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Yin Yang 1 Is a Negative Regulator of p53

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Summary

Yin Yang 1 (YY1) is a transcription factor that plays an essential role in development. However, the full spectrum of YY1's functions and mechanism of action remains unclear. We find that YY1 ablation results in p53 accumulation due to a reduction of p53 ubiquitination in vivo. Conversely, YY1 overexpression stimulates p53 ubiquitination and degradation. Significantly, recombinant YY1 is sufficient to induce Hdm2-mediated p53 polyubiquitination in vitro, suggesting that this function of YY1 is independent of its transcriptional activity. We identify direct physical interactions of YY1 with Hdm2 and p53 and show that the basis for YY1-regulating p53 ubiquitination is its ability to facilitate Hdm2-p53 interaction. Importantly, the tumor suppressor $p14^{ARF}$ compromises the Hdm2-YY1 interaction, which is important for YY1 regulation of p53. Taken together, these findings identify YY1 as a potential cofactor for Hdm2 in the regulation of p53 homeostasis and suggest a possible role for YY1 in tumorigenesis.

Introduction

Yin Yang 1 (YY1) is a multifunctional transcription factor that can act as a transcriptional repressor, activator, or initiator element binding protein (reviewed in Shi et al. [1996] and Thomas and Seto [1999]). The transcriptional activity of YY1 can be regulated by viral oncoproteins such as adenovirus E1A, suggesting a possible role for YY1 in cell proliferation (Shi et al., 1991). Consistent with this hypothesis, previous studies identified a myriad of potential YY1 target genes whose products are important for proliferation and differentiation (reviewed in Shi et al. [1996] and Thomas and Seto [1999]). Furthermore, $YY1^{-/-}$ mouse embryos die during early embryogenesis at around the time of implantation, which is a developmental stage characterized by rapid cell proliferation and differentiation (Donohoe et al., 1999). However, the exact cellular functions of YY1 remained unclear. To further investigate the role and mechanism of action of YY1 in vivo, we ablated YY1 in cultured cells using both homologous recombination-based gene knockout and RNA-mediated interference (RNAi) (Fire et al., 1998). These studies have led to the discovery of a previously unsuspected connection between YY1, Hdm2, and p53 and a crucial role for YY1 in p53 regulation (see below).

p53 is a tumor suppressor protein and a transcriptional regulator that plays an important role in cellular responses to various stress signals (reviewed in Levine [1997] and Ryan et al. [2001]). p53 protein is drastically induced under conditions such as DNA damage. The rise in p53 either activates transcription of the cell cycle regulator p21, which induces cell growth arrest, or the proapoptotic gene *bax*, which induces apoptosis (reviewed in Oren [2003]). Similarly, p53 is also induced in response to oncogenic insults, and activation of p53 is an important mechanism that helps prevent tumor development (reviewed in Sherr and Weber [2000] and Lowe [1999]). The importance of p53 as a tumor suppressor is highlighted by the fact that over 50% of all tumors carry inactivating mutations in *p53* gene (reviewed in Lane [1994]).

p53 is regulated primarily at the level of protein stability by its interacting partner, the mouse double minute 2 protein (Mdm2, or Hdm2 for the human ortholog). Mdm2-deficient mice die during early embryogenesis. This early lethal phenotype can be suppressed completely by simultaneous inactivation of p53, suggesting that the main function of Mdm2 in vivo is to regulate p53 (Jones et al., 1995; Montes de Oca Luna et al., 1995). At the molecular level, Mdm2 functions as an E3 ubiquitin ligase, which mediates p53 ubiquitination and proteasomal degradation in vivo (Honda et al., 1997). Thus, factors that regulate the physical interaction of Mdm2 with p53 or Mdm2 activity will directly impact the p53 steady-state level in the cell. For instance, posttranslational modifications such as phosphorylation and acetylation of p53 have been shown to regulate p53 stability in part by controlling the accessibility of p53 to Mdm2 (Ito et al., 2002; Prives, 1998). Likewise, multiple mechanisms may also be in place to modulate Mdm2 activity and/or its ability to access p53 for ubiquitination. For instance, the tumor suppressor $p14^{ARF}$ ($p19^{ARF}$ in mouse) directly interacts with and functionally inactivates Mdm2, by either relocating Mdm2 to the nucleolus and/or forming a tripartite complex with Mdm2 and p53, which abrogates Mdm2 regulation of p53 (reviewed in Sherr and Weber [2000] and Zhang and Xiong [2001]). Similarly, the human ribosomal protein L11 can also interact with Hdm2 and participate in Hdm2 relocalization to the nucleolus (Lohrum et al., 2003). Recently, cyclin G has been shown to regulate p53 level through modulation of Hdm2 via the associated phosphatase PP2A (Okamoto et al., 2002). Taken together, these findings suggest that both p53 and Hdm2 can be subjected to multiple levels of regulation, which affect the intracellular level of p53.

In this study, we found that loss of YY1 results in a significant increase in p53 level. Conversely, overexpression of YY1 significantly decreased endogenous p53 level. Multiple lines of evidence suggest that YY1

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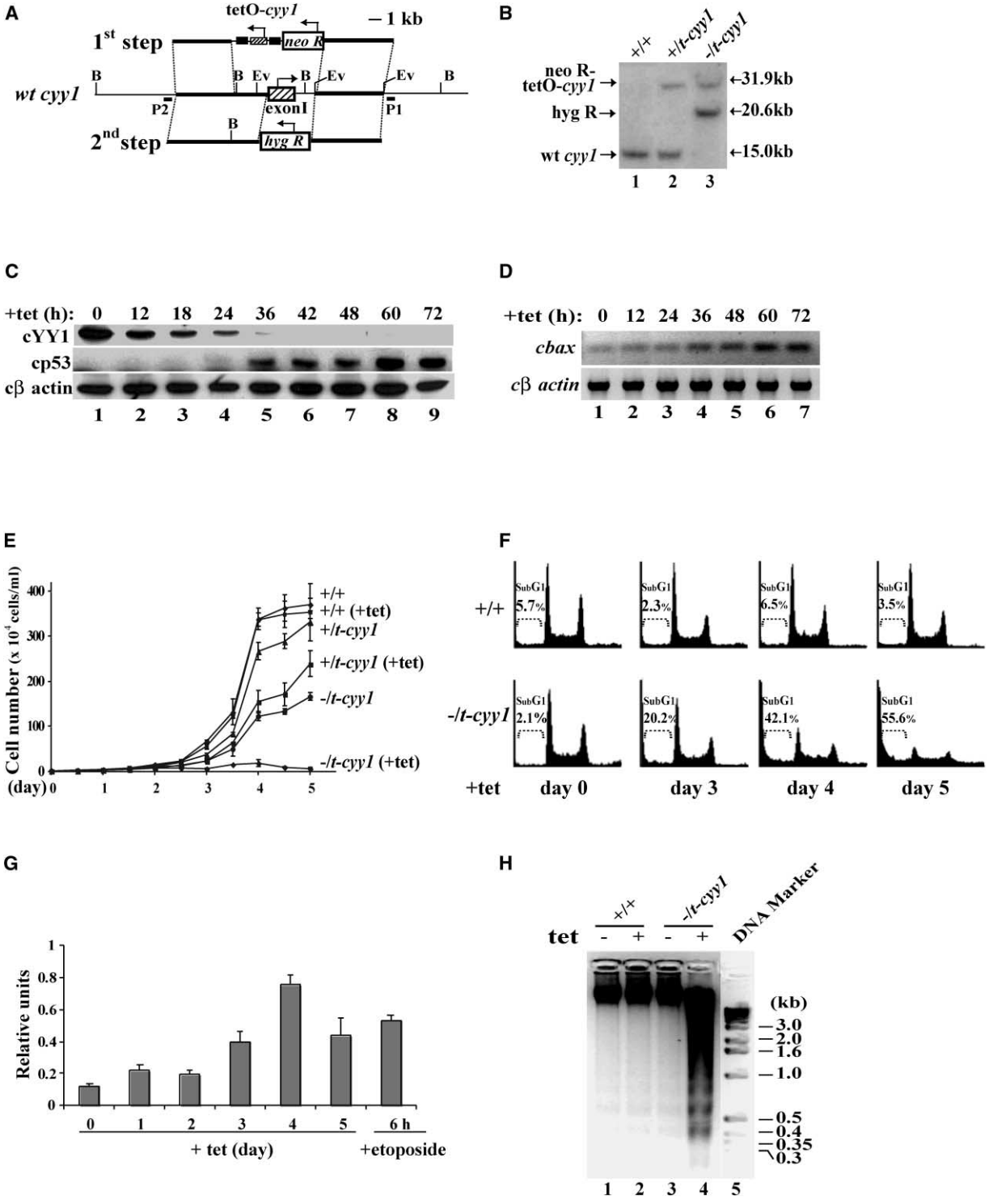


Figure 1. Generation of a Targeted Mutation at the *cyy1* Locus in DT40 Cells and the Characterization of the *cyy1* Nullizygous Cells

(A) Genomic organization of the *cyy1* locus and design of the targeting constructs. For the knockin/knockout step, a 4.9 kb *Bam*HI/*Eco*RV fragment containing *cyy1*'s entire exon I and its ~2 kb promoter region was replaced by the *neo R* expression cassette and the *cyy1* transgene driven by tet-repressive promoter (*t-cyy1*). The second knockout step replaces the entire exon I plus a few hundred base pairs of the proximal promoter of *cyy1*. External probes P1 and P2 were used to identify homologous recombination by Southern blot. *B*, *Bam*HI; *Ev*, *Eco*RV.

