

Differential basal levels of *MDM-2* transcription induced by p53-P72 and p53-R72

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Human wild-type p53 comes in two common variants: a primordial one with a proline residue at position 72 within the regulatory proline-rich domain, and a more recent one with an arginine residue instead. The two p53s differ in their ability to regulate basal functions of the human organism. For example, p53-R72 is a better transactivator of the *LIF* gene.¹ Since LIF is crucial for the efficient implantation of the embryo into the uterus,¹ increased fertility among p53-R72 females may have been the primary force behind the rapid fixation of the *R72*-allele in the human population. On the other hand, p53-R72 individuals have a reduced life expectancy compared to p53-P72 individuals for unknown reasons not related to tumor suppression.² *MDM-2* is the major negative regulator of p53; its gene is transactivated by p53.

Resting unstressed primary cells express p53 at steady, very low basal levels. Cycling unstressed cells, in contrast, exhibit brief pulses of p53-overexpression in the G₁ and S/G₂ phases of cell cycle that are, however, non-productive with respect to transactivation of p53 target genes.³ One notable exception is the *MDM-2* gene; it is transactivated in response to unstressed p53 pulses in cycling cells.³ To study differences between the two wild-type p53 variants in unstressed primary human cells and to avoid imponderability due to the G₁- and S/G₂-pulses in asynchronously cycling cultures, we analyzed the expression of p53 target genes by RT-qPCR in resting contact-inhibited cultures of human diploid fibroblasts

(HDF). HDF32 are homozygous for *p53-P72* whereas HDF10 are homozygous for *p53-R72*; in addition, both fibroblast lines are from age-matched individuals and are heterozygous for the regulatory SNP309 T/G (rs2279744) in the *MDM-2* gene promoter 2 that affects *MDM-2* expression.⁴ In a typical experiment, both cultures were grown to confluency and were then exposed to culture medium with one-tenth growth factor content for 48 h. Flow cytometry showed that the vast majority of cells had exited the cell cycle. In addition, the HDF cultures were similar with respect to their levels of reactive oxygen species (oxygen stress; Fig. 1A). Both p53 proteins were expressed to similar low steady-state levels in both cultures (Fig. 1B). Nonetheless, the p53-R72 cultures consistently contained the *MDM-2* transcript at levels approximately two-fold higher than those in the p53-P72 cultures (Fig. 1B). This transcript consisted of spliced RNA (mRNA) as intronic primers that were productive on genomic DNA and failed to amplify sequences from the reversely transcribed mRNA pools. In contrast to *MDM-2*, *FAS* and *hTERT* were expressed at similar levels in both HDF cultures; *NOXA* and *PUMA* transcripts were undetectable. The RT-qPCR quantifications were performed by two colleagues (M.C. and D.D.) independently and at least five times. Since the overall levels of *MDM-2* transcript were low (approximately seven PCR cycles above the number of cycles for *gapdh*), we did not expect—and actually failed—to see any *MDM-2* protein in standard

western immunoblots. However, *MDM-2* protein became visible upon treatment of the cultures with the proteasome inhibitor MG132 for 6 h. In agreement with the transcript quantifications, the p53-R72 cultures consistently harbored more *MDM-2* under these conditions (Fig. 1B). Similar differential basal *MDM-2* expression was observed with our fibroblast lines HDF25 (p53-P72) and HDF22 (p53-R72) (Fig. 1C). These were, in contrast to HDF32 and HDF10, homozygous for the *MDM-2* SNP309 G allele.

Unknown genetic differences between the HDF may have uncontrollably impacted the basal regulation of *MDM-2*. To test whether the two p53s can differentially affect *MDM-2* expression in an isogenic background, we generated stably bulk-transfected cell lines in which the levels of p53-P72 or p53-R72 could be fine-tuned to equal steady-state levels by the drug doxycycline from p53-deficient human H1299 cells (Fig. 1D). In these unstressed, exponentially growing cultures p53-R72 expression again gave rise to *MDM-2* transcript levels that were approximately 2.8-fold higher than the levels in the p53-P72 cultures (Fig. 1D). *FAS* and *hTERT* were expressed to similar levels. Absence of p53 reduced the *MDM-2* transcript levels to undetectable. Additional treatment of the cultures with the DNA-damaging drug adriamycin (0.34 μM) caused a p53-dependent rise in *MDM-2* transcript to indistinguishable levels in both cultures. Combined, these results point to the possibility that p53-R72 individuals have elevated basal

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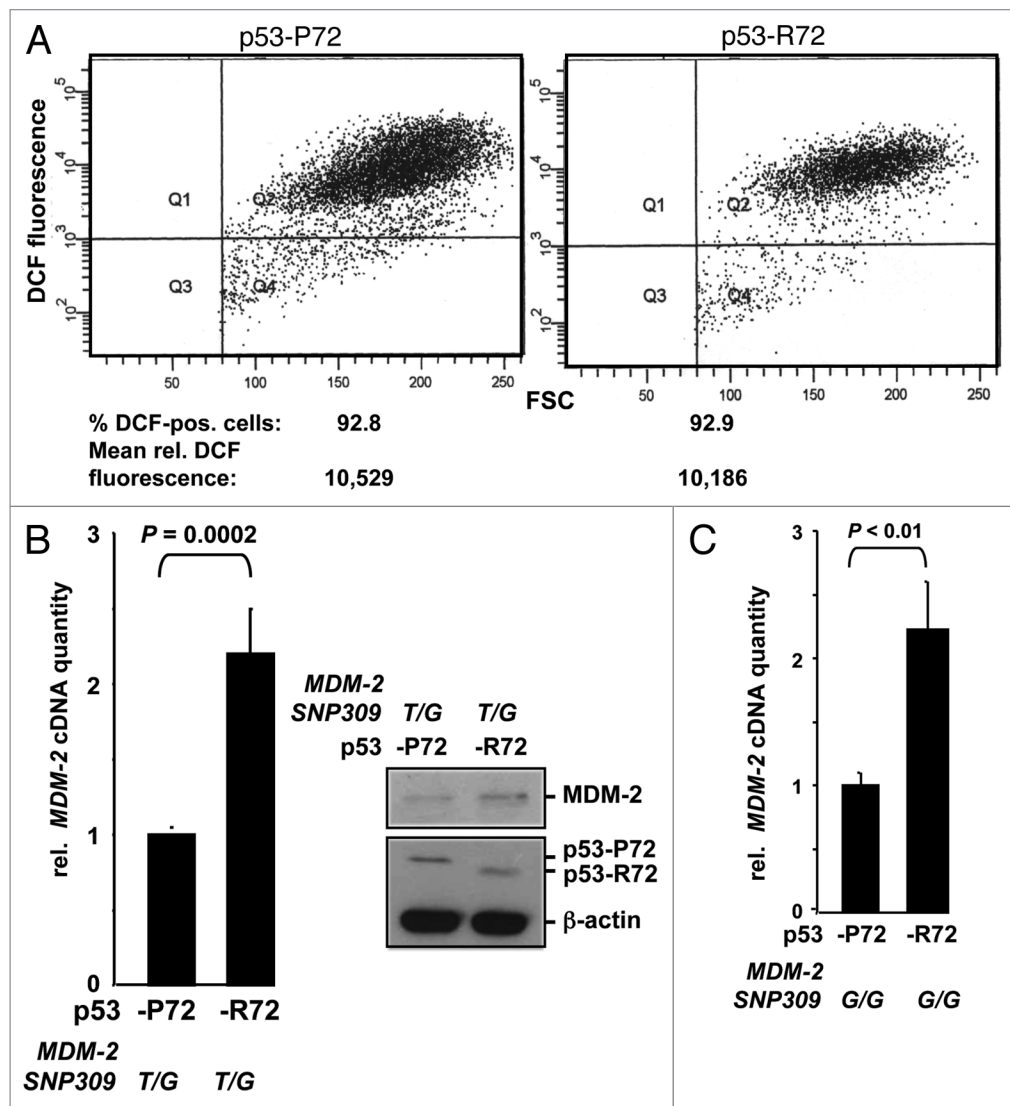


Figure 1A–C. Differential expression of *MDM-2* by p53-P72 and p53-R72. (A) Flow-cytometric detection of DCF fluorescence produced by the oxidation of DCFH-DA through cellular reactive oxygen species (peroxides) in resting, contact-inhibited HDF expressing either p53-P72 or -R72. Cells were loaded with DCFH-DA (10 μ M) for 30 min at 37°C. Cells exhibiting background fluorescence were arbitrarily set as $<10^3$ rel. Fluorescence intensity (window Q4). FSC: forward scatter. (B and C) Quantitative RT-PCR (RT-qPCR) analysis of *MDM-2* mRNA in a background of total RNA isolated from resting, contact-inhibited fibroblast lines HDF32 (p53-P72) and HDF10 (p53-R72) (left diagram) or HDF25 (p53-P72) and HDF22 (p53-R72) (right diagram), that had been determined to carry the indicated *MDM-2* SNP309 genotypes. The following primers were employed for the detection of *MDM-2* transcript; forward: 5'-ATC GAA TCC GGA TCT TGA TG-3'; reverse: 5'-TCT TGT CCT TCT TCA CTA AGG C-3'. The relative *MDM-2* mRNA levels were determined by normalization for the quantity of *gapdh* transcript (forward: 5'-TGG TAT CGT GGA AGG ACT CAT GAC-3'; reverse: 5'-AGT CCA GTG AGC TTC CCG TTC AGA-3'). Details on the SNP309 genotyping can be obtained upon request. Error bars denote standard deviations calculated from at least three quantifications. *p*-values were determined by the *t*-test (two-tailed); values ≤ 0.05 were considered significant. For western immunoblot analysis, 15 μ g of total cellular protein were run on a standard 8% SDS polyacrylamide gel. Anti-*MDM-2* monoclonal Ab-1 (Calbiochem) was used at 1:500 dilution; anti-p53 monoclonal DO-1 (Calbiochem) at 1:2,000; and anti- β -actin monoclonal (Sigma) at 1:5,000.

