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PML, YAP, and p73 Are Components of a Proapoptotic Autoregulatory Feedback Loop

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SUMMARY

p73 has been identified as a structural and functional homolog of the tumor suppressor p53. The transcriptional coactivator Yes-associated protein (YAP) has been demonstrated to interact with and to enhance p73-dependent apoptosis in response to DNA damage. Here, we show the existence of a proapoptotic autoregulatory feedback loop between p73, YAP, and the promyelocytic leukemia (PML) tumor suppressor gene. We demonstrate that *PML* is a direct transcriptional target of p73/YAP, and we show that *PML* transcriptional activation by p73/YAP is under the negative control of the proto-oncogenic Akt/PKB kinase. Importantly, we find that PML and YAP physically interact through their PVPVY and WW domains, respectively, causing PML-mediated sumoylation and stabilization of YAP. Hence, we determine a mechanistic pathway in response to DNA damage that could have relevant implications for the treatment of human cancer.

INTRODUCTION

The main activities of the p53 family occur through the transcriptional activation or repression of a plethora of target genes that encode for key proteins involved in cell growth inhibition, apoptosis, senescence, and differentiation (Vousden and Lu, 2002). Transcriptional activity is frequently governed by the formation of large protein complexes, including transcription factors, coactivators or corepressors, and acetylases or deacetylases, whose spatial and temporal integration imparts gene selectivity and specificity (Naar et al., 2001). The transcriptional coactivator Yes-associated protein (YAP) has been demonstrated to interact with and to enhance p73-dependent apoptosis in response to DNA damage (Strano et al., 2001, 2005). It has been reported that YAP is phosphorylated by AKT, and such modification impairs YAP-nuclear translocation and attenuates p73-mediated apoptosis (Basu et al., 2003). Recently, we demonstrated that p73 is required for the nuclear translocation of endogenous YAP in cells exposed to cisplatin and that YAP is recruited by PML into the nuclear bodies to promote p73 transcriptional activity. We found that YAP contributes to p73 stabilization in response to DNA damage and promotes p73-dependent apoptosis through the specific and selective coactivation of apoptotic p73 target genes and potentiation of p300-mediated acetylation of p73 (Strano et al., 2005). Collectively, these results identify YAP as an important determinant for p73 target gene specificity through p300 recruitment and p73 acetylation.

Recently, Rossi et al. have shown that Itch, a human ubiquitin-protein ligase that belongs to the Nedd4-like E3 family containing a WW domain, binds and ubiquitinates p73 and determines its rapid proteosome-dependent degradation (Rossi et al., 2005). More recently, Levy et al. have shown that YAP competes with Itch for binding to p73 at the PPPY motif, and this prevents Itch-mediated ubiquitination and subsequent degradation of p73 (Levy et al., 2007).

The *PML* tumor suppressor gene, involved in the t(15;17) chromosomal translocation of acute promyelocytic leukemia (APL), encodes a protein that localizes to the PML-nuclear body. PML has been shown to be involved in apoptosis; it is markedly upregulated upon a number of cellular stresses and proapoptotic stimuli, such as ionizing radiation (Ferbeyre et al., 2000; Pearson et al., 2000). In addition, $PML^{-/-}$ mice are resistant to the lethal effects of both γ irradiation and CD95 (Salomoni and Pandolfi, 2002). Moreover, the importance of PML in p73-mediated apoptosis has already been demonstrated (Strano et al., 2005).

Here, we show the existence of a proapoptotic autoregulatory feedback loop between p73, YAP, and the promyelocytic leukemia (PML) tumor suppressor gene. We performed microarray analysis on cisplatin-treated HCT116 cells, where the endogenous expression of p73 or YAP was silenced through specific siRNAs, and found that *PML* is a direct transcriptional target of p73/YAP. We demonstrate that PML contributes to the p73-dependent apoptotic response by regulating YAP stability.

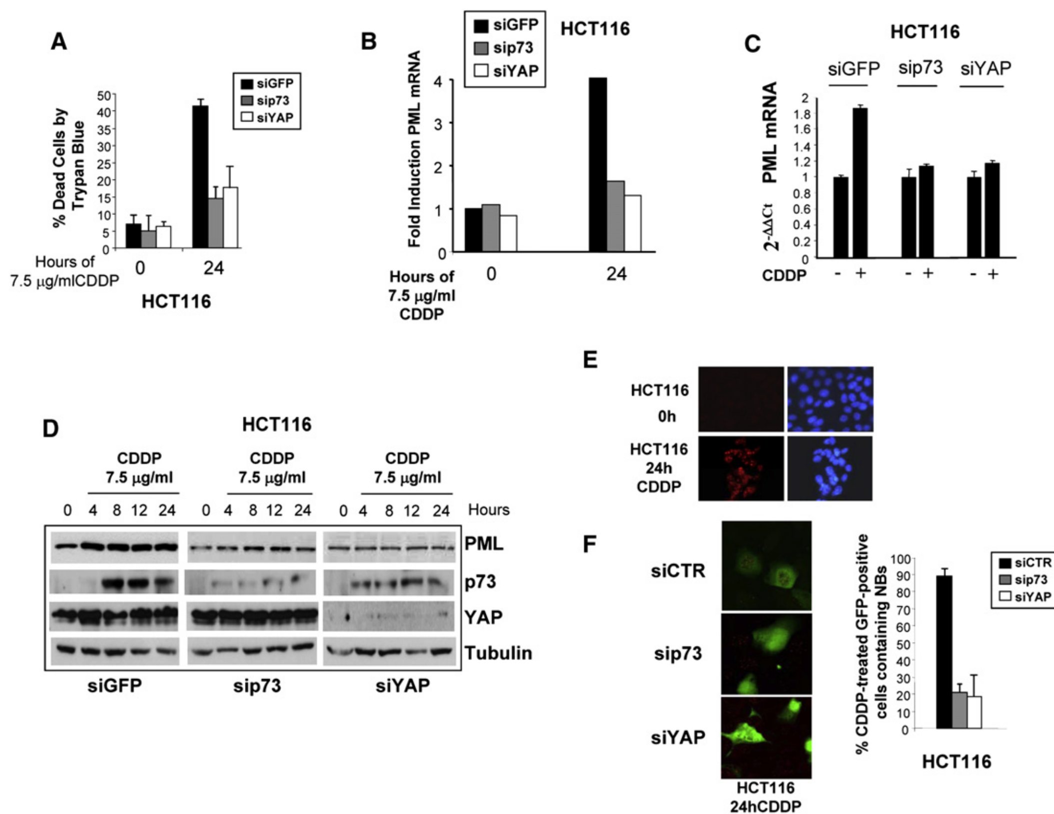

Figure 1. *PML* Expression Is Modulated by the Protein Complex p73/YAP upon Cisplatin Treatment

(A) HCT116 cells were transfected with specific anti-p73, anti-YAP, or control anti-GFP siRNAs and treated with cisplatin (CDDP), 7.5 μg/ml for 24 hr. Floating and attached cells were collected and counted for trypan blue exclusion. Histograms show the mean of three experiments; bars indicate SD.

(B) Histogram showing PML expression in HCT116 cells transfected, treated, and processed as in (A), obtained from the microarray data.

(C) Quantitative real-time PCR analysis of PML transcripts in HCT116 cells processed as in (A). Histograms show the mean of three experiments; bars indicate SD.

(D) Proteins were extracted from HCT116 cells; transfected with specific anti-p73, anti-YAP, or control anti-GFP siRNAs at the indicated time points after treatment with 7.5 μg/ml CDDP; and subjected to WB analysis.

(E) HCT116 cells, after 24 hr of CDDP treatment, were fixed and stained with an anti-PML antibody.

(F) Proliferating HCT116 cells were processed as in (A). Twenty-four hours after treatment, cells were fixed and stained with an anti-PML antibody. The graph shows the percentage of GFP-positive cells containing PML NBs. Histograms show the mean of three experiments; bars indicate SD.

Importantly, we find that PML and YAP physically interact through their PVPVY and WW domains, respectively, causing YAP stabilization upon cisplatin treatment, which occurs through PML-mediated sumoylation.

RESULTS

Microarray Analysis Reveals that *PML* Expression Is Modulated by the Protein Complex p73/YAP upon Cisplatin Treatment

To dissect the transcriptional events regulating the proapoptotic activity of the protein complex p73/YAP, we performed a microarray analysis on cisplatin (CDDP)-treated HCT116 cells in which the expression of p73 or YAP was knocked down by specific siRNAs (Figure 1 and Figure S1 available online). p73- or YAP-deficient expression resulted in a severe reduction of CDDP-induced apoptosis in HCT116 cells (Figure 1A). Bioinformatic analysis revealed that among the 393 genes upregulated in response to CDDP, 332 genes showed no upregulation in the samples where p73 or YAP expression was inhibited by specific siRNAs (Figure S1). This list was further reduced to 156 genes upregulated above 2-fold in at least three time points in the control sample (siGFP) (Table S1) that showed no upregulation above 2-fold at any of the time points in the sip73 or siYAP cells lines. We subsequently focused our investigation on one of these genes, the *promyelocytic leukemia* (*PML*) tumor suppressor gene, since previous findings have highlighted the potential existence of a transcriptional and functional crosstalk with the protein complex p73/YAP. (Figure 1B).