(B) Identification of DT40 cells containing different *cyy1* alleles. Probe P1 was hybridized to the *Bam*HI digested genomic DNA from wt and mutant DT40 cells (genotypes indicated at the top), and genotypes were confirmed by probe P2 (data not shown).

(C) cYY1 and cp53 expression in the presence of tet. Aliquots of *cyy1* nullizygous cells (*−/t-cyy1*) grown in the medium with 0.5 μg/ml of tet over a period of 72 hr were collected. Whole cell lysates were analyzed by Western blotting using YY1 (top panel), p53 (middle panel), and β actin (bottom panel) antibodies.

directly regulates the ability of Hdm2 to ubiquitinate p53 through protein-protein interactions. These include the findings that YY1 physically interacts with Hdm2 and p53 and that wild-type (wt) YY1 but not an Hdm2 binding-defective mutant stimulates Hdm2-mediated p53 ubiquitination both in vivo and in vitro using purified protein components. We further demonstrate that this YY1 regulation of Hdm2-p53 is likely to be the result of the ability of YY1 to promote Hdm2-p53 physical interaction, which is a prerequisite for Hdm2 regulation of p53 ubiquitination. Consistently, we found a reduction of Hdm2-p53 interaction upon reduction of YY1 protein level. Significantly, $p14^{ARF}$ also interacts with YY1 and can disrupt the Hdm2-YY1 interaction, which is important for YY1 regulation of Hdm2-mediated p53 ubiquitination. Taken together, our findings identify YY1 as a potential cofactor for Hdm2 and support the model that YY1 plays an important role in Hdm2-mediated p53 ubiquitination and degradation. These findings not only provide important insights into YY1 function but also shed light on the increasingly complex regulation of Hdm2-mediated p53 ubiquitination that may be important for understanding tumorigenesis.

Results

Genetic Ablation of YY1 in Chicken DT40 Cells

Chicken B lymphoma DT40 cells support efficient homologous recombination and thus are well suited for gene function studies through targeted gene disruption (Buerstedde and Takeda, 1991). To knock out *yy1* in DT40 cells, we isolated a full-length chicken *yy1* cDNA (*cyy1*), which shares 88% amino acid similarity with that of human YY1 (hYY1) (see Supplemental Figure S1A at http://www.cell.com/cgi/content/full/117/7/859/DC1). The C-terminal half of cYY1, which includes the four zinc fingers, is identical to that of hYY1, suggesting that cYY1 and hYY1 recognize the same DNA sequence elements. We next isolated a *cyy1* genomic fragment that contains the promoter as well as the entire exon I, which represents 55% of the *cyy1* coding region. Using this genomic fragment, we generated a targeting vector, which is essentially the same as the *yy1* mouse knockout construct described earlier (Donohoe et al., 1999) (Supplemental Figure S1B). Replacement of the wt allele with this targeting vector is predicted to generate a *cyy1* null allele (Donohoe et al., 1999). Using this strategy, we first isolated *cyy1* heterozygous cells (Supplemental Figure S1C). We then attempted to mutate the second *cyy1* allele without success, although we observed homologous recombination between the targeting vector and the mutant *cyy1* allele (data not shown), suggesting that lack of cYY1 is likely to be cell lethal.

To obtain a *cyy1* null cell line, we turned to a knockin/knockout strategy, outlined in Figure 1A. Essentially, exon I of *cyy1* was first replaced by a tet-regulatable (*tet-off*) *cyy1* transgene, referred to as *t-cyy1* (Figure 1A, "1st step," and Figure 1B, lane 2). The combined cYY1 expression from the remaining wt and the *t-cyy1* alleles upon induction was comparable to that of the wt DT40 cells (data not shown). Under the *tet-off* condition in which the *cyy1* transgene was induced, we successfully mutated the remaining wt *cyy1* allele (Figure 1B, lane 3) using the constitutive *cyy1* knockout vector depicted in Figure 1A ("2nd step").

Loss of YY1 Leads to an Increase in p53 Level and DT40 Cell Apoptosis

As shown in Figure 1C, upon tet addition, ectopically provided cYY1 was significantly depleted by 24 hr and was undetectable by 48 hr (top panel). As a consequence, growth of these cells was significantly compromised (Figure 1E, −/*t-cyy1*, +tet). We analyzed a total of three independently isolated cell lines and obtained the same results. Interestingly, the *cyy1* heterozygous cells (+/− and +/*t-cyy1* [+tet]) also displayed significant growth retardation (Figure 1E, +/*c-yy1*, +tet; and see Supplemental Figure S2 at *Cell* web site). The growth potential of these heterozygous cells can be restored close to the wt level by turning on *cyy1* transgene expression (Figure 1E, compare +/*t-cyy1* [+tet] with +/*t-cyy1*), suggesting that YY1 dosage may be important for cell proliferation. To determine the basis of this growth defect, we analyzed the *cyy1* null cells by FACS. As shown in Figure 1F, upon tet addition, the sub G1 cell population continued to accumulate in the *cyy1* nullizygous but not in wt cells, coinciding with the depletion of the cYY1 proteins in the cell. This suggested that cells lacking cYY1 may be undergoing apoptosis. Consistent with this, we found caspase activation in a time course that paralleled cYY1 decrease and the onset of apoptosis (Figure 1G). By day 5, the majority of the YY1-negative cells were disintegrated, which may account for the decrease in the caspase activity at this time point (Figure

(D) YY1 depletion induces *bax* transcription in DT40 cells. The DT40 *cyy1* nullizygous cells (−/*t-cyy1*) were collected at the different time points post tet addition for mRNA preparation. Semiquantitative RT-PCR was performed to detect expression of *cbax* and *cβ actin*. The sequences of the oligos used in RT-PCR are the following: *cbax*, 5′-CAGAAGGCGGAGGACCTCGTGAAG-3′ and 5′-ACAGCCTTGTGCTTCCG CAGCCACC-3′; and *cβ actin*, 5′-CTGCGCTCGTTGTTGACAATGGCTC-3′ and 5′-GTAGCCTTCATAGATGGGCACAGTG -3′.

(E) Proliferation of wt, *cyy1* heterozygous, and nullizygous cells. Wt DT40, +/*t-cyy1*, and −/*t-cyy1* cells (triplicate samples) were analyzed for the presence of live cells using trypan blue every 12 hr over a period of 5 days in the presence and absence of tet, with the starting cell density at 20,000 cells/ml. The proliferation curves were obtained by plotting cell density over time with standard deviation.

(F) FACS analysis of wt and *cyy1* nullizygous cells (−/*t-cyy1*). Wt DT40 and −/*t-cyy1* cells were grown in the absence or the presence of tet. Every 24 hr, aliquots of cells were collected, fixed in 50% ethanol, and stained by propidium iodide before FACS analysis. The percentages of sub G1 (apoptotic) cells are indicated.

(G) Depletion of cYY1 expression is correlated with an increase in caspase activity. Whole cell lysates prepared from −/*t-cyy1* cells grown in the presence of tet over a period of 5 days were assayed for caspase 3 activity. Lysates from cells treated with 10 μM etoposide were used as a positive control.

(H) DNA laddering in DT40 cells depleted of YY1. Wt DT40 (+/+) and the *cyy1* nullizygous (−/*t-cyy1*) cells were grown in the presence or absence of tet for 5 days before the cells were collected. Genomic DNA was prepared and analyzed on a 1.5% agarose gel.

1G). Furthermore, DNA isolated from these cells displayed internucleosomal DNA degradation characteristic of apoptotic cells (Figure 1H). Taken together, these findings showed that DT40 cells undergo apoptosis in the absence of cYY1.

The tumor suppressor p53 plays an essential role in cell growth arrest and apoptosis in response to DNA damage or oncogenic insult (Levine, 1997). We asked whether removal of cYY1 may have affected p53 regulation. As shown in Figure 1C, the gradual depletion of cYY1 upon tet addition was paralleled by p53 (cp53) accumulation. Significantly, transcription of the apoptotic gene *bax*, which is a downstream target of p53 (Miyashita and Reed, 1995), was also activated in a time course that parallels cp53 accumulation (Figure 1D), suggesting that cp53 is likely to be functional in DT40 cells. p53 expression in DT40 cells has been a controversial issue, with two published studies reporting opposite results (Takao et al., 1999; Tanikawa et al., 2001). In addition to the Western blotting result (Figure 1C), we were able to detect and subclone p53 mRNA from DT40 cells by RT-PCR, thus providing unequivocal evidence demonstrating p53 expression in DT40 cells (our unpublished data). Taken together, these findings identify a previously unsuspected role for cYY1 in regulating p53 protein level in vivo. This regulation is not unique to DT40 cells, because we also observed a similar increase in p53 protein level and its downstream target genes *p21*, *bax*, and *gadd45* in mammalian cells upon depletion of YY1 by RNAi (Figures 2A and 2B). As expected, the p53 mRNA level remained unchanged, but mRNA levels of *p21, bax*, and *gadd45* were increased (Supplemental Figure S3), which could account for the rise of p21, Bax, and GADD45 at the protein levels. Significantly, global profiling of YY1 target genes identified a large number of p53 target genes whose expression is affected by the loss of YY1 (our unpublished data), suggesting that YY1 plays a critical and widespread role in regulating p53 target gene expression. The functional consequence of YY1 knockdown was further analyzed by RNAi. As shown in Figure 2C, the number of puromycin-resistant colonies was significantly reduced as a result of YY1 depletion, suggesting that YY1 is essential for U2OS cell growth. Taken together, these findings suggest that YY1 regulation of p53 is conserved in mammalian cells and that depletion of YY1 can result in either apoptosis or growth suppression.

Since YY1 can function as a transcriptional repressor (Shi et al., 1991), we next asked if the increase in cp53 level in the absence of cYY1 was due to an increase in *cp53* mRNA. Northern blot analysis found comparable *cp53* mRNA levels in the presence and absence of cYY1 (Supplemental Figure S4), suggesting that regulation of cp53 by cYY1 is not at the level of *cp53* transcription or RNA processing. In addition, c-*myc*, a known activator of *p53*, is only modestly affected if at all (Supplemental Figure S4). As discussed below, the effect of YY1 on p53 (stimulation of ubiquitination) appears to be separable from YY1's transcriptional activities and may be mediated by direct protein-protein interactions.

To investigate mechanisms by which YY1 regulates DT40 apoptosis, we first carried out genetic rescue experiments to identify domains of YY1 that are important for YY1 function in DT40 cells. YY1 contains a number

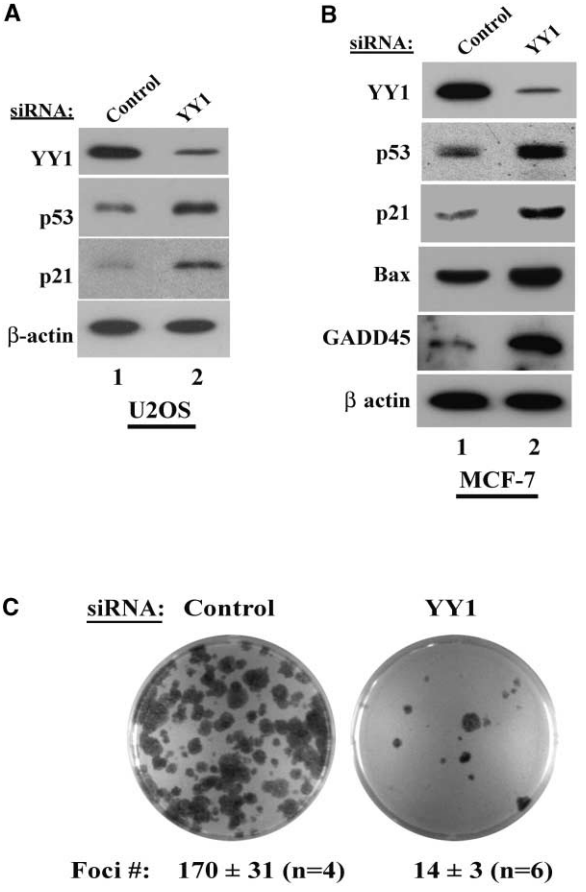


Figure 2. YY1 Regulation of p53 in Mammalian Cells

(A and B) Expression of YY1, p53, and p53 target genes in *yy1* knockdown cells. Cells were either infected with retrovirus containing U6/*gfp* or U6/*yy1* siRNA DNA templates (U2OS) (A) or transfected with pBS/U6/*gfp* or pBS/U6/*yy1* siRNA plasmids (MCF-7) (B). Four days after infection or transfection, total cell lysates were analyzed by Western blot using the antibodies indicated on the left. (C) YY1 depletion causes cell growth retardation. U2OS cells were transfected with pBS/U6/*gfp* or pBS/U6/*yy1* siRNA plasmids containing the puromycin expression cassette and selected with 1.0 μg/ml of puromycin for about 10 days. The foci were detected with crystal violet, and the numbers were the average of four (control) or six (*yy1*) independently transfected plates.

of motifs, including the bipartite acidic regions at the N terminus, the histidine cluster, the glycine/alanine (G/A)- and the glycine/lysine (G/K)-rich regions, and the spacer region, which contains an element that is uniquely conserved from *Drosophila* to human (reviewed in Thomas and Seto [1999]). While the acidic regions are important for transcriptional activation (Bushmeyer et al., 1996; Lee et al., 1995b), the G/K region binds histone deacetylases and is important for YY1 repression function (Yang et al., 1996). We analyzed cYY1 mutants carrying these various deletions for their ability to rescue cYY1-depleted DT40 cells. As shown in Figure 3, wt cYY1 restored growth assayed by the colony numbers, indicating that apoptosis observed in DT40 cells is a result of cYY1 depletion. Importantly, deletions that removed the HDAC-interacting or the acidic domains of cYY1 had only a mild effect on the rescuing ability of cYY1,

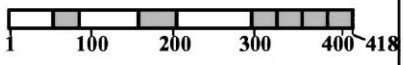
cYY1			Colony numbers		
	Deletion	Diagram	Exp. #1	Exp. #2	Average
vector	--	--	3	1	2
wt	--		82	115	98
Δ55-71	His-cluster		46	26	36
Δ71-160	Acidic region		51	72	61
Δ161-204	GA/GK-rich region		40	80	60
Δ181-204	GK-rich region		70	102	86
Δ205-299	Spacer region		5	2	3

Figure 3. Genetic Rescue Experiments in DT40 Cells

Wt and deletion mutants of chicken *yy1* driven by the endogenous chicken *yy1* promoter were individually transfected into *cyy1* nullizygous cells (−/*t-cyy1*). After 2 days, the cells were plated to 96-well plates in the medium containing 1.0 μg/ml of *tet* to suppress *cyy1* expression from the *t-cyy1* allele. Cell colonies were counted 2 weeks later. Results from two independent experiments and the average are shown.

consistent with the hypothesis that individual transcriptional activities may not be essential for this biological function of YY1 (Figure 3). Deletion of the histidine cluster had a significant impact on the rescuing ability, but the basis for this defect remains to be determined. However, removal of the spacer region (Δ205–299) essentially abrogated the ability of cYY1 to rescue (Figure 3). Control experiments indicate that all the cYY1 mutants tested can be expressed at comparable levels (data not shown). As will be discussed later, this region of YY1 appears to be necessary for the physical interaction with Hdm2 and for regulating p53 ubiquitination.

YY1 Physically Interacts with Hdm2 and p53 In Vivo and In Vitro

p53 is controlled primarily by Mdm2-mediated ubiquitination and degradation by the proteasome. Recent studies suggest that additional proteins may regulate Mdm2-mediated p53 ubiquitination (Cummings et al., 2004; Grossman et al., 2003; Kamijo et al., 1998; Li et al., 2004; Lohrum et al., 2003; Okamoto et al., 2002; Pomerantz et al., 1998; Zhang et al., 1998). Given that p53 protein level is inversely correlated with that of YY1 and that p53 RNA level appears unaffected by YY1 depletion, we asked whether YY1 plays a role in regulating Hdm2-mediated p53 ubiquitination in vivo through protein-protein interactions. We first investigated whether YY1 physically interacts with Hdm2 and p53. As shown in Figure 4A, a YY1 but not two unrelated antibodies coimmunoprecipitated (CoIP) Hdm2 (compare lane 4 with lanes 2 and 3). Likewise, reciprocal CoIP with an Hdm2 but not the unrelated antibodies also pulled down YY1 (Figure 4B, compare lane 4 with lanes 2 and 3), suggesting an interaction between endogenous YY1 and Hdm2. Similarly, we also detected a weak endogenous YY1-p53 interaction in U2OS cells, which was significantly enhanced when p53 level was raised with the treatment of the proteasome inhibitor AdaAhx$_3$L$_3$VS (Ada, [Kessler et al., 2001]) (Figure 4C, compare lane 6 with lane 3). These experiments suggest that YY1 interacts with both Hdm2 and p53 in vivo. The interactions of YY1 with Hdm2 and p53 are likely to be direct, since these interactions can be recapitulated with bacterially purified proteins in vitro (Figures 4D–4G).

To better understand the interactions among YY1, Hdm2, and p53, we carried out in vitro binding experiments to identify domains involved in the interactions. As shown in Figures 4D and 4E, amino acids (aa) 150–290 of Hdm2 and the C-terminal region of p53 (aa 290–393) are important for the direct interactions with YY1, respectively. Previous studies showed that the N-terminal regions of Hdm2 and p53 are involved in direct physical interactions with one another (Chen et al., 1993), which are distinct from the domains that mediate their interactions with YY1. We also investigated domains within YY1 that interact with p53 and Hdm2. Two regions in YY1 (aa 142–224 and aa 331–414) appear to interact with p53 (Figure 4F). We next analyzed the interaction between Hdm2 and YY1. As shown in Figure 4G, the human YY1 spacer deletion mutant (Δ200–295), which is equivalent to the chicken mutant (Δ205–299) defective in the rescue experiment (Figure 3), displayed a greatly reduced ability to interact with Hdm2 (compare lane 7 with lane 3). However, this mutant interacted with p53 (Figure 4G, compare lane 8 with lane 4) and can repress transcription (Supplemental Figure S5), similar to wt YY1. Thus, the reduced ability to interact with Hdm2 is unlikely to be due to a gross structural alteration. Taken together, these findings identify nonoverlapping domains involved in YY1, Hdm2, and p53 interactions, strongly suggesting that these three proteins can form a ternary complex. To test this hypothesis, we subjected bacterially purified YY1, Hdm2, and p53 to glycerol gradient centrifugation. As shown in Supplemental Figure S6, only when all three proteins were present did we observe the appearance of YY1, Hdm2, and p53 simultaneously in high molecular weight factions (MW greater

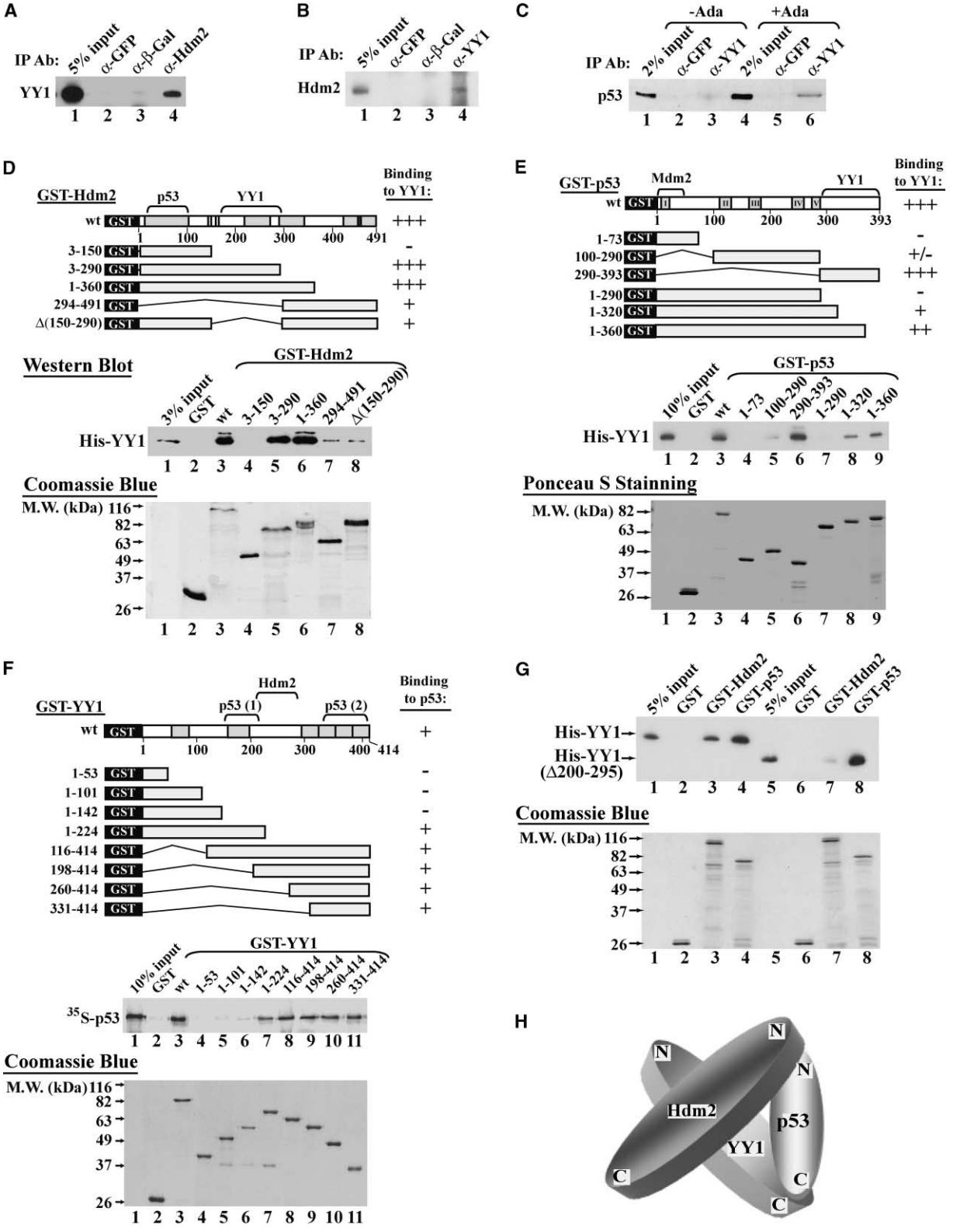


Figure 4. YY1 Interacts with Both Hdm2 and p53

(A) YY1 interacts with Hdm2 in vivo. Total U2OS cell lysates were immunoprecipitated with Hdm2 (N-20), GFP, or β-gal polyclonal antibodies. The immunoprecipitates were analyzed by Western blot using a YY1 monoclonal antibody (H-10).

(B) Reciprocal IP confirms YY1 and Hdm2 interaction in vivo. Total U2OS cell lysates were immunoprecipitated with YY1 polyclonal (H-414) and two control antibodies, respectively, followed by Western blot analysis using a Hdm2 antibody (Smp-14).

(C) YY1 and p53 interact in vivo. U2OS cells were either untreated or treated with 10 μM of Ada (Kessler et al., 2001) for 6 hr. Cell lysates

than 150 KD; Figures S6d, S6g, and S6j, fractions 31–37), suggesting a possible ternary or higher order complex. Although the stoichiometry remains to be determined, these findings, together with the domain mapping information, support the model that YY1, Hdm2, and p53 can form ternary or higher order complexes (Figure 4H).

YY1 Can Stimulate Hdm2-Mediated p53 Polyubiquitination and Degradation

We next investigated the possibility that YY1 may directly regulate p53 ubiquitination and proteasome-mediated degradation. As shown in Figure 5B, YY1 downregulation by RNAi significantly reduced endogenous p53 polyubiquitination in a human diploid fibroblast cell line (WI-38) (compare lane 2 with lane 1), suggesting that YY1 is required for efficient p53 ubiquitination in vivo. In contrast to YY1 depletion, YY1 overexpression led to an enhanced endogenous p53 ubiquitination in the presence of the proteasome inhibitor Ada (Figure 5B, compare lane 5 with lane 4). Consistently, in the absence of Ada, overexpression of YY1 resulted in a significant reduction of the p53 steady-state level (Figure 5C, middle panel, compare lanes 4–6 with lane 1). Given that YY1 is a transcription factor, we asked whether this effect of YY1 on p53 ubiquitination was linked to its ability to regulate transcription. We analyzed a YY1 chimeric protein (Chi17), in which zinc finger 2 was replaced by a similar C_2H_2-type finger from GFI-1 (Figure 5A and Galvin and Shi [1997]). Chi17 is unable to bind YY1 consensus sequences or to repress transcription as a GAL4 fusion protein (Galvin and Shi, 1997). However, similar to wt YY1, Chi17 can interact with Hdm2 and p53 (Supplemental Figure S7) and stimulate p53 ubiquitination (Figure 5B, lane 6). Taken together, these findings suggest that the ability of YY1 to stimulate p53 ubiquitination is likely to be independent of YY1 target gene regulation. Supporting this model, the YY1 spacer deletion mutant (Δ200–295), which is inactive in the genetic rescue experiments (Figure 3) and defective for crucial protein interactions with Hdm2 (Figure 4G), was compromised in its ability to stimulate p53 ubiquitination (Figure 5B, compare lane 7 with lane 5).

Ubiquitin (Ub) chain extension can occur on a number of different lysine (K) residues on the Ub molecule, but only chain polymerization at K48 is important for substrate degradation by the proteasome (Pickart, 1997). We compared YY1-stimulated p53 polyubiquitination using either wt or the K48R (arginine) mutant Ub. As shown in Figure 5D, the ability of YY1 to stimulate p53 polyubiquitination was significantly compromised using the mutant Ub (K48R), suggesting that YY1-stimulated p53 polyubiquitination occurred predominantly on K48 of the Ub moiety (compare lanes 5 and 6 with lanes 2 and 3). We confirmed that these slower-migrating moieties are polyubiquitinated p53 by reprobing the same blot with a p53 monoclonal antibody (Figure 5D, middle panel). These findings support the model that YY1 plays an important role in p53 ubiquitination and degradation by the proteasome.

We next determined whether YY1 stimulation of p53 ubiquitination is mediated by Hdm2 in vivo. YY1, p53, and HA-tagged Ub together with either wt or a catalytically inactive Hdm2 were transfected into $p53^{-/-}$/$mdm2^{-/-}$ mouse embryonic fibroblast (MEF) cells. As shown in Figure 5E, YY1 overexpression alone had very little effect on p53 ubiquitination (compare lanes 3 and 4 with lane 2). In contrast, YY1 significantly enhanced p53 ubiquitination in the presence of transfected wt but not the catalytically inactive Hdm2 (Figure 5E, compare lanes 6 and 7 with lane 5 and with lanes 8 and 9). Taken together, these findings argue strongly that YY1 stimulates p53 ubiquitination through Hdm2 and provide a possible molecular explanation for the increase in p53 level observed in the YY1 nullizygous cells.

