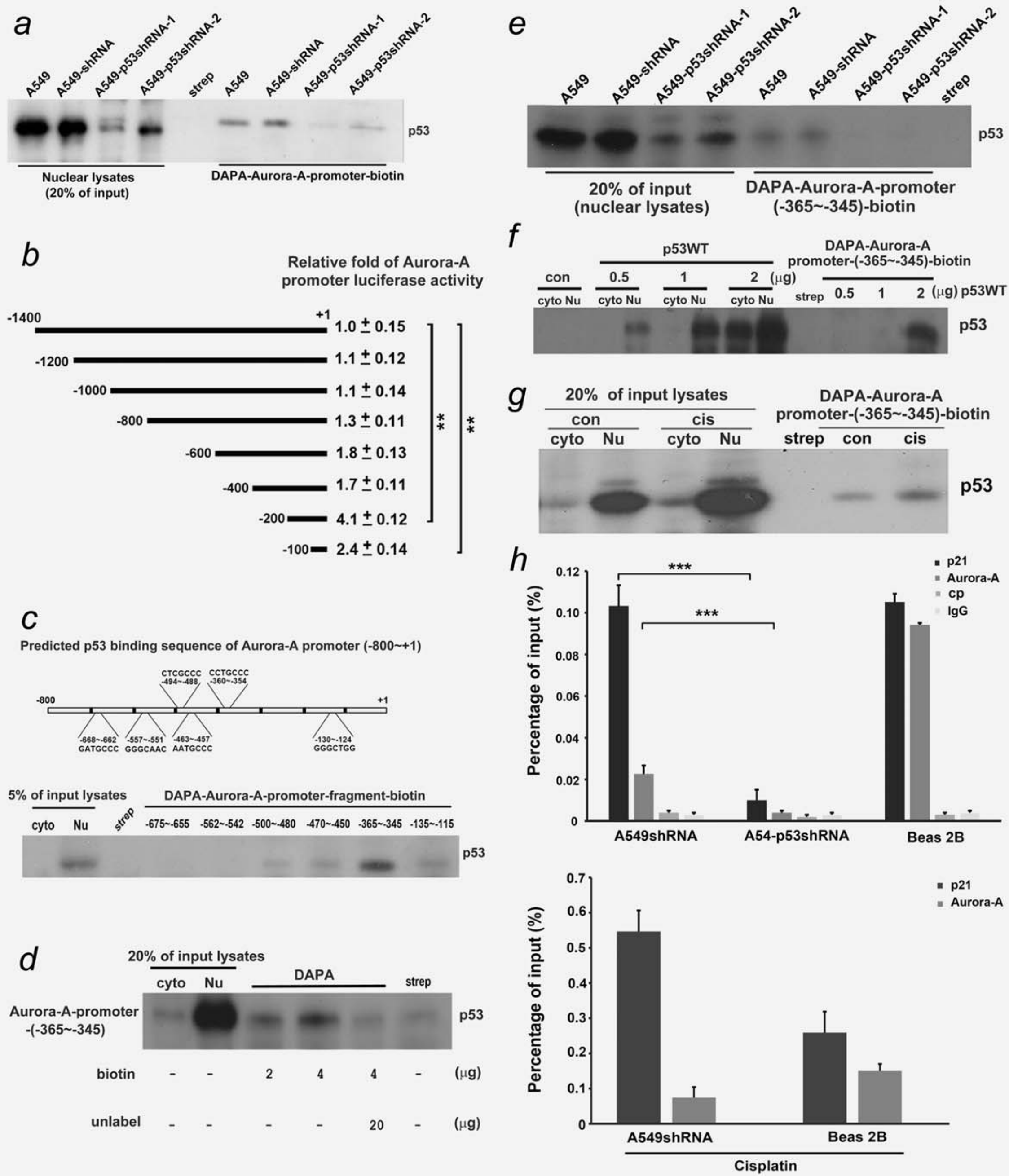

Figure 3. Direct binding of p53 to *Aurora-A* promoter *in vitro* and *in vivo*. (*a*) Determination of p53 direct binding in *Aurora-A* promoter by DAPA analysis. The 1.4 kb upstream region of *Aurora-A* promoter DNA fragment was labelled with biotin prior to incubate with the nuclear lysates of A549, A549-shRNA, A549-p53shRNA-1 and A549-p53shRNA-2 cells. The reaction mixtures were then analyzed by DAPA assay using anti-p53 antibody. (*b*) A repressor element located within −200 to −400 upstream of *Aurora-A* promoter. The HEK293 cells transfecting with the 5'-serial-deleted pGL3-*Aurora-A* promoter luciferase were harvested and the relative luciferase activities were assessed. (*c*) Define the precise p53 binding sequence. The DNA probes contained various predicted binding regions of *Aurora-A* promoter for p53 (upper panel) were biotinylated and incubated with A549 nuclear lysates. The DAPA analysis was conducted using anti-p53 antibody (lower panel). (*d*) p53 binding sequences located at −365 to −345 region. The biotinylated −365 to −345) x3 *Aurora-A* promoter probe was incubated with A549 nuclear lysates alone or with 20-fold excess of same probe without biotinylation. The DAPA analysis was then performed using anti-p53 antibody. (*e*) Knockdown of endogenous p53 reduces p53 binding to −365 to −345region. The biotinylated −365 to −345) x3-*Aurora-A* promoter probe was and incubated with the nuclear lysates of wild type or p53-knockdown A549 cells, respectively. The DAPA analysis was carried out using anti-p53 antibody. (*f*) Ectopic expressed wild-type p53 binds to −365∼-345 region of *Aurora-A* promoter in H1299 cells. The wild-type p53-transfected H1299 cells were harvested and the nuclear lysates were extracted followed by incubating with biotinylated (–365 to −345) x3-*Aurora-A* promoter probe. The DAPA assay was done using anti-p53 antibody. (*g*) DNA damage agent cisplatin increases p53 binding to *Aurora-A* promoter −365∼-345 region. The nuclear lysates of parental or cisplatin treated A549 cells were incubated with biotinylated-(–365 to −345) x3-*Aurora-A* promoter probe to perform DAPA using p53 antibody. (*h*) Identification of p53 directly binds to *Aurora-A* promoter by chromatin immunoprecipitation assay. Upper panel, the A549shRNA, A549-p53shRNA-1 and non-tumorous Beas 2B cells were subjected to chromatin immunoprecipitation using anti-p53 antibody. The quantitative PCR of Aurora-A or p21 promoter was then performed using eluted DNA fragments from immunoprecipitated complex; the primers were listed in materials and methods. Bottom panel, the cisplatin treated A549 or Beas 2B cells were subjected to chromatin immunoprecipitation using anti-p53 antibody followed by quantitative-PCR. IgG represented as immunoprecipitation control and cp represented as randomized primers of Aurora-A promoter. For all experiments, data are representative or expressed as the mean ± SEM of three independent experiments. ***$p < 0.01$, compared with control group.

probe (Fig. 3*d*). These results indicated that −365∼-345 region of *Aurora-A* promoter was a p53 direct binding site *in vitro*.

The p53-binding site in *Aurora-A* promoter is responsible for p53-mediated suppression of *Aurora-A* promoter activity

Next, the DAPA was conducted to compare the binding ability of p53 onto full (-1400∼+15) or fragment (-365∼-345 region) of *Aurora-A* promoter. The results showed the −365∼-345 DNA fragment of *Aurora-A* promoter exhibited strong interaction with p53 in A549 cells in a higher level than that of A549-p53shRNAp53 cells, similar with that of the full length (-1400∼+15) of *Aurora-A* promoter (Figs. 3*e* and 3*a*). Besides, the ectopic expression of p53 also interacted with −365∼-345 region of *Aurora-A* promoter in a dose-dependent manner (Fig. 3*f*). Our previous study has shown that a DNA damage agent cisplatin induces p53 activation while reduces the expression and promoter activity of *Aurora-A*[47] (Fig. 2*e*); thus, to test the role of −365∼-345 *Aurora-A* promoter region in p53-mediated repression under DNA damage stress, the A549 cells were treated with cisplatin followed by the DAPA. Figure 3*h* showed that cisplatin increased the interaction between p53 and −365∼-345 *Aurora-A* promoter *in vitro* (Fig. 3*g*). To examine whether p53 binds to the *Aurora-A* promoter *in vivo*, the chromatin-immunoprecipitation (ChIP) assay was performed. The complex of p53 with *Aurora-A* promoter could be detected in both A549 and Beas 2B cells; however, such interaction was limited in p53-knockdown A549 cells (Fig. 3*h*, upper panel). Besides, our results also showed that p53 bind to p21 promoter in a higher degree than Aurora-A promoter in both A549 and Beas 2B cells. The treatment with cisplatin increased the bindings of p53 onto both Aurora-A and p21 promoter in these two cell lines. In A549 cells, the cisplatin induced about a five-folds binding affinity of p53 onto p21 and three-folds of that onto Aurora-A promoter, while only two-folds increase of binding of p53 onto both p21 and Aurora-A promoter upon cisplatin treatment in Beas 2B cells (Fig. 3*h* bottom panel). Finally, our combining results suggested that p53 directly binds to Aurora-A promoter and acts as a negative regulator in controlling *Aurora-A* gene transcription.

p53 Binds in a sequence-specific manner to the *Aurora-A* promoter

Next, we introduced mutation into −365∼-345 *Aurora-A* promoter region by PCR (Fig. 4*a* upper panel), and then the DAPA was carried out. The results showed that the interaction of p53 with the mutated *Aurora-A* promoter was dramatically decreased in comparison with the wild-type *Aurora-A* promoter (Fig. 4*a* lower panel). Besides, we found that the mutated promoter-driven luciferase activity was higher than that of wild-type-driven in HEK293, HeLa and A549 cells (Fig. 4*b*), suggesting that −365∼-345 region was responsible for the repression. Next, the wild-type or mutated *Aurora-A* promoter was cotransfected with wild-type p53 into H1299 cells followed by the luciferase assay. Figure 4*c* showed the mutated but not the wild-type *Aurora-A* promoter was significantly resistant to p53-mediated repression. Besides, treatment with cisplatin reduced the activity of wild-type *Aurora-A* promoter more significant than that of mutated promoter (Fig. 4*d*). These results indicated that the −360∼-354 core region was required for p53-mediated repression of *Aurora-A* gene.

Trichostatin a inhibits p53-mediated repression of *Aurora-A* gene

Histone deacetylase 1 (HDAC1) is responsible for the stringent interaction between histone and chromatin to regulate gene expression.[48] Previously, p53-mediated transcriptional repression has been identified to associate with mSin3a and HDAC1.[22] To analyze whether transcriptional repression of *Aurora-A* gene by p53 involves a recruitment of histone deacetylases to its promoter, the effect of trichostatin A (TSA) on the *Aurora-A* gene repression was examined. In the presence of TSA, the levels of protein, mRNA and promoter activity of *Aurora-A* was upregulated (Fig. 5*a*). On the contrary, the ectopic expression of HDAC1 decreased the levels of protein, mRNA and promoter activity of *Aurora-A* (Fig. 5*b*). Next, the wild-type or mutated *Aurora-A* promoter-luciferase vector were transfected into A549 cells 24 hrs prior to TSA administration. As indicated in Figure 5*c*, TSA treatment led to increase in the activity of the luciferase gene driven by the *Aurora-A* promoter, indicating that this promoter was quite sensitive to TSA. However, the TSA did not affect the activity of mutated *Aurora-A* promoter significantly. Next, the effect of TSA on the interaction between p53 and *Aurora-A* promoter −365∼-345 region was examined by DAPA. As expected, TSA attenuated the binding of p53 onto the *Aurora-A* promoter (-365∼-345) region dose-dependently (Fig. 5*d*). Moreover, treatment with TSA disrupted the interaction of p53, together with mSin3a and HDAC1, onto the *Aurora-A* promoter −365∼-345 region (Fig. 5*e*). Data from ChIP assay also showed that TSA effectively attenuated the complex formation of p53, mSin3a and *Aurora-A* promoter (Fig. 5*f*). To confirm the direct interaction among p53, mSin3a, HDAC1 and *Aurora-A* promoter, the DAPA was performed using the biotinylated *Aurora-A* promoter (-365∼-345) as a probe with nuclear lysates from wild type or A549-p53shRNA cells. The results showed that large amount of p53, HDAC1 and mSin3a bound to the *Aurora-A* promoter (-365∼-345) region in A549 cells but limited in p53-knockdown A549 cells (Fig. 5*g*), suggesting that a complex of p53, mSin3a, and HDAC-1 bound onto the *Aurora-A* promoter. However, the mSin3a, HDAC1, and p53, all of these three molecules lost their ability to bind to the mutated *Aurora-A* promoter (-355 mutant) (Fig. 5*h*). In the following experiment, we analyze the binding of HDAC1 and mSin3a onto Aurora-A promoter in the absence of p53. The parental or p53 transient-knockdown A549 cells were subjected to ChIP assay using indicated antibody. The results showed that knockdown of p53 indeed significantly decreased

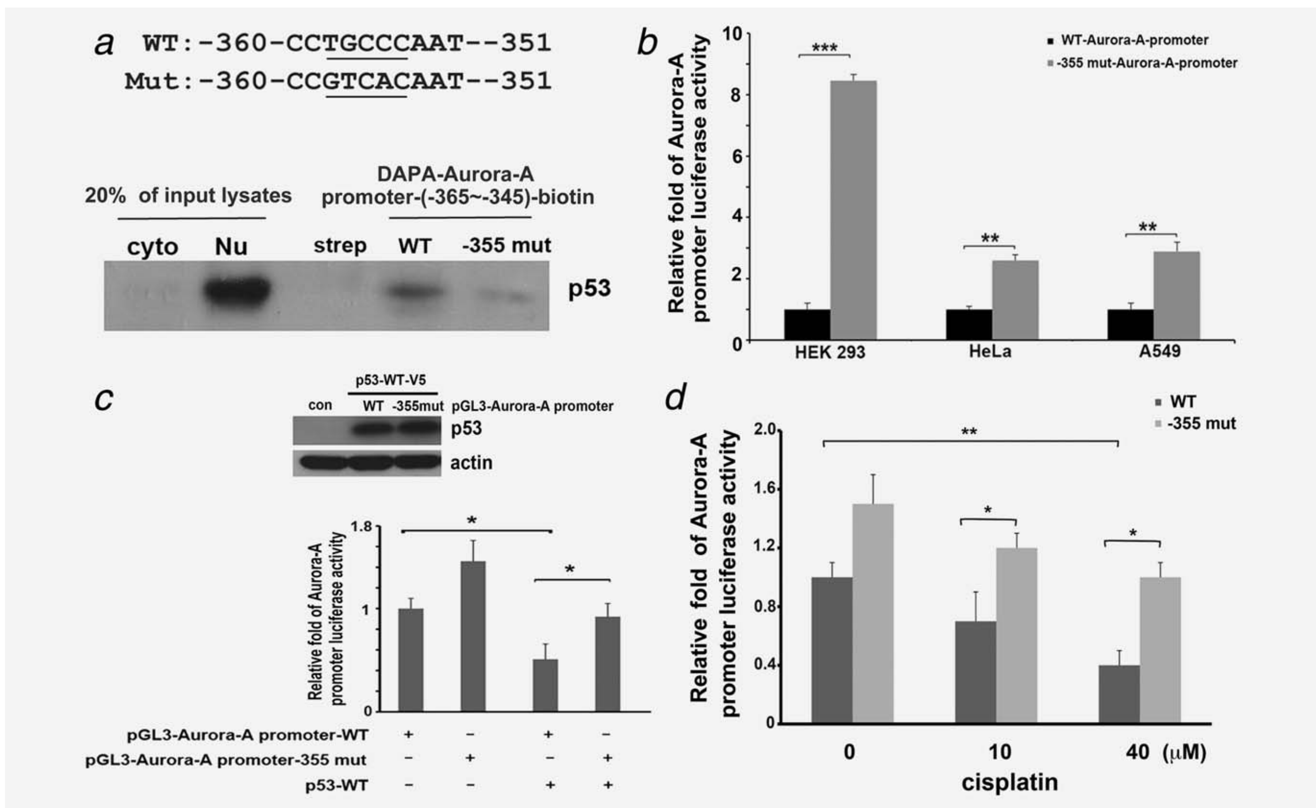

Figure 4. **p53 binds and represses the activity of *Aurora-A* promoter in a sequence-specific manner.** (*a*) Sequence of the *Aurora-A* promoter in the minimal p53-binding site. The sequence of wild type or −355 mutated *Aurora-A* promoter was depicted (upper panel). The biotinylated wild-type (WT) or −355 mutant (mut) of *Aurora-A* promoter-(−365 to −345) x3 probes were incubated with A549 cell nuclear lysates, the DAPA assay was conducted using anti-p53 antibody (lower panel). (*b*) Mutated −355 *Aurora-A* promoter abrogates *Aurora-A* promoter repression activity. The control or WT or −355 mut of pGL3-*Aurora-A* promoter (1.4 kb)- transfected HeLa, HEK293 or A549 cells subjected to luciferase activity assay. The luciferase activity of *Aurora-A*-355 mutant promoter was normalized with that of *Aurora-A* wild-type promoter in HeLa, HEK293 and A549 cells, respectively. (*c*) Mutated −355 *Aurora-A* promoter sequence attenuates p53-mediated repression of *Aurora-A* promoter activity. The WT or −355 mut of pGL3-*Aurora-A* promoter-transfected H1299 cells were transfected without or with p53-WT-V5 vector followed by Western blot or luciferase activity assay, respectively. (*d*) Mutated −355 *Aurora-A* promoter attenuates cisplatin-triggered repression of *Aurora-A* promoter activity. The WT or −355 mutant *Aurora-A* promoter luciferase-transfected A549 cells were administrating with cisplatin and subjected to luciferase activity assay. For all experiments, data are representative or expressed as the mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$ or ***$p < 0.01$, compared with control group.

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its binding capacity onto both Aurora-A and p21 promoter. Furthermore, the absence of p53 attenuated about 30% of the binding affinity of HDAC1 onto Aurora-A promoter while the knockdown of p53 almost blocked the binding of HDAC1 onto p21 promoter (Fig. 5*i*), similar results were also observed in mSin3a (data not shown). These results clearly demonstrated that p53, HDAC1 and mSin3a indeed forms complex with Aurora-A promoter in a physiological manner.

Knockdown of mSin3a blocks p53-mediated repression of *Aurora-A* promoter activity and gene expression

Next, we examined the role of mSin3a in p53-meditaed *Aurora-A* repression, as shown in Figure 6*a*, down-regulation of misn3a by siRNA dose-dependently increased the protein, mRNA and promoter activity of *Aurora-A*. Data from DAPA indicated that knockdown of mSin3a prevented the binding of both p53 and HDAC1 to the −365∼-345 region of *Aurora-A* promoter, suggesting that mSin3a is an important corepressor involving in p53-mediated repression of *Aurora-A* gene expression (Fig. 6*b*). Overall, these results revealed that the −365∼-345 region of the *Aurora-A* promoter might serve as a response element for the binding and repressive regulation of *Aurora-A* expression by the cooperation of p53, mSin3a and HDAC1.

Aurora-A and p53 exhibit an inverse correlation in expression and prognosis of NSCLC patients

To further address whether such inverse correlation of Aurora-A and p53 expression could be found in NSCLC patients, two different human lung cancer tissue arrays, which totally contain 99 cases (198 duplicated cores), were applied to examine the expression relation between p53 and Aurora-A by IHC staining. The results showed that the expression patterns between Aurora-A and p53 exhibited a mutually-exclusive like manner (Fig. 7*a*). The quantitative results indicated that the low Aurora-A expression (<25%

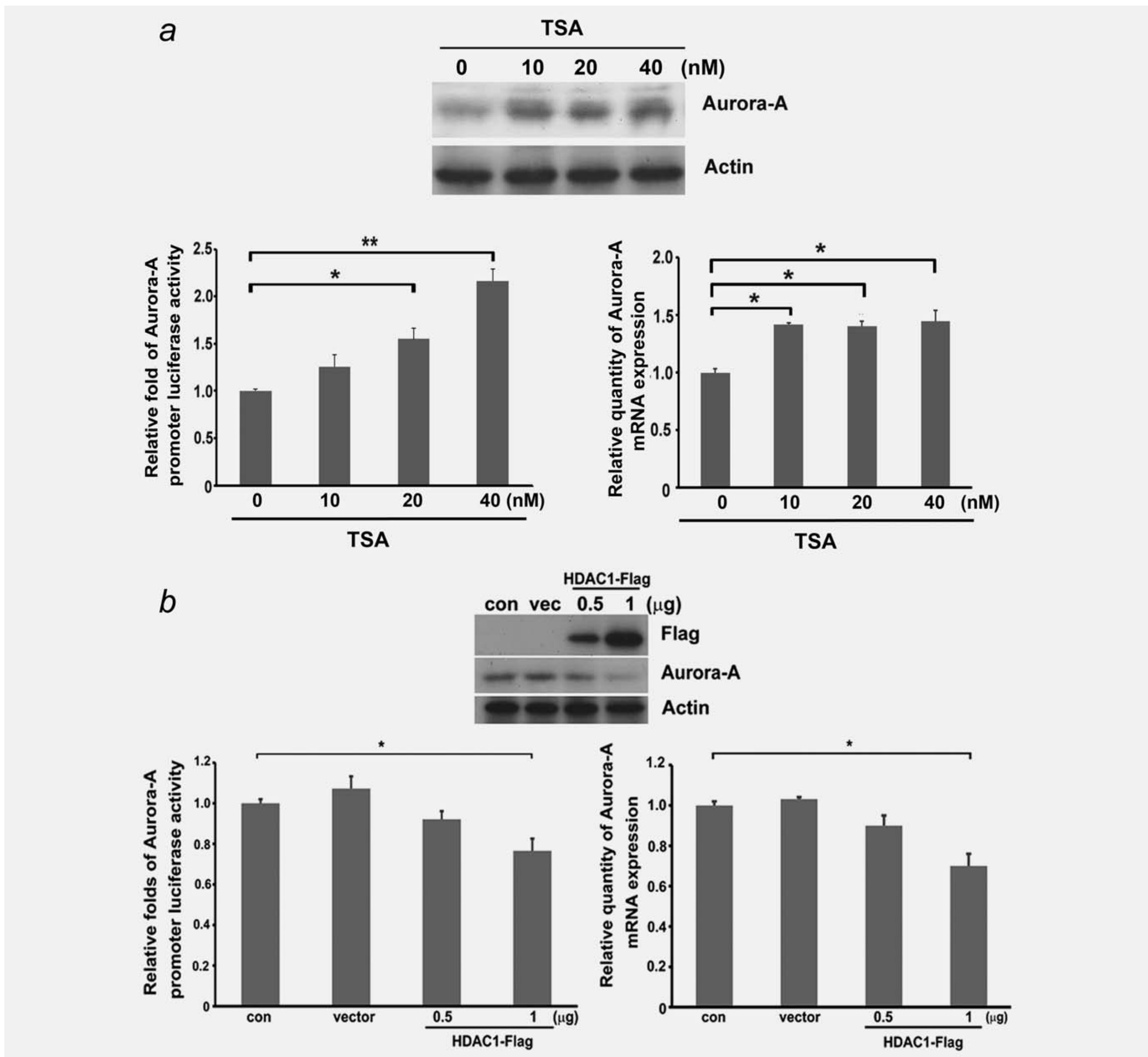

Figure 5. HDAC1 is a co-repressor with p53 in reducing expression and promoter activity of *Aurora-A*. (*a*) HDAC inhibitor TSA increases *Aurora-A* promoter activity and gene expression. The A549 cells were untreated or pretreated with TSA (20 nM) for 16 hrs. The cells were then collected and subjected to Western blot and real-time PCR to examine the protein (upper panel) and mRNA (lower left panel) levels of *Aurora-A*. Regarding to the promoter activity, the pGL3-*Aurora-A* promoter luciferase-transfected A549 cells were administrating with different dose of TSA and then subjected to luciferase assay (lower right panel). (*b*) Ectopic expression of HDAC1 reduces the levels of promoter, mRNA and protein of *Aurora-A*. The different dosages of HDAC1-Flag -transfected A549 cells were collected and subjected to the real-time PCR and Western blot, regarding to the promoter activity, the pGL3-*Aurora-A* promoter luciferase-transfected A549 cells were administrating with different dose of TSA for indicated time period, collected and subjected to luciferase assay (lower left panel). (*c*) TSA does not affect the Mutated −355 *Aurora-A* promoter activity. The WT or −355mut *Aurora-A* promoter luciferase-transfected A549 cells were treated with TSA followed by the luciferase assay. (*d*) TSA inhibits p53 binding to *Aurora-A* (−365 to −345) promoter region. The nuclear lysates of parental or TSA-treated A549 cells were subjected to DAPA using biotinylated *Aurora-A* promoter-(-345∼-365) x3 probes using indicated antibody. (*e*) TSA prevents HDAC1 and mSin3a binding to *Aurora-A* (−365 to −345) promoter region. The parental or TSA-treated A549 cells were subjected to DAPA with biotinylated *Aurora-A* promoter-(−365 to −345) x3 and anti-p53, mSin3a or HDAC1 antibody, respectively. (*f*) Chromatin immunoprecipitation assay to confirm abrogation of p53 and mSin3a binding to *Aurora-A* promoter region by TSA. The parental or TSA-treated A549 cells were fixed and subjected to chromatin immunoprecipitation using anti-p53 or anti-mSin3a antibody, respectively. IgG represented as immunoprecipitation control and cp represented as randomized primers of Aurora-A promoter. Data are representative or expressed as the mean ± SEM of three independent experiments. ***$P < 0.001$, compared with control group. (*g*) Knockdown of p53 blocks HDAC1, mSin3a, and p53 binding to *Aurora-A* promoter. The nuclear lysates of A549-shRNA or A549-p53shRNA cells were extracted and incubated with biotinylated *Aurora-A* promoter-(−365 to −345) x3 probes followed by DAPA analysis using anti-p53, mSin3a or HDAC1 antibody, respectively. (*h*) Mutation of *Aurora-A* promoter blocks the binding of p53, HDAC1 and mSin3a. The biotinylated WT or −355 mut *Aurora-A* promoter was incubated with A549 nuclear lysates followed by DAPA analysis using antibodies against p53, mSin3a, and HDAC1, respectively. (*i*) Knockdown of p53 disrupts the p53 repressive complex onto Aurora-A promoter. The wild-type or p53-siRNA transfected A549 cells were subjected to Chromatin IP assay using p53 or HDAC1 antibody, respectively. The occupancy of p53 or HDAC1 onto Aurora-A or p21 promoter were then determined via quantitative PCR method. IgG represented as immunoprecipitation control. Data are representative or expressed as the mean ± SEM of three independent experiments. **$p < 0.01$ or ***$p < 0.01$, compared with control group.

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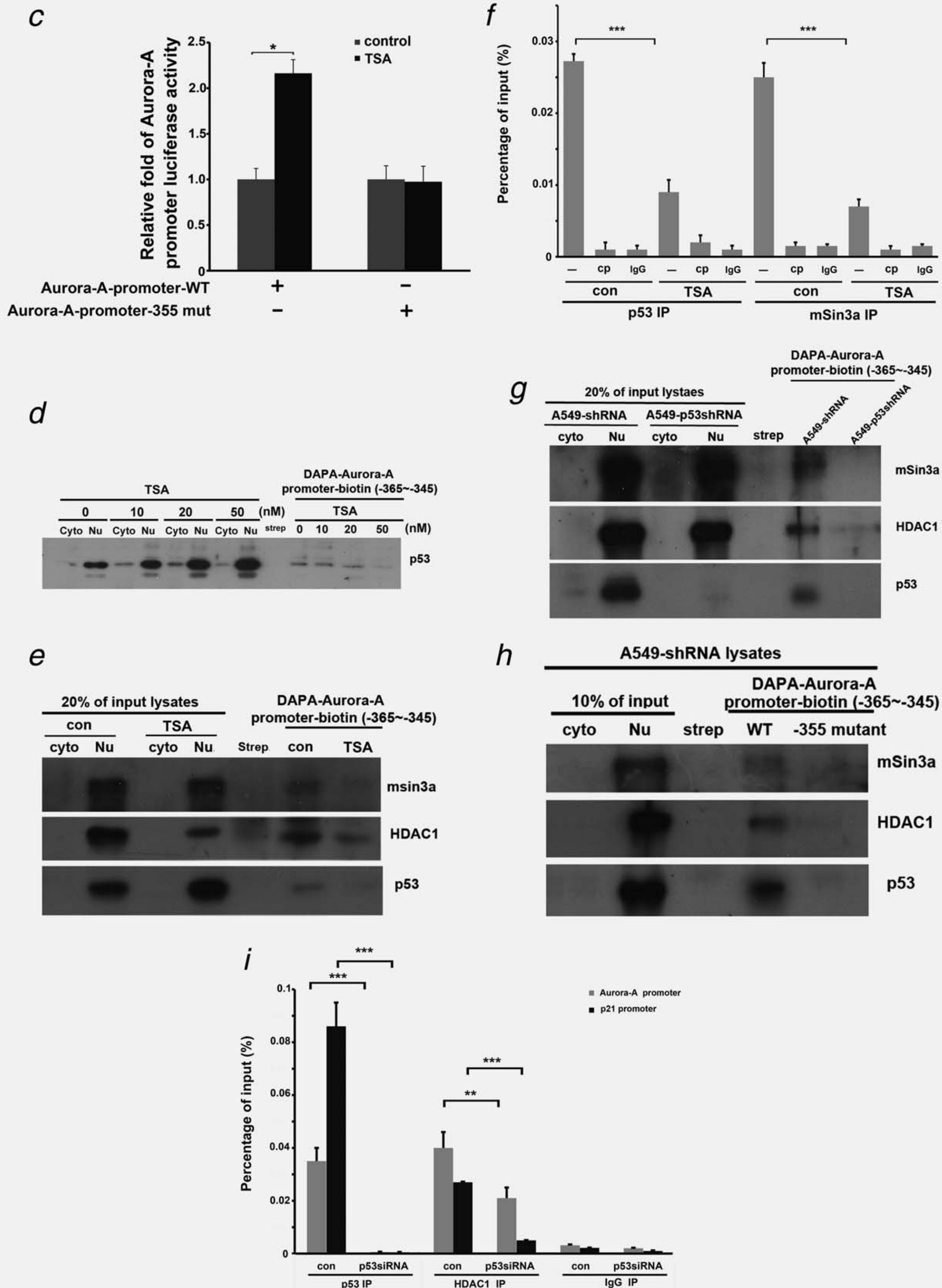

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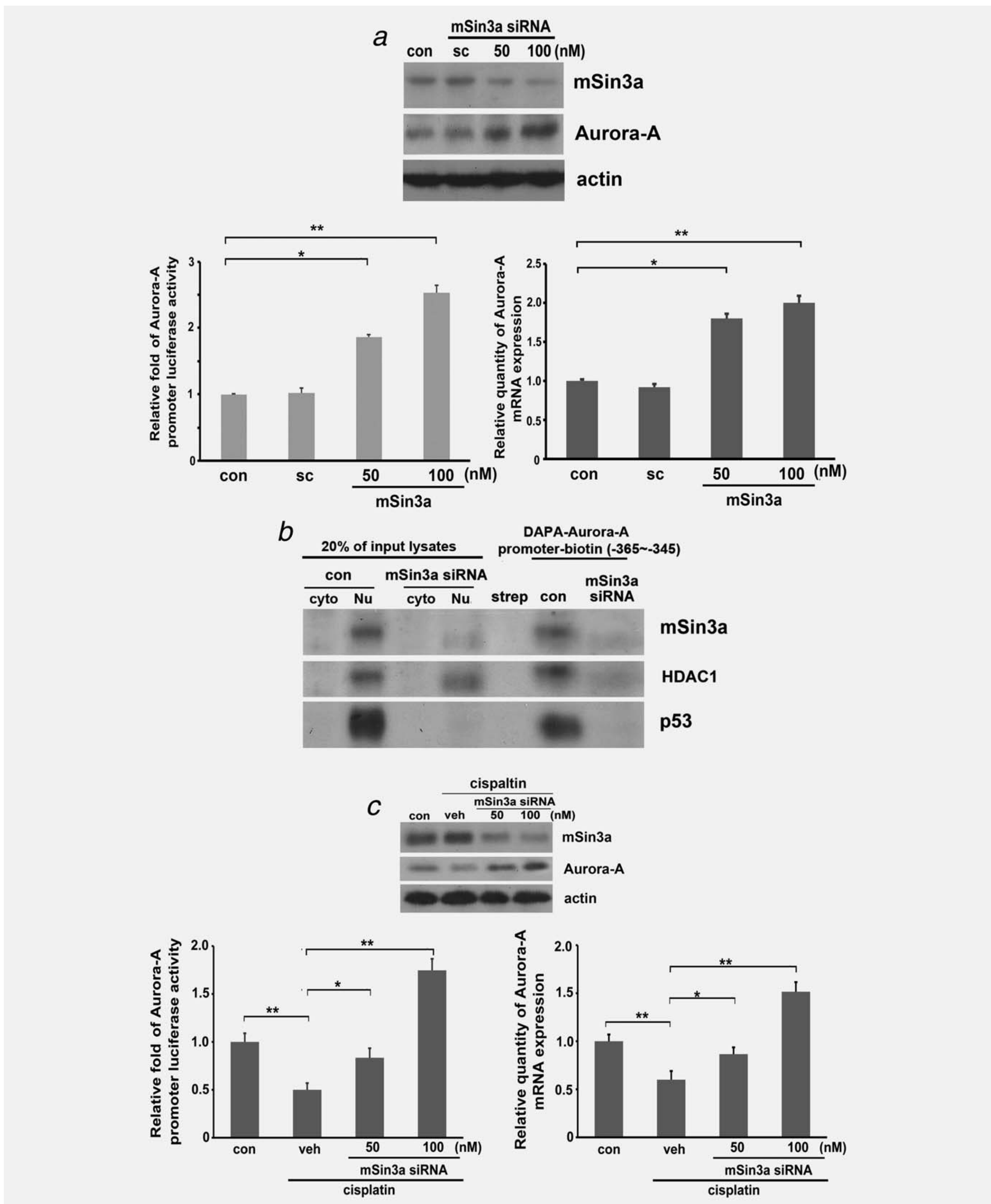
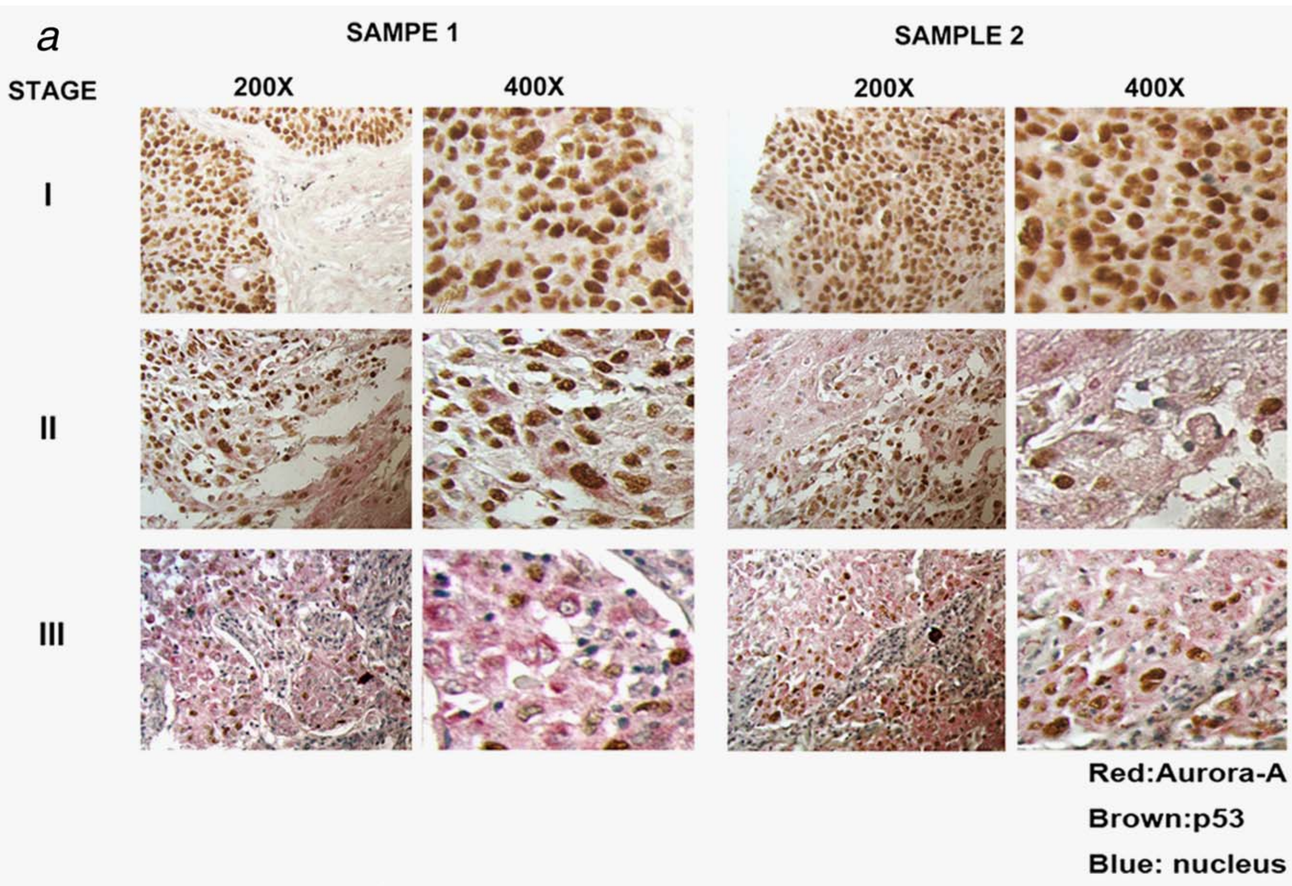


Figure 6. **mSin3a involves in p53-mediated repression of *Aurora-A* promoter activity and gene expression.** (*a*) mSin3a siRNA increases the expression of *Aurora-A* protein and mRNA levels and enhances *Aurora-A* promoter activity. The pGL3-*Aurora-A* promoter luciferase-transfected A549 cells were transfected with various dosages of mSin3a siRNA and then subjected to western blot, RT-PCR and luciferase assay, respectively. (*b*) Knockdown of mSin3a abrogates p53, mSin3a, and HDAC1 interaction with *Aurora-A* promoter. The nuclear lysates of parental or mSin3a-knockdown cells were extracted and incubated with biotinylated *Aurora-A* promoter-(−365 to −345) x3 probe followed by DAPA analysis using anti-p53, mSin3a or HDAC1 antibody, respectively.



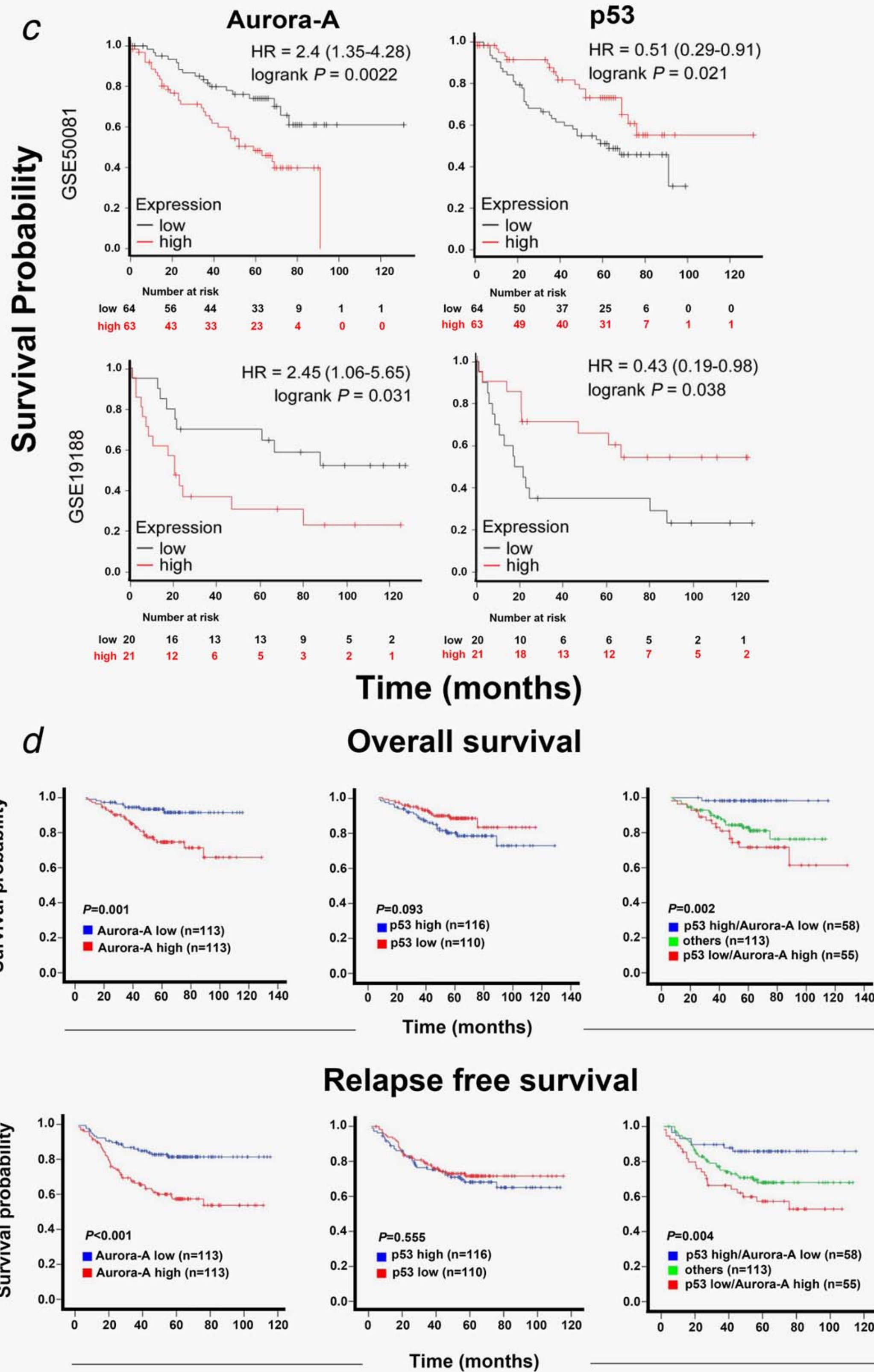
b

Expression Area			
Aurora-A	Stage I	Stage II	Stage IIIa
<25%	57.7	32.7	24.8
25-50%	17.1	61.1	25.1
>50%	26.2	7.2	50.1

Expression Area			
p53	Stage I	Stage II	Stage IIIa
<25%	48.5	67.2	50.1
25-50%	31.7	24.7	24.1
>50%	19.8	8.1	24.8

Figure 7. **Aurora-A level negatively correlates with p53 and associates with poor outcome in lung adenocarcinoma patients.** (*a*) The two Human lung cancer tissue microarrays were applied to stain p53 (brown) and Aurora-A (red) simultaneously coupled with H&E staining (Blue) as described in the materials and methods. (*b*) The quantitative percentage of p53 and Aurora-A expression area of each core of two lung cancer tissue arrays were calculated. (*c*) Kaplan-Meier plotter analysis displayed the overall survival of lung adenocarcinoma patients with indicated *Aurora-A* and *p53* status in GSE50081 and GSE19188. Case numbers after grouping *Aurora-A* and *p53* according to expression level were listed below. HR: hazard ratio. (*d*) Kaplan-Meier plot demonstrated the overall survival and relapse-free survival of lung adenocarcinoma cases separated into high *p53*/low *Aurora-A* and low *p53*/high *Aurora-A* groups. The data were retrieved and analyzed from microarray dataset GSE31210. (*e*) Relative *Aurora-A* and *p53* expression patterns in The Cancer Genome Atlas (TCGA) lung adenocarcinoma cohort was showed in the plot (upper), and the correlation in expression was statistically analyzed by Spearman's rho (lower). [Color figure can be viewed at wileyonlinelibrary.com].

area of each core) is about 58%, while the middle to high Aurora-A expression (>25% area of each core) is only about 42% of stage I adenocarcinoma tissues. However, the ratio of the middle to high expression of Aurora-A increased to about 70∼80% of stage II and III lung adenocarcinoma tissues (Fig. 7*b*), suggested that the expression of Aurora-A might be



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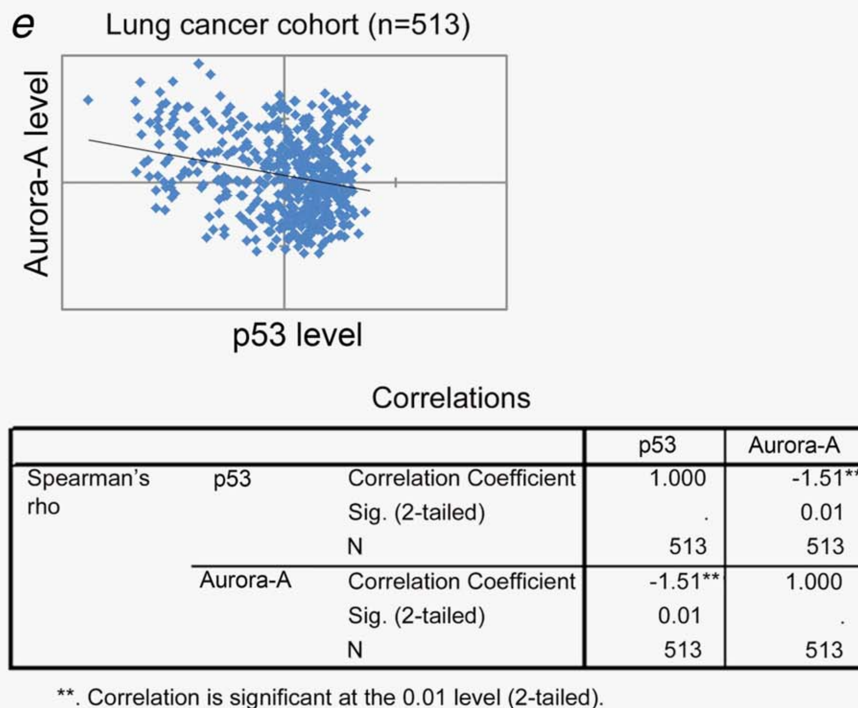

			p53	Aurora-A
Spearman's rho	p53	Correlation Coefficient	1.000	-1.51**
		Sig. (2-tailed)	.	0.01
		N	513	513
	Aurora-A	Correlation Coefficient	-1.51**	1.000
		Sig. (2-tailed)	0.01	.
		N	513	513

**. Correlation is significant at the 0.01 level (2-tailed).

Figure 7. (Continued).

positively correlated with the advanced stage of lung cancer. On the contrary, in the stage I lung adenocarcinoma tissues, the low expression of p53 (<25% are of each core) was about 48%, while the middle to high expression was about 52%. In stage II lung adenocarcinoma tissues, the ratio of low p53 expression samples increased to 70% while the ratio from middle to high p53 expressed samples decreased to 30%. It should be noticed that the expression of p53 increased in stage IIIa lung adenocarcinoma tissues. Whether this result might be caused by the presence of an oncogenic mutant form of p53 remains to be studied, since the antibody applied in this assay was unable to distinguish between wild-type and mutated p53 (Fig. 7*b*). Overall, these results implied that the expression of p53 exhibited an inverse correlation with that of Aurora-A in lung adenocarcinoma tissues that we investigated.

To explore the clinical relevance of the relationship between *Aurora-A*, *p53* and the prognosis of lung cancer patients, online databases of lung adenocarcinoma cancer cohort were studied. Results from Kaplan-Meier plotter analysis showed that *Aurora-A* expression significantly correlated with poor prognosis, whereas *p53* was associated with good prognosis in cancer patients, respectively, in GSE50081 and GSE19188 (Fig. 7*c*). The combination of low *p53*/high *Aurora-A* status displayed the correlation with poor outcome including overall survival and relapse-free survival, which was validated in another lung cancer cohort (GSE31210, Fig. 7*d*). In GSE50081 dataset, the patients with high *Aurora-A*/low *P53* status are statistically associated with poor outcome. The GSE19188 result also shows the similar pattern in survival curve (Supporting Information Fig. 2) In addition, *Aurora-A* level was inversely correlated with *p53* in lung adenocarcinoma patients (Spearman's rho $\rho = -1.51$, $P = 0.01$, Fig. 7*e*).

These results revealed that *Aurora-A* level was negatively correlated with *p53* in tumor and the lung cancer patients with low *p53*/high *Aurora-A* status displayed the poorest outcome.

Discussion

Despite the composition or orientation of the sequences are different to some degree, one common characteristic of transactivated or repressed element for p53 binding is they are all composed of four half sites (RRRCW or WGYYY). It was suggested that each whole-site of the p53 response element can be bound by p53 tetramer, with each half-site bound by p53 dimer.[49] In this study, the −360 to −354 core region of the *Aurora-A* promoter was identified as a p53 binding site which only contained a quarter of the consensus p53- binding element (-358∼TGCCC∼-354, WGYYY) (Fig. 4*a*). This is the first report demonstrating that p53 can interact with only a quarter site of its consensus element of target gene promoter. According to previous descriptions about the consensus binding element and p53 tetramerization, our finding opens a new question about whether p53 only exerts its direct transcriptional regulation to target promoter in a tetramer manner? Since the relative smaller −360∼-354 region of the *Aurora-A* promoter obviously can't be bound by the p53 tetramer. Unexpectedly, our luciferase reporter assay showed that the mutation within the −360∼-354 region of the

Aurora-A promoter only partially interfered but not complete abolished the p53-mediated transcriptional repression (Figs. 4*c* and 4*d*). Furthermore, the results from the DAPA showed that p53 was able to interact with some other regions located at the upstream of *Aurora-A* promoter (Fig. 3*c*). These results suggested that p53 might bind to multiple but not yet canonical binding sequence of the *Aurora-A* promoter.

On contrary to wild-type p53, various tumor-derived mutant forms of p53 protein are defective in transactivation of target genes.[50] Examination of the effect of mutated p53 on *Aurora-A* gene expression, we found that the *Aurora-A* promoter activity was repressed by wild-type p53 while the R175H mutant form of p53 had no effect on *Aurora-A* promoter activity; interestingly, p53-R280K mutant promoted the activity of *Aurora-A* promoter (Fig. 2*d*). Unlike other loss-of-function mutations of p53, R280K mutated p53 can act as an oncogene to promote or maintain tumorigenesis by activating several genes such as *MAP2K3* (Mitogen-activated protein kinase kinase 3), *c-myc* and *MDR1*, etc.[51–53] Consistent with those findings, our results revealed that *Aurora-A*, an oncogene, was transactivated by R280K mutated p53. This finding might extend the mechanisms of oncogenic mutated p53-R280K in promoting tumorigenesis. However, the mechanisms of how p53-R280K upregulates certain oncogenes need to be further explored.

Nowadays, three direct p53-mediated gene repression are characterized: first, binding-site overlap (steric interference); second, p53 squelching of transcriptional activators; and third, p53-mediated recruitment of mSin3a and HDACs.[54] Previous reports demonstrated that p53 negatively regulates *MAP4* and *stathmin* gene expression through the recruiting mSin3a and HDAC1 on their promoter region.[22] Consistently, our studies showed that p53-mediated *Aurora-A* gene repression was via an mSin3a and HDAC1-dependent pathway. Besides, we also detect a complex formation between p53 and HDAC1 (Supporting Information Fig. 3), support the previous studies. However, the related p53 binding elements within the *MAP4* or *stathmin* promoter are not identified yet. Notably, the p53-HDAC1-mSin3a complex binding site located at the *Aurora-A* promoter −365∼-345 region was identified in this study (Figs. (3 and 4) and 5).

Previous report suggested that the effect of TSA might be *via* acetylation of Histones followed by recruiting positive regulator SP1 and basic transcription machine to the promoter region of target gene and turn on its transcription.[55] We have previously showed that E2F3 is a positive regulator of *Aurora-A* transcription;[43] besides, we also spotted a potential binding of E2Fs around −365∼-345 region, and more interestingly, the binding of p53 onto *Aurora-A* promoter was interfered by the presence of E2F3 (data not shown). Whether p53 repressive complex acts antagonistically with E2F3 is worthy to be elucidated. On the other hand, knockdown of mSin3a also disrupted the formation of p53 repressive complex onto *Aurora-A* promoter, and thus enhanced the promoter activity, and increased the levels of *Aurora-A* mRNA and protein, and moreover, abrogated cisplatin-mediated *Aurora-A* repression (data not shown). Previous report indicates that mSin3a interacts with p53 and protects it from proteasome-mediated degradation;[56] this study showed that knockdown of mSin3a resulted in a decrease of p53 expression (Fig. 6*b*); whether the loss of p53 expression in mSin3a knockdown cells attributes to proteasome-mediated degradation remains to be elucidated. Taken together, the results of the present study and our previous studies[43,47] have revealed a possibility that p53 might utilize both direct and indirect pathways to regulate the expression of oncogenic kinase *Aurora-A*.

Accumulating evidence have demonstrated the association of the expression level of *Aurora-A* alone or p53 alone with prognosis in vary types of cancer.[57,58] However, limited reference takes into consideration of these two molecules together with cancer patient's outcome. Jeng *et al.* shows that the overexpression of *Aurora-A* combined with the mutated p53 associates with poor prognosis of HCC patients.[41] The mutations of p53 are more usually observed and been focused in lung cancer patients.[59,60] We showed that *Aurora-A* and p53 indeed exhibited an inversely correlation in NSCLC patients (Fig. 7). Additionally, our results clearly indicated that combining consideration of the expression levels of p53 and *Aurora-A* is able to provide a more precise predictive value and to identify poor prognostic NSCLC patients who may benefit from specific and individual therapy.

In this study, we provide the first evidence that p53 may use an evolutionarily conserved mechanism for transcriptional repression of *Aurora-A* by recruitment of mSin3a and HDAC1 complex and directly binding to a non-classical p53-response element located at 5′-end of *Aurora-A* promoter. This study links p53 and *Aurora-A* in a previously unidentified regulatory mechanism that may underlie the relevance of the tumor suppressing ability of p53 in cancer to associate with the repression of certain oncogene expression.

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