Runt-related transcription factor 2 attenuates the transcriptional activity as well as DNA damage-mediated induction of pro-apoptotic TAp73 to regulate chemosensitivity

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Although runt-related transcription factor 2 (RUNX2) is known to be an essential key transcription factor for osteoblast differentiation and bone formation, RUNX2 also plays a pivotal role in the regulation of p53-dependent DNA damage response. In the present study, we report that, in addition to p53, RUNX2 downregulates pro-apoptotic TAp73 during DNA damage-dependent cell death. Upon adriamycin (ADR) exposure, human osteosarcoma-derived U2OS cells underwent cell death in association with an upregulation of TAp73 and various p53/TAp73-target gene products together with RUNX2. Small interfering RNA-mediated silencing of p73 resulted in a marked reduction in ADR-induced p53/TAp73-target gene expression, suggesting that TAp73 is responsible for the ADR-dependent DNA damage response. Immunoprecipitation and transient transfection experiments demonstrated that RUNX2 forms a complex with TAp73 and impairs its transcriptional activity. Notably, knockdown of RUNX2 stimulated ADR-induced cell death accompanied by a massive induction of TAp73 expression, indicating that RUNX2 downregulates TAp73 expression. Consistent with this notion, the overexpression of RUNX2 suppressed ADR-dependent cell death, which was associated with a remarkable downregulation of TAp73 and p53/TAp73-target gene expression. Collectively, our present findings strongly suggest that RUNX2 attenuates the transcriptional activity and ADR-mediated induction of TAp73, and may provide novel insights into understanding the molecular basis behind the development and/or maintenance of chemoresistance. Thus, we propose that the silencing of RUNX2 might be an attractive strategy for improving the chemosensitivity of malignant cancers.

Structured digital abstract
• p73 and RUNX2 colocalize by fluorescence microscopy (View interaction)
• RUNX2 physically interacts with p73 by anti bait coip (1, 2)

Abbreviations
ADR, adriamycin; DAPI, 4',6-diamidino-2-phenylindole; HDAC, histone deacetylase; HRP, horseradish peroxidase; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; RUNX2, runt-related transcription factor 2; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.
Introduction

The appropriate DNA damage response that monitors and maintains genomic integrity is considered to be a critical barrier against tumorigenesis. Tumor suppressor p53 plays a pivotal role in the regulation of the DNA damage response. p53 is divided into three characteristic functional domains, including the NH2-terminal transactivation domain (TA), the central sequence-specific DNA-binding domain (DB) and the COOH-terminal oligomerization domain (OD). As expected from its structural property, p53 acts as a transcriptional factor and has the ability to transactivate numerous target genes implicated in the induction of cell cycle arrest \((p21^{\text{WAF1}} \text{ and } 14-3-3\alpha)\), DNA repair \((GADD45)\), cellular senescence \((p21^{\text{WAF1}})\) and/or apoptotic cell death \((BAX, PUMA \text{ and } NOXA)\) [1–3]. Among these cellular processes, the promotion of apoptotic cell death has a prominent role in tumor suppression by eliminating cells with seriously damaged DNA. Upon cellular insult, such as DNA damage, oncogene activation and hypoxia, p53 is quickly activated by sequential post-translational modifications, including phosphorylation \((\text{Ser-15, Ser-20 and Ser-46})\) and acetylation \((\text{Ace-373 and Ace-382})\), thereby exerting its pro-apoptotic function via the subsequent transactivation of its target genes [1–3].

Unfortunately, p53 is mutated and loses its tumor-suppressive function in approximately half of human cancerous cells [4–6]. Over 90% of p53 mutations are detectable within the genomic region encoding its central sequence-specific DNA-binding domain, suggesting that the sequence-specific transactivation activity of p53 is tightly linked to its pro-apoptotic function. By contrast to a short-lived wild-type p53, mutant p53 shows a prolonged half-life [3]. Additionally, mutant p53 acquires an oncogenic potential and also displays a dominant-negative behavior against wild-type p53 [7].