YY1 Stimulates p53 Polyubiquitination in an In Vitro Ubiquitination System

To seek further evidence that YY1 regulates Hdm2-mediated p53 ubiquitination via direct physical interaction, we asked whether recombinant YY1 protein can stimulate Hdm2-mediated p53 ubiquitination in vitro using purified Hdm2 and p53 (Grossman et al., 2003). It has been suggested that Mdm2 mainly mediates monoubiquitination of p53 in vitro (Grossman et al., 2003; Lai et al., 2001; Li et al., 2003). As shown in Figure 6A, bacterially purified His-YY1 stimulated the production of slower-migrating p53 (lane 2), while a mock-purified fraction did not (lane 1). These slower-migrating p53 proteins are likely to be polyubiquitinated p53 species, because the use of methylated Ub, which does not sup-

were immunoprecipitated with GFP (FL) and YY1 (H-414) antibodies and analyzed by Western blot using a p53 antibody (DO-1).

(D) Identification of Hdm2 domain that interacts with YY1. GST-Hdm2 fusion proteins were diagrammed in the top panel. Purified GST-Hdm2 proteins (3 μg each) were individually incubated with purified His-YY1 (1.5 μg) and Western blotted using a His epitope antibody (H-3). The results of the GST pulldown experiment are summarized on the right of the top panel. The domains identified to be necessary for the physical interaction are indicated on the top. The input GST proteins are shown in the bottom panel.

(E) Identification of p53 domains that interact with YY1. Wt and mutant GST-p53 fusion proteins are diagramed in the top panel. Interactions of His-YY1 with various GST-p53 fusion proteins were analyzed by GST pulldown assays (middle panel). The input GST fusion proteins are shown in the bottom panel. The results are summarized on the right, and the interacting domains for Hdm2 and YY1 are indicated on the top.

(F) Identification of YY1 domains that interact with p53. GST-YY1 fusion proteins are diagramed in the top panel. The purified GST-YY1 proteins (3 μg each) were incubated with in vitro translated, ^{35}S-labeled p53 followed by SDS-PAGE analysis. The autoradiography of the bound ^{35}S-p53 and the Coomassie blue staining of the gel are shown in the middle and the bottom panels, respectively. The results are summarized on the right, and the binding sites for p53 and Hdm2 are indicated at the top.

(G) YY1 interacts with Hdm2 and p53 in vitro. Equal amounts (3 μg) of purified GST, GST-Hdm2, or GST-p53 were incubated with purified His-YY1 or His-YY1 (Δ200–295) (1.5 μg each) at 4°C for 4 hr. After extensive washing, the samples were analyzed by Western blot using the His epitope antibody.

(H) Schematic diagram of the YY1-Hdm2-p53 ternary complex. The N-terminal regions of Hdm2 and p53 are involved in their interaction with one another (Chen et al., 1993). Two regions of YY1 (middle and C-terminal) can interact with the C-terminal region of p53. A middle, nonoverlapping region of YY1 interacts with Hdm2.

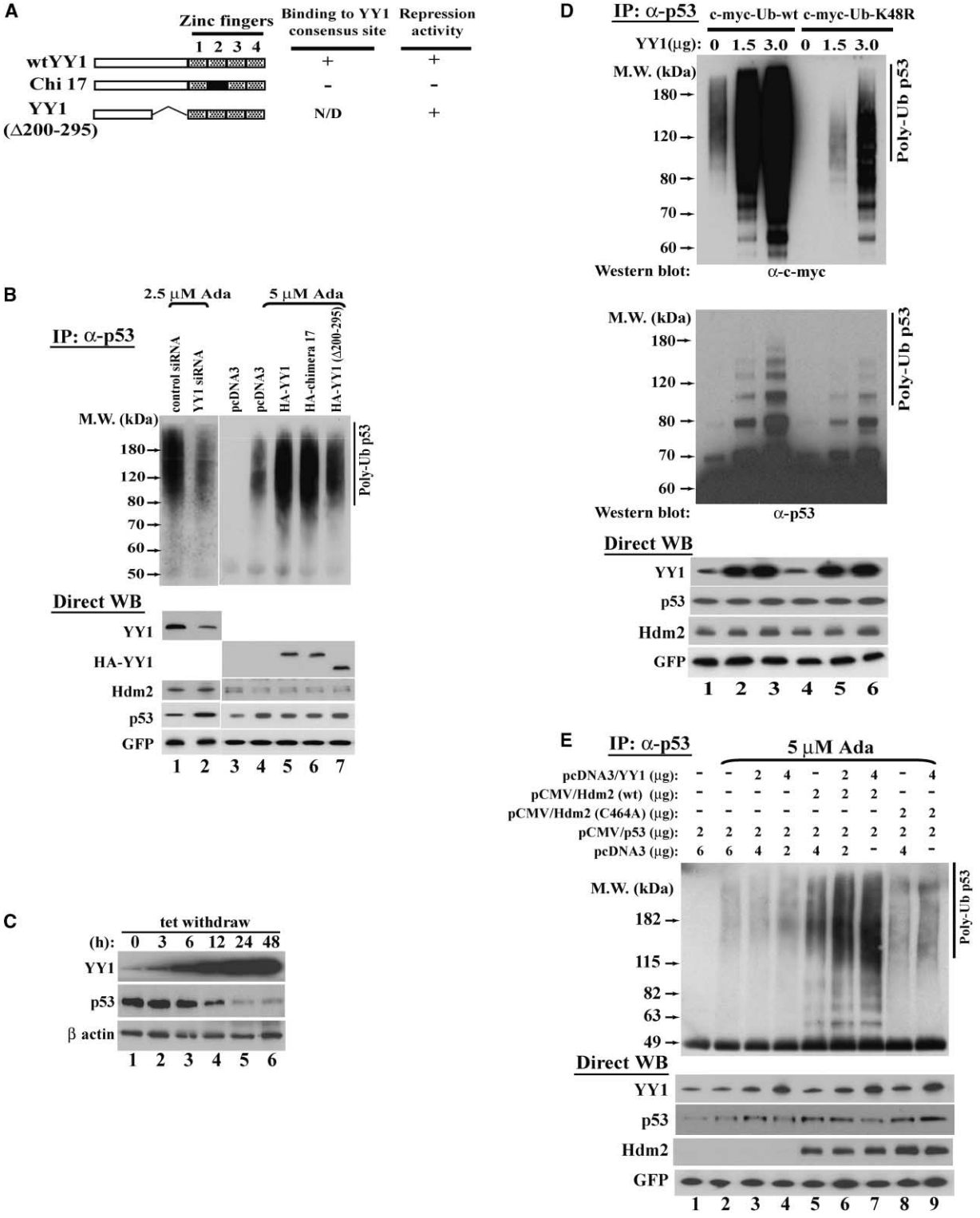


Figure 5. YY1 Is Necessary for Optimal Hdm2-Mediated p53 Ubiquitination and Degradation In Vivo

(A) Schematic diagrams of wt, YY1 chimera 17 (Chi17), and the spacer deletion mutant. The four zinc fingers at the C terminus are boxed. The zinc finger from GFI-1 is indicated by a solid box. Their binding ability to YY1 consensus sites and the repression activity are indicated by the plus and minus signs. N/D, not determined.

(B) Endogenous p53 ubiquitination is regulated by YY1. WI-38 cells were either infected by retroviruses containing an U6/*gfp* or U6/*yy1* siRNA (lanes 1 and 2) or transfected by pcDNA3 vector alone (lanes 3 and 4) and HA-tagged wt YY1, Chi17, or YY1 (Δ200–295) (lanes 5–7). Cells were grown in medium containing 2.5 μM of Ada for 3 days (lanes 1 and 2) or 5.0 μM Ada for 24 hr (lanes 4–7). Cell lysates were immunoprecipitated with a p53 antibody and Western blotted with a Ub antibody (MAB1510, Chemicon). The direct Western blots for YY1, Hdm2, p53, and cotransfected

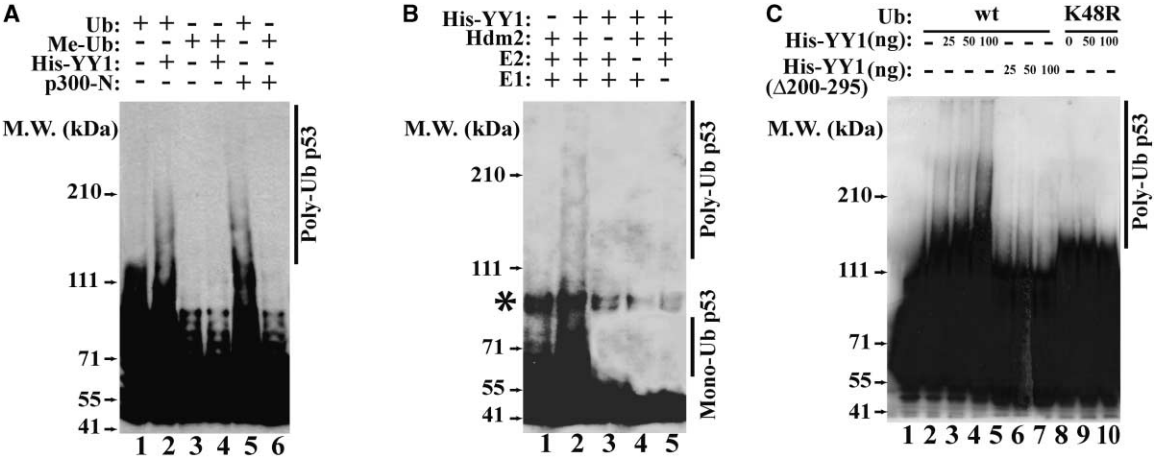


Figure 6. YY1 Stimulates p53 Polyubiquitination In Vitro

(A) YY1 stimulates p53 ubiquitination using native but not methylated Ub. In vitro ubiquitination assays were performed in the presence of purified HA-p53, purified FLAG-Hdm2 (E3), E1, UbcH5a (E2), and ATP. p53 polyubiquitination in the presence of Ub (compare lanes 1 and 2) or methylated Ub (Me-Ub) (compare lanes 3 and 4) was determined in the presence or absence of His-YY1. Lane 5 is the positive control for polyubiquitination (Grossman et al., 2003). The reaction mix was analyzed by Western blot using a p53 antibody (DO-1).