Microarray data were further validated by analyzing PML transcript expression and the level and subcellular localization of PML protein in HCT116 cells, treated as described above (Figures 1C–1E). As shown in Figure 1C–1D, PML transcript and protein levels are upregulated upon CDDP treatment in siGFP cells, but not in sip73 or siYAP cells. PML levels were affected in similar way in MCF7 and SKBR3 cells after treatment with CDDP and doxorubicin (Figures S2A–S2C; Figure 2C; data not shown), showing conservation between cell types and different stresses.

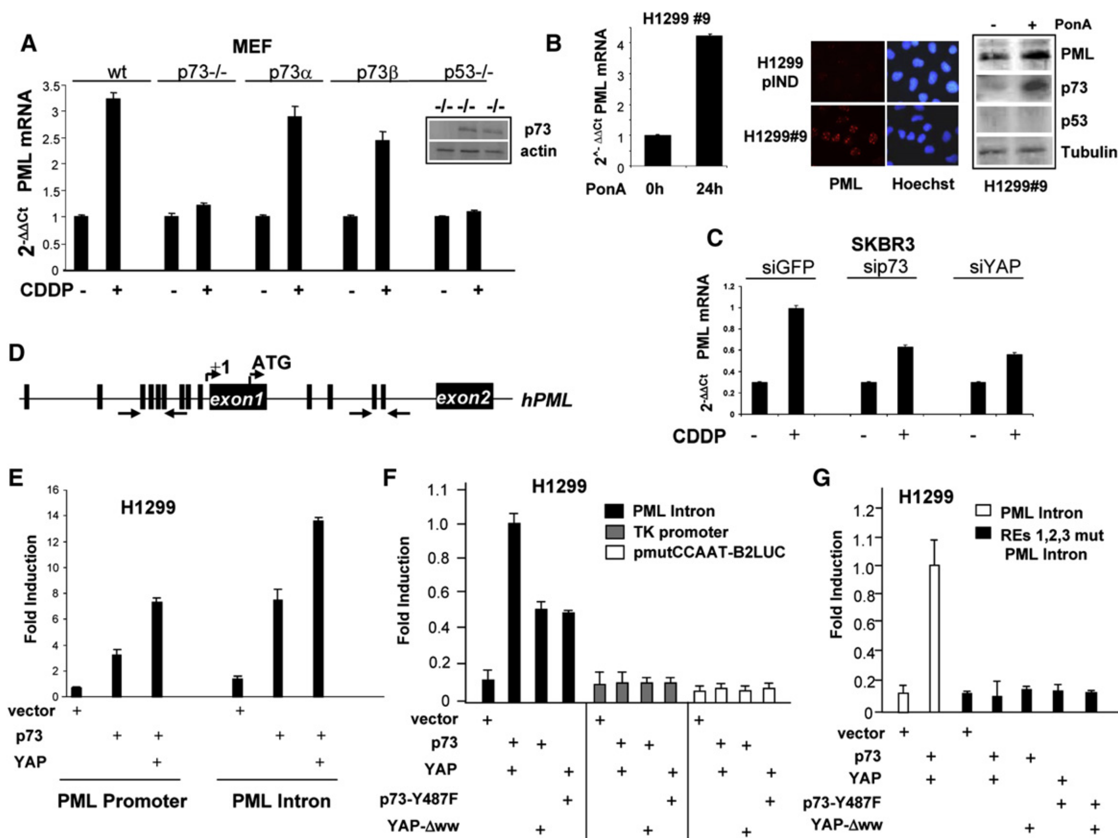

Figure 2. *PML* Is a Transcriptional Target of p73 and YAP

(A) Quantitative real-time PCR analysis of PML transcripts in wild-type, p73$^{-/-}$, p73$^{-/-}$ reconstituted with p73alpha, p73$^{-/-}$ reconstituted with p73beta, and p53$^{-/-}$ MEFs exposed to 7.5 μg/ml CDDP for 24 hr. Histograms show the mean of three experiments; bars indicate SD.

(B) H1299 cells with ponasterone A-inducible p73 expression (H1299#9) and H1299 stably transfected with the pIND control vector (H1299 pIND) were treated with 2.5 μM ponasterone A. After 24 hr, RNA was extracted and subjected to real-time PCR analysis (left panel; histograms show the mean of three experiments; bars indicate SD.), cells were fixed and stained with an anti-PML antibody (middle panel), and proteins were extracted and subjected to WB analysis (right panel).

(C) Quantitative real-time PCR analysis of PML transcripts in SKBR3 cells transfected with specific anti-p73, anti-YAP, or control anti-GFP siRNAs and exposed to 7.5 μg/ml CDDP for 24 hr.

(D) Schematic diagram depicting the genomic regions spanning 5000 bp upstream of the predicted *PML* transcriptional start site and 5000 bp into the first and the second exons of human *PML*. Predicted p53-responsive elements, identified using Mat-Inspector Professional software, are represented by black boxes. The arrows represent the position of the primers used in the ChIP analysis.

(E–G) H1299 cells were transiently transfected with the plasmids indicated in the figures together with constructs carrying the luciferase reporter gene driven by human *PML* promoter, *PML* first intron, *thymidine kinase* (*TK*) promoter, *cyclin B2* promoter mutated in the three CCAAT boxes (pmutCCAAT-B2LUC), and *PML* first intron mutated in the three p53-consensus sequences. An equal amount of CMV-βgal was added to each transfection. Luciferase activity was determined relative to total proteins and β-gal activity. Results are presented as a fold of induction over the control. Histograms show the mean of three experiments each performed in duplicate; bars indicate SD.

PML is ubiquitously expressed, albeit at very low levels, and PML NBs are detected in almost any cell in the developing embryo or the adult organism. Nonetheless, PML is markedly upregulated upon a number of cellular stresses, including inflammation, oncogenic transformation, and proapoptotic stimuli (Ferbeyre et al., 2000). Under these conditions, the number and the size of the PML NBs, as well as the soluble nonmatrix PML nuclear and cytosolic fractions, increase. Immunofluorescence performed in HCT116 cells reveals that upon CDDP treatment, PML protein levels and the number and size of PML-containing nuclear bodies (NBs) were increased (Figure 1E). To further define the contribution of p73 and YAP in the formation of NBs, we knocked down p73 or YAP expression and evaluated the number of PML NBs by immunofluorescence. We found that the number of PML-containing NBs in the GFP-positive p73 or YAP siRNAs-transfected cells, treated with CDDP, was clearly diminished when compared to that of the surrounding GFP-negative untransfected cells. No modulation in the number of NBs was seen in the cells transfected with the unrelated siRNAs (Figure 1F).

PML Is a p73 and YAP Transcriptional Target

To provide genetic evidence for *PML* as a transcriptional target of p73 upon DNA damage, we treated wild-type MEFs, p73$^{-/-}$ MEFs, and p73$^{-/-}$ MEFs reconstituted with p73α or p73β with CDDP. As shown in Figure 2A, the upregulation of PML observed

in WT MEFs after DNA damage was impaired in p73$^{-/-}$ MEFs and was partially restored in p73α-p73β MEFs.

It has been previously demonstrated that *PML* is a p53 target gene (de Stanchina et al., 2004), so it is not surprising that we found the upregulation of *PML* in p53$^{-/-}$ MEFs also impaired (Figure 2A). Moreover, HCT116 cells have wild-type p53, which is activated under the experimental conditions used (Figure S2D), and this can synergize with p73/YAP in the transcriptional regulation of *PML*. Indeed, using HCT116 p53$^{-/-}$ cells we observed the same extent of upregulation in PML mRNA after CDDP treatment, but both PML mRNA basal level and the level after DNA damage were lower than in HCT116 harboring wild-type p53 (Figure S2E).

To underline the importance of the p73/YAP-mediated induction of PML upon CDDP treatment, we asked whether PML is induced in cells that lack p53 or in cells expressing mutant p53. We used H1299 cells (p53 null) with ponasterone A-inducible p73 expression (H1299#9) and H1299 cells stably transfected with the pIND vector (H1299 pIND) as a control (Fontemaggi et al., 2002). In agreement with previous findings, H1299 cells showed a low level and a diffused nuclear-cytoplasmic expression of PML that was clearly upregulated and relocalized in nuclear bodies (NBs) following overexpression of p73 (Figure 2B), indicating that overexpression of p73 alone is sufficient to induce PML mRNA (Figure 2B, left panel) and protein (Figure 2B, right panel) upregulation and NBs formation (Figure 2B, middle panel). Moreover, we found that siRNA-mediated knockdown of p73 and YAP expression prevents PML induction in mutant p53-expressing breast cancer cells (SKBR3) upon CDDP treatment, thereby implying that WT p53 is dispensable for p73/YAP-mediated transcriptional activation of PML in response to CDDP (Figure 2C). Thus, it is clear that p53 has a role in regulating *PML* expression, synergizing with p73/YAP in particular cellular contexts; however, notably, we demonstrated that p73 expression alone is sufficient to induce PML expression and NB formation in cells that lack p53 (H1299) or in cells expressing mutant p53 (SKBR3), highlighting a potential alternative pathway in response to DNA damage that works in cells lacking functional p53 protein.