Moreover, cancerous cells expressing p53 mutant sometimes exhibit chemoresistant phenotypes [7,8]. Of note, mutant p53 transactivates myriad of genes involved in many different aspects of tumorigenesis such as \(\text{MYC, EGFR \text{ and } MDR1} \) [8]. p73 is a member of p53 tumor suppressor family and acts as a negative regulator of tumorigenesis by inducing cell cycle arrest and/or apoptotic cell death via its ability to transactivate overlapping set of p53-target genes [9,10]. Similar to p53, p73 has been shown to accumulate in response to certain DNA damaging agents such as cisplatin and Adriamycin \((\text{ADR})\) and to play a crucial role in the regulation of DNA damage response [11]. It has been reported that DNA damage-mediated transcriptional activation of \(p73\) is modulated by E2F-1 [12–14]. Importantly, \(p73\) produces two distinct gene products, including a transactivation-domain-containing isoform \((\text{TAp73})\) and a transactivation-deficient \(\text{NH}_2\)-terminally truncated isoform \((\Delta\text{Np73})\). \(\Delta\text{Np73}\) arises from the alternative promoter usage [15]. Although \(p73\) is rarely mutated in human cancerous cells [16], \(\text{TAp73}\)-deficient mice showed an increased susceptibility to spontaneous and carcinogen-induced tumors with a peculiar promotion of lung adenocarcinomas, implying that \(\text{TAp73}\) possesses a tumor-suppressive capability [17]. It is worth noting that \(\text{TAp73}\) is required for p53-dependent apoptotic cell death, whereas \(\text{TAp73}\) alone has the ability to induce apoptotic cell death in the absence of wild-type p53 [18]. By contrast, \(\Delta\text{Np73}\) lacking the \(\text{NH}_2\)-terminal transactivation domain has an oncogenic function and acts as a dominant-negative inhibitor toward \(\text{TAp73}\), as well as wild-type p53 [15,19]. Thus, the intracellular balance between the amounts of anti-apoptotic \(\Delta\text{Np73}\) and pro-apoptotic p53/\(\text{TAp73}\) might be a critical determinant of cell fate. The mammalian runt-related transcription factor \((\text{RUNX})\) family is composed of three members, including \(\text{RUNX1, RUNX2 \text{ and } RUNX3}\). \(\text{RUNX}\) family members contain an evolutionarily conserved 128-amino acids runt domain, which is required for specific DNA-binding \((\text{ACC(A/G)CA})\) and heterodimerization with its cofactor \(\text{CBF}\beta \) [20,21]. \(\text{CBF}\beta \) does not bind directly to DNA but enhances \(\text{RUNX}\) proteins binding to DNA [22]. Among the \(\text{RUNX}\) family members, \(\text{RUNX2}\) was shown to play a key role in the regulation of proper osteoblast differentiation and bone formation [23,24]. By contrast to \(\text{RUNX2}\), \(\text{RUNX1}\) and \(\text{RUNX3}\) are known to act as essential regulators of hematopoietic and gastric development, respectively [25–28]. Indeed, \(\text{RUNX2}\)-deficient mice completely failed to form mineralized bone [23,24] and mice overexpressing \(\text{RUNX2}\) displayed osteopenia with multiple fractures [29]. Consistent with these observations, \(\text{RUNX2}\) transactivates various target genes involved in the promotion of osteogenic differentiation, such as type 1 collagen, osteopontin and osteocalcin [30,31].