(B) YY1 stimulation of p53 polyubiquitination requires E1, E2, and E3 (Hdm2). In vitro p53 ubiquitination assays were performed in the presence of E1, UbcH5a (E2), and Hdm2 (E3) without (lane 1) or with His-YY1 (lanes 2–5, 100 ng), followed by p53 immunoblotting. Hdm2, UbcH5a (E2), or E1 were respectively omitted in lanes 3–5. The blot was relatively underexposed to better highlight the region where monoubiquitination would be observed. p53 immunoreactive signal is absent in the 61–100 kDa range within lanes 3–5 where monoubiquitinated p53 species would appear (Grossman et al., 2003), indicating that YY1 cannot substitute for E1, E2, or Hdm2 (E3) functions for the ubiquitination of p53. Mono- and polyubiquitinated p53 are indicated on the right. Asterisk indicates a covalent dimer (nonubiquitinated) form of purified p53 seen when purified p53 is incubated at 37°C (Grossman et al., 2003).

(C) Wt His-YY1 but not His-YY1 (Δ200–295) stimulates p53 polyubiquitination through K48 of Ub. In vitro ubiquitination assays were performed as described above in the presence of Ub (lanes 1–7) or Ub (K48R) (lanes 8–10) at 37°C for 30 min in the absence or presence of either wt or mutant YY1 (Δ200–295) as indicated on the top. The reaction mixes were analyzed by Western blot using a p53 antibody.

port Ub chain polymerization, blocked their formation (Figure 6A, lane 4). Incubation of YY1 with p53 alone in the absence of E1, UbcH5a (E2), or Hdm2 (E3) had no effect (Figure 6B). Furthermore, the K48R Ub failed to support YY1 stimulation of p53 polyubiquitination (Figure 6C, lanes 8–10). These results are consistent with the cell culture data (Figures 5D and 5E), providing further support that YY1 stimulation of p53 requires Mdm2 and that the polyubiquitinated p53 is destined for proteasome-mediated degradation. Importantly, the YY1 mutant (Δ200–295), which is defective for binding Hdm2, was significantly compromised in its ability to stimulate p53 ubiquitination both in vitro (Figure 6C, lanes 5–7) and in vivo (Figure 5B, lane 7). Taken together, these experiments demonstrated that YY1 is sufficient to induce Hdm2-mediated p53 polyubiquitination in the absence of transcription, and this regulation is likely to be mediated by direct physical interactions. The ability of YY1 to stimulate p53 polyubiquitination in vitro suggests that YY1 may function either as a cofactor for Hdm2 or may itself have an intrinsic enzymatic activity (E4 ligase) that promotes p53 polyubiquitination (Grossman et al., 2003). Since YY1 plays an important role in facilitating Hdm2-p53 physical interaction (discussed below), we favor the model that YY1 functions as a cofactor for Hdm2 to induce p53 polyubiquitination in vivo.

YY1 Regulates Hdm2-p53 Physical Interaction

To address the consequence of YY1's physical interaction with Hdm2, we analyzed the effect of YY1 overexpression on the Hdm2-p53 interaction by CoIP. Increasing amounts of wt but not the Hdm2 binding-defective

GFP are shown in the lower panel. The reason that no significant decrease of p53 was observed is most likely due to low transfection efficiency of the WI-38 cells.

(C) YY1 overexpression leads to decreased steady level of endogenous p53. U2OS cells containing *tet-off* responsive *yy1* were first maintained in medium with 0.5 μg/ml of tet. Aliquots of cells were collected at a number of time points as indicated after tet withdrawal, followed by Western blotting using the antibodies indicated on the left.

(D) YY1 enhances p53 polyubiquitination through K48 of Ub. Myc-epitope tagged wt Ub or the Ub mutant (K48R) were cotransfected with increased amounts of pcDNA3/*yy1* (0, 1.5, and 3.0 μg), pCMV/*hdm2* (1.5 μg), and pCMV/*p53* (1.5 μg) into U2OS cells. Two days after transfection, p53 was isolated by IP and analyzed by Western blot using myc-epitope antibody (top panel). The same blot was reprobed with a p53 monoclonal antibody (middle panel). The direct Western blots for YY1, p53, Hdm2, and cotransfected GFP are shown in the lower panels. The reason that no significant decrease of p53 level was observed is probably due to overexpression of p53 and saturation of the proteasome.

(E) YY1 overexpression stimulates Hdm2-mediated p53 ubiquitination. Increasing amounts (indicated on the top) of pcDNA3/*yy1* were cotransfected into $p53^{-/-}$/$mdm2^{-/-}$ double knockout MEF cells with plasmids encoding wt Hdm2 or a mutant Hdm2 (C464A), p53, and poly HA-Ub. p53 was isolated by IP and analyzed by an HA antibody.

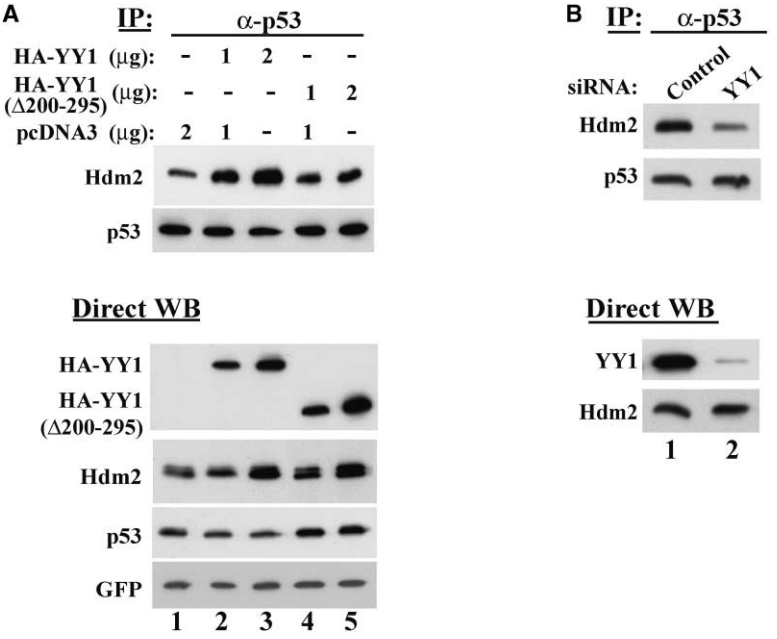


Figure 7. YY1 Regulates Hdm2-p53 Interaction

(A) Overexpression of YY1 enhances Hdm2-p53 interaction. pCMV/*hdm2* and pCMV/*p53* (2 μg of each) were transfected into U2OS cells with either pcDNA3/*HA-yy1* or pcDNA3/*HA-yy1* (Δ200–295) (1.0 and 2.0 μg of each). Hdm2 was coimmunoprecipitated by a p53 antibody (FL-393) and detected by Western blot using an Hdm2 antibody (Smp-14). Direct Western blot of the samples used are shown in the lower panels.

(B) *yy1* RNAi decreases the interaction between Hdm2 and p53. U2OS cells were infected by retroviruses containing U6/*gfp* siRNA or U6/*yy1* siRNA. Two days after infection, cells were cotransfected with pCMV/*hdm2* and pCMV/*p53* (2.0 μg each). Cell lysates were normalized using p53 as the reference, immunoprecipitated with a p53 antibody, and analyzed by Western blot. Direct Western blot of the samples used are shown in the lower panels.

YY1 mutant (Δ200–295) caused a reduction in p53 level (Figure 7A). Importantly, despite the decrease in p53, a p53 antibody brought down significantly more Hdm2 when YY1 was overexpressed (Figure 7A, compare lanes 2 and 3 with lane 1). This stimulatory activity of YY1 was dependent on YY1 interaction with Hdm2, since the Hdm2 binding-defective YY1 mutant (Δ200–295) was significantly compromised in its ability to stimulate Hdm2-p53 interaction (Figure 7A, compare lanes 4 and 5 with lane 1). This strongly suggests that wt YY1 promotes Hdm2-p53 physical interaction via a protein-protein interaction mechanism. Consistently, RNAi knockdown of YY1 resulted in a decrease in the interaction between Hdm2 and p53 (Figure 7B, top panel). In this experiment, the immunoprecipitated p53 from the control and *yy1* siRNA-treated cells was normalized to equal levels (Figure 7B, top panel), and an equal amount of Hdm2 was used (Figure 7B, bottom panel). Taken together, these findings suggest that YY1 is likely to be required for optimal Hdm2-p53 interaction and p53 ubiquitination in vivo.