p73 and YAP Promote *PML* Transcriptional Activation

To further validate *PML* as a direct transcriptional target of p73 and YAP, we first assessed the ability of the p53 binding sites contained in the *PML-IV* regulatory regions (Figure 2D) (de Stanchina et al., 2004) to confer p73/YAP responsiveness to a heterologous reporter. Unlike WT p53, which exerts its transcriptional activity by binding directly to the p53 consensus in the first intron of *PML-IV* (de Stanchina et al., 2004), we found that p73α promotes transcriptional activation of both *PML-IV* promoter and intron (Figure 2E). This effect was further enhanced by the concomitant expression of YAP (Figure 2E). The synergistic effect between p73 and YAP was prevented using a p73 mutant in which the tyrosine in the YAP-WW binding consensus was mutated to phenylalanine (p73-Y487F, Strano et al., 2001) that consequently cannot bind YAP, and a YAP mutant deleted of the WW domain (YAP-ΔWW) that consequently cannot bind p73 (Figure 2F). p73/YAP transactivation activity was lost using a *PML-IV* first intron carrying three mutated p53 responsive elements (Figure 2G, de Stanchina et al., 2004). The *thymidine kinase* (*TK*) promoter and the *cyclin B2* promoter carrying three mutated CCAAT boxes (pmutCCAAT-B2LUC, Di Agostino et al., 2006), which does not contain any p53 consensus, were not activated by p73/YAP complex (Figure 2F). Moreover, to demonstrate the role of DNA damage in p73-dependent induction of *PML*, a luciferase assay after treatment with CDDP was performed. As shown in Figure S2F, *PML-IV* first intron was transactivated after CDDP treatment, and the transactivation activity was lost with the *PML-IV* first intron carrying three mutated p53 responsive elements. Altogether, these results demonstrate that p73/YAP complex is capable of transcriptionally regulating the *PML-IV* promoter and first intron and this activity requires the integrity of the p53 consensus sequences of the target regulatory regions.

p73 and YAP Bind *PML* Regulatory Regions

To determine whether p73 and YAP occupy *PML* regulatory regions we performed chromatin immunoprecipitation (ChIP) experiments. As shown in Figure 3A, p73 and YAP were recruited on both the promoter and first intron of *PML* in the presence of CDDP. Moreover, we found that p300 is preferentially recruited on *PML* regulatory regions upon CDDP treatment, and this correlates with the transcriptional activation of the gene as shown by the increase of H4 histones acetylation (Figure 3A). It has been previously shown (Basu et al., 2003; Strano et al., 2005) that Akt-mediated phosphorylation of YAP causes its cytoplasmic retention and impairs its cotranscriptional activity. Moreover, PML has recently been shown to orchestrate a nuclear tumor suppressor network for inactivation of nuclear pAKT (Trotman et al., 2006). Interestingly, performing the ChIP assay in cells transfected with a constitutively active mutant of AKT (CA-AKT) that phosphorylates YAP in a constitutive manner, we found no changes in the degree of histone H4 acetylation on both the *PML* promoter and first intron in response to CDDP treatment. This was accompanied by less p300 bound on the same regulatory regions (Figure 3B). Overexpression of CA-AKT decreased the binding between YAP and p73 (Figure 3C), impairing p73/YAP-mediated transcriptional activation of PML mRNA (Figure 3D) and NBs formation (Figure 3E) in response to CDDP. This highlights once again that YAP, together with p73, is essential for the activation of *PML* after treatment with CDDP and demonstrates that the proto-oncogenic Akt/PKB kinase plays a role in *PML* transcriptional activation.

PML Contributes to p73/YAP-Mediated Apoptosis

Next, we aimed to investigate the contribution of PML induction to the transcriptional activity of p73 and YAP. We have previously shown that the induction of Bax and p21waf1 in response to CDDP was reduced in cells where p73 and YAP expression is knocked down by specific siRNAs (Strano et al., 2005). PML is essential for multiple stress/DNA damage-activated apoptotic pathways, and its importance in p73-mediated apoptosis has already been demonstrated (Strano et al., 2005). In agreement with these findings, RNA interference-mediated decrease of PML expression in HCT116 treated with CDDP impairs both basal and induced mRNA and protein expression of Bax and p21waf1 (Figure 4A; Figure S3A), associated with the reduction

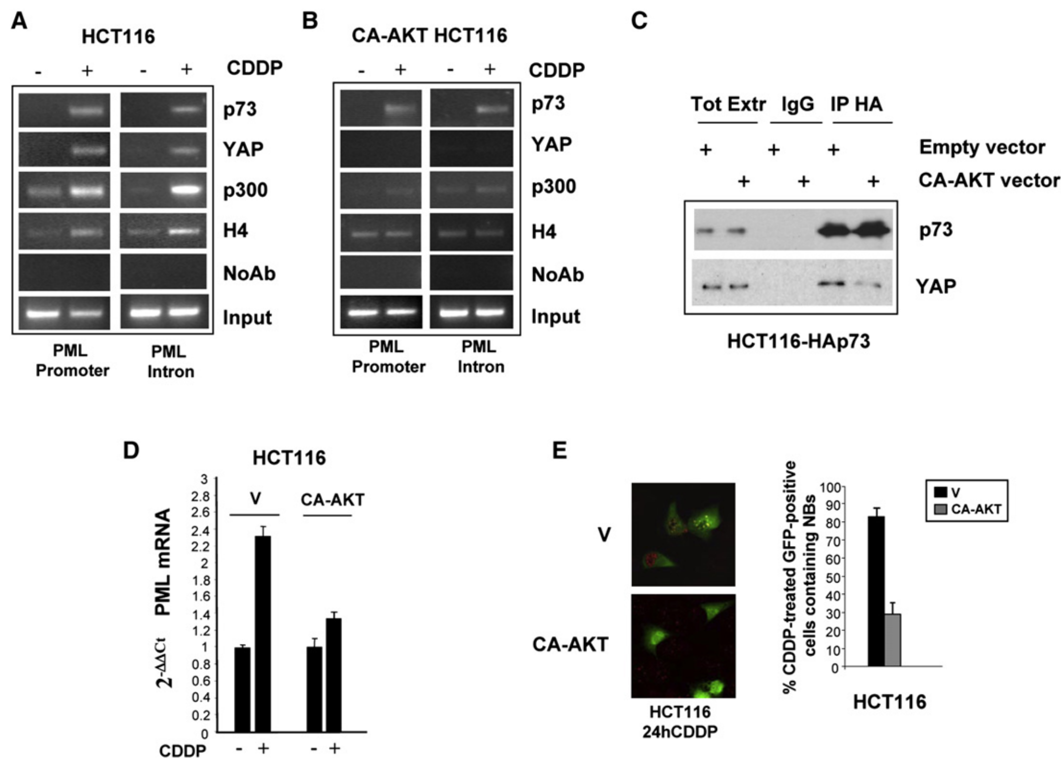

Figure 3. p73 and YAP Bind PML Regulatory Regions

(A) Crosslinked chromatin derived from HCT116 cells untreated or treated with 7.5 μg/ml CDDP for 24 hr was immunoprecipated with antibodies against p73, YAP, p300, acetylated histone H4, or in the absence of antibody and analyzed by PCR with specific primers for the indicated regulatory regions. Input, nonimmunoprecipitated crosslinked chromatin.

(B) Crosslinked chromatin derived from HCT116 cells, stably transfected with a mutant constitutively active of AKT (CA-AKT), untreated or treated with 7.5 μg/ml CDDP for 24 hr, was immunoprecipated with antibodies against p73, YAP, p300, acetylated histone H4, or in the absence of antibody and analyzed by PCR with specific primers for the indicated regulatory regions. Input, nonimmunoprecipitated crosslinked chromatin.

(C) Total cell lysates (1 mg) derived from HCT116 cells stably expressing HA-p73, transfected with CA-AKT or with the empty vector, were immunoprecipitated with anti-HA and control (IgG) antibodies. The immunoprecipitates and an aliquot (50 μg) of total cell lysates were subjected to WB analysis.

(D) Quantitative real-time PCR analysis of PML transcripts in HCT116 cells transfected with CA-AKT or with the empty vector and exposed to 7.5 μg/ml CDDP for 24 hr. Histograms show the mean of three experiments; bars indicate SD.

(E) Proliferating HCT116 cells were transfected with CA-AKT vector or the empty vector together with a GFP-expressing vector and treated with CDDP. Twenty-four hours after treatment, cells were fixed and stained with an anti-PML monoclonal antibody. The graph shows the percentage of GFP-positive cells containing PML NBs. Histograms show the mean of three experiments; bars indicate SD.

of PARP fragmentation, a hallmark of apoptosis (Figure 4A). These findings strongly indicate the existence of a close functional network among p73, YAP, and PML in the execution of CDDP-induced apoptosis.

