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Concerted Activation of the Mdm2 Promoter by p72 RNA Helicase and the Coactivators p300 and P/CAF

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Abstract A scarcely studied and under-recognized feature of RNA helicases is their ability to regulate gene transcription. In particular, very little is known about the role of p72 RNA helicase in gene regulation. Here, we have analyzed how this helicase may enhance promoter activity. We demonstrate that p72 RNA helicase forms complexes with the homologous coactivators p300 and CBP in vitro and in vivo, especially leading to an enhancement of the transactivation potential of their C-termini. In addition, we show that the p300/CBP-associated protein (P/CAF) also interacts with p72 RNA helicase, and both this interaction and the binding to p300/CBP are mediated by the N-terminal 63 amino acids of p72 RNA helicase. p300, P/CAF and p72 RNA helicase synergize to stimulate selected promoters, including the Mdm2 one. Notably, downregulation of p72 RNA helicase leads to reduced Mdm2 transcription. Furthermore, our data suggest that p72 RNA helicase activates the Mdm2 promoter in a p53 dependent and independent manner. Collectively, our results have unraveled a mechanism of how p72 RNA helicase can regulate gene transcription, namely by cooperating with p300/CBP and P/CAF. Thereby, p72 RNA helicase may not only be involved in the p53-Mdm2 regulatory loop, but also profoundly impact on the transcriptome through various CBP/p300 and P/CAF interacting proteins. J. Cell. Biochem. 101: 1252–1265, 2007.

Key words: CBP; Mdm2; p300; p72 RNA helicase; P/CAF; transcription

RNA helicases constitute a large superfamily of conserved proteins that perform many essential functions. They participate in virtually all biological processes involving RNAs, including RNA splicing, editing, nuclear export, translation, nonsense-mediated decay, turnover, ribosome biogenesis and RNA interference. Mechanistically, RNA helicases can act by unwinding duplex RNA, disrupting RNA:protein interactions or assisting in the correct folding of RNA [Tanner and Linder, 2001; Silverman et al., 2003; Rocak and Linder, 2004]. In addition, RNA helicases are also involved in gene transcription, possibly by stabilizing nascent transcripts or releasing completed transcripts from the template [Eisen and Lucchesi, 1998]. Alternatively, RNA helicases can function as transcriptional cofactors [Nakajima et al., 1997; Endoh et al., 1999; Aratani et al., 2001; Westermarck et al., 2002; Rajendran et al., 2003; Yan et al., 2003; Bates et al., 2005].

p72 belongs to the DEAD box family of RNA helicases characterized by a conserved Walker B motif containing the sequence Asp-Glu-Ala-Asp (D-E-A-D) that is involved in ATP hydrolysis [Caruthers and McKay, 2002]. Several enzymatic activities have been ascribed to p72. (i) It hydrolyzes ATP, and this ATPase activity is stimulated in the presence of RNA [Lamm et al., 1996]. (ii) It is an RNA helicase that unwinds small regions of dsRNA [Rössler et al., 2001; Lee, 2002; Uhlmann-Schiffler et al., 2002]. (iii) It has an RNA annealing activity, which together with the RNA helicase activity rearranges secondary RNA structures [Rössler et al., 2001]. One or more of these enzymatic activities are required for p72 RNA helicase to regulate alternative splicing [Hönig et al., 2002].

Apart from its role in RNA metabolism, p72 RNA helicase acts in the regulation of gene transcription. Specifically, it binds to estrogen receptor-α and thereby stimulates its

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transactivation function [Watanabe et al., 2001]. Furthermore, p72 interacts with AIB1, a coactivator overexpressed in the majority of all breast tumors [Anzick et al., 1997], and with SRA, an RNA with coactivating properties [Lanz et al., 1999]. It is thought that p72 RNA helicase, SRA and AIB1 synergistically stimulate estrogen receptor-α dependent transcription [Watanabe et al., 2001]. Similarly, p72 RNA helicase has recently been shown to interact with the tumor suppressor p53 and thereby augment, albeit reportedly very inefficiently, its transactivation function [Bates et al., 2005]. However, p72 may also interact with a histone deacetylase and thereby promoter-specifically repress gene transcription [Wilson et al., 2004]. Collectively, these results suggest that p72 RNA helicase may be a transcriptional cofactor.

Here, we have analyzed if p72 RNA helicase regulates gene transcription by interacting with CBP and p300 that are homologous coactivators and acetyltransferases capable of modulating chromatin structure as well as the function of a variety of different transcription factors [Janknecht and Hunter, 1996a,b; Goodman and Smolik, 2000; Janknecht, 2002]. In addition, we have studied whether p72 RNA helicase may cooperate with another coactivator and histone acetyltransferase, p300/CBP-associated protein (P/CAF) [Yang et al., 1996].

MATERIALS AND METHODS

Antibody Production

Antibodies were raised in rabbits against an epitope peptide (CGQTAYQYPPPPPPPPPSRK) that contains amino acids 632–650 of human p72 RNA helicase and an N-terminal cysteine utilized for coupling to keyhole limpet hemocyanin. Antiserum obtained was affinity purified on AffiGel 10 (Bio-Rad) to which the epitope peptide had been coupled. After elution with 4 M $MgCl_2$, affinity purified antibodies were dialyzed against 10 mM Hepes pH 7.4, 100 mM NaCl, 50% glycerol and stored at −20°C.

Immunostaining

Cells were grown on coverslips in 12-wells [Knebel et al., 2006]. Where indicated, they were transiently transfected with 1 μg of expression vector plus 1.2 μg of carrier DNA (pBluescript KS^+, Stratagene) by the calcium phosphate coprecipitation method [Bosc et al., 2001]. Cells were stained as described before [Papoutsopoulou and Janknecht, 2000; De Haro and Janknecht, 2002] utilizing a 1:50 dilution of anti-p72 antibody and a 1:200 dilution of goat anti-rabbit antibodies coupled to rhodamine or fluorescein isothiocyanate.

Coimmunoprecipitations

For immunoprecipitation of endogenous p72 RNA helicase, MDA-MB-468 or SW620 cells were grown in 6 cm dishes and lysed at 4°C in 650 μl of 3.3 mM Tris, 10 mM $Na_4P_2O_7$, 16.6 mM NaCl, 16.6 mM NaF, 0.33% Triton X-100 (pH 7.1) supplemented with 0.2 mM dithiothreitol (DTT), 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 mM Na_3VO_4. The cell lysate was vortexed for 10 s, tumbled for 45 min and debris was removed by centrifugation (8 min, 20,800*g*). The supernatant was precleared with 15 μl of protein A-agarose beads (Repligen) for 1 h with continuous tumbling. After removal of the protein A-agarose beads by centrifugation (5 min, 20,800*g*), 8 μl of affinity purified antibodies were added and 2 h later 15 μl of protein A-agarose beads. After another hour of tumbling, beads were recovered by centrifugation (1 min, 960*g*) and then washed four times in lysis buffer. Finally, the beads were resuspended in Laemmli sample-buffer, boiled and denatured proteins subjected to SDS–polyacrylamide gel electrophoresis. Proteins were detected on Western blots with indicated antibodies employing enhanced chemiluminescence [Janknecht et al., 1998].