Regulation of YY1-Hdm2 Interaction by the Tumor Suppressor p14^{ARF}

As a tumor surveillance mechanism, p14^{ARF} inactivates Hdm2 either by relocating it into the nucleolus or by forming a tripartite complex with Hdm2 and p53, which prevents p53 export and degradation (reviewed in Zhang and Xiong [2001]). Given the findings discussed above, we wished to investigate whether p14^{ARF} also interacts with YY1 and regulates the physical and functional interaction of YY1 with Hdm2-p53. As shown in Figure 8A, CoIP experiments identified physical interactions between endogenous p14^{ARF} and YY1 in the p14^{ARF}-expressing HeLa cells. This interaction is likely to be direct, since bacterially purified YY1 can also interact with the in vitro translated p14^{ARF} (Figure 8B). We next carried out domain mapping experiments and identified aa 216–224 of YY1 to be important for the p14^{ARF} interaction (Figure 8B). Given that p14^{ARF} interacts with both YY1 and Hdm2, we asked whether the Hdm2-YY1 interaction can be regulated by p14^{ARF}. As shown in Figure 8C, the Hdm2-YY1 interaction was significantly impaired by p14^{ARF} in a dose-dependent manner (top panel, compare lanes 2–5 with lane 1). Conversely, overexpression of YY1 can antagonize the ability of p14^{ARF} to stabilize p53 (Figure 8D, compare lane 3 with lane 2). The fact that p14^{ARF} can disrupt the YY1-Hdm2 interaction lends further support to the idea that the YY1-Hdm2 physical interaction is likely to be functionally important, and the disruption of this physical interaction by p14^{ARF} may be an important event during oncogenesis.

Discussion

We have provided genetic and biochemical evidence revealing an essential requirement of YY1 for regulation of p53 in vivo. We have shown that knocking out YY1 results in a significant increase in p53 level and a reduction of endogenous p53 ubiquitination (Figures 1C, 2A, 2B, and 5B). Consistently, an increase in YY1 leads to enhanced p53 ubiquitination mediated by Hdm2 both in vivo and in vitro and a decreased endogenous p53 steady-state level (Figures 5 and 6). These findings suggest that YY1 regulates p53 through modulation of the ability of Hdm2 to ubiquitinate p53. Our findings further suggest that this newly identified YY1 function is independent of its transcriptional activity. Instead, strong evidence is provided that supports the model of YY1 regulation of Hdm2-p53 via direct protein-protein interactions. First, similar to the wt protein, the YY1 mutant that is unable to recognize YY1 consensus sites and therefore presumed to be defective in activating or repressing YY1 target genes can still stimulate p53 ubiquitination (Figure 5B). Second, YY1 physically interacts with Hdm2 and p53 in vivo and can directly bind these

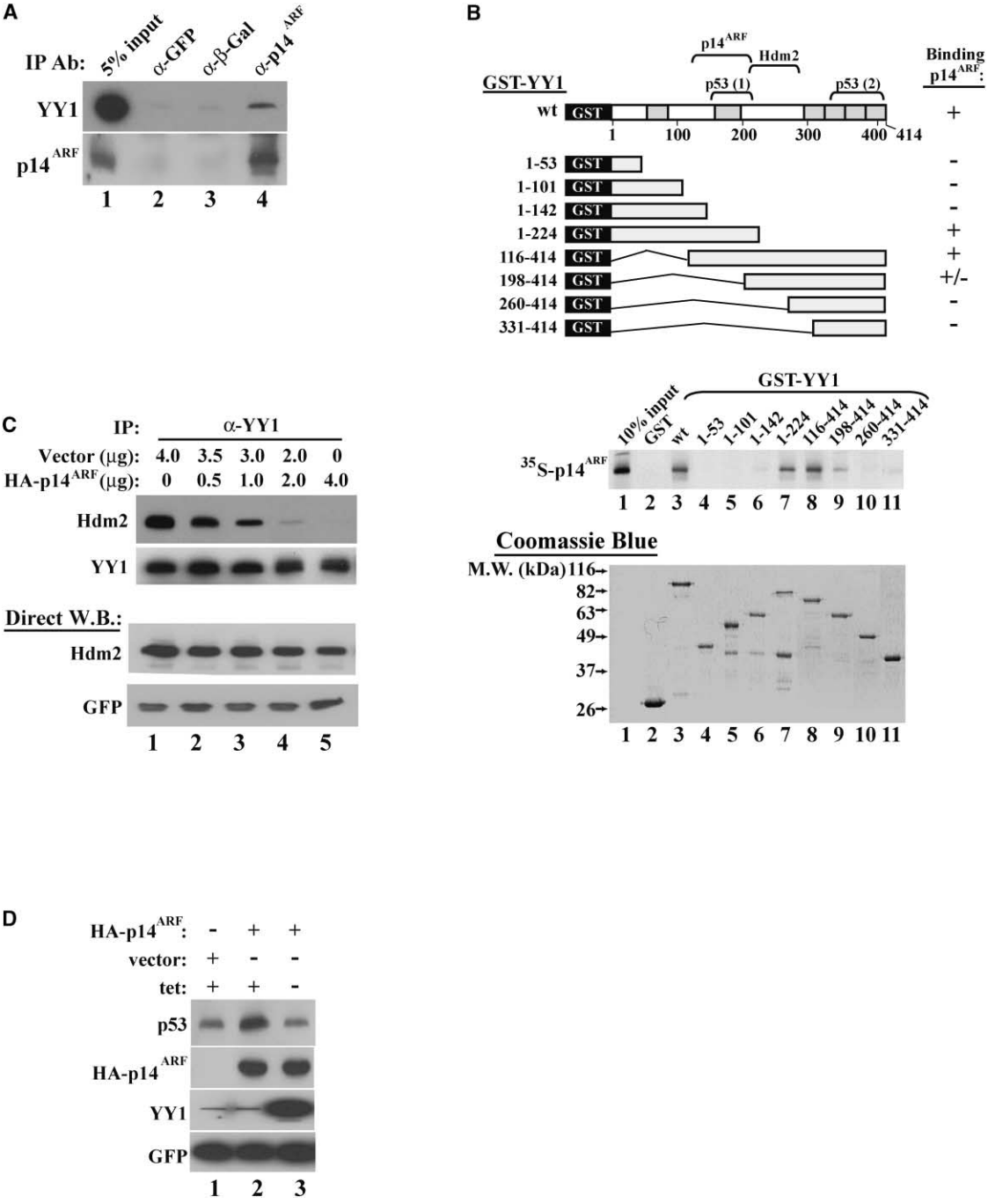


Figure 8. p14ARF Regulation of YY1-Hdm2 Interaction

(A) YY1 interacts with p14ARF in vivo. HeLa cell lysates were respectively incubated with GFP, β-gal, and p14ARF antibodies for immunoprecipitation and Western blotted with YY1 (top panel) and p14ARF antibody (bottom panel).

(B) Identification of a p14ARF-interacting domain in YY1. GST-YY1 fusion proteins are diagramed in the top panel. The purified GST-YY1 proteins (3 μg each) were incubated with in vitro translated p14ARF labeled by ^{35}S-methionine, followed by SDS-PAGE analysis. The autoradiography and the Coomassie blue staining of input GST fusion proteins are shown in the middle and the lower panels, respectively. The interaction results are summarized on the right of the diagram, and the interacting domains for p53, Hdm2, and p14ARF are indicated at the top.

(C) Overexpression of p14ARF disrupts Hdm2-YY1 interaction. Equal amounts of pcDNA3/*yy1* and pCMV/*hdm2*, together with increasing amounts of pcDNA3/*HA-p14ARF*, were cotransfected to HeLa cells (indicated on the top). A YY1 antibody (H-414) was used to CoIP Hdm2. The top panels show the immunoprecipitated Hdm2 and YY1 in the presence of increasing amounts of p14ARF. Direct Western blots for Hdm2 and cotransfected GFP are shown in the bottom panels.

(D) Overexpression of YY1 can antagonize the ability of p14ARF to stabilize p53. U2OS cells containing *tet-off* responsive *yy1* were cultured in the presence of tet to suppress expression of the *tet-off* yy1 transgene prior to transfection with 4 μg of pcDNA3 vector (lane 1) or pcDNA/*HA-p14ARF* (lanes 2 and 3). The cells were then grown in medium with (lanes 1 and 2) or without 0.5 μg/ml of tet (lane 3). Thirty-six hours posttransfection, cells were collected and analyzed by Western blot using the antibodies indicated on the left.

two proteins in vitro (Figure 4). Significantly, the Hdm2 binding-defective YY1 is compromised in its ability to promote p53 ubiquitination, substantiating the functional importance of the physical interaction (Figure 5B). Third, wt recombinant YY1 but not the Hdm2 binding-defective mutant induces p53 polyubiquitination in an in vitro ubiquitination system (Figure 6). Taken together, these results strongly support direct protein-protein interactions as a mechanism by which YY1 regulates p53 in vivo. These findings, however, do not exclude the possibility that the functional interaction between YY1 and Hdm2-p53 could occur on DNA. Our recent microarray data suggest that YY1 regulates a large number of p53 target genes (our unpublished data). Future experiments will determine whether these genes are direct targets for both YY1 and p53 and whether YY1 may promote p53 ubiquitination and degradation on promoters.

How, then, does YY1 promote p53 ubiquitination via physical interactions? We have shown that overexpression of wt but not the Hdm2 binding-defective YY1 results in a dose-dependent increase in the physical interaction between Hdm2 and p53. Consistently, a reduction of YY1 level leads to a decrease in Hdm2-p53 interaction. Previous studies of the Hdm2-interacting protein $p14^{ARF}$ ($p19^{ARF}$ for the mouse homolog) suggest that $p14^{ARF}$ can inactivate Hdm2 by relocalizing it to the nucleolus under certain circumstances (Weber et al., 1999). However, depletion of YY1 did not cause Hdm2 relocalization under our assay conditions (our unpublished data). Since YY1 can interact with both Hdm2 and p53 to form a ternary complex (Supplemental Figure S6), we speculate that YY1 probably helps stabilize the physical interaction between Hdm2 and p53 in vivo. Consistent with this, as revealed by the YY1 spacer mutant (Δ200–295) that binds p53 but is compromised in its ability to interact with Hdm2, YY1 binding to p53 alone is not sufficient for YY1 to promote p53 ubiquitination or efficient Hdm2-p53 physical interaction (Figures 5B, 6C, and 7A). A recent report suggests that Hdm2 at a high concentration is sufficient to mediate p53 polyubiquitination, but, at a low concentration, Hdm2 only monoubiquitinates p53 (Li et al., 2003). Based on this model, we speculate that, by enhancing the Hdm2-p53 interaction, YY1 may have increased the effective local concentration of Hdm2, thus enabling Hdm2 to mediate p53 polyubiquitination. Taken together, these findings suggest that YY1 is essential for optimal Hdm2-p53 physical interaction in vivo, which is a prerequisite for Hdm2 to be able to ubiquitinate p53.

In addition to the physical interaction model for YY1 regulation of Hdm2-p53 interaction discussed above, other mutually nonexclusive possibilities must also be considered. For instance, previous studies showed that p53 stability can be regulated by various posttranslational modifications such as phosphorylation and acetylation (Ito et al., 2002; Prives, 1998). Deacetylation of p53 by HDAC1 has been shown to facilitate p53 ubiquitination and degradation (Ito et al., 2002). Since YY1 interacts with HDAC1 (Yang et al., 1996), it is possible that YY1 may be targeting HDAC1 to the Hdm2-p53 complex for p53 modification. However, we found that the HDAC binding-defective YY1 (Yang et al., 1996) had comparable activity in stimulating Hdm2-mediated p53 ubiquitination, suggesting that recruitment of HDAC1 is not the main mechanism by which YY1 promotes p53 ubiquitination (our unpublished data). Alternatively, since YY1 interacts with p300 (Lee et al., 1995a), which has recently been shown to function as an E4 ligase for p53 polyubiquitination (Grossman et al., 2003), it is also possible that YY1 may recruit p300 to promote p53 ubiquitination. Our in vitro ubiquitination result shows, however, that YY1 is sufficient to promote p53 ubiquitination without p300 (Figure 6). Lastly, it is possible that additional posttranslational modifications of Hdm2 and/or p53 may take place in the absence of YY1 that could contribute to the regulation of the Hdm2-p53 interaction.

We also investigated whether blocking p53 expression can rescue cell death or growth arrest caused by the loss of YY1. Preliminary results show that simultaneous inhibition of YY1 and p53 expression by RNAi failed to rescue the YY1 depletion phenotype (our unpublished data). This is perhaps not surprising, because YY1 may play multiple roles in cells, including the well-established transcription function. Our recent microarray results suggest that YY1 not only regulates p53 target genes but also genes involved in cell cycle progression, mitosis, and differentiation (our unpublished data), thus offering a possible molecular explanation for the failure of the rescue experiments discussed above.

Taken together, our findings uncovered a crucial role for YY1 in controlling p53 homeostasis via regulating Hdm2-mediated p53 ubiquitination through a direct physical interaction mechanism. The physical interaction of YY1 with Hdm2 appears to be important for efficient Hdm2-p53 interaction in vivo, which in turn is necessary for p53 ubiquitination. These findings highlight the complexity of the Hdm2-p53 ubiquitination process in vivo and support the emerging paradigm that p53 ubiquitination and degradation are subjected to regulation at multiple levels via both direct and indirect mechanisms. Furthermore, these findings suggest that alteration of YY1 expression and/or activity may be an important event during oncogenesis. Consistent with this hypothesis, YY1 expression is found to be elevated in tumors such as lung adenocarcinoma (our unpublished data) and some cases of human acute myeloid leukemia (Erkeland et al., 2003). The finding that $p14^{ARF}$ can disrupt Hdm2-YY1 interaction (Figure 8C), which is important for efficient polyubiquitination of p53 mediated by Hdm2 (Figure 5), further supports a role for YY1 in tumorigenesis.

Experimental Procedures

Cell Culture

Standard conditions and procedures were used for culturing mammalian and DT40 cells, respectively (Sambrook et al., 1990; Wang et al., 1996).

Plasmids

A human *yy1* zinc finger probe was used to isolate a chicken *yy1* cDNA, which was then used to obtain c*yy1* genomic fragments from a chicken spleen genomic library (Stratagene). The tTA plasmid was from J. Manley. The tet-repressive *cyy1* plasmid was constructed using pUHD/10-3 (Gossen and Bujard, 1992) with the Kozak sequence altered from AAC to TTC to dampen cYY1 expression. pcDNA3/*yy1* (Δ200–295) was constructed by PCR-based mutagenesis. Mutant GST-p53 and GST-Hdm2 are from W. Gu, while other

GST- and His-fusion plasmids were generated in this lab. pCMV/*p53*, pCMV/*hdm2*, pCMV/*hdm2* (C464A), pcDNA3/*poly HA-Ub*, and pcDNA3/*HA-p14^{ARF}* are from Y. Xiong. BS/U6 *yy1* and *gfp* siRNA vectors were constructed as described (Sui et al., 2002). The sequences of *yy1* and the *gfp* siRNAs are 5′-GGGAGCAGAAGCAG GUGCAGAU-3′ and 5′-GGGCCAUGGCACGUACGGCAAG-3′.

Homologous Recombination-Based Knockout of *cyy1* in DT40 Cells

A DT40 cell line expressing tTA was used for the knockin/knockout transfection ("1st step") shown in Figure 1A. G418 (2.0 mg/ml) and hygromycin B (1 mg/ml) were used for selection, respectively. Limited serial dilution was performed to isolate single cells, which were expanded into cell lines. Southern blots were conducted following the standard protocol (Sambrook et al., 1990).

Growth Curves, FACS Analysis, and Caspase Activity Determination for DT40 Cells

DT40 cells were grown at a starting density of 2000 cells/ml with each treatment (without or with 0.5 μg/ml tet) comprising triplicate samples. Small aliquots from each sample were taken at 12 hr intervals and stained by trypan blue (Sigma) for live cells. FACS analysis was performed by sorting 20,000 cells per sample using FACSCalibur and analyzed by CellQuest software (Becton Dickinson). The caspase activity assay was performed as described (Gurtu et al., 1997).

Transfection and Infection

Mammalian cells were transfected using either Lipofectamine 2000 (Invitrogen) or FuGene 6 (Roche). To infect cells by retrovirus, 8 μg/ml of polybrene (Sigma) was added together with the retrovirus and was replaced by fresh medium after 12 hr incubation.

Generation of a Human *yy1* Inducible Cell Line

A tTA-expressing cell line derived from U2OS cells (Englert et al., 1995) was transfected with linearized *tet-off* plasmid pUHD10-3/*yy1* containing a puromycin cassette. Medium with 1.0 μg/ml of puromycin and 1.0 μg/ml of tet was used in the selection, and YY1 expression upon tet withdrawal was analyzed by Western blotting.

In Vitro Ubiquitination Assay

The assay was performed essentially as described (Grossman et al., 2003). Purified HA-tagged p53 and FLAG-tagged Hdm2 were incubated together for 30 min on ice before wt or mutant His-YY1 was added, which was then followed by 1 hr incubation on ice. Ubiquitination reactions were allowed to occur at 37°C for 30 min. The reaction mixes were analyzed by Western blot using a p53 antibody (DO-1).

Antibodies

The following antibodies were gifts from S. Tronick (Santa Cruz Biotech): YY1 (H-414 and H-10), p53 (DO-1, Pab240, and FL-393), Hdm2 (Smp-14 and N-20), p21 (F-5), c-*myc* epitope (9E10), HA epitope (F-7), GAL4 (DBD), β-gal (H-80), His epitope (H-3), and GFP. Antibodies for β actin (MAB1501) and Ub (MAB1510), goat α mouse and goat α rabbit IgG horseradish peroxidase (HRP) conjugated secondary antibodies, were purchased from Chemicon International, Inc.

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