PML and YAP Physically Interact In Vivo

To further dissect the functional crosstalk between p73, YAP, and PML, we looked for the presence of protein complexes involving these three proteins. As shown in Figure 4B, coprecipitation experiments performed in HCT116 cells revealed that endogenous YAP, PML, and p73 can physically interact under physiological conditions. As shown in Figure S3B, p73/YAP protein complexes could be observed in $PML^{-/-}$ MEFs, and the reconstitution of PML expression in $PML^{-/-}$ cells allowed the detection of a more abundant endogenous p73/YAP protein complex (Figure S3C). The protein complex YAP/PML could also be observed in $p73^{-/-}$ MEFs (Figure S3D). Altogether, these findings indicate that protein complexes p73/YAP and PML/YAP can be found independently from PML or p73 presence, respectively, suggesting reasonably that a mixture of both protein complexes might elicit the apoptotic effects. This, of course, does not rule out the possibility that under peculiar conditions, a triple complex can be formed and recruited for the full activation of p73/YAP-mediated apoptosis.

A computer-assisted search for modular protein domains allowed the identification of a WW domain binding motif (PVPVY) in PML that might potentially bind to the WW domain of YAP. To test this hypothesis, we generated a human YAP mutant deleted for its WW domain (YAP-ΔWW), which consequently was unable to bind to PML in coprecipitation assays (Figure 4C). It has been previously shown that the terminal tyrosine (Y) of the PPxY motif is critical for the binding to class I of WW domain-containing proteins (Sudol and Hunter, 2000). In agreement with this, we found that a PML mutant, whose tyrosine Y455

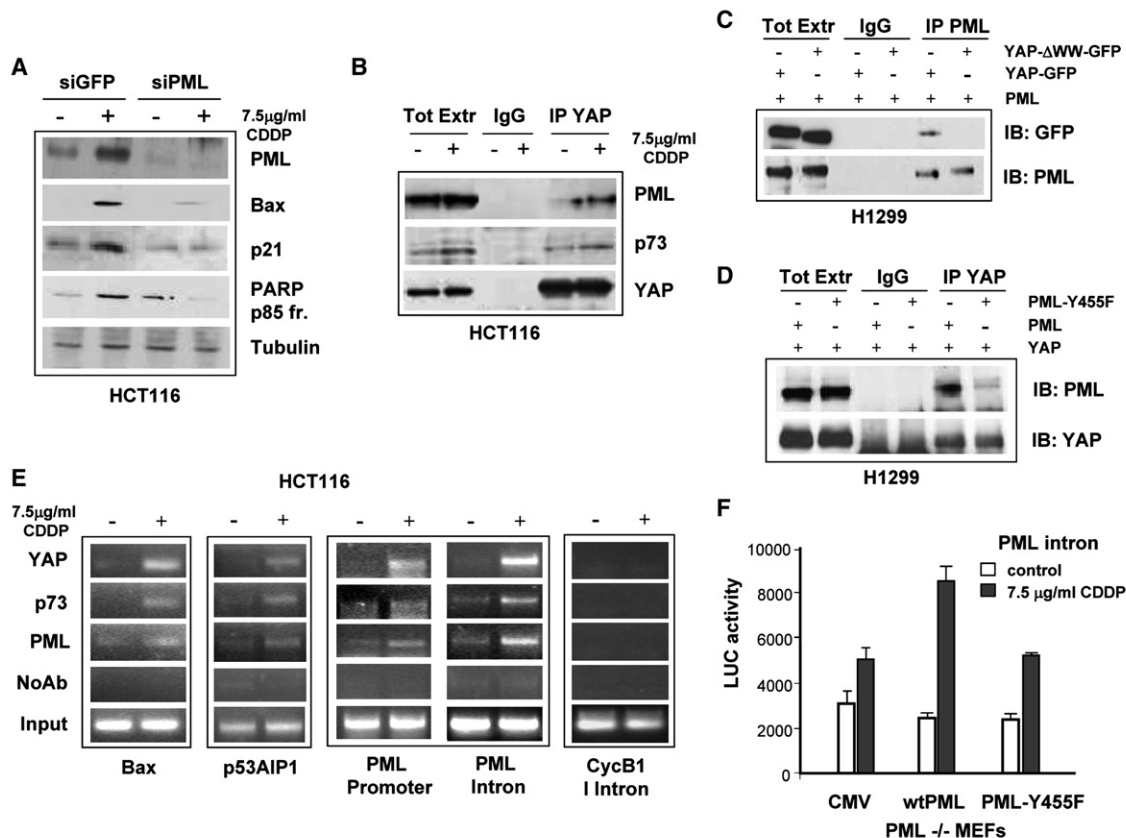

Figure 4. PML and YAP Physically Interact In Vivo

(A) Total cell lysates (35 μg) derived from HCT116 cells, transfected with specific anti-PML and control anti-GFP siRNAs, untreated or treated with 7.5 μg/ml CDDP for 24 hr, were subjected to WB analysis.

(B) Total cell lysates (1 mg) derived from HCT116 cells, untreated or treated with 7.5 μg/ml CDDP for 24 hr, were immunoprecipitated with anti-YAP and control (IgG) antibodies. The immunoprecipitates and an aliquot (50 μg) of total cell lysates were subjected to WB analysis.

(C) H1299 cells were transfected with plasmids encoding for PML, YAP-GFP, or the mutant deleted of the WW domain (YAP-ΔWW-GFP). Total cell lysates (500 μg) were immunoprecipitated with anti-PML and control (IgG) antibodies. Immunoprecipitates and an aliquot (50 μg) of total cell lysates were subjected to WB analysis.

(D) H1299 cells were transfected with plasmids encoding for PML, YAP, and a PML mutant containing a Y455 to F455 substitution (PML-Y455F). Total cell lysates (500 μg) were immunoprecipitated with anti-YAP and control (IgG) antibodies. Immunoprecipitates and an aliquot (50 μg) of total cell lysates were subjected to WB analysis.

(E) Crosslinked chromatin derived from HCT116 cells, untreated or treated with 7.5 μg/ml CDDP for 24 hr, was immunoprecipitated with anti-YAP, anti-p73, and anti-PML antibodies and subjected to PCR analysis by using specific primers spanning a region that included p73 binding sites. Input, nonimmunoprecipitated crosslinked chromatin. The occupancy of p73, YAP, and PML of the amplified regulatory regions of *PML*, *BAX*, *p53AIP1*, and *Cyclin B1* first intron as a control are shown.

(F) $PML^{-/-}$ MEFs were transiently transfected with plasmids encoding wild-type PML or PML-Y455F together with constructs carrying the luciferase reporter gene driven by murine PML first intron. An equal amount of CMV-βgal was added to each transfection. Cells were treated with 7.5 μg/ml CDDP for 24 hr, and cell extracts were prepared 36 hr after transfection. Luciferase activity was determined relative to total proteins and β-gal activity. Histograms show the mean of three experiments each performed in duplicate; bars indicate SD.

was mutated to phenylalanine F455, lost its ability to bind to YAP (Figure 4D). Moreover, this mutant was unable to stabilize the endogenous p73/YAP complex when exogenously transfected in $PML^{-/-}$ MEFs (Figure S3C).

PML Binds, Together with p73 and YAP, Its Own Regulatory Regions and Promotes Its Own Transcriptional Activation

To investigate whether PML, YAP, and p73 could jointly play a role in the transcriptional control of specific gene targets, we analyzed their in vivo occupancy on *Bax*, *p53AIP1*, and *PML* regulatory regions. As shown in Figure 4E (two left panels), PML, YAP, and p73 can be recruited on *Bax* and *p53AIP1*, which contain p73-binding sites within their promoter regions, in response to CDDP. Notably, PML binds to its own promoter and first intron where YAP and p73 were also recruited (Figure 4E, middle panel) and promotes its own transcriptional activation (PML-IV intron), after CDDP treatment, when transfected in $PML^{-/-}$ MEFs (Figure 4F). Interestingly, *PML* transcriptional activation was abrogated when we transfected the Y455F PML