Alternatively, there is growing evidence to suggest that \(\text{RUNX2}\) has a pro-oncogenic function in a wide variety of cells. For example, overexpression of \(\text{RUNX2}\) in transgenic mice was found to predispose mice to lymphoma [32]. Intriguingly, Browne et al. [33] reported that \(\text{RUNX2}\) induces the expression of pro-survival \(\text{Bel-2}\) and thus contributes to the development of malignant phenotypes in prostate cancer cells. Furthermore, current evidence indicates that \(\text{RUNX2}\) is a critical pathological factor in human metastatic breast, pancreatic, thyroid, prostate and colon cancer cells [34–37]. Thus, it
is likely that RUNX2 is closely involved in malignant cancer cell proliferation, chemoresistance, migration and invasion. To overcome the malignant phenotypes of human cancers, a precise understanding of the molecular mechanism(s) with respect to how aggressive cancers develop and maintain resistance to chemotherapeutic agents is required. As noted above, chemoresistance of cancerous cells is often attributed to an impaired functioning of p53 as a result of genetic mutation and/or sequestration by the other proteins. Recently, we described that RUNX2 attenuates p53-dependent apoptotic cell death in response to DNA damage [38], indicating that RUNX2 contributes to chemoresistance by inhibiting p53.

In the present study, we report that RUNX2 has the ability to suppress transcriptional activity as well as ADR-mediated induction of TAp73, thereby inhibiting the proper DNA damage response of cancerous cells.

Results

Simultaneous induction of TAp73 and RUNX2 during ADR-mediated cell death

To determine whether a functional interaction exists between pro-apoptotic TAp73 and pro-oncogenic RUNX2 during the DNA damage response, we investigated the expression level of TAp73 and RUNX2 in response to ADR. Accordingly, human osteosarcoma-derived \( p53 \)-proficient U2OS cells were exposed to increasing doses of ADR. Consistent with our recent observations [38], U2OS cells underwent cell death after ADR treatment (Fig. 1). Under our experimental conditions, non-apoptotic cell death was barely detectable, as examined by annexin V and propidium iodide (PI) double-staining experiments. Whole cell lysates and total RNA were then prepared and analyzed by immunoblotting and RT-PCR, respectively. As shown in Fig. 2A, ADR-mediated accumulation of \( \gamma H2AX \) and cleaved poly(ADP-ribose) polymerase (PARP) was detectable, indicating that DNA damage-induced cell death takes place in response to ADR. As expected, ADR treatment led to an induction of TAp73, as well as RUNX2, in association with a remarkable upregulation of p53/TAp73-target gene products, such as p21\(^{\text{WAF1}}\) and BAX. Additionally, RT-PCR analysis revealed that ADR-dependent induction of TAp73 and RUNX2 is regulated at the transcription level (Fig. 2B).

Because p53 was also induced and phosphorylated at Ser-15 in response to ADR, it is likely that U2OS cells undergo cell death in a p53 and/or a TAp73-dependent manner. To confirm whether TAp73 could play a central role in the regulation of ADR-mediated cell death, U2OS cells were transfected with control small interfering RNA (siRNA) or with siRNA targeting \( p73 \). Transfected cells were then treated with or without ADR. Twenty-four hours after treatment, whole cell lysates and total RNA were extracted and analyzed for the expression of TAp73, thereby inhibiting the proper DNA damage response of cancerous cells.

Fig. 1. U2OS cells undergo cell death in response to ADR. (A) Phase-contrast micrograph. U2OS cells were treated with the indicated concentrations of ADR or left untreated. Twenty-four hours after treatment, phase-contrast micrographs were taken. Scale bar = 100 µm. (B) Trypan-blue exclusion assay. U2OS cells were treated as described in (A). Twenty-four hours after treatment, cells were trypsinized and stained with 0.4% of trypan blue. Cells restricting trypan-blue entry were considered viable. (C) Annexin V and PI double staining. U2OS cells were treated with 0.5 µM of ADR (filled boxes) or left untreated (gray boxes). Twenty-four hours after treatment, cells were subjected to annexin V and PI double staining.
required for ADR-mediated DNA damage response (Fig. 3B).