In case of ectopically expressed proteins, 293 T cells were grown in 6 cm dishes and transiently transfected with 3 μg of p300-HA expression plasmid, 2 μg of Flag-P/CAF expression plasmid and 0.5 μg of 6Myc-p72 or empty vector $pCS3^+$-6Myc as indicated. Total DNA amount was made up with pBluescript KS^+ to 9 μg. Coimmunoprecipitations were performed 36 h after transfection as described above, but the lysis buffer contained changed amounts of Tris (5 mM), $Na_4P_2O_7$ (15 mM), NaCl (25 mM), NaF (25 mM) and Triton X-100 (0.5%). Mouse monoclonal antibodies anti-HA (12CA5), anti-Myc (9E10) or anti-Flag (M2) were employed to detect the respectively tagged proteins after Western blotting [Janknecht, 2001].

Protein Extracts

293 T cells grown in 6 cm dishes were transiently transfected with 3 μg p300-HA,

9 μg CBP-HA or 4.5 μg 6Myc-p72 [Janknecht and Hunter, 1997]. Cells were washed with phosphate-buffered saline and then incubated for 3 min in 0.6 ml of 40 mM Hepes pH 7.4, 10 mM EDTA, 150 mM NaCl before detachment from the 6 cm dish. Cells were collected by centrifugation (2 min, 960*g*) and then lysed in 100 μl of 10 mM Tris, 30 mM $Na_4P_2O_7$, 50 mM NaCl, 50 mM NaF, 1 mM DTT, 1% Triton X-100, 0.5 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A (pH 7.1). After 1 h on ice, debris was removed by centrifugation (5 min, 20,800*g*, 4°C), the supernatant frozen in liquid nitrogen and stored at −80°C.

Pull-Down Assays

Glutathione *S*-transferase (GST) fusion proteins were produced in *E. coli* and purified on glutathione-agarose beads according to standard procedures. After elution with glutathione, proteins were dialyzed against 10 mM Hepes pH 7.4, 50 mM NaCl, 1 mM DTT, 0.2 mM PMSF, 10% glycerol, dispensed into aliquots and stored at −80°C after freezing in liquid nitrogen. For the pull-down assays, ~1 μg of GST fusion protein was loaded onto 20 μl of glutathione-agarose beads (Sigma) and then incubated with 7 μl of protein extract in 700 μl of 20 mM Hepes pH 7.4, 25 mM NaCl, 0.01% Tween-20, 1 mM DTT, 0.2 mM PMSF, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A. After 2 h tumbling at 4°C, beads were collected by centrifugation (1 min, 960*g*), washed three times and then boiled in Laemmli sample buffer before applying onto SDS–polyacrylamide gels [Wu and Janknecht, 2002].

Luciferase Assays

CV-1 cells were grown in 12 wells to 30% confluency and then transiently transfected by the calcium phosphate coprecipitation method [Janknecht, 1996]. 0.2 μg reporter gene plasmid, 0.5 μg empty vector pEV3S or 0.5 μg HA-p72 or 0.5 μg HA-p72-K142N, 150 ng pEV3S or 150 ng p300-HA, and 150 ng pEV3S or 150 ng Flag-P/CAF expression vectors were utilized as indicated. The luciferase reporter plasmids TORU-luc [Monte et al., 1995], Mdm2-luc [Ries et al., 2000], MLP-luc [Dennler et al., 1998] and c-fos-luc [Goueli and Janknecht, 2004] were as reported, and the MMP7-luc reporter plasmid contains the −301 to +52 promoter region of human MMP7 cloned into pGL2-Basic (Promega). The total DNA amount was adjusted to 2.2 μg with 1.2 μg of pBluescript KS^+. In case of 293 T cells, 200 ng $GAL4_2$-tk80-luc [Janknecht et al., 1993] reporter plasmid, 100 ng control vector GAL4-linker, indicated GAL4-CBP construct [Janknecht, 2003] or GAL4-p72 expression vector, 200 ng pEV3S or 200 ng HA-p72, and 1.7 μg of pBluescript KS^+ were employed for transfecting cells. Thirty-six hours after transfection, cells were lysed and luciferase activity was determined as described [Bosc and Janknecht, 2002; De Haro and Janknecht, 2005].

ChIP Assays

Chromatin immunoprecipitation (ChIP) assays were performed essentially as described before [Goueli and Janknecht, 2003]. The following primers were utilized to amplify a 338 bp fragment of the human Mdm2 promoter utilizing iProof high-fidelity DNA polymerase (Bio-Rad): 5′-GGCAGGTTGACTCAGCTTTTCCTC-3′ and 5′-CATGTTGGTATTGCACATTTGCCTAC-3′. Similarly, the following primers were utilized to amplify a 328 bp fragment of the human MMP7 promoter: 5′-GTCCTGAATGATACCTATGAGAGC-3′ and 5′-CCAGAGACAATTGTTCTTGGACC-3′.

RT-PCR

To downregulate p72, shRNA targeting the sequence GAGACGCTGTGATGATCTG was cloned into pSIREN-RetroQ (Clontech). RKO and RKO-E6 cells were infected with retrovirus produced from this construct according to standard procedures. RNA was prepared with Trizol reagent (Invitrogen) and reverse transcribed and amplified with the iScript one-step RT-PCR kit with SYBR green (Bio-Rad). Production of a 284 bp human Mdm2, a 642 bp human MMP7 or a 226 bp human GAPDH cDNA fragment was measured in real-time with a Mini Opticon system (Bio-Rad). The following primers were utilized for amplification: Mdm2: 5′-GTATCAGGCAGGGGAGAGTGATAC-3′ and 5′-CACATGACTCTCTGGAATCATTCAC-3′; MMP7: 5′-TGTGGAGTGCCAGATGTTGCAG-3′ and 5′-CTAAATGGAGTGGAGGAACAGTGC-3′; GAPDH: 5′-GAGCCACATCGCTCAGACACC-3′ and 5′-TGACAAGCTTCCCGTTCTCAGC-3′.

RESULTS

Characterization of Anti-p72 RNA Helicase Antibodies

In order to characterize endogenous p72 RNA helicase, we generated and affinity purified polyclonal antibodies directed against a C-terminal peptide of human p72 RNA helicase. In Western blot experiments with human SW620 colon cancer cells, MDA-MB-468 breast cancer cells and 293 T transformed embryonal kidney cells, two polypeptides of 72 and 82 kDa apparent molecular weight were detected with our anti-p72 RNA helicase antibody (Fig. 1A). This is consistent with the fact that p72 RNA helicase mRNA is translated into two different isoforms due to the utilization of two different start codons [Uhlmann-Schiffler et al., 2002].

To demonstrate the specificity of our anti-p72 RNA helicase antibody, we performed peptide competition experiments (Fig. 1B). Simultaneous incubation of the anti-p72 antibody with an excess of the epitope peptide against which the antibody was raised resulted in total suppression of the detection of p72 and p82 polypeptides, whereas a control peptide was without effect. Furthermore, we assessed if the anti-p72 RNA helicase antibody might cross-react with other RNA helicases. The closest relative of p72 RNA helicase is p68 RNA helicase that displays 70% identity at the amino acid sequence level [Lamm et al., 1996]. Therefore, we tested whether p68 RNA helicase is also detected by our anti-p72 RNA helicase antibody. This is not the case since the anti-p72 RNA helicase antibody neither recognized endogenous nor ectopically expressed p68 RNA helicase (Fig. 1C). Thus, we conclude that our anti-p72 RNA helicase antibodies specifically recognize both the p72 and p82 isoforms derived from p72 RNA helicase mRNA.

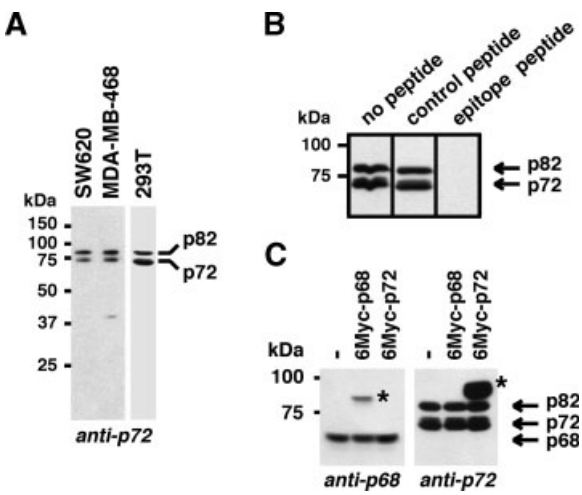

Fig. 1. Specific detection of p72 and p82 polypeptides by anti-p72 RNA helicase antibody. **A**: Anti-p72 Western blots with SW620, MDA-MB-468, and 293 T cell extracts. **B**: Peptide competition experiments. Epitope peptide, an unrelated control peptide or no peptide were utilized at the same time as anti-p72 RNA helicase antibody to challenge a Western blot of 293 T cell extracts. **C**: Absence of cross-reactivity. A Western blot of extracts from 293 T cells (untransfected or transfected with 6Myc-tagged p68 or p72) was probed with anti-p68 RNA helicase antibodies and, after stripping, with anti-p72 RNA helicase antibodies. Asterisks mark ectopically expressed proteins and arrows endogenous ones. Please note that the 6Myc-tag causes an increase in MW of ~20 kDa.

Nuclear Localization of p72 RNA Helicase

A requirement for p72 RNA helicase to act as a transcriptional cofactor is its presence in the cell nucleus. Previously, it has been shown that ectopically expressed p72 RNA helicase is localized within the cell nucleus [Lamm et al., 1996]. However, it has remained unresolved whether this nuclear localization is an overexpression artefact. Therefore, we endeavored to assess whether endogenous p72 RNA helicase is also localized within the cell nucleus. To this end, we stained three different human cell lines with our anti-p72 RNA helicase antibody. In all three cell lines, we observed a nearly complete nuclear staining pattern (Fig. 2A), best seen with human Ovcar-3 ovarian cancer cells as they have more cytoplasm compared to SW620 and MDA-MB-468 cells; also, the nucleoli showed very little p72 staining. Thus, p72 RNA helicase is a nuclear resident protein and fulfills thereby one condition for being a transcriptional cofactor.

To delineate regions within p72 RNA helicase that may be responsible for nuclear localization, we generated several truncations of this 650 amino acid long protein and replaced the start methionine with a Myc-tag that allows their detection with anti-Myc antibodies. As expected, full-length $p72_{2-650}$ was localized exclusively in the nucleus of Ovcar-3 cells (Fig. 2B). However, all of the p72 RNA helicase truncations tested were localized to both the cytoplasm and the nucleus, albeit the ratio of cytoplasmic to nuclear staining was different (Fig. 2B). Comparable results were obtained with MDA-MB-468 cells and also when replacing the Myc-tag with an HA-tag (data not shown). Altogether, these results suggest that

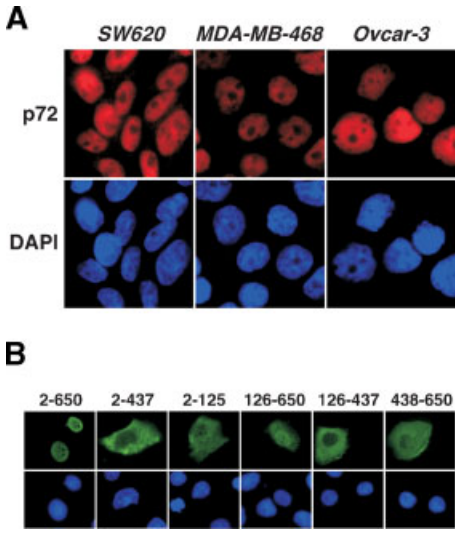

Fig. 2. Nuclear localization of p72 RNA helicase. **A**: Immunostaining of endogenous p72 RNA helicase in SW620, MDA-MB-468, and Ovcar-3 cells. The bottom panels show staining of DNA with DAPI. **B**: Indicated 6Myc-tagged p72 RNA helicase amino acids were transiently expressed in Ovcar-3 cells. The top panels show staining of transfected cells with anti-Myc antibodies, and the lower panels staining with DAPI. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

nuclear localization of p72 RNA helicase is determined by many different regions within this molecule.

Interaction of p72 RNA Helicase With p300 and CBP

Due to our longstanding interest in the coactivators p300 and CBP, we analyzed whether they might interact with p72 RNA helicase. First, we analyzed whether a GST-p72 RNA helicase fusion protein would interact with p300 and CBP. As shown in Figure 3A, both p300 and CBP interacted with GST-p72 but not with the GST moiety itself. To extend these in vitro findings, we ectopically expressed HA-tagged p300 and Myc-tagged p72 RNA helicase in 293 T cells. After anti-HA immunoprecipitation, we screened for any coimmunoprecipitated p72 RNA helicase by anti-Myc Western blotting. Indeed, p72 RNA helicase coimmunoprecipitated with p300 (Fig. 3B, top left panel). Similarly, in a reverse order immunoprecipitation experiment, p300 coimmunoprecipitated with p72 RNA helicase (Fig. 3B, top right panel).

In order to prove that p300 and p72 RNA helicase also form complexes under physiological conditions, we analyzed whether immunoprecipitation of endogenous p72 RNA helicase

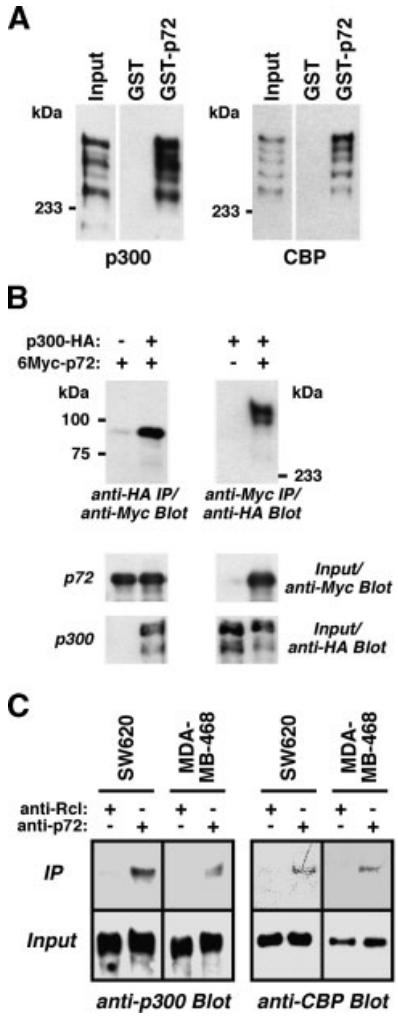

Fig. 3. Binding of p72 RNA helicase to p300 and CBP. **A**: GST pull-down assay. Extracts from 293 T cells transfected with HA-tagged p300 or HA-tagged CBP were incubated with comparable amounts of GST or GST-p72 prebound to glutathione-agarose beads. p300 or CBP retained on the beads was detected by anti-HA Western blotting. **B**: Coimmunoprecipitation of p300 and p72 RNA helicase. HA-tagged p300 and 6Myc-tagged p72 were coexpressed as indicated in 293 T cells. After anti-HA (on the left) or anti-Myc (on the right) immunoprecipitation, coprecipitated proteins were detected by anti-Myc or anti-HA Western blotting, respectively. The bottom panels show input levels of p300-HA and 6Myc-p72 RNA helicase. **C**: Coimmunoprecipitation of endogenous proteins in SW620 and MDA-MB-468 cells. After immunoprecipitation with anti-p72 RNA helicase antibody or irrelevant anti-Rcl antibody, p300 and CBP were detected by Western blotting with a 1:1,000 dilution of anti-p300 C-20 (sc-585, Santa Cruz) or with a 1:500 dilution of each anti-CBP A-22 and C-20 antibodies (sc-369 and sc-583, Santa Cruz).

would coprecipitate endogenous p300. In two different cell lines, SW620 and MDA-MB-468, we observed that immunoprecipitation with our anti-p72 RNA helicase antibody, but not with an irrelevant anti-Rcl antibody, led to the

coprecipitation of p300 (Fig. 3C, left panels). Moreover, the p300 homolog, CBP, also coimmunoprecipitated with p72 RNA helicase (Fig. 3C, right panels). We conclude that endogenous p72 RNA helicase can form complexes with endogenous p300 and CBP.

We next wished to determine which regions within p72 RNA helicase may interact with p300. p72 RNA helicase contains a central helicase domain (see Fig. 4A for a sketch of p72 RNA helicase) that is characterized by nine conserved motifs [Rocak and Linder, 2004]. The central helicase domain appears not to be required for the interaction of p72 RNA helicase with p300, since amino acids 2–125, but not 126–437 and 439–650 were bound by p300 (Fig. 4B, left panels). Since p72 amino acids 2–125 still contain the conserved Q domain of helicases, we determined if this domain is required for interaction with p300. However, only amino acids 2–63 but not 64–125 were able

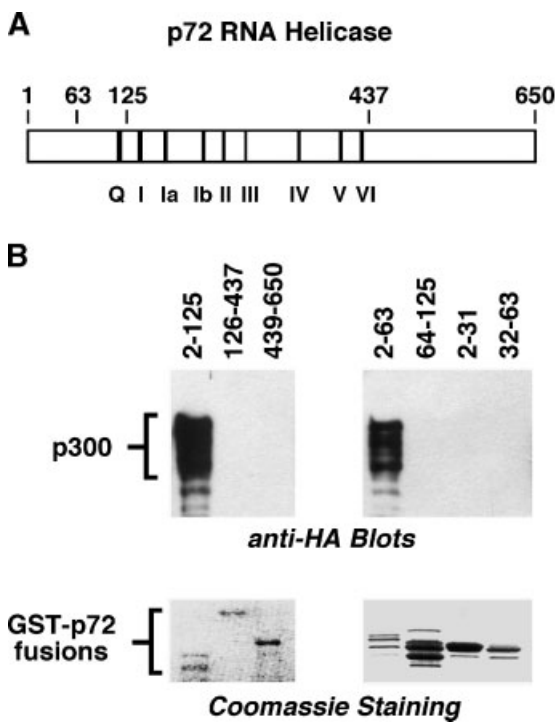

Fig. 4. p300 binds to the N-terminus of p72 RNA helicase. **A**: Sketch of human p72 RNA helicase. Conserved motifs in the central helicase domain are thought to be involved in ATP binding (Q, I, II, and probably VI) or RNA binding (Ia, Ib, IV, and V). **B**: GST pull-down assays. Indicated p72 RNA helicase amino acids were fused to GST and employed to test the binding of p300-HA. The top panels show anti-HA Western blots, the bottom panels Coomassie Blue-stained protein gels demonstrating that comparable amounts of GST fusion proteins were employed.

to interact with p300 (Fig. 4B, right panels), indicating that the Q domain is not involved in p300 binding. Further subdividing amino acids 2–63 into 2–31 and 32–63 resulted in abolition of p300 interaction. Thus, the N-terminal 63 amino acids of p72 interact with p300.

Expression of p300-HA or CBP-HA always led to the appearance of smaller degradation products in anti-HA Western blots, which represent C-terminal portions of p300 or CBP as the HA-tag resides at the C-terminus. Such smaller degradation products can also interact with GST-p72 as shown above in Figure 3A, implying that the C-termini of p300 and CBP are involved in the interaction with p72 RNA helicase. However, since we ran 5% polyacrylamide gels to focus on full-length p300 or CBP, degradation products of a size less than 200 kDa were run off the gel and were initially not analyzed. Therefore, we repeated the pull-down experiment with GST-p72 and p300-HA and ran 9% polyacrylamide gels (please note that due to the inefficient transfer of large proteins in 9% polyacrylamide gels during Western blotting, smaller p300 degradation products are highly overrepresented when compared to full-length p300). Even the smallest degradation product observable at ~60 kDa was bound by GST-p72 (Fig. 5A), suggesting that the last 500–600 amino acids of p300 interact with p72 RNA helicase.

We then performed more systematic analyses with defined domains of CBP. Consistent with the fact that the last 500–600 amino acids of p300 interact with p72 RNA helicase, CBP amino acids 1,891–2,441 did so, too, and subdividing this region into two halves revealed that specifically amino acids 1,891–2,175 but not 2,175–2,441 bind to p72 RNA helicase (Fig. 5B, right panels). However, we found additional binding to CBP amino acids 1–451 and 451–721 (Fig. 5B, left panels). Thus, p72 RNA helicase is capable of interacting with multiple regions within CBP.

Interestingly, the regions of CBP that bind to p72 RNA helicase are located within the N- or C-terminal activation domain or within the KIX domain (see Fig. 5C for a sketch of CBP), all three of which have been shown to be able to stimulate transcription [Goodman and Smolik, 2000; Janknecht, 2002]. Accordingly, when fused to the GAL4 DNA binding domain and assessed with a GAL4 binding site-driven luciferase reporter, CBP amino acids 1–451,

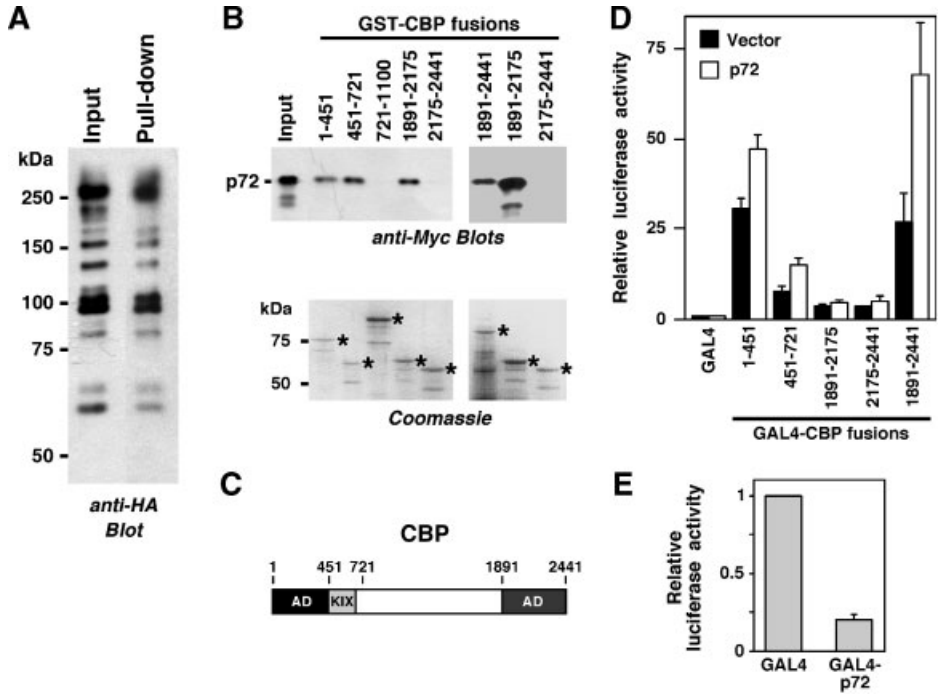

Fig. 5. p72 RNA helicase interacts with multiple domains in CBP/p300. **A**: p300-HA bound to GST-p72 was revealed by anti-HA blotting. 5% of the input is shown. **B**: GST-CBP fusion proteins were employed to pull-down 6Myc-p72 RNA helicase. The top panels show anti-Myc Western blots, the bottom panels demonstrate by Coomassie Blue staining that comparable amounts of GST-CBP proteins were employed. Please note that the left and right panels derive from different experiments. **C**: Sketch of CBP. AD, activation domain. **D**: Indicated GAL4-CBP fusion proteins were cotransfected without or with p72 RNA helicase into 293 T cells. Luciferase activities derived from a cotransfected GAL4 binding site-driven luciferase reporter are depicted. **E**: The GAL4 DNA binding domain or GAL4-p72 were transfected into 293 T cells and luciferase activity from the cotransfected GAL4 binding site-driven luciferase reporter determined.

451–721 and 1,891–2,441 strongly activated transcription (Fig. 5D). When p72 RNA helicase was coexpressed, we observed no effect on the GAL4 DNA binding domain itself, but GAL4-$CBP_{1,891-2,441}$ was stimulated 2.5-fold by p72 RNA helicase, and the ability of GAL4-$CBP_{451-721}$ and GAL4-CBP_{1-451} to activate transcription was enhanced by ~100% or ~50%, respectively. Although p72 RNA helicase binds to CBP amino acids 1,891–2,175 at least as avidly as to CBP amino acids 1,891–2,441 (see Fig. 5B, right panels), it does not significantly activate the respective GAL4-$CBP_{1,891-2,175}$ fusion protein, indicating that binding of p72 RNA helicase per se does not lead to transcriptional activation. Moreover, a GAL4-p72 fusion protein that directly tethers p72 to DNA repressed transcription (Fig. 5E), possibly through the reported ability of p72 to recruit histone deacetylases [Wilson et al., 2004]. Altogether, these data indicate that especially the physical interaction of p72 RNA helicase with the C-terminus of CBP/p300 is functionally relevant.

Interaction of P/CAF and p72 RNA Helicase

A coactivator that can cooperate with p300 and CBP is P/CAF [Yang et al., 1996]. Therefore, we tested whether p72 RNA helicase and P/CAF might also form complexes in vivo. When jointly overexpressing Myc-tagged p72 RNA helicase and Flag-tagged P/CAF in 293 T cells, we observed that P/CAF coimmunoprecipitated with p72 RNA helicase (Fig. 6A). Similarly, endogenous P/CAF specifically coimmunoprecipitated with endogenous p72 RNA helicase in SW620 and MDA-MB-468 cells (Fig. 6B).

In addition, P/CAF bound to GST-p72 in vitro (Fig. 6C), but not to the GST moiety itself. Furthermore, we established that the same N-terminal 63 amino acids of p72 RNA helicase that are required for the interaction with p300 are also needed for the in vitro interaction with P/CAF (Fig. 6D). In conclusion, p72 RNA helicase can interact both in vitro and in vivo with P/CAF.

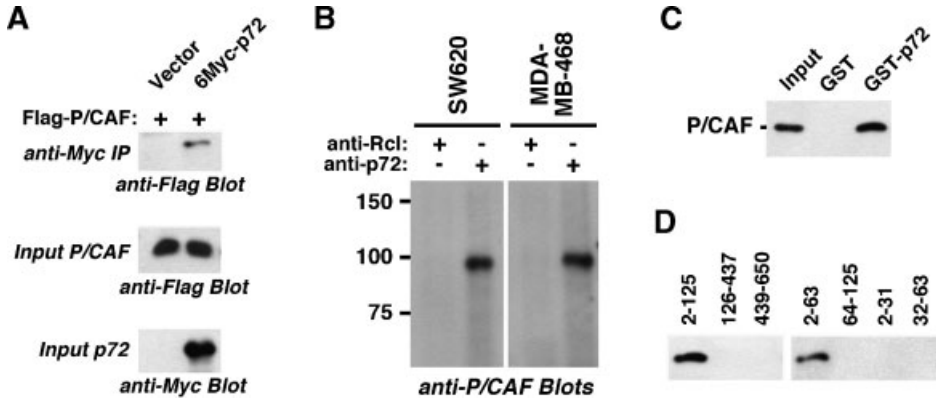

Fig. 6. Binding of P/CAF to p72 RNA helicase. **A**: Coimmunoprecipitation of P/CAF and p72 RNA helicase. Flag-tagged P/CAF and 6Myc-tagged p72 were coexpressed as indicated in 293 T cells. After anti-Myc immunoprecipitation, coprecipitated P/CAF was detected by anti-Flag Western blotting. The bottom panels show input levels of Flag-P/CAF and 6Myc-p72 RNA helicase. **B**: Immunoprecipitation of endogenous p72 RNA helicase leads to the coprecipitation of endogenous P/CAF. **C**: GST pull-down experiments with Flag-tagged P/CAF. P/CAF bound to GST-p72 was detected by anti-Flag Western blotting. **D**: Analogous, pull-down experiments with truncations of p72 RNA helicase.

Functional Cooperation Between p72 RNA Helicase, p300 and P/CAF

Next, we assessed whether the physical interaction between p72 RNA helicase, p300 and P/CAF is reflected by a functional collaboration. To this end, we employed the TORU-luc reporter plasmid that can be stimulated by p300 and P/CAF in conjunction with other transcription factors [Gutman and Wasylyk, 1990; Goel and Janknecht, 2003]. Alone, p300 and/or P/CAF were basically unable to stimulate the TORU-luc reporter, whereas p72 RNA helicase on its own activated luciferase activity by 3.5-fold (Fig. 7A). However, p300 or P/CAF augmented p72 RNA helicase dependent transcription 1.7- or 2-fold, and most significantly, p300 and P/CAF together had a synergistic effect (5.8-fold stimulation of p72 RNA helicase). These effects were not due to alterations in p72 RNA helicase protein levels upon p300 and P/CAF overexpression, since p72 RNA helicase protein levels were comparable in the absence or presence of p300 and P/CAF (Fig. 7B). Thus, p300 and P/CAF synergize in activating the ability of p72 RNA helicase to stimulate transcription.

We then asked the question if p72 RNA helicase indiscriminately activates gene transcription by testing various luciferase reporter genes (Fig. 7C). We observed a promoter specific behavior: both the MMP7 (encoding the matrix metalloproteinase 7 promoter) as well as the MLP (encoding the TATA-box and initiator sequence of adenovirus major late promoter) luciferase reporter plasmids were unaffected by p72 RNA helicase. On the other hand, the Mdm2 and c-fos promoter were activated by p72 RNA helicase, and this activation was synergistically stimulated by p300 and P/CAF. Altogether, these results indicate that p72 RNA helicase, p300 and P/CAF promoter-specifically activate gene transcription.

To enquire whether enzymatic activity is required for p72 RNA helicase to stimulate gene transcription, we also assessed its K142N mutant; mutation of homologous lysine residues in other RNA helicase has been shown to disable ATP binding and therefore helicase activity [Pause and Sonenberg, 1992; Rossow and Janknecht, 2003]. The K142N mutant was expressed at levels comparable to wild-type p72 RNA helicase (Fig. 7B) and displayed an interesting behavior on the TORU-luc promoter: on its own, the K142N mutant activated transcription to the same extent as wild-type p72 RNA helicase but was less active in the joint presence of p300 and P/CAF (Fig. 7A). In contrast, there was no difference between wild-type and K142N p72 RNA helicase on the Mdm2 and c-fos promoters (Fig. 7C). These data show that the helicase activity of p72 is required at some, but not all promoters for efficient synergism with p300 and P/CAF.

Mdm2 Activation by p72 RNA Helicase

Finally, we assessed whether p72 RNA helicase can affect endogenous gene transcription.

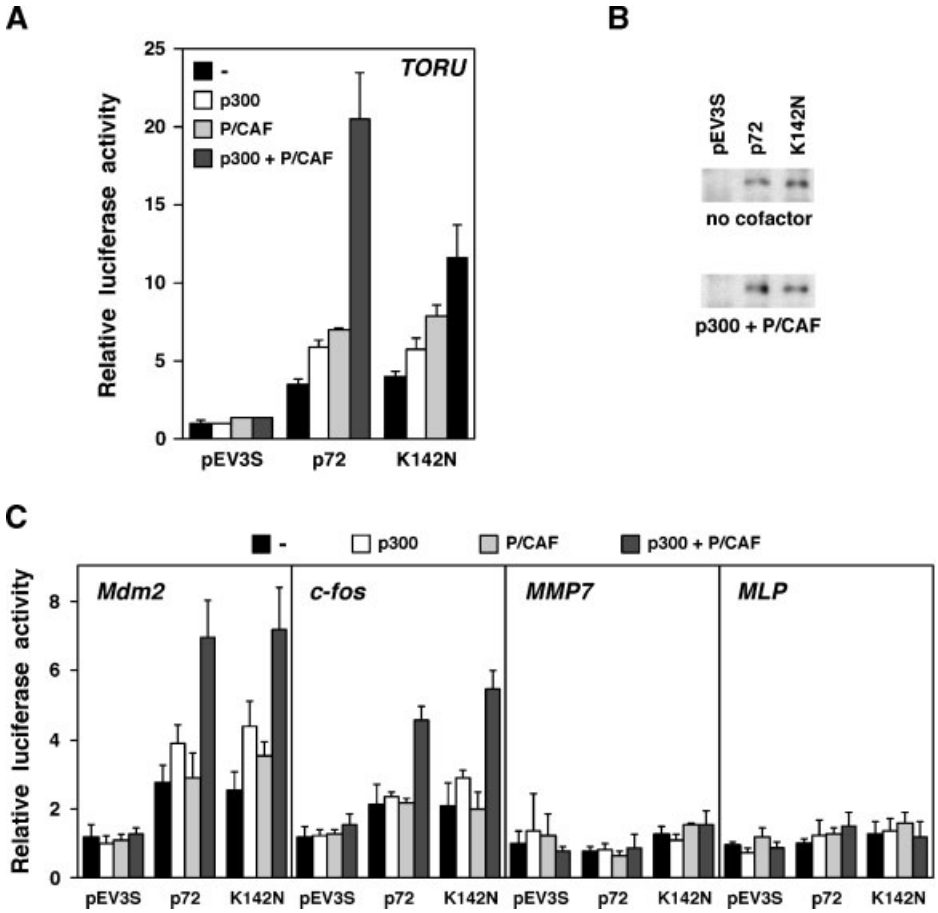

Fig. 7. Luciferase reporter gene assays in CV-1 cells. **A**: Cells were transfected with the TORU-luc reporter and 500 ng of either empty vector pEV3S, expression vector for HA-tagged p72 RNA helicase or its helicase-deficient mutant, K142N. As indicated, expression vectors for p300 and/or P/CAF were cotransfected. **B**: anti-HA Western blot after anti-HA immunoprecipitation of CV-1 cells transfected with indicated HA-tagged proteins. **C**: Activation of Mdm2-, c-fos-, MMP7-, and MLP-luc reporter genes.

We elected to study the Mdm2 gene, since (i) its promoter was stimulated by p72 (see Fig. 7C), (ii) the Mdm2 promoter is upregulated by the tumor suppressor p53 [Bond et al., 2005], (iii) p53 binds to and is activated by p300/CBP and P/CAF [Scolnick et al., 1997; Grossman, 2001], and (iv) p72 interacts with p53 [Bates et al., 2005]. Thus, it is conceivable that p53, p72, p300/CBP, and P/CAF form a multi-protein complex that controls Mdm2 transcription.

To support this conjecture, we first asked the question whether p72 RNA helicase localizes to the Mdm2 P2 promoter that is bound by p53. To this end, we performed ChIP assays utilizing human RKO cells that carry wild-type p53. We split RKO cell extract after formaldehyde cross-linking and sonication into three identical samples, ascertaining that equivalently treated chromatin is present in all samples. Then, we utilized no antibody, a control anti-Rcl antibody or anti-p72 RNA helicase antibodies to pull down chromatin. As shown in Figure 8A (top panel), only the anti-p72 antibodies were capable of immunoprecipitating the P2 promoter of the Mdm2 gene. Thus, p72 RNA helicase does bind to the human Mdm2 promoter in vivo at the same location as p53.

Next, we assessed how downregulation of endogenous p72 RNA helicase affects Mdm2 transcription. To this end, we employed p72 shRNA in RKO cells. Efficient downregulation of endogenous p72 and its p82 isoform was achieved with our p72 shRNA, while actin or p68 RNA helicase or p53 levels were unaffected (Fig. 8B). Then, we studied by real-time PCR Mdm2 mRNA and normalized this to GAPDH

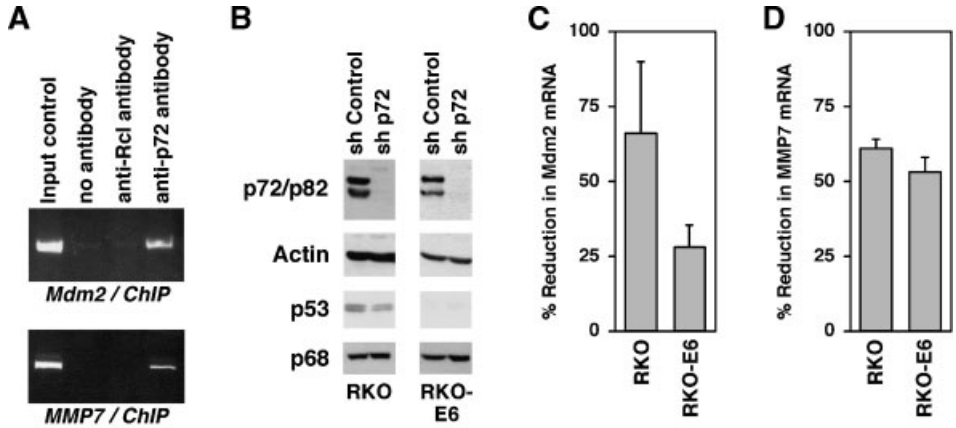

Fig. 8. Impact of p72 RNA helicase on the human Mdm2 and MMP7 promoters. **A**: ChIP assay in RKO cells. Indicated antibodies were utilized to immunoprecipitate chromatin. Shown are agarose gels after PCR amplification of the Mdm2 P2 or MMP7 promoter. **B**: Western blots showing the downregulation of p72/p82 by control or p72 shRNA in RKO and RKO-E6 cells. **C**: Reduction of Mdm2 mRNA in RKO and RKO-E6 cells upon p72 shRNA expression compared to control shRNA. Real-time PCR was employed to measure Mdm2 mRNA levels that were normalized to GAPDH levels. **D**: Analogous for MMP7 mRNA.

mRNA levels. Downregulation of p72 RNA helicase led to a strong reduction in Mdm2 mRNA (Fig. 8C), demonstrating that p72 RNA helicase is indeed contributing to Mdm2 gene transcription.

Further, we employed a cell line, that is, isogenic to RKO cells, namely RKO-E6. The only difference between RKO and RKO-E6 cells is that the latter express the human papilloma virus E6. This viral protein leads to the degradation of p53 [Mantovani and Banks, 2001] and therefore RKO-E6 cells are essentially devoid of p53 (see Fig. 8B). If p72 RNA helicase solely acts via p53 in the upregulation of Mdm2 transcription, we would expect that p72 RNA helicase downregulation leads to no reduction of Mdm2 transcription in RKO-E6 cells. However, if p72 RNA helicase cooperates with other transcription factors, Mdm2 transcription should be reduced upon p72 downregulation. We observed that p72 shRNA, which efficiently downregulated p72/p82 in RKO-E6 cells (Fig. 8B), still led to a reduction in Mdm2 mRNA (Fig. 8C). This indicates that p72 RNA helicase regulates the Mdm2 promoter by a to-be-identified transcription factor(s) other than p53. However, since the reduction of Mdm2 transcription upon p72 downregulation was significantly less in RKO-E6 compared to RKO cells (Fig. 8C), p72 appears to also regulate the Mdm2 promoter in a p53 dependent manner.

Finally, we also assessed whether p72 might interact with the MMP7 promoter. A respective ChIP assay indicated that p72 can bind to the MMP7 promoter (Fig. 8A, lower panel) and downregulation of p72 led to a reduction of MMP7 mRNA in RKO cells (Fig. 8D). This was surprising, since our luciferase reporter gene assays in CV-1 cells (see Fig. 7C) suggested that p72 is not involved in MMP7 promoter regulation. This discrepancy might be explained by the different cell types utilized (RKO vs. CV-1); by the fact that the MMP7 promoter is maximally induced in CV-1 cells so that ectopic p72 expression is without effect; by the fact that the MMP7 luciferase reporter construct may not contain all relevant MMP7 promoter elements; or by the fact that the episomal luciferase reporter construct does not have the necessary chromatin organization as the endogenous MMP7 gene. Nevertheless, in contrast to Mdm2 transcription, transcription of the MMP7 gene that is not regarded as a p53 target gene was not differentially affected by p72 downregulation in RKO and RKO-E6 cells (Fig. 8D), further lending support to our hypothesis that Mdm2 transcription is regulated by p72 in both a p53 dependent and independent manner.

DISCUSSION

In this report, we have shown that p72 RNA helicase interacts with the coactivators p300 and CBP. In addition, we demonstrate for the first time that the coactivator P/CAF can bind to

an RNA helicase. Significantly, p300 and P/CAF are capable of synergizing with p72 RNA helicase to stimulate transcription.