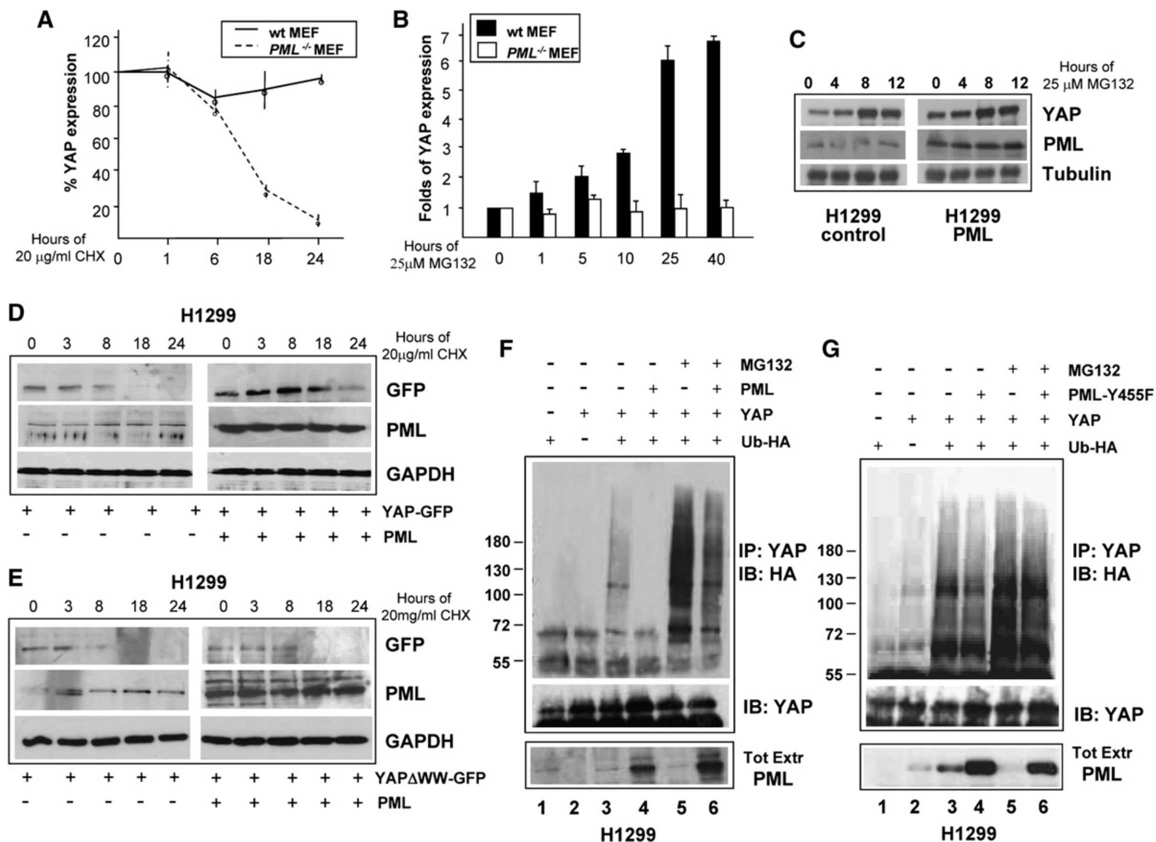

Figure 5. YAP Degradation Occurs through the Ubiquitin-Proteasome Pathway and Is Negatively Regulated by PML

(A) Total cell lysates (50 μg) derived from wild-type and PML$^{-/-}$ MEFs, treated with 20 μg/ml CHX and harvested at the indicated time points, were subjected to WB analysis. Quantification by densitometry and normalization on β-tubulin expression were performed. Histograms show the mean of three experiments; bars indicate SD.

(B) Total cell lysates (50 μg) derived from wild-type and PML$^{-/-}$ MEFs, treated with 25 μM MG132 and harvested at the indicated time points, were subjected to WB analysis. Quantification by densitometry and normalization on β-tubulin expression were performed. Results represent the fold induction over the 0 hr time point. Histograms show the mean of three experiments; bars indicate SD.

(C) Proteins were extracted from H1299 cells, previously transfected with a plasmid encoding for PML or an empty vector as control, at the indicated time points after treatment with 25 μM MG132 and subjected to WB analysis.

(D and E) Proteins were extracted from H1299 cells, previously transfected with a plasmid encoding for PML or an empty vector as control and with a plasmid encoding YAP-GFP or YAP-ΔWW-GFP, at the indicated time points after treatment, with 20 μg/ml CHX, and subjected to WB analysis.

(F and G) H1299 cells were cotransfected with the indicated plasmids for 24 hr. Some cultures were incubated with 25 μM MG132 (lanes 5 and 6). YAP-Ubiquitin immunocomplexes were immunoprecipitated with anti-YAP antibody and analyzed by IB analysis with anti-HA antibody.

mutant, which is unable to bind to YAP, demonstrating that the effect of WT PML is YAP-dependent (Figure 4F). These data clearly indicate that a transcriptional autoregulatory feedback loop involving p73, YAP, and PML takes place in the execution of CDDP-induced apoptosis.

YAP Degradation Occurs through the Ubiquitin-Proteasome Pathway and Is Negatively Regulated by PML

As we have previously shown that YAP-mediated *p73* transcriptional activation was diminished in cells lacking PML (Strano et al., 2005), we set out to further define the contribution of PML to YAP transcriptional coactivation. PML isoform IV protects both p53 and p73 from proteasome-mediated degradation (Bernassola et al., 2004; Bernardi et al., 2004; Guo et al., 2000). We therefore investigated whether PML might regulate YAP stability. For this purpose, we followed YAP half-life in PML$^{-/-}$ and wild-type MEFs upon treatment with cycloheximide (CHX). We observed that the half-life of YAP was markedly shortened in PML$^{-/-}$ compared with wild-type MEFs (Figure 5A and Figure S4A). Moreover, the accumulation of endogenous YAP upon proteasome inhibitor (MG132) treatment was abrogated in PML$^{-/-}$ MEFs compared to that of wild-type counterparts (Figure 5B; Figure S4B), while YAP was stabilized after ectopic expression of PML in H1299 cells (Figure 5C). These findings strongly suggest that YAP turnover is directly regulated by proteasome-mediated proteolysis and that PML is participating in controlling YAP's half life. To discriminate between a transcriptional/translational and a posttranslational control of PML on YAP, we followed the half-life of overexpressed YAP-GFP, with

or without PML overexpression, upon treatment with CHX (Figure 5D). We observed that YAP-GFP was stabilized, and its half-life was longer in cells overexpressing PML compared to control cells (Figure 5D). On the other hand, no stabilization was observed using the mutant YAP-ΔWW-GFP that is unable to bind PML (Figure 5E). These data suggest that the presence of PML may protect YAP from degradation. To explore this hypothesis in vivo, ubiquitylation assays were performed. We observed that YAP was clearly polyubiquitinylated in vivo, and its ubiquitylation levels were significantly reduced in PML-overexpressing cells (Figure 5F). PML also prevented YAP ubiquitylation in MG132-treated cells (Figure 5F, lane 6), indicating that PML acts upstream of the proteasome degradation process. The binding of PML to YAP was necessary for the protective effect on YAP ubiquitylation. Indeed, a PML-Y455F mutant unable to bind YAP was inefficient in impairing YAP ubiquitylation (Figure 5G). These findings identify PML as a critical regulator of YAP stability and, consequently, as an important determinant in the execution of apoptosis, induced by the protein complex p73/YAP.

YAP Is Stabilized by Sumoylation after CDDP Treatment

It has been demonstrated that PML enhances p53 and p73 acetylation, leading to the potentiation of p53 transcriptional activity (Bernassola et al., 2004; Pearson et al., 2000) and to the inhibition of p73 ubiquitin-dependent degradation (Strano et al., 2005). Competition between ubiquitylation, sumoylation, and acetylation of overlapping lysine residues constitutes a mechanism to regulate protein stability (Desterro et al., 1998). Moreover, it has been shown that PML contains a SUMO-binding motif that mediates PML-SUMO1 interaction independently of sumoylation (Shen et al., 2006) and that PML stimulates SUMO conjugation in yeast (Quimby et al., 2006). We therefore investigated whether YAP can be sumoylated and whether PML has a role in modulating YAP posttranslational modifications.

We found that, after protein extraction with 8 M urea buffer, an electrophoretically slower migrating YAP form was recognized by a specific anti-YAP antibody upon overexpression of PML-IV and SUMO-1 (Figure 6A). To determine whether this form represented YAP conjugated to SUMO-1, NETN lysates derived from the same cell line were immunoprecipitated with an antibody against YAP. The immunocomplexes were analyzed by western blot with an anti-SUMO-1 antibody, in parallel with an anti-YAP antibody, to demonstrate that the SUMO-1 crossreactive protein was indeed a modified YAP form (Figure 6B). In both experiments, PML-IV expression together with SUMO-1 expression enhanced YAP sumoylation (Figures 6A and 6B). Furthermore, the concomitant expression of PML and SUMO-1 reduced YAP polyubiquitylation (Figure 6C, lane 5). These findings may implicate PML-mediated YAP sumoylation as a key molecular event that stabilizes the autoregulatory feedback loop involving p73, YAP, and PML in the execution of DNA damage-induced apoptosis. Interestingly, endogenous YAP protein was elevated upon CDDP treatment (Figure 6D), which is not the result of transcriptional regulation since there was no change in the level of YAP mRNA upon CDDP treatment (Figure 6E). This observation suggests that CDDP treatment is likely to influence YAP expression at the protein level and that posttranslational modifications might be involved in the stabilization of YAP protein. Therefore, we checked whether there was an increase in YAP sumoylation and/or decrease in YAP ubiquitylation after CDDP treatment of HCT116 cells. As shown in Figure 6F there was an increase in YAP sumoylation after CDDP treatment, together with an inhibition of YAP ubiquitylation, as shown by the ubiquitylation assay in Figure 6G. This could explain the stabilization of YAP protein that takes place after the induction of apoptosis. Moreover overexpression of SUMO-1 enhanced the transcriptional activation exerted by the p73/YAP complex on the proapoptotic Bax promoter, as shown in the luciferase assay reported in Figure S5A, confirming SUMO's role in amplifying p73/YAP-mediated apoptotic response.

PML Mediates YAP-SUMO Conjugation

To demonstrate the key role of PML in the regulation of YAP during the execution of p73/YAP-mediated apoptosis, we checked YAP accumulation and posttranslational modifications after treatment with CDDP in wild-type and $PML^{-/-}$ MEFs.