**Complex formation between TAp73 and RUNX2 in response to ADR**

The above observations showing that TAp73 and RUNX2 are simultaneously induced after ADR treatment prompted us to examine whether TAp73 could form a complex with RUNX2. Accordingly, U2OS cells were stimulated with ADR or left untreated and then subjected to indirect immunofluorescence staining followed by subsequent confocal microscopy analysis. As shown in Fig. 4A, TAp73 and RUNX2 were induced to accumulate and co-localize in cell nucleus in response to ADR, suggesting that TAp73 might interact with RUNX2 in the cell nucleus. To further confirm this possibility, we performed co-immunoprecipitation experiments using whole cell lysates prepared from ADR-treated U2OS cells. As shown in Fig. 4B, the anti-p73 immunoprecipitates contained the endogenous RUNX2. Intriguingly, we failed to detect RUNX2/TAp73 complex in the absence of ADR (data not shown). Thus, our present results imply that TAp73 forms a complex with RUNX2 in cell nucleus in response to DNA damage.

**RUNX2 inhibits the transcriptional activity of TAp73**

We next investigated a functional consequence of the complex formation between TAp73 and RUNX2. Accordingly, U2OS cells were transfected with or without increasing amounts of RUNX2 expression plasmid. Forty-eight hours post-transfection, whole cell lysates and total RNA were isolated and analyzed by immunoblotting and RT-PCR, respectively. As described previously [38], RUNX2 had an undetectable effect on the expression level of p53 (Fig. 5A). Overexpression of RUNX2 caused a marked decrease in the expression level of p53/TAp73-target genes, and similar results were also obtained in p53-deficient human lung carcinoma H1299 cells (data not shown). Of note, overexpression of RUNX2 in U2OS cells
resulted in an accelerated cell proliferation in a dose- and time-dependent fashion (Fig. 5B,C), raising the possibility that RUNX2-mediated downregulation of p53/TAp73-target gene products might contribute to the promotion of cell proliferation. Because U2OS and H1299 cells expressed wild-type TAp73, it is likely that, in addition to p53, RUNX2 might also inhibit the transcriptional activity of TAp73, thereby enhancing their unregulated cell proliferation.

To adequately address this issue, H1299 cells were transfected with the indicated combinations of the expression plasmids. Forty-eight hours post-transfection, whole cell lysates and total RNA were prepared and processed for immunoblotting and RT-PCR, respectively. As clearly shown in Fig. 6, overexpression of TAp73α stimulated the expression of p53/TAp73-target genes, such as BAX, PUMA and NOXA, whereas co-expression of TAp73α with RUNX2 markedly reduced their TAp73α-dependent transcriptional activation. Consistent with these results, luciferase reporter assays demonstrated that RUNX2 remarkably decreases TAp73α-mediated enhancement of luciferase activities driven by p53/TAp73-responsive human BAX and NOXA promoters in a dose-dependent manner (data not shown). These observations strongly suggest that RUNX2 inhibits the transcriptional activity of TAp73.

**Knockdown of RUNX2 enhances ADR-mediated cell death in association with the transcriptional induction of TAp73**

Recently, we reported that depletion of RUNX2 in U2OS cells augments p53-dependent cell death after ADR exposure [38]. These observations led us to examine a possible effect of RUNX2 knockdown on TAp73 expression. Accordingly, U2OS cells were transfected with control siRNA or with siRNA targeting RUNX2, followed by incubation with or without ADR for 24 h. In accordance with our recent observations [38], RUNX2 was successfully knocked down under our experimental conditions (Fig. 7A) and RUNX2-depleted cells underwent cell death in response to ADR (42%) much more efficiently than ADR-treated control cells (24%), as examined by DNA ladder formation (Fig. 7B) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (Fig. 8). To check whether silencing of
**RUNX2** could affect the expression level of TAp73 after ADR treatment, total RNA was prepared and analyzed by RT-PCR. As clearly shown in Fig. 7C, ADR-mediated induction of p53/TAp73-target genes, such as p21WAF1, BAX and PUMA, was further enhanced in RUNX2-knockdown cells. Of note, knockdown of RUNX2 had a negligible effect on p53 in cells exposed to ADR, whereas ADR-mediated upregulation of TAp73 was further enhanced upon the silencing of RUNX2 expression. Additionally, depletion of RUNX2 resulted in a slight increase in the steady-state expression level of TAp73. These observations suggest that RUNX2 has an ability to suppress ADR-dependent induction of TAp73, and TAp73 contributes at least in part to the higher sensitivity to ADR of RUNX2-depleted cells.

**RUNX2** attenuates ADR-mediated cell death in association with the downregulation of TAp73

To further confirm whether RUNX2 could downregulate the expression of TAp73 during ADR-mediated cell death, U2OS cells were transfected with the empty plasmid or with RUNX2 expression plasmid. Twenty-four hours post-transfection, cells were incubated with or without 0.5 μM of ADR. As expected, overexpression of RUNX2 suppressed ADR-mediated cell death (Fig. 9), whereas ADR-mediated cell death was further stimulated in the presence of exogenous p53. Similar results were also obtained in a trypan-blue exclusion assay (data not shown). Under these experimental conditions, whole cell lysates and total RNA were prepared and analyzed for the expression level of TAp73 by immunoblotting and RT-PCR, respectively. As shown in Fig. 10A, ADR-mediated proteolytic cleavage of PARP, caspase-9 and induction of p53/TAp73-target products were notably suppressed by exogenous RUNX2. Similar results were also obtained in RT-PCR analysis (Fig. 10B). Intriguingly, ADR treatment led to a remarkable induction of TAp73, whereas ADR-mediated upregulation of TAp73 was impaired in cells expressing exogenous RUNX2. As expected, overexpression of RUNX2 also abrogated ADR-dependent transcriptional induction of TAp73 and various p53/ TAp73-target genes in H1299 cells (Fig. 11).

Taken together, our present findings strongly suggest that RUNX2 helps the resistance of cancerous cells to DNA damage by downregulation of TAp73.
In the present study, we report that the transcriptional activity and ADR-mediated induction of pro-apoptotic TAp73 are significantly impaired by RUNX2. Because RUNX2 prohibits both wild-type p53 and TAp73, it is likely that the inhibition of RUNX2 might be a promising anti-cancer therapy for chemoresistant malignant cancers.

It is well established that, similar to p53, TAp73 is activated through DNA damage-mediated post-translational modifications such as phosphorylation and acetylation. For example, TAp73 was tyrosine phosphorylated at Tyr-99 by nonreceptor tyrosine kinase c-Abl in response to DNA damage, and c-Abl-mediated phosphorylation of TAp73 enhanced its stability as well as its transcriptional activity [39-41]. Alternatively, Costanzo et al. [42] demonstrated that DNA damage-induced acetylation of TAp73 at Lys-321, Lys-327 and Lys-331 stimulates its pro-apoptotic activity.

According to our immunoprecipitation experiments, we successfully detected RUNX2/TAp73 complex in cells exposed to ADR (Fig. 4). By contrast, we failed to detect RUNX2/TAp73 complex in untreated cells, which might result from the low expression level of RUNX2 and TAp73 or the absence of post-translationally modified active TAp73. Because RUNX2 significantly reduced the transcriptional activity of TAp73, it is likely that RUNX2 could preferentially associate with the transcriptionally active form of TAp73. Although plausible, this possibility remains to be tested experimentally.

According to the results of the present study, overexpression of RUNX2 suppressed the ADR-dependent induction of TAp73 and siRNA-mediated knockdown of RUNX2 further enhanced ADR-dependent upregulation of TAp73, indicating that RUNX2 might repress TAp73 expression at the transcriptional level. In support of this notion, luciferase reporter assays demonstrated that luciferase activity driven by human TAp73

![Image](RUNX2-mediated suppression of pro-apoptotic TAp73.png)
promoter (-2713/+20) is remarkably reduced in response to increasing amounts of exogenous RUNX2 (data not shown). Intriguingly, we identified several putative RUNX2-recognition sites (ACC(A/G)CA) within this promoter region (data not shown). RUNX/CBFβ complex is known to activate or repress the transcription of key regulators of growth, survival and differentiation pathways [43]. Recently, McGee-Lawrence et al. [44] reported that RUNX2 represses the transcription of its target genes by recruiting histone deacetylases (HDACs) to their promoters. We have also found that RUNX2 interacts with HDAC6, thereby inhibiting the transcription of p53/TAp73-target genes in response to DNA damage [38]. It is possible that RUNX2 downregulates the transcription of TAp73 by recruiting HDACs to the putative RUNX2-binding site located within TAp73 promoter region; however, additional experiments are required to adequately address this issue.

Alternatively, it has been demonstrated that transcriptional repressor ZEB1 which has anti-apoptotic potential, inhibits the transcription of TAp73 [45,46]. Aghdassi et al. [47] reported that ZEB1-mediated recruitment of HDAC1 and HDAC2 onto the E-cadherin promoter results in its downregulation. Under our experimental conditions, the expression level of ZEB1 was markedly increased in response to exogenous RUNX2, and siRNA-mediated knockdown of RUNX2 resulted in a massive downregulation of ZEB1 in U2OS cells (data not shown), raising the possibility that the expression of ZEB1 is regulated under the control of RUNX2. Based on these observations, it is conceivable that RUNX2-mediated transcriptional activation of ZEB1 causes a marked repression of TAp73 or that RUNX2 facilitates the efficient recruitment of the repressor complex containing ZEB1 onto the putative RUNX2-binding site within 5'-upstream region of TAp73 and/or ZEB1-binding site located in the first intron of TAp73, thereby attenuating TAp73 transcription.

As described previously [18], TAp73 was required for p53-dependent cell death, whereas TAp73 was capable of promoting cell death in a p53-independent manner. In support of these observations, the overexpression of TAp73 alone in p53-deficient pancreatic cancer cells triggered cell death [48]. In addition, Willis et al. [49] demonstrated that TAp73 exerts its growth-suppressive activity in cancerous cells expressing mutant p53. Previously, Chen et al. [50] found that p53 transactivates TAp73 through a functional p53-responsible element located within its 5'-upstream region. Under our experimental conditions, overexpression of FLAG-p53 stimulated transcription of TAp73 in U2OS cells (data not shown). Given that TAp73 acts as an essential cofactor for p53 [18], RUNX2 knockdown-mediated enhancement of pro-apoptotic activity of p53 might be at least in part attributed to p53-dependent induction of TAp73. It was worth noting that siRNA-mediated knockdown of p73 strongly suppresses ADR-dependent upregulation of p53/TAp73-target gene expression even in the presence of active p53 (Fig. 3), implying that TAp73 plays a critical role during ADR-mediated cell death. Because TAp73 is rarely mutated in a variety of human cancers [16], it is likely that silencing of RUNX2 enhances chemosensitivity of malignant cancers expressing functionally impaired-p53 through up-regulation of TAp73.

In summary, we provided evidence showing that RUNX2 downregulates TAp73 in response to DNA damage. Therefore, we propose that silencing of RUNX2 might be a novel strategy for improving chemosensitivity of malignant cancers.
Experimental procedures

Cells and transient transfection

*p53*-proficient human osteosarcoma-derived U2OS and *p53*-deficient human lung carcinoma-derived H1299 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with heat-inactivated 10% FBS (Invitrogen, Carlsbad, CA, USA) and penicillin-streptomycin at 37 °C in 5% CO₂. For transient transfection, cells were transiently transfected with the indicated combinations of the expression plasmids by using Lipofectamine 2000 reagent in accordance with the manufacturer’s instructions (Invitrogen).

The expression plasmids used in the present study were: pcDNA3 empty plasmid, pcDNA3-TAp73α and pcDNA3-RUNX2.

Trypan-blue exclusion assay

The number of dead cells was determined by the trypan-blue exclusion assay. Cells were exposed to the indicated concentrations of ADR. Twenty-four hours after the treatment, cells were trypsinized and stained with trypan-blue (0.4%) at room temperature for 10 min. Cells restricting trypan-blue entry were considered viable.
Annexin V and PI double staining

U2OS cells were treated with the indicated concentration of ADR or left untreated. Twenty-four hours after treatment, cells were washed in PBS and incubated with annexin V-FITC solution containing PI (50 µg/mL) at room temperature for 5 min in accordance with the manufacturer’s instructions (PromoKine, Heiderberg, Germany). After the incubation, cells were washed in PBS, fixed in 2% formaldehyde at room temperature for 30 min and then observed under a confocal microscope (Leica, Milton Keynes, UK).

RNA extraction and RT-PCR

Total RNA was prepared from the indicated cells using RNeasy mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer’s instructions. The integrity of total RNA was verified by visualization of rRNAs after electrophoresis in agarose gel. One microgram of total RNA for each sample was reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen) in the presence of random primers. The resultant cDNA was subjected to PCR-based amplification using the specific primer sets for TAp73, 5’-TCTGGAACCAGACAGCCT-3’ (sense) and 5’-GTGCTGGACTGGAAGT-3’ (antisense); p53, 5’-CTGCCTCAACAAGATGTTTTG-3’ (sense) and 5’-CTATCTGAGCAGCGCTCATGG-3’ (antisense); RUNX2, 5’-TCTGGCCCTTCCACTCTCAGT-3’ (sense) and 5’-GACTGGGCTGGTGTTAAAGTCAA-3’ (antisense); p21WAF1, 5’-ATGAAATTCACCCCCTTTCC-3’ (sense) and 5’-CCCTAGGCTGTCCTCACTTCAGT-3’ (antisense); 14-3-3, 5’-GAGCGAAACCTGCTCTCAGT (sense) and 5’-CTCCTTGAGTGGTGCTG-3’ (antisense); BAX, 5’-TTTTGGTCAGAGGTCTTAACTCC-3’ (sense) and 5’-CAGTTGAAGTTGGCTGCACTCA-3’ (antisense); PUMA, 5’-GCCCCAGACTGGAATCCTGTG-3’ (sense) and 5’-TCCTCCCTCTCAGAGATTT-3’ (antisense);
NOXA, 5' - CTGGAGCTGATGCTACT-3' (sense) and 5' - TCAGGTTCCTGAGAAGAG-3' (antisense); GAPDH, 5' - ACCTGACCTGCCGTCTGAA-3' (sense) and 5' - TCCACCACCTGGTCTGTA-3' (antisense). The PCR products were electrophoresed on 1.5% agarose gels and their amounts were evaluated by staining with ethidium bromide. GAPDH was used as an internal control.

**Immunofluorescence**

Cells were exposed to 0.5 μM of ADR or left untreated. Twenty-four hours after treatment, cells were washed twice in ice-cold PBS, fixed in 3.7% formaldehyde in PBS at room temperature for 30 min, permeabilized with 0.1% Triton X-100 in PBS at room temperature for 5 min and then blocked with 3% BSA in PBS at room temperature for 1 h. After blocking, cells were washed in ice-cold PBS and simultaneously incubated with mouse monoclonal anti-RUNX2 (8G5; Medical & Biological Laboratories, Nagoya, Japan) and rabbit polyclonal anti-p73 (Bethyl Laboratories, Montgomery, TX, USA) antibodies at room temperature for 1 h followed by the incubation with rhodamine-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG (Invitrogen) at room temperature for 1 h. After the extensive washing, cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Peterborough, UK). Fluorescent images were captured using a confocal microscope.

**Immunoblot analysis**

Cells were washed twice in ice-cold PBS and immediately lysed in Laemmli buffer as described previously [38]. Equal amounts of cell lysates were resolved by 10% SDS/PAGE and electro-transferred onto poly(vinylidene difluoride) membrane. Immunoblot analyses were performed as
described previously [19] using the primary antibodies: mouse monoclonal anti-p53 (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-p73 (Ab-4; NeoMarkers, Fremont, CA, USA), mouse monoclonal anti-γH2AX (2F3; BioLegend, San Diego, CA, USA), rabbit polyclonal anti-p21WAF1 (Santa Cruz Biotechnology), rabbit polyclonal anti-phospho-p53 at Ser-15 (Cell Signaling Technology, Beverly, MA, USA), rabbit polyclonal anti-RUNX2 (Cell Signaling Technology), rabbit polyclonal anti-E2F-1 (Cell Signaling Technology), rabbit polyclonal anti-PARP (Cell Signaling Technology), rabbit polyclonal anti-caspase-9 (Cell Signaling Technology) and rabbit polyclonal anti-Actin (20–33; Sigma, St Louis, MO, USA) antibodies. Immunoreactive bands were visualized by an enhanced chemiluminescence system (ECL; GE Healthcare, Little Chalfont, UK) in accordance with the manufacturer’s instructions.

### Immunoprecipitation

For immunoprecipitation, cells were exposed to 0.5 μM of ADR for 24 h and then lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2.7 mM KCl, 1% Triton X-100 and protease inhibitor mixture (Sigma). Equal amounts of cell lysates (500 μg of protein) were first precleared with 30 μL of protein G-Sepharose beads for 1 h at 4 °C and then incubated with monoclonal anti-p73 antibody overnight at 4 °C followed by the incubation with 30 μL of protein G-Sepharose beads for 2 h at 4 °C. The beads were extensively washed with lysis buffer and the immunoprecipitates were eluted by boiling in SDS-sample buffer. The bound proteins were separated by 10% SDS/PAGE.

### siRNA interference

Cells were transfected with control siRNA or with siRNA directed against RUNX2 (10 nM) using Lipofectamine 2000 transfection reagent in accordance with the manufacturer’s recommendations. Twenty-four hours after transfection, cells were treated with 0.5 μM of ADR or left untreated. Twenty-four hours after treatment, total RNA was prepared and analyzed for the expression level of RUNX2 by RT-PCR.

### Luciferase reporter assay

Cells were seeded at a density of 5 x 10⁴ cells per 12-well plate and allowed to attach overnight. Cells were then...
transfected with the constant amount of TAp73 expression plasmid (25 ng), 100 ng of luciferase reporter plasmid carrying p53/p73-responsible human BAX or NOXA promoter and 10 ng of renilla luciferase expression plasmid, together with or without increasing amounts of the expression plasmid for RUNX2. Equal DNA concentration in each experiment (500 ng per well) was maintained by adding the appropriate amounts of the empty plasmid to the DNA mixture. Forty-eight hours after transfection, cell lysates were prepared and their luciferase activities were measured in accordance with the protocol for the dual-luciferase system (Promega, Madison, WI, USA). The resultant firefly luciferase values were normalized by those of renilla luciferase. The experiments were performed in parallel and repeated at least three times.

**TUNEL assay**

TUNEL assay was performed by using an In Situ Cell Detection Kit (Roche Applied Science, Mannheim, Germany) in accordance with the manufacturer’s protocol. In brief, cells were washed twice in PBS, fixed in freshly prepared 4% paraformaldehyde for 1 h at room temperature, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min at room temperature, and then incubated with the enzyme solution in a humidified atmosphere for 1 h at 37 °C in the dark. After the reaction, cells were washed in PBS three times and fluorescent images were taken using a confocal microscope.

**Statistical analysis**

Statistical significance was determined by two-sided paired Student’s t-test. P < 0.05 was considered statistically significant.

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**Author contributions**

TO and HN planned the experiments. TO, HS, MN, KH and HY performed the experiments. TO analyzed the data. MS and KF contributed reagents or other essential materials. TO wrote the paper.

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