The interaction of p72 RNA helicase with p300/CBP and P/CAF is mediated by its N-terminal 63 amino acids. These amino acids lie outside the enzymatic core of p72 RNA helicase and are not conserved amongst RNA helicases. Thus, CBP/p300 and P/CAF are not expected to indiscriminately interact with all RNA helicases via their conserved enzymatic cores. Hitherto only two other helicases, p68 RNA helicase and RNA helicase A, have been shown to bind to CBP/p300 [Nakajima et al., 1997; Endoh et al., 1999; Rossow and Janknecht, 2003]. Similar to p72 RNA helicase, RNA helicase A utilizes its non-conserved N-terminus to bind to CBP/p300, whereas p68 RNA helicase employs part of its helicase domain encompassing the conserved motifs Ib-IV to do so. Thus, although p68 and p72 RNA helicases share extensive homology [Lamm et al., 1996], they interact through different domains with CBP/p300.

Conversely, CBP interacts through three different regions with p72 RNA helicase. This is not an unusual behavior of CBP and has been observed before with other transcription factors including p53, MyoD, Elk-1, or YY1 [Goodman and Smolik, 2000; Janknecht, 2002]. However, only the interaction of p72 RNA helicase with the C-terminus of CBP appears to have a larger functional effect. Other proteins have been shown to bind to the same C-terminal region of CBP as p72 RNA helicase, including R-Smads and YY1 [Goodman and Smolik, 2000; Janknecht, 2002]. Thus, p72 RNA helicase may interfere with these proteins' function by preventing them from binding to CBP/p300, which may be one possible explanation for why p72 RNA helicase can promoter-specifically repress transcription [Wilson et al., 2004]. However, it remains to be studied whether binding of p72 RNA helicase, R-Smads or YY1 to CBP/p300 is indeed mutually exclusive.

Whereas the enzymatic activity of RNA helicases is required for most, if not all, of their functions in RNA metabolism, their enzymatic activity may be dispensable for their role as coactivators. For instance, the ability of p68 RNA helicase to coactivate estrogen-dependent transcription does not require enzymatic activity [Endoh et al., 1999]. However, activation of the TORU reporter gene by p68 RNA helicase has been shown to be absolutely dependent on its helicase activity [Rossow and Janknecht, 2003], suggesting a promoter specific requirement for helicase activity. In contrast to p68, our data indicate that p72 on its own does not require its helicase activity to stimulate the TORU reporter gene. However, helicase activity was necessary for synergizing with p300 and P/CAF on the TORU promoter. Yet this is not true for all promoters, since the Mdm2 and c-fos promoters were equally responsive to wild-type p72 and the helicase deficient K142N mutant in the absence and presence of p300 and P/CAF, reiterating that helicase activity is promoter-specifically required. In conclusion, p72 RNA helicase employs helicase activity dependent and independent mechanisms to stimulate gene expression.

Our results have shown that p72 RNA helicase binds to the human Mdm2 P2 promoter in vivo and that it is required for efficient Mdm2 transcription. Notably, our results with RKO (p53 wild-type) and RKO-E6 (p53 devoid) cells indicate that p72 RNA helicase acts both p53 dependently and independently. p53 and p72 form complexes in vivo [Bates et al., 2005], and therefore p72 RNA helicase is likely to be recruited by p53 to the Mdm2 P2 promoter that contains two binding sites for the p53 tumor suppressor [Bond et al., 2005].

But how may p72 activate Mdm2 transcription p53 independently? Both the mouse and human Mdm2 promoter are not only regulated by p53 but also by AP1 and ETS transcription factors [Ries et al., 2000; Phelps et al., 2003]. AP1 and ETS binding sites are in close proximity to the p53 binding sites in the P2 promoter of the Mdm2 gene, and it is this region that was probed for p72 RNA helicase binding in our ChIP assays. Also, AP1 and ETS proteins are known to recruit p300/CBP [Goodman and Smolik, 2000; Janknecht, 2002] and therefore likely candidates that are coactivated by p72 RNA helicase. This would be consistent with our observation that the TORU promoter, which is regulated by AP1 and ETS proteins [Gutman and Wasylyk, 1990; Goel and Janknecht, 2003], and the c-fos promoter, which is regulated by ETS proteins [Janknecht and Nordheim, 1993; Janknecht et al., 1995], are stimulated by p72 RNA helicase in CV-1 cells; it would additionally be consistent with the observed decrease of MMP7 transcription upon p72 knock-down in RKO and RKO-E6 cells, since the MMP7

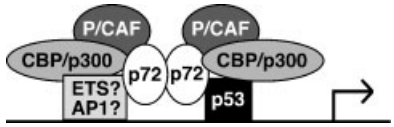

Fig. 9. Model of Mdm2 activation by p72 RNA helicase. Please note that p72 may not directly contact AP1 or ETS factors, but indirectly through CBP/p300 and/or P/CAF. Also, since p53 is a tetramer, multiple p72 dimers might be recruited at the same time to the Mdm2 promoter.

promoter is also regulated by ETS and AP1 proteins [Crawford et al., 2001].

One may even imagine that a complex consisting of p72, CBP/p300, and P/CAF is forming contacts with both p53 and AP1/ETS transcription factors on the Mdm2 promoter (see Fig. 9); this would even be possible if p53 and AP1/ETS bind to the same amino acids in p72, since p72 is capable of forming homodimers [Ogilvie et al., 2003] and thus could accommodate simultaneous binding to two proteins competing for the same interaction interface. Moreover, p72 can form heterodimers with the related p68 RNA helicase [Ogilvie et al., 2003] and thus a heteromeric p72/p68 complex (or possibly even a p68 dimer) might also be capable of activating the Mdm2 promoter.

Mdm2 is an E3 ubiquitin ligase that targets p53 for degradation. Since its expression is regulated by p53, it is part of an autoregulatory loop controlling the levels of p53, the guardian of the genome [Bond et al., 2005; Brooks and Gu, 2006]. Our results showing that p72 RNA helicase is involved in Mdm2 regulation point at an important function of p72 RNA helicase in cell proliferation and survival control. Also, one would predict that p72 RNA helicase overexpression may lead to enhanced expression of the Mdm2 oncogene, thus promoting tumorigenesis. And indeed, our unpublished data indicate that p72 RNA helicase is overexpressed in several different tumors.

In addition, p72 RNA helicase may influence the transcriptome by interacting with other transcription factors. For instance, one of the first transcription factors shown to physically and functionally interact with both p300/CBP and P/CAF is MyoD, a key regulator of muscle differentiation [Puri et al., 1997]. Thus one may speculate as to whether the p300/CBP and P/CAF interacting p72 RNA helicase is also involved in MyoD dependent physiological processes. Similarly, other transcription factors, including c-Myc, hepatocyte nuclear factor-1, fetal Krüppel-like factor 2 and Notch intracellular receptor domain have been shown to cooperate with p300/CBP and P/CAF [Soutoglou et al., 2000; Song et al., 2002; Wallberg et al., 2002; Vervoorts et al., 2003; Patel et al., 2004] and could thus also be regulated by p72 RNA helicase. In conclusion, we have unraveled p72 RNA helicase to be a novel coactivator that may profoundly affect gene transcription in conjunction with p300/CBP and P/CAF.

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