Endogenous YAP protein was elevated upon CDDP treatment (Figure 7A), but no change in the level of YAP mRNA upon CDDP treatment was observed (Figure S5B). Interestingly, YAP stabilization after treatment with CDDP was lost in $PML^{-/-}$ MEFs (Figure 7A), as well as YAP sumoylation (Figure 7B), and as a consequence, treatment with CDDP was no longer able to decrease YAP ubiquitylation in $PML^{-/-}$ MEFs (Figure 7C). These data demonstrate that the presence of PML mediates YAP sumoylation, protecting YAP from degradation and reinforcing its transcriptional coactivation in CDDP induced p73-mediated apoptosis.

To determine the sites at which YAP is modified by SUMO-1, a computer-assisted search for putative sumoylated lysines was performed and puntiform mutants on Lys97 and Lys242 were generated. As shown in Figure 7D, the double mutant YAP-K97/242A-GFP, but not the single mutants (data not shown), showed less sumoylation upon SUMO-1 overexpression. Moreover, this mutant showed reduced ubiquitylation levels compared with wild-type YAP (Figure 7E), indicating that the same lysines within YAP protein are subject to either sumoylation or ubiquitylation depending on the stimulus. Indeed, the double mutant YAP-K97/242A-GFP showed a longer half-life compared with wild-type YAP (Figure S5C); however, it was unable to be readily upregulated following CDDP treatment (Figure 7F), since it lost its SUMO-mediated regulation.

DISCUSSION

In this study, our findings have led to two major conclusions. First, *PML* is a direct target gene of the p73/YAP complex during apoptosis triggered by anticancer drugs. We found that *PML* belongs to a group of genes that are upregulated following CDDP treatment in HCT116 cells transfected with control siRNA but remained constant in cells transfected with either p73 or YAP siRNA. *PML* is not a specific p73 target gene, since it has also been demonstrated to be a p53 target gene (de Stanchina et al., 2004), and in fact, p53 can synergize with p73/YAP in the transcriptional regulation of *PML* during the apoptotic response of HCT116 cells. However, interestingly, we found

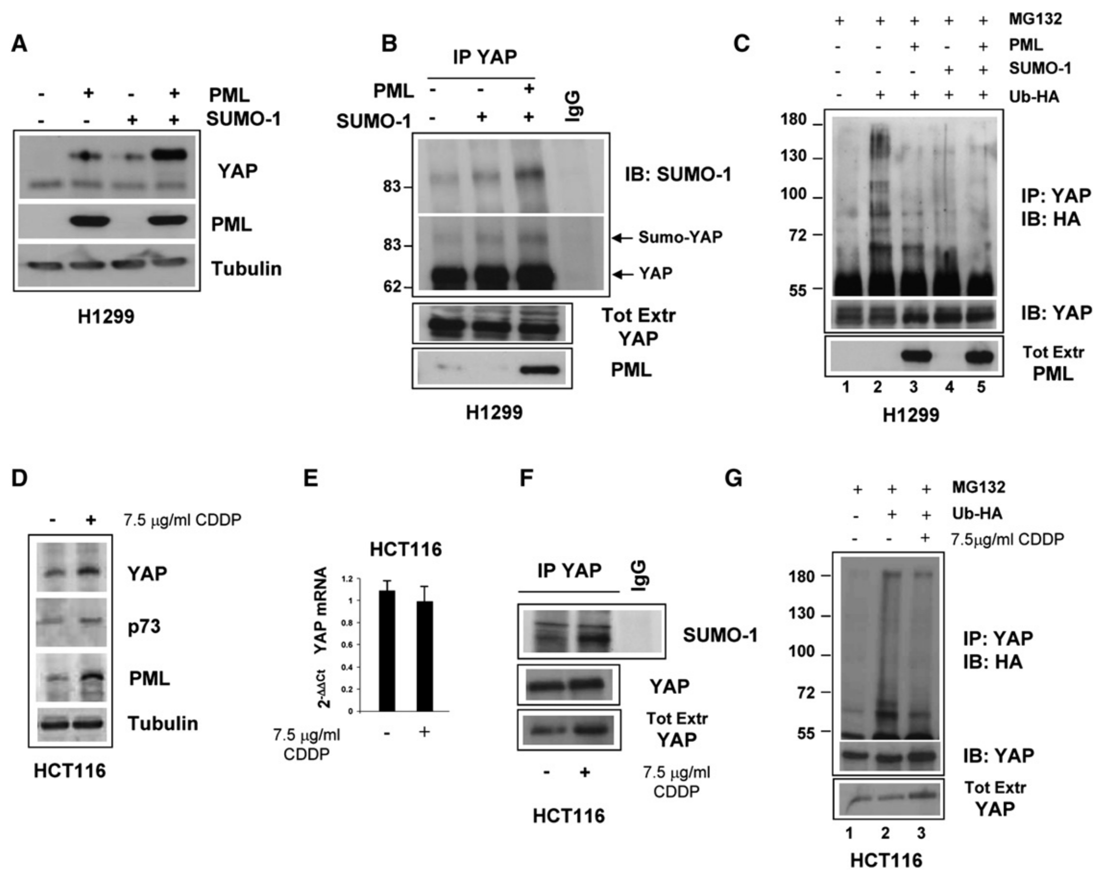

Figure 6. YAP Is Stabilized by Sumoylation after CDDP Treatment

(A) Proteins were extracted from H1299 cells, previously transfected with plasmids encoding for PML and/or SUMO-1 for 24 hr, and subjected to WB analysis.

(B) H1299 cells were transfected with plasmids encoding for PML and/or SUMO-1 for 24 hr. Total cell lysates (500 μg) were immunoprecipitated with anti-YAP and control (IgG) antibodies. Immunoprecipitates and an aliquot (50 μg) of total cell lysates were subjected to WB analysis.

(C) H1299 cells were cotransfected with the indicated plasmids for 24 hr and incubated with 25 μM MG132. YAP-Ubiquitin immunocomplexes were immunoprecipitated with anti-YAP antibody and analyzed by IB analysis with anti-HA antibody.

(D) HCT116 cells were treated with CDDP (7.5 μg/ml) for 24 hr. Total cell lysates (35 μg) were subjected to WB analysis.

(E) Quantitative real-time PCR analysis of YAP transcripts in HCT116 cells treated with CDDP (7.5 μg/ml) for 24 hr. Histograms show the mean of three experiments; bars indicate SD.

(F) HCT116 cells were treated with CDDP (7.5 μg/ml) for 24 hr. Total cell lysates (1 mg) were immunoprecipitated with anti-YAP and control (IgG) antibodies. Immunoprecipitates and an aliquot (50 μg) of total cell lysates were subjected to WB analysis.

(G) HCT116 cells were cotransfected with Ub-HA plasmid for 24 hr, incubated with 25 μM MG132, and treated with CDDP (7.5 μg/ml) for 12 hr, where indicated. YAP-Ubiquitin immunocomplexes were immunoprecipitated with anti-YAP antibody and analyzed by IB analysis with anti-HA antibody.

that in specific cellular contexts where p53 is absent or mutated, p73 alone is able to induce PML expression and NBs formation. This finding could highlight a potential alternative pathway in response to DNA damage that works in cells lacking functional p53 protein. Moreover, we underlined the importance of YAP as a coactivator of p73 in the transactivation of *PML*. We have previously demonstrated that YAP is required for recruitment of p300 to the regulatory regions of the apoptotic target gene *p53AIP1* (Strano et al., 2005). Here, we show that when YAP is sequestered into the cytoplasm by a constitutively active mutant of AKT, there is a reduction of p300 recruitment to the *PML* regulatory regions, and this correlates with a reduction in histone acetylation and a reduction in *PML* expression. There is already growing evidence in the current literature saying that p53 can be found on the regulatory regions of its target genes without delivering any transcriptional activity. In response to diverse stimuli, p53 transcriptional activity is turned on through the recruitment of coactivators and acetylases. We believe that YAP recruitment is a critical event in turning on the transcriptional activity of p73 by favoring the formation of transcriptionally active competent complexes that play a pivotal role in eliciting apoptosis in response to anticancer treatment (Strano et al., 2005; Levy et al., 2008).

