Regulation of Expression of the Rat Orthologue Of Mouse Double Minute 2 (Mdm2) By H_2O_2-Induced Oxidative Stress in Neonatal Rat Cardiac Myocytes*

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The Mdm2 ubiquitin ligase is an important regulator of p53 abundance and p53-dependent apoptosis. *Mdm2* expression is frequently regulated by a p53-*Mdm2* autoregulatory loop whereby p53 stimulates *Mdm2* expression and hence its own degradation. Though extensively studied in cell lines, relatively little is known about *Mdm2* expression in heart where oxidative stress (exacerbated during ischemia-reperfusion) is an important pro-apoptotic stimulus. We demonstrate that *Mdm2* transcript and protein expression is induced by oxidative stress (0.2 mM H_2O_2) in neonatal rat cardiac myocytes. In other cells, constitutive *Mdm2* expression is regulated by the P1 promoter (5' to exon 1), with inducible expression regulated by the P2 promoter (in intron 1). In myocytes, H_2O_2 increased *Mdm2* expression from the P2 promoter which contains two p53 response elements (REs), one AP-1 RE and two Ets REs. H_2O_2 did not detectably increase expression of *p53* mRNA or protein but did increase expression of several AP-1 transcription factors. H_2O_2 increased binding of AP-1 proteins (c-Jun, JunB, JunD, c-Fos, FosB, Fra-1) to an *Mdm2* AP-1 oligodeoxynucleotide probe, and chromatin immunoprecipitation assays showed it increased binding of c-Jun or JunB to the P2 AP-1 RE. Finally, antisense oligonucleotide-mediated reduction of H_2O_2-induced Mdm2 expression increased caspase 3 activation. Thus, increased *Mdm2* expression is associated with transactivation at the P2 AP-1 RE (rather than the p53 or Ets REs), and Mdm2 induction potentially represents a cardioprotective response to oxidative stress.

Exposure of cardiac myocytes to sufficiently-high levels of reactive oxygen species (ROS[2]) such as H_2O_2 leads to their death (1-3) and this probably involves a continuum from apoptosis to necrosis, depending the severity of the oxidative stress (4). In aerobic tissues such as the heart, the mitochondria probably represent a significant source of ROS and increased ROS production by these organelles during hypoxia and ischemia-reperfusion injury may be particularly important in myocardial injury (5,6). However, at lower concentrations, ROS have been reported to promote either growth of the cardiac myocyte (7) or to induce 'preconditioning' (8), either of which potentially increases the ability of the cardiac myocyte to survive cytotoxic stresses. H_2O_2-induced oxidative stress simultaneously stimulates a number of potentially pro-apoptotic and cytoprotective signaling pathways in the whole heart or cardiac myocytes (9), and the final outcome (cell death or survival) could depend on which signaling pathway(s) predominates and endures.

As shown by our microarray studies, H_2O_2 can positively and negatively regulate global gene expression in cardiac myocytes (3,10). One gene consistently upregulated by H_2O_2 in rat cardiac myocytes at toxic and non-toxic concentrations is the orthologue of transformed mouse 3T3 cell double minute 2 (*Mdm2*) (3,10), a protooncogene (11,12), to which we will refer as *Rdm2*[3]. The human orthologue will be abbreviated to *HDM2*. The Mdm2 protein binds to the pro-apoptotic p53

tumor suppressor transcription factor to inhibit its transactivating activity (13). Perhaps more importantly, Mdm2 is an E3-ubiquitin ligase which ubiquitinates p53 and other proteins (13), thus promoting their proteasomal degradation. Indeed, the stability of p53 protein appears to be of major importance in controling its abundance (13). In addition, Mdm2 may autoubiquitinate to promote its own degradation (14,15).

Regulation of *Mdm2* expression is complex and involves two alternative promoters. The P1 promoter lies 5' to exon 1 and the P2 promoter lies within intron 1 (16). P1 primarily regulates constitutive expression of *Mdm2* with P2 contributing to a lesser extent. Inducible expression of *Mdm2* is regulated by P2 which contains two p53 response elements and, through these, p53 itself regulates stress-induced expression of *Mdm2* as part of a negative feedback loop (16). Transcripts from the P1 promoter may be translated more slowly than those from P2 because of the presence of short (but differing) upstream open reading frames (two in each) in exon 1 of both *HDM2* and *Mdm2* transcripts (17,18). However, P2 contains additional response elements (AP-1, Ets) that may allow induction of *Mdm2* expression in a p53-independent manner (19-21). The P1- and the P2-regulated transcripts can each give rise to two identical Mdm2 proteins with translation from the initiation codon in exon 3 encoding an Mdm2 species that migrates at about 90 kDa on SDS-PAGE. A truncated 76 kDa species can also be expressed from a second in-frame initiation codon in exon 4 either following removal of exon 3 by alternative splicing or by internal ribosomal entry (22,23). Expression of p76-Mdm2 protein is more favored with transcription from P1 than from P2, without there being any change in absolute p90-Mdm2 protein expression (23). p76-Mdm2 cannot bind to p53 or inhibit its transactivating activity, and it may in fact inhibit the actions of p90-Mdm2 (22). Here, we characterize the *Rdm2* gene and the expression of *Rdm2* mRNA and protein in response to exposure to ROS (H_2O_2) in neonatal rat cardiac myocytes. We demonstrate that upregulation of *Rdm2* expression by ROS is largely independent of p53 but correlates instead with transcriptional activation at the AP-1 response element. Furthermore, Rdm2 is cytoprotective against ROS-induced apoptosis (caspase 3 cleavage) in cardiac myocytes.

EXPERIMENTAL PROCEDURES

Materials - H_2O_2 was standardized volumetrically before use. It was stable at 4EC for at least 6 months. Antibodies against Rdm2 [sc-965, a mouse monoclonal antibody raised to residues 154-167 of HDM2 corresponding to residues 169-173 in Rdm2 (Supplemental Material Fig. 1) in which region Rdm2 and HDM2 are identical in primary sequence], c-Jun (sc-45X, for supershifting), JunB (sc-46X), JunD (sc-74X), phospho-Elk-1(Ser-383) (sc-7979X), Fli-1 (sc-356X), c-Fos (sc-52X), FosB (sc-48X), Fra-1 (sc-605X), Fra-2 (sc-171X) and ATF2 (sc-187X) were from Santa Cruz Biotechnology Inc. Antibodies against c-Jun (9162, used for immunoblotting) and 17 kDa caspase-3 (9661) were from Cell Signaling Technology. Sarcomeric "-actin antibodies (A2172) were from Sigma Aldrich and appropriate horseradish peroxidase-linked secondary antibodies were from DAKO.

Six Santa Cruz Biotechnology Inc. anti-p53 antibodies were tested. These were 2B2.71 (sc-71819, a mouse monoclonal IgG_{2b} raised against residues 370-378 of human p53), DO-1 (sc-126, a mouse monoclonal IgG_{2a} raised against residues 11-25 of human p53), FL-393 (sc-6243, a rabbit polyclonal IgG raised against full-length human p53), Pab 240 (sc-99, a mouse monoclonal IgG_1 raised against residues 156-214 of mammalian p53), Pab 246 (sc-100, a mouse monoclonal IgG_1 raised against residues 88-93 of mouse p53), and R-19 (sc-1313, a goat polyclonal IgG raised against an epitope mapping to the C-terminus of rat p53). With the exception of DO-1 which is not explicitly specified by the supplier as being able to recognize rat p53, all other antibodies are supposed to recognize rat p53 on immunoblotting. As negative and positive controls, we used lysates from non-transfected 293T cells (sc-117752) and from 293T cells transfected with mouse *p53* (sc-125766), respectively, and extracts of daunorubicin-treated cardiac myocytes.

The p53 electrophoretic mobility shift assay (EMSA) oligodeoxynucleotide probe was from Santa Cruz Biotechnology Inc. (sc-

2579). Other probes were synthesized by Sigma Genosys (Sigma Aldrich). Phosphorothioate-protected ODNs and standard oligonucleotides were from MWG Biotech. Lipofectin was from Invitrogen. SMART RACE cDNA amplification kits were from Clontech/BD Bioscience.

Primary culture of neonatal rat cardiac myocytes - Myocytes (yield of 3.5 ! 4.5 million cells/heart) were dissociated from the ventricