A Regulatory Circuit Controlling Itch-mediated p73 Degradation by Runx*

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The members of the tumor suppressor p53 family are under tight regulation by distinct ubiquitin-protein isopeptide (E3) ligases. The level of p73 is regulated by the E3 ligase Itch. Itch levels are sharply reduced in response to DNA damage with concomitant p73 accumulation and activation. The mechanism of controlling Itch level is not known. We show that the *Itch* promoter is a target of the transcription activator Runx. Yes-associated protein (Yap1) is a shared transcription co-activator of Runx and p73. Under normal conditions, the Runx-Yap1 complex binds the *Itch* promoter and supports its transcription and p73 degradation. In response to DNA damage, Yap1 is phosphorylated by c-Abl at the position Tyr-357. The modified Yap1 does not co-activate Runx in supporting *Itch* transcription. The subsequent reduction in the Itch level gives rise to p73 accumulation. These results demonstrate how Yap1 supports degradation of p73 via Runx and how it plays an opposite role in response to DNA damage.

The level of many regulatory proteins is determined also at the level of their decay. The E3[2] ligases select the substrate to be degraded by the process of ubiquitin-dependent proteasomal degradation. For example, the level of the p53 tumor suppressor protein is regulated by a number of E3 ligases, the major one being Mdm2 (1–5). The mode of cross-talk between the E3 ligase, as was learned from p53 and Mdm2, has a number of attributes, some with broader implications. Firstly, in this process, p53 undergoes constitutive Mdm2-dependent degradation unless p53 escapes Mdm2 interaction. A second characteristic of this pathway is that the promoter of the *Mdm2* gene is a target of p53 (6, 7), generating a minimal regulatory circuit whereby the substrate supports the production of its destroyer. A third characteristic of this pathway relies on the fact that Mdm2 binds the N-terminally positioned transcription activation domain of p53 (8). This endows the Mdm2 E3 ligase with the capacity to regulate p53 transcription activity as well (3, 9).

Overall, the hallmark of this regulatory circuit is that protein degradation is directly linked to transcription activation.

Despite the fact that the level of other members of the p53 family, such as p73 and p63, is regulated at the level of protein stability (10, 11), no such simple regulatory circuit was identified for either p73 or p63. Similar to p53, the level of p73 is increased in response to DNA damage insults, possibly by escaping the cognate E3 ligases (12). One of the known p73 E3 ligases is Itch, which belongs to the Nedd4-like E3 family containing a WW domain (13). Itch, via its WW domain, binds p73 by interacting with the p73 PPPPY motif, a prerequisite step in p73 ubiquitination and sensitization to rapid proteasome-dependent degradation (11). We have recently shown that the Yes-associated protein (Yap1) stabilizes p73 by competing with Itch for the binding to p73, which allows p73 to escape Itch-mediated ubiquitination (14). A similar mechanism was also suggested for the Nedd4-binding partner 1 (N4BP1) (15). Interestingly, in response to DNA damage, Itch protein level is down-regulated, which gives rise to p73 stabilization (11). However, the question of how Itch expression is regulated under normal and stress conditions was not addressed.

As a transcription factor, p73 has an important role in induction of pro-apoptotic and cell cycle arrest genes following DNA damage. An important player in this process is the non-receptor tyrosine kinase c-Abl (16–20). In response to DNA damage, c-Abl is activated, binds p73 via the SH3 domain, and tyrosine-phosphorylates p73 at the residue Tyr-99. Under this condition, p73 accumulates and induces expression of pro-apoptotic genes (12, 17). Interestingly, c-Abl also phosphorylates Yap1 on Tyr-357. The modified Yap1 accumulates to high levels and displays higher affinity to p73 and selectively co-activates the p73 pro-apoptotic target genes (21). Yap1 binds other transcription factors, such as all the members of the Runx family. Remarkably, modified Yap1 shows an increased association with p73 but a decreased association with Runx (21). We hypothesized that this molecular logic may also regulate Itch expression.

In this study, we identified a functional Runx-binding site at the *Itch* promoter. We show that Yap1 under normal conditions enhances the transcriptional activation of *Itch* via Runx and gives rise to p73 degradation. This process is blunted under DNA damage stress because of the phosphorylation-dependent promoter switching behavior of Yap1 (21). In response to DNA damage signaling, Yap1 is phosphorylated by c-Abl, and the phosphorylated Yap1 escapes the *Itch* promoter-associated Runx. These results provide evidence for Runx to negatively regulate p73 stability by enhancing Itch-mediated proteasomal degradation.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—The cell lines used were HEK293 and HEK293T human embryonic kidney cells, human non-small cell lung carcinoma H1299 p53-null cells, human HCT-116 p53$^{-/-}$ colorectal carcinoma cells (22), and the human breast adenocarcinoma cell line MCF-7. Cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 8% fetal bovine serum (Invitrogen), 100 units/ml penicillin, 100 μg/ml streptomycin and cultured at 37 °C in a humidified incubator with 5.6% CO_2. H1299 cells were cultured under the same conditions with RPMI medium.

Plasmids and Transfection—Overexpression of p73 was achieved by transfection of pSG5-HA-p73α and pEFIRES FLAG-p73α. FLAG pCMV-FLAG-Yap1 was a gift from M. Sudol. pCDNA FLAG wild type, Y357F, and Y357E Yap1 were previously described in Ref. 21. pCGN HA-Runx 1b was a gift from Y. Groner. pCDNA FLAG Runx was subcloned by PCR and the addition of EcoRI and NotI linkers. shRNA targeting c-*Abl* was described previously (21). All transfections were done by the calcium phosphate method as described in Levy *et al.* (21) or with jetPEI (Polyplus Transfection) according to the manufacturer's protocol. To generate Runx and Itch shRNA plasmids, the pSUPER vector was ligated with annealed oligonucleotides, containing a 19-nucleotide sequence derived from the human Runx transcript (CGCACTGGCGCTGCAACAT), which down-regulates all Runx family members, or from the human *Itch* transcript (CCAGATCACCTGAAATATT), a 9-nucleotide spacer and the reverse complement of the same 19-nucleotide sequence. The *Itch* promoter was produced by PCR using genomic DNA from HEK293 human embryonic kidney cells after sonication. Primer sequences for production of the promoter were 5′-ATCCCATCCGCACTCCACC-3′ and 5′-AGGCCTCCGGAAGCCTGTG-3′. The PCR product was cloned into the SacI and XhoI sites of pGL3-basic vector (Promega).

Luciferase Assay—Cells were transiently transfected with the indicated combinations of plasmids. The total amount of transfected DNA in each dish was kept constant by the addition of empty vector wherever necessary. Cell extracts were prepared 36 h later and subjected to determination of luciferase activity using the Lucy3 luminometer (Anthos) as described in Ref. 23. Results are represented as -fold induction of luciferase activity (normalized to *Renilla*) when compared with the control cells transfected with an empty expression vector.

Immunoblot and Co-immunoprecipitation Studies—Immunoblot and co-immunoprecipitation assays were done as described previously in Ref. 14. The antibodies used were anti-HA, monoclonal anti-β-tubulin, anti-FLAG M5 (Sigma); anti-p73 BL906 (Bethyl Laboratories Inc.); anti-Itch antibody (BD Biosciences); and polyclonal anti-Yap1 H-125 (Santa Cruz Biotechnology), and anti-Runx antibody was kindly provided by Y. Groner.

Half-life Determination—HEK293 and HCT-116 p53$^{-/-}$ cells were transfected with the indicated plasmids. 24 h after transfection, cells were treated with 20 μg/ml cycloheximide (Sigma) for different time points. Cells were harvested, and cell extracts were immunoblotted with the indicated antibodies. ^{35}S pulse-chase experiments were done as described in Ref. 14.

RNA Extraction and Reverse Transcriptase Reaction—RNAs were isolated and reverse transcribed as described previously in Ref. 23. The sequences of the primers used were 5′-CCTTAC-GTAGAGGTCACAGTAG-3′ and 5′-CTCCAAGCTGCAAA-GTCAC-3′ for *Itch* and 5′-ACCGCGAGAAGATGACCCAG and 5′-CCATCTCGTTCTCGAAGTCCA-3′ for β-*actin*.

Formaldehyde Cross-linking and Chromatin Immuno-precipitation—Chromatin immunoprecipitation (ChIP) was performed according to the protocol of Ainbinder *et al.* (24). Briefly, formaldehyde cross-linked protein-DNA complexes were precipitated by incubation overnight with the indicated antibodies or with Protein A beads (Amersham Biosciences) for negative control. Precipitated DNA fragments were extracted with Chelex 100 resin (Bio-Rad) as described (25) and amplified by PCR or Real-Time PCR (LightCycler 480, Roche Applied Science) with specific primers. The sequences of the primers used were 5′-TGATTTACCACCGCAGCCAG-3′ and 5′-TCCCTCTCCCGAGAACTGC-3′ for the *Itch* promoter when analyzed in semiquantitative PCR and 5′-CTCA-GGCGCCTGCGCTTTAGCTGCT-3′ and 5′-CGACTCCTC-AGTCCCTAGACTAAGATG-3′ for the *Itch* promoter when analyzed by Real-time PCR.

RESULTS

Inspection of the sequence of the *Itch* promoter revealed a consensus TGTGGT-binding site for the transcription factor Runx (26) located between positions 28 and 33 upstream of its first exon and the *Itch* transcription start site (Fig. 1*A*). The genomic region between positions +291 and −742 of the *Itch* promoter containing the putative Runx-binding site was cloned upstream of the luciferase reporter gene to evaluate the effect of Runx on the *Itch* promoter activity. H1299 and HEK293 cells were transfected with the Itch-Luc reporter plasmid together with an increasing amount of Runx. Runx activated the *Itch* promoter reporter plasmid in a dose-dependent manner in both cell lines (Fig. 1, *B* and *C*). Next we examined the effect of overexpressed Runx on the endogenous *Itch* promoter. Cells were transfected with Runx, and the level of *Itch* mRNA transcript was analyzed by RT-PCR. Remarkably, Runx was sufficient to support *Itch* expression (Fig. 1*D*). To demonstrate that endogenous Runx binds the endogenous *Itch* promoter at the predicted site, we performed ChIP analysis by immunoprecipitating DNA cross-linked protein complexes from HEK293T cells either with specific anti-Runx or with a control antibody. The ChIP analysis revealed that Runx is associated with the *Itch* promoter at the predicted site (Fig. 1*E*).

Since Yap1 is a known transcription modulator of Runx (27), we next asked whether Yap1 regulates *Itch* activation by Runx. ChIP analysis in HEK293T cells revealed that endogenous Yap1 is associated with the *Itch* promoter at the region that binds Runx (Fig. 2*A*). We then analyzed the effect of overexpressed Yap1 and Runx on endogenous levels of *Itch* mRNA (Fig. 2*B*). As expected, the level of endogenous *Itch* transcript was elevated by the transfected Runx. Interestingly, the level of *Itch* mRNA was also increased by Yap1 alone and had an additive effect when both Runx and Yap1
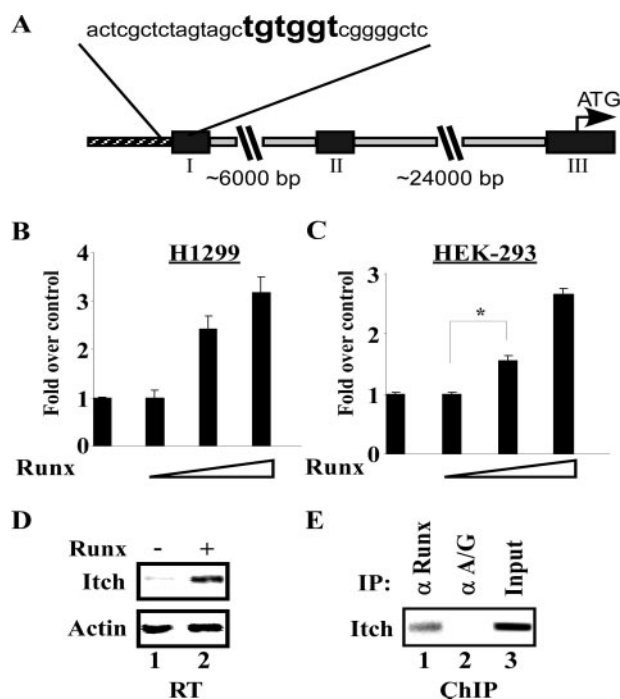
FIGURE 1. Runx binds and activates the Itch promoter. *A*, schematic representation of the Itch promoter. Exons are shown as *black boxes*, and introns are shown as *narrow rectangles* separated by *two diagonal lines* with their length below them. The Runx-binding site location and sequence are in *bold*. *B* and *C*, H1299 and HEK293 cells were transfected with the Itch promoter luciferase construct, control *Renilla*, and increasing amounts of Runx. Luciferase expression from the Itch promoter was measured 36 h after transfection. *Error bars* represent S.D. of triplicate samples. *, $p < 0.0006$ (calculated by using Student's *t* test). *D*, HEK293T cells were transiently transfected with an empty vector or Runx. RT-PCR was performed using Itch-specific primers and β-actin-specific primers as controls. *E*, cross-linked chromatin derived from HEK293T was immunoprecipitated (*IP*) with the anti-Runx antibody and was analyzed by PCR for the Itch promoter. Immunoprecipitation using only the protein-A/G beads served as a control. Non-immunoprecipitated cross-linked chromatin served as the input.
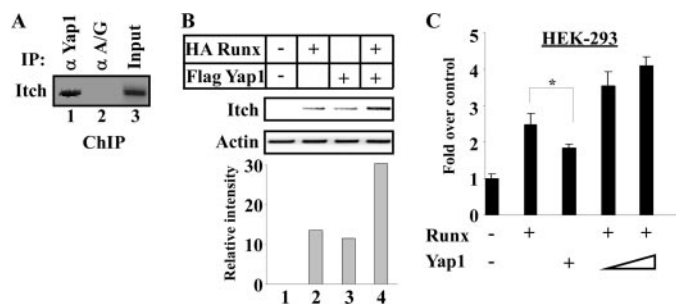
FIGURE 2. Yap1 enhances the transcriptional activation of Itch by Runx. *A*, cross-linked chromatin derived from HEK293T was immunoprecipitated (*IP*) with the anti-Yap1 antibody and was analyzed by PCR for the Itch promoter. Immunoprecipitation using only the protein-A/G beads served as a control. Non-immunoprecipitated cross-linked chromatin served as the input. *B*, HEK293T cells were transiently transfected with the indicated plasmids. RT-PCR was performed using Itch-specific primers and β-actin-specific primers as controls. Relative intensity of Itch mRNA level bands normalized to actin was measured using the Tina 2.0 software. *C*, HEK293 cells were transfected with Runx and Yap1 as indicated, and luciferase expression from the Itch promoter was measured. *Error bars* represent S.D. of triplicate samples. *, $p < 0.045$ (calculated by using Student's *t* test).

were overexpressed. The effect of Yap1 alone is likely to be mediated via the endogenous Runx. Similar results were obtained in the context of the Itch-Luciferase reporter plasmid. Overexpression of Runx and Yap1 each increased the

luciferase activity of the *Itch* promoter, which was further augmented when both proteins were overexpressed together (Fig. 2*C*). Taken together, these data show that Yap1 enhances the transcriptional activation of *Itch* by Runx.

Given the fact that Itch induces p73 degradation (11), we predicted that the obtained increased expression of *Itch* by Runx would result in p73 destabilization. To challenge this hypothesis, HEK293 cells were transfected with p73 alone or in combination with Runx. Runx overexpression resulted in a dramatic reduction in p73 protein level (Fig. 3*A*). Moreover, endogenous p73 was decreased by Runx overexpression in H1299 cells (Fig. 3*B*), or conversely, induced by specific Runx shRNA in HEK293T cells (Fig. 3*C*).

To examine the possibility that the Runx-mediated decrease in the p73 protein level is the result of increased p73 degradation, we measured p73 protein half-life in the presence and absence of co-transfected Runx. HEK293 cells were transiently co-transfected with p73 either alone or together with Runx. 24 h after transfection, cells were treated with cycloheximide to inhibit protein synthesis. Whole cell lysates were prepared at different time points and subjected to immunoblotting with the anti-HA antibody. This experiment revealed that overexpression of Runx dramatically decreases the half-life of the transfected p73 (Fig. 3*D*). Similar results were obtained in HCT-116 p53$^{-/-}$ where the level of the endogenous p73 was monitored (Fig. 3*E*). Interestingly, and in accord with a previous report (17), the endogenous p73 half-life is much shorter than the overexpressed one. In a reciprocal approach, knocking down of endogenous Runx by a specific shRNA resulted in a prolonged endogenous p73 protein level in a pulse-chase experiment (Fig. 3*F*).

Next we checked whether p73 destabilization by Runx occurs via the Itch-p73 protein degradation pathway (11). To this end, we knocked down endogenous *Itch* expression by co-expressing a control or a plasmid encoding an *Itch*-specific shRNA. Runx-dependent decrease in the endogenous p73 protein level was diminished when Itch expression was knocked down (Fig. 3*G*), suggesting that Runx-mediated p73 degradation is Itch-dependent. In a reciprocal experiment, we knocked down the endogenous Runx and measured the level of the endogenous Itch and p73 proteins. Runx knock down resulted in a down-regulation of Itch and accumulation of the endogenous p73 both in H1299 and in HCT-116 cell lines (Fig. 3*H*). Thus, activation of *Itch* by Runx gives rise to enhanced degradation of p73.

Next we investigated the molecular mechanism of activation of Itch by Runx and Yap1 under DNA damage. We have previously shown that induction of DNA damage by cisplatin or γ irradiation (IR) gives rise to Yap1 phosphorylation on the Tyr-357 residue (21). Phosphorylated Yap1 shows high affinity to p73 but low affinity to Runx. Thus, it is very likely that the observed activation of *Itch* transcription by the Runx-Yap1 complex is compromised under DNA damage insults. To challenge this prediction, we first measured Runx-Yap1 physical interaction under DNA damage. HEK293 cells were transfected with FLAG-Yap1 and Runx, and co-immunoprecipitation was performed before and after exposure to IR. Interestingly, the level of Yap1-Runx complex was dramatically reduced after
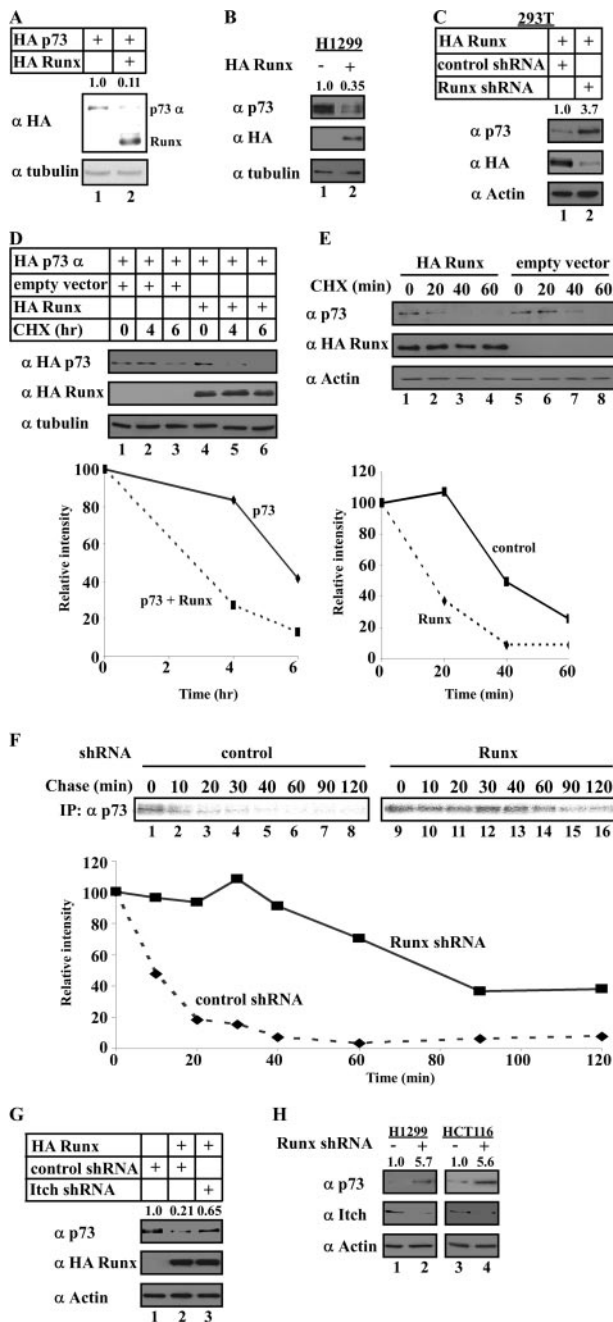
FIGURE 3. **Runx down-regulates p73 protein level.** *A*, HEK293 cells were co-transfected with the indicated expression vectors and were harvested 24 h after transfection for immunoblotting analysis with the indicated antibodies. *Numbers* shown above bands represent the relative -fold of p73 band intensity, normalized to tubulin, as measured by the Tina 2.0 software. *B*, H1299 cells were transfected with HA-Runx and were treated as in *A*. *C*, HEK293T cells were transfected with HA-Runx without or with Runx shRNA. 48 h after transfection, cells were harvested and were treated as in *A*. Tina 2.0 software was used to measure the relative -fold of p73 band intensity, normalized to actin. *D*, HEK293 cells were transfected with plasmids expressing HA p73 with or without Runx. 24 h after transfection, cells were treated with 20 μg/ml cyclo-heximide (*CHX*) for different time points. Equal amounts of total protein lysates were subjected to Western blotting analysis with the indicated antibodies. Relative intensity of p73, normalized to the total protein loaded (anti-tubulin) shown in the graph, was measured using the Tina 2.0 software. *E*, HCT-116 cells were transfected with or without HA-Runx and were treated as in *D*. *F*, HCT-116 cells were transfected with a control of Runx shRNA. 48 h after transfection, cells were starved in a Met-free medium and then were labeled with [^{35}S]methionine. Unlabeled Met (2%) was added, and cells were collected at the indicated time points. Immunoprecipitates (*IP*) were washed, resolved by SDS-PAGE, and detected by autoradiography. The graph shows
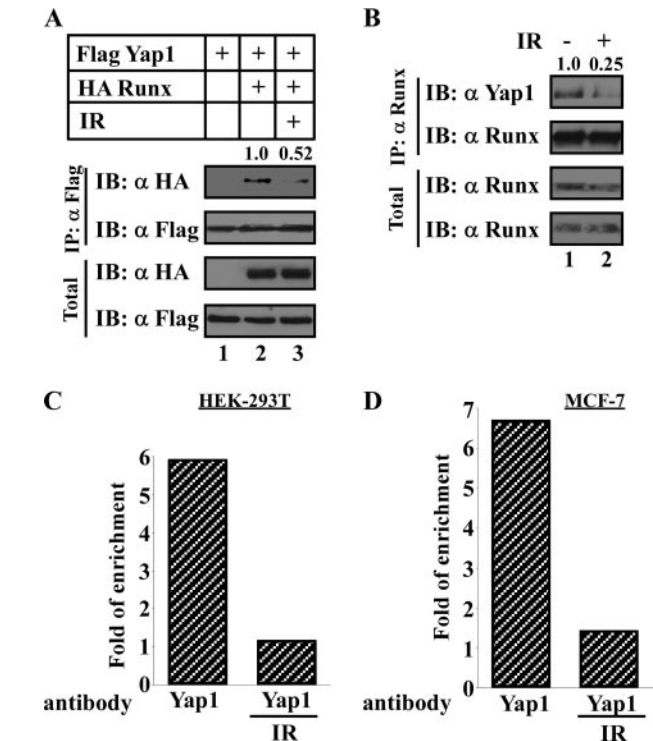

FIGURE 4. **Tyrosine-phosphorylated Yap1 does not bind Runx.** *A*, HEK293 cells were transfected with the indicated plasmids. 24 h after transfection, cells were γ-irradiated and collected 4 h after IR. Cell extracts were then immunoprecipitated using anti-FLAG antibody. Co-precipitating proteins were detected with the indicated antibodies. *Numbers* shown above bands represent the relative -fold of Yap1-Runx interaction intensity as measured by the Tina 2.0 software. *IB*, immunoblot. *B*, H1299 cells were γ-irradiated and collected 4 h after IR. Cell extracts were then immunoprecipitated with antibody to endogenous Runx and were subjected to Western blot analysis with the indicated antibodies. *Numbers* shown above bands represent the relative -fold of Runx-Yap1 interaction intensity as measured by the Tina 2.0 software. *C* and *D*, γ-irradiated HEK293T (*C*) or MCF-7 (*D*) cells were collected 10 h after IR, chromatin-immunoprecipitated with the indicated antibodies, and subjected to quantitative real-time PCR using primers for the Itch promoter.

DNA damage stress (Fig. 4*A*). Similar results were obtained when the levels of the endogenous proteins were measured (Fig. 4*B*). These results suggest that in response to DNA damage, Yap1 escapes Runx. Next we asked whether under this condition Yap1 is in association with the *Itch* promoter. ChIP analysis revealed that in two different cell lines, HEK293T (Fig. 4*C*) and MCF-7 (Fig. 4*D*), the endogenous Yap1 bound the *Itch* promoter. However, in response to IR, the amount of Yap1 in association with the *Itch* promoter was reduced by over 6-fold. These results suggest that in response to DNA damage, Yap1 is no longer functional in supporting *Itch* gene expression.

Itch protein level is down-regulated in response to DNA damage (11), but the mechanism is not known. The results of the ChIP assay, demonstrating that Yap1 dissociated from the *Itch* promoter following DNA damage, suggested that the reduction in *Itch* level following DNA damage may be due to loss of Yap1 co-activation and a reduction in transcription of

densitometric analysis of endogenous p73 protein. *G*, HEK293 cells were transfected with HA-Runx without or with pSUPER Itch. 48 h after transfection, cells were harvested and subjected to Western blot analysis. *Numbers* shown above bands represent the relative -fold of p73 band intensity, normalized to actin, as measured by the Tina 2.0 software. *H*, H1299 or HCT-116 cells were transfected with Runx shRNA and were treated as in *G*.
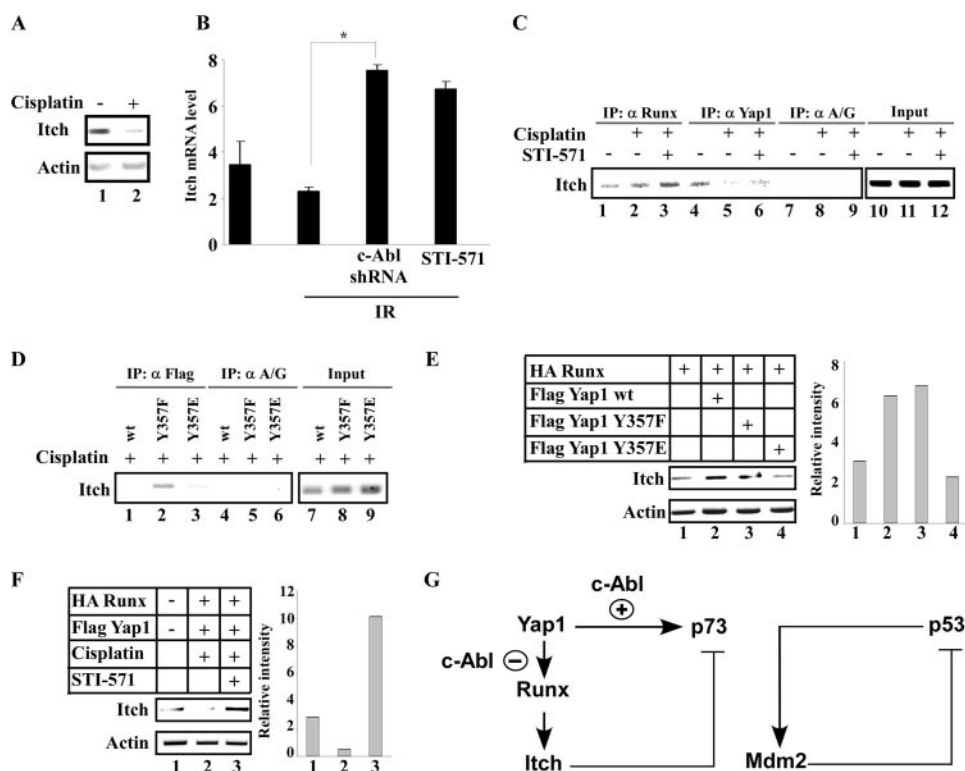
FIGURE 5. **Phosphorylated Yap1 dissociates from the Itch promoter under DNA damage.** *A*, HEK293T cells were treated with 25 μM cisplatin for 24 h where indicated. RT-PCR was done using Itch-specific primers or β-actin-specific primers as controls. *B*, γ-irradiated HEK293T were transfected with pSUPER c-Abl encoding c-Abl-specific shRNA or were treated with 10 μM STI-571 for 24 h. 48 h after transfection, RNA from the cells was extracted and subjected to reverse transcription analysis by real-time RT-PCR with specific Itch primers, and results were normalized to actin. *Error bars* represent S.D. of triplicate samples. *C*, cross-linked chromatin derived from HEK293T cells treated for 24 h with 25 μM cisplatin and 10 μM STI-571 was immunoprecipitated (*IP*) with the indicated antibodies and was analyzed by PCR for the Itch promoter. Immunoprecipitation using only the protein-A/G beads served as a control. Non-immunoprecipitated cross-linked chromatin served as the input. *D*, HEK293T cells were transfected with the indicated plasmids and were treated as in *C*. *E*, HEK293T cells were transfected with the indicated plasmids and were subjected to RT-PCR analysis. Relative intensity of Itch mRNA level bands normalized to actin were measured using the Tina 2.0 software. *wt*, wild type. *F*, cells were transfected with the indicated plasmids. 24 h after transfection, cells were treated with 25 μM cisplatin and 10 μM STI-571 for 24 h and were then treated as in *A*. Bands intensity was calculated as in *D*. *G*, a schematic model representing a regulatory circuit controlling p73 degradation by Runx when compared with the p53-Mdm2 loop.

presence of STI-571 (Fig. 5*B*). Thus, the kinase activity of c-Abl negatively regulates *Itch* expression.

These results predict that under DNA damage, phosphorylated Yap1 at Tyr-357 is poorly associated with the *Itch* promoter. We used ChIP analysis to test this prediction. ChIP analysis revealed that both endogenous Runx and endogenous Yap1 are in association with the *Itch* promoter prior to cisplatin treatment in HEK293T cells (Fig. 5*C*). However, after cisplatin treatment, whereas Runx binding to the *Itch* promoter was not affected, Yap1 was no longer detected. Remarkably, however, Yap1 binding to the *Itch* promoter was at least partially resumed when cells were treated with STI-571 to inhibit c-Abl kinase activity. These results suggest that the modified Yap1 no longer binds and co-activates Runx, the *Itch* promoter activator.

To confirm this conclusion, we tested the effect of different Yap1 mutants at the Tyr-357 residue on the recruitment to the *Itch* promoter by ChIP analysis (Fig. 5*D*). As expected, in response to cisplatin treatment, both wild type Yap1, which is phosphorylated in response to cisplatin treatment, and the phospho-mimetic Y357E Yap1 mutant are not associated with the *Itch* promoter. In sharp contrast, the Y357F mutant, which cannot be

the *Itch* gene. To test this possibility, we checked the effect of cisplatin on the *Itch* mRNA level. Interestingly, the level of *Itch* mRNA, as determined by RT-PCR, was dramatically decreased after cisplatin treatment (Fig. 5*A*), suggesting that the reduction in Itch protein after DNA damage is due to a reduction in transcription. As shown above, DNA damage reduced the association of Yap1 with Runx (Fig. 4). Our previous work has shown that DNA damage-induced phosphorylation of Yap1 by c-Abl prevents its association with Runx (21). Therefore, the reduction in Runx-mediated transcription of *Itch* following DNA damage is likely to be due to the dissociation of phosphorylated Yap1 from Runx. Therefore, an immediate prediction is that inhibition of c-Abl would improve *Itch* transcription. In HEK293T IR-treated cells, knockdown of the endogenous c-Abl by expressing c-Abl-specific shRNA resulted in an ~3-fold increase in the level of the endogenous *Itch* mRNA, as determined by real-time PCR (Fig. 5*B*). To test whether the kinase activity of c-Abl is involved in this process, we utilized STI-571, the c-Abl-specific kinase inhibitor (28). The level of the endogenous *Itch* mRNA was increased by ~3-fold in the

phosphorylated by c-Abl (21), is associated. To confirm this conclusion, we next tested the effect of different Yap1 constructs on transcription of the endogenous *Itch* in the presence of Runx (Fig. 5*E*). As expected, both wild type and Y357F Yap1 augmented *Itch* mRNA expression, whereas the phospho-mimetic Y357E Yap1 did not. This observation was also tested under DNA damage conditions. Overexpression of Runx and Yap1 in the presence of cisplatin resulted in reduction of *Itch* mRNA, which was completely restored after the addition of the c-Abl kinase inhibitor STI-571 (Fig. 5*F*). These results suggest that the modified Yap1 at Tyr-357 escapes the *Itch* promoter, and as a result, *Itch* transcription is down-regulated.

DISCUSSION

In this study, we provide evidence for the Itch promoter to be a novel target of Runx. This conclusion is based on the following findings. Firstly, the *Itch* promoter reporter plasmid was transcriptionally induced by overexpression of Runx. Secondly, overexpressed Runx stimulated the transcription activation of the resident *Itch* promoter. Thirdly, ChIP analysis revealed that

Runx bound the endogenous *Itch* promoter. Finally, Yap1, a known transcription co-activator of Runx (27), augmented the Runx-mediated *Itch* promoter activation. Although Runx1 is the only family member that was tested, at this stage, we do not discriminate between the different Runx paralogs (Runx2 and Runx3) since they all appear to be functional in this process (data not shown).

It has been documented that c-Abl tyrosine kinase activity is increased in response to DNA double strand breaks induced by ionizing radiation or by drugs, such as cisplatin (reviewed in Ref. 19). At least two relevant substrates, p73 and Yap1, then undergo tyrosine phosphorylation by c-Abl (16, 17, 20, 21). Unlike the naive Yap1, the modified Yap1 does not bind Runx and does not co-activate *Itch* and possibly other Runx target genes. We found this process to be responsible for diminished *Itch* transcription and the consequent p73 accumulation.

Our data demonstrate that the transcription induction of *Itch* by Runx as a transcription activator and Yap1 as a transcription co-activator is of functional significance. We show that induction of the endogenous *Itch* transcription by over-expression of Runx augments p73 degradation, and conversely, knockdown of Runx induces p73 accumulation. Significantly, this regulatory circuit is down-regulated in response to DNA damage, when there is a demand for p73 accumulation and activation of the pro-apoptotic genes such as *Bax* (16, 20, 21, 29). However, in other systems, Yap1 overexpression induces anti-apoptotic genes and supports cell proliferation and oncogenesis (30–32). Our findings provide an explanation to the observed double-edged sword activity of Yap1. Yap1 acts via Runx to down-regulate p73 and its pro-apoptotic function, but in response to DNA damage, Yap1, by undergoing tyrosine phosphorylation, plays an opposite role of co-activating p73. Thus, our finding that c-Abl helps determine which transcription factor Yap1 will co-activate is an important step in understanding the various aspects of Yap1 activity.

Previously, we have reported on another mechanism of p73 accumulation by Yap1 (14). Yap1 directly binds p73 at the region that Itch binds to polyubiquitinate p73. Under DNA damage stress, Yap1 is tyrosine-phosphorylated by c-Abl and accumulates to high levels. The accumulated Yap1 in turn is effective in competing with Itch for binding to p73 to allow p73 to escape degradation. Here we describe a mechanism whereby Runx plays an important role in destabilizing p73. Yap1 increases *Itch* expression and p73 degradation via co-activation of *Itch* promoter-associated Runx. Paradoxically, Yap1 may increase p73 level by inhibiting Itch-p73 complex formation (14) (Fig. 5*F*). This paradoxical behavior is resolved by c-Abl. c-Abl is activated in response to DNA damage and tyrosine-phosphorylates p73 (16, 17, 20) and Yap1 (21). The modified Yap1 loses its interaction with Runx but increases its interaction with p73 (21). Under DNA damage stress, therefore, the circuit is shifted toward p73 accumulation by negating *Itch* expression and eliminating Itch-p73 complex formation. At the moment, the question of how this process is terminated is open, but a number of models can be proposed. The most trivial one is the involvement of tyrosine phosphatases that would inactivate c-Abl and dephosphorylate Yap1.

Itch, the HECT E3 ligase, has a number of other important substrates such as Notch (33) c-FLIP (34), and c-Jun and Jun-B. The latter proteins are important regulators of the immune response (35, 36). Interestingly, c-Jun escapes Itch by a mechanism that involves c-Abl. c-Abl directly modifies c-Jun at the region that blocks c-Jun-Itch interaction (37), and this process eliminates c-Jun ubiquitination and degradation in T cells. Our finding that c-Abl also down-regulates *Itch* expression provides another and a complementary explanation for c-Jun stabilization. Furthermore, it has been reported that c-Jun supports *Yap1* expression (38). Thus, under the regulatory circuit described here, the stabilized c-Jun is likely to further support Yap1 accumulation.

Although p73 and p53 share a significant structural and functional homology, the molecular circuits regulating their stabilization are substantially different (39). Each has its own unique E3 ligase. Although Mdm2, the E3 ubiquitin ligase of p53 (3), binds to p73, it does not promote p73 degradation (40). Itch, the p73 E3 ligase, does not bind p53 (11). Also, Mdm2 is a downstream target of p53 (41), but *Itch* gene expression appears not to be p53/p73-responsive, although at the moment, we cannot unequivocally exclude this possibility. Notably, it has been shown that Runx1 and -2 are possible targets of p63 (42) and presumably of p73, generating a direct feedback loop between Runx and p73. The cross-talk between p73 and Itch is mediated by a few other components (Fig. 5*G*). These differences are substantial, and each is predicted to generate a unique output. The p73-Itch regulatory circuit permits a fine-tuning regulation mechanism controlled by c-Abl as a rheostat, with minimal oscillated output. This is not the case with the p53-Mdm2 loop, which acts as a binary switch, giving rise to an oscillating pulse mode of outputs (43).

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