Second, our data suggest a role for PML in the regulation of YAP stability. YAP is emerging as a very intriguing protein due to its critical role in regulating p73 accumulation and function following DNA damage (Strano et al., 2005) and its recent identification as a tumor suppressor in breast (Yuan et al., 2008),

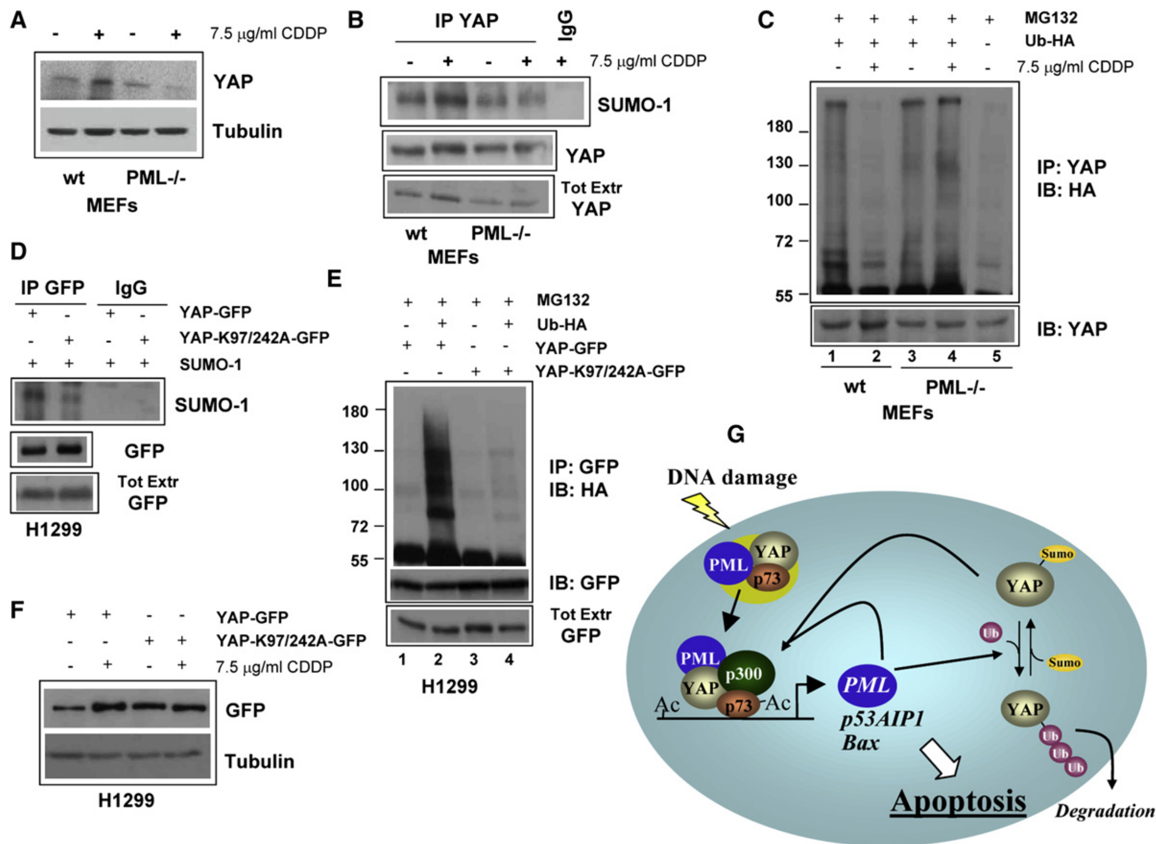

Figure 7. PML Mediates YAP-SUMO Conjugation

(A) Total cell lysates (50 μg) derived from wild-type and PML$^{-/-}$ MEFs, treated with CDDP (7.5 μg/ml) for 24 hr, were subjected to WB analysis.

(B) Wild-type and PML$^{-/-}$ MEFs were treated with CDDP (7.5 μg/ml) for 24 hr. Total cell lysates (1 mg) were immunoprecipitated with anti-YAP and control (IgG) antibodies. Immunoprecipitates and an aliquot (50 μg) of total cell lysates were subjected to WB analysis.

(C) Wild-type and PML$^{-/-}$ MEFs cells were cotransfected with Ub-HA plasmid for 24 hr, incubated with 25 μM MG132, and treated with CDDP (7.5 μg/ml) for 12 hr, where indicated. YAP-Ubiquitin immunocomplexes were immunoprecipitated with anti-YAP antibody and analyzed by IB analysis with anti-HA antibody.

(D) H1299 cells were transfected with plasmids encoding for YAP-GFP or YAP-K97/242A-GFP and SUMO-1 for 24 hr. Total cell lysates (500 μg) were immunoprecipitated with anti-GFP and control (IgG) antibodies. Immunoprecipitates and an aliquot (50 μg) of total cell lysates were subjected to WB analysis.

(E) H1299 cells were cotransfected with the indicated plasmids for 24 hr and incubated with 25 μM MG132. YAP-Ubiquitin immunocomplexes were immunoprecipitated with anti-GFP antibody and analyzed by IB analysis with anti-HA antibody.

(F) H1299 cells were cotransfected with the indicated plasmids for 24 hr and treated with CDDP (7.5 μg/ml) for 24 hr where indicated. Total cell lysates (35 μg) were subjected to WB analysis.

(G) Proposed model for the autoregulatory feedback loop involving p73, YAP, and PML in the execution of DNA damage-induced apoptosis. DNA damage causes p73 accumulation and YAP relocalization into the nucleus. PML is required to localize YAP into the NBs to coactivate p73. Under apoptotic conditions, the transcriptionally active complex that contains acetylated p73α, YAP, and p300 assembles onto the regulatory regions of the proapoptotic target genes. p73 and YAP are also required for the transcriptional activation of PML during the apoptotic response and for the subsequent accumulation of PML protein and formation of nuclear bodies. As a consequence, PML can contribute to the p73-dependent apoptotic response by promoting both p300-mediated acetylation of p73 and stabilizing YAP by inhibiting its ubiquitin-mediated degradation.

but very little is known about its regulation. Here we show that YAP is polyubiquitinylated in vivo and degraded via the ubiquitin-proteasome pathway. We found that YAP and PML physically interact through the YAP WW domain and the new identified PML PVPVY motif. We also showed that PML plays a role in the regulation of YAP half-life by enhancing YAP sumoylation and thus preventing its ubiquitylation and subsequent degradation.

Notably, it has been demonstrated that PML stimulates hSUMO-1 modification in yeast (Quimby et al., 2006), however, to date, no evidence existed for such activity in mammalian cells. We identified YAP as the first target for a PML-mediated sumoylation in a physiological context.

It has also been demonstrated that PML contains a SUMO-binding motif that is independent of its sumoylation sites and is necessary for PML-NB formation (Shen et al., 2006). According to the proposed model, in interphase, PML is sumoylated and the PML SUMO-binding motif can mediate interactions with nearby sumoylated PML molecules, hence allowing the formation of orderly PML networks. As a large number of proteins associated with the PML NBs are sumoylated and/or contain SUMO-binding motifs, it is possible that these proteins are

recruited to the PML networks through noncovalent interactions mediated by covalently bound SUMO and SUMO-binding motifs present in PML. YAP sumoylation could, therefore, not only stabilize YAP, preventing its ubiquitylation, but it may also be important in mediating the recruitment of YAP/p73 complex to the PML NBs. The timing of these events needs to be further investigated.

It has already been described that endogenous YAP protein is elevated upon CDDP treatment (Levy et al., 2007); however, the molecular mechanism was not described. Here, we show that the upregulation of YAP protein, upon CDDP treatment, is not due to transcriptional regulation, but it is a posttranslational event that correlates with an increase in YAP sumoylation mediated by PML, since it is completely abrogated in $PML^{-/-}$ MEFs. Elevation of YAP levels in response to DNA damage demonstrates another level of regulation of this pathway, suggesting that p73 activation must be tightly controlled to ensure quick and efficient activation of p73 target genes in response to stress conditions.

Our findings demonstrate the existence of a positive regulatory loop between the p73/YAP protein complex and PML during apoptosis triggered by CDDP in HCT116 cells. It has been previously demonstrated that YAP requires PML and NBs localization to coactivate p73 (Strano et al., 2005). Here, we show that p73 and YAP are required for the transcriptional activation of *PML* during the apoptotic response and for the subsequent accumulation of PML protein and formation of nuclear bodies. As a consequence, PML can contribute to the p73-dependent apoptotic response by promoting both p300-mediated acetylation of p73 (Bernassola et al., 2004) and stabilizing YAP by inhibiting its ubiquitin-mediated degradation.

EXPERIMENTAL PROCEDURES

Cell Culture

Human epithelial non-small cell lung carcinoma (NSCLC) cell line H1299, human colon carcinoma cell line HCT116, mouse embryo fibroblasts MEFs, human breast cancer cell line SKBR3, and human breast cell line MCF7 were cultured in Dulbecco's modified medium (DMEM) with 10% fetal bovine serum (FBS).

Immunoprecipitation and Western Blot Analysis

Cells were lysed and immunoprecipitated as previously described (Strano et al., 2001) by using anti-PML antibody PG-M3 (Santa Cruz), with anti-YAP antibody H-125 (Santa Cruz) or anti-GFP (Abcam). For the detection of YAP-Ubiquitin immunocomplexes, cells were lysed in PBS containing 1% NP40, 1% SDS, 5 mM EDTA, 10 mM iodoacetamide, and Protease Inhibitor Cocktail (Roche). After lysis, extracts were incubated at 60 degrees for 10 min, and 10 mM of DTT was added and diluted 10 times with PBS-NP40. Immunocomplexes were precipitated with protein G-agarose (KPL, Guilford, CA). For immunoblotting, the following antibodies were used: p73 (Ab4, Neomarker), p21 (C19, Santa Cruz), bax (N20, Santa Cruz), PARP p85 fragment (Promega), p53 (Cell Signaling), actin (Abcam), GAPDH (Abcam), SUMO-1 (Abcam), tubulin (Sigma), YAP (H125, Santa Cruz), PML (PG-M3, Santa Cruz), GFP (Abcam), and HA (Covance).

Plasmids

PML-Y455F, YAP-ΔWW-GFP, and YAP-K97/242A-GFP were obtained by site-directed mutagenesis followed by subcloning into pcDNA3 or GFP vector. Sequences of the primers are available on request.