MDM-2 levels in at least some tissues compared to p53-P72 individuals with the same *MDM-2* SNP309 genotype. Chromatin immunoprecipitation failed to detect differences in binding of the two p53s to the *MDM-2* promoter in H1299 cells (Fig. 1E).

Consistent with our findings, Jeong and colleagues have recently reported that

MDM-2 is transcribed in human Saos-2 cells to 1.5-fold higher levels when they express p53-R72 compared to p53-P72 for 6 h.⁵ p53-R72 is a better inducer of apoptosis than p53-P72.⁶ An increased basal level of *MDM-2* in p53-R72 cells may help rapidly enhance mono-ubiquitination of p53-R72 and thereby support p53's direct mitochondrial cell

death program in case of fatal damage.⁷ Finally, *MDM-2* possesses functions that are not dependent of p53. For instance, *MDM-2* can regulate the internalization of the β 2-adrenergic receptor (*ADRB2*) via β -arrestin ubiquitination.⁸ *ADRB2* polymorphisms have been correlated with myocardial infarction and cancer and with overall/healthy longevity in

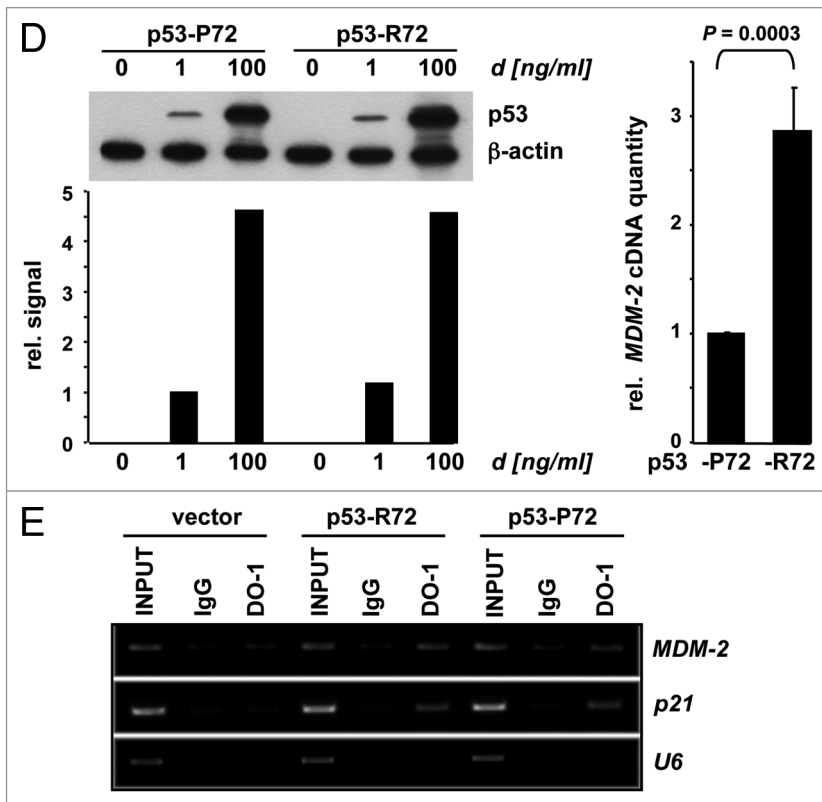


Figure 1D and E. Differential expression of *MDM-2* by p53-P72 and p53-R72. (D) Human H1299 cultures were stably bulk-transfected with the episomal plasmid pRTS-1 carrying either the *p53-P72* or *-R72* gene. Transfected cells were selected and maintained in 400 μg/ml hygromycin. The western blot, performed as described above, shows the steady-state p53 levels in cultures that had been induced to express the respective transgene with the indicated quantities of doxycyclin (d) for 24 h. The p53 signal intensities, normalized to the β-actin signals, were determined by densitometry; they document equal p53 expression levels. The RT-qPCR analysis of *MDM-2* mRNA was performed as described above. (E) Chromatin immunoprecipitation on the H1299 cultures with vector only or with inducible p53, shown in (D), performed as suggested by the Upstate ChIP protocol, with modifications as reported by us earlier (Heyne et al. Nucl Acids Res 2010; 38:3159–71). ChIP primers for *MDM-2* promoter were (forward: 5'-GGT TGA CTC AGC TTT TCC TCT TG-3'; reverse: 5'-GGA AAA TGC ATG GTT TAA ATA GCC-3'); for *p21* (forward: 5'-ACC TTT CAA CCA TTC CCC TAC-3'; reverse: 5'-GCC CAA GGA CAA AAT AGC CA-3'); and for the *U6* control (forward: 5'-GGC CTA TTT CCC ATG ATT CC-3'). Antibody DO-1 precipitates p53; IgG is an irrelevant control antibody.

the Framingham Heart Study Offspring cohort.⁹ MDM-2 may thus have a direct, not p53-mediated effect on longevity in humans.

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