Indirect Immunofluorescence

HCT116 and H1299 cells were fixed in 4% PBS-paraformaldeyde for 10 min; incubated in 0.5% Triton for 10 min, then in 10% FBS with 0.3% Triton; and then stained for 1 hr with an anti-PML antibody (PG-M3 Santa Cruz) used at a 1:100 dilution in 2% FBS with 0.1% Triton (Strano et al., 2005).

Transfections and Luciferase Assays

Transient reporter assays were performed and normalized as described (Strano et al., 2005).

RNA Extraction and Reverse Transcriptase Reaction

RNAs were isolated and reverse transcribed as previously described (Fontemaggi et al., 2002). Polymerase Chain Reaction (PCR) analyses were carried out by using oligonucleotides specific for the following genes: $p21^{waf1}$, Bax, hPML, and the housekeeping aldolase A (available upon request).

Microarray Hybridization and Clustering Analysis

Preparation of biotinylated cRNA targets and microarray hybridization, using Genechip HG-U133A containing 22,215 probes, were carried out according to the instruction of the supplier (Affymetrix, Santa Clara, CA). Scanned output files were analyzed by the probe level analysis package MAS 5.0. Gene-expression values < 10 were adjusted to 10 to eliminate noise from the data, and then all values were log_2 transformed. The expression ratio of each gene at each time point was determined separately for each group (GFPi, p73i, and YAPi) relative to expression at a time point of 0 hr for the particular group. Lists of upregulated genes were derived for each of the conditions (GFPi, p73i, and YAPi) by selecting only genes with expression ratios above or below 2-fold in at least three out of the four time points of each treatment. The modulated gene lists were presented in Venn diagrams that compared the common and specific genes in each treatment group. The Sorting Points Into Neighborhoods (SPIN) algorithm was applied to the data (Tsafrir et al., 2005).

Real-Time RT-PCR

PCR was performed on the cDNA samples using an ABI PRISM 7500 Sequence Detector (PE Applied Biosystems). Specific primers for *hPML*, *mPML*, and *mYAP* were used. GAPDH gene was used as endogenous control to standardize the amount of RNA in each reaction (Taqman GAPDH control reagents).

Formaldehyde Crosslinking and Chromatin Immunoprecipitation

Chromatin immunoprecipitation experiments were performed as in Fontemaggi et al. (2002). The following antibodies were used: a mixture of p73 antibodies (sc-7237 and sc-7238, Santa Cruz), YAP (H125, Santa Cruz), a mixture of p300 antibodies (sc-584 and sc-585, Santa Cruz), PML (PG-M3, Santa Cruz), and HA (Covance). Enrichment in PML promoter, PML intron, Bax, p53AIP1, and Cyclin B1 first intron sequences was evaluated by PCR. The sequences of the oligonucleotides are available upon request.

siRNA Studies

HCT116, SKBR3 and MCF7 cells were transiently transfected as previously reported in Strano et al. (2005) with either 1 μg of the control small interfering RNAs or with sip73, siYAP, or siPML (Dharmacon Inc.). Twenty-four hours after the transfection the cells were treated with 7.5 μg/ml CDDP.

SUPPLEMENTAL DATA

The Supplemental Data include five figures and one table and can be found with this article online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(08)00841-1.

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REFERENCES

Basu, S., Totty, N.F., Irwin, M.S., Sudol, M., and Downward, J. (2003). Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14–3–3 and attenuation of p73-mediated apoptosis. Mol. Cell *11*, 11–23.

Bernardi, R., Scaglioni, P.P., Bergmann, S., Horn, H.F., Vousden, K.H., and Pandolfi, P.P. (2004). PML regulates p53 stability by sequestering Mdm2 to the nucleolus. Nat. Cell Biol. *6*, 665–672.

Bernassola, F., Salomoni, P., Oberst, A., Di Como, C.J., Pagano, M., Melino, G., and Pandolfi, P.P. (2004). Ubiquitin-dependent degradation of p73 is inhibited by PML. J. Exp. Med. *199*, 1545–1557.

de Stanchina, E., Querido, E., Narita, M., Davuluri, R.V., Pandolfi, P.P., Ferbeyre, G., and Lowe, S.W. (2004). PML is a direct p53 target that modulates p53 effector functions. Mol. Cell *27*, 523–535.

Di Agostino, S., Strano, S., Emiliozzi, V., Zerbini, V., Mottolese, M., Sacchi, A., Blandino, G., and Piaggio, G. (2006). Gain of function of mutant p53: the mutant p53/NF-Y protein complex reveals an aberrant transcriptional mechanism of cell cycle regulation. Cancer Cell *10*, 191–202.

Desterro, J.M., Rodriguez, M.S., and Hay, R.T. (1998). SUMO-1 modification of IkappaBalpha inhibits NF-kappaB activation. Mol. Cell *2*, 233–239.

Ferbeyre, G., de Stanchina, E., Querido, E., Baptiste, N., Prives, C., and Lowe, S.W. (2000). PML is induced by oncogenic ras and promotes premature senescence. Genes Dev. *14*, 2015–2027.

Fontemaggi, G., Kela, I., Amariglio, N., Rechavi, G., Krishnamurthy, J., Strano, S., Sacchi, A., Givol, D., and Blandino, G. (2002). Identification of direct p73 target genes combining DNA microarray and chromatin immunoprecipitation analyses. J. Biol. Chem. *277*, 43359–43368.

Guo, A., Salomoni, P., Luo, J., Shih, A., Zhong, S., Gu, W., and Pandolfi, P.P. (2000). The function of PML in p53-dependent apoptosis. Nat. Cell Biol. *2*, 730–736.

Levy, D., Adamovich, Y., Reuven, N., and Shaul, Y. (2007). The Yes-associated protein 1 stabilizes p73 by preventing Itch-mediated ubiquitination of p73. Cell Death Differ. *14*, 743–751.

Levy, D., Adamovich, Y., Reuven, N., and Shaul, Y. (2008). Yap1 phosphorylation by c-Abl is a critical step in selective activation of proapoptotic genes in response to DNA damage. Mol. Cell *29*, 350–361.

Naar, A.M., Lemon, B.D., and Tjian, R. (2001). Transcriptional coactivator complexes. Annu. Rev. Biochem. *70*, 475–501.

Pearson, M., Carbone, R., Sebastiani, C., Cioce, M., Fagioli, M., Saito, S., Higashimoto, Y., Appella, E., Minucci, S., Pandolfi, P.P., and Pelicci, P.G. (2000). PML regulates p53 acetylation and premature senescence induced by oncogenic Ras. Nature *406*, 207–210.

Quimby, B.B., Yong-Gonzalez, V., Anan, T., Strunnikov, A.V., and Dasso, M. (2006). The promyelocytic leukemia protein stimulates SUMO conjugation in yeast. Oncogene *18*, 2999–3005.

Rossi, M., De Laurenzi, V., Munarriz, E., Green, D.R., Liu, Y.C., Vousden, K.H., Cesareni, G., and Melino, G. (2005). The ubiquitin-protein ligase Itch regulates p73 stability. EMBO J. *24*, 836–848.

Salomoni, P., and Pandolfi, P.P. (2002). The role of PML in tumor suppression. Cell *108*, 165–170.

Shen, T.H., Lin, H.K., Scaglioni, P.P., Yung, T.M., and Pandolfi, P.P. (2006). The mechanisms of PML-nuclear body formation. Mol. Cell *24*, 331–339.

Strano, S., Munarriz, E., Rossi, M., Castagnoli, L., Shaul, Y., Sacchi, A., Oren, M., Sudol, M., Cesareni, G., and Blandino, G. (2001). Physical interaction with Yes-associated protein enhances p73 transcriptional activity. J. Biol. Chem. *276*, 15164–15173.

Strano, S., Monti, O., Pediconi, N., Baccarini, A., Fontemaggi, G., Lapi, E., Mantovani, F., Damalas, A., Citro, G., Sacchi, A., et al. (2005). The transcriptional coactivator Yes-Associated Protein drives p73 gene target specificity in response to DNA damage. Mol. Cell *18*, 447–459.

Sudol, M., and Hunter, T. (2000). New wrinkles for an old domain. Cell *103*, 1001–1004.

Trotman, L.C., Alimonti, A., Scaglioni, P.P., Koutcher, J.A., Cordon-Cardo, C., and Pandolfi, P.P. (2006). Identification of a tumour suppressor network opposing nuclear Akt function. Nature *441*, 523–527.

Tsafrir, D., Tsafrir, I., Ein-Dor, L., et al. (2005). Sorting points into neighborhoods (SPIN): data analysis and visualization by ordering distance matrices. Bioinformatics *21*, 2301–2308.

Vousden, K.H., and Lu, X. (2002). Live or let die: the cell's response to p53. Nat. Rev. Cancer *2*, 594–604.

Yuan, M., Tomlinson, V., Lara, R., Holliday, D., Chelala, C., Harada, T., Gangeswaran, R., Manson-Bishop, C., Smith, P., Danovi, S.A., et al. (2008). Yes-associated protein (YAP) functions as a tumour suppressor in breast. Cell Death Differ. *15*, 1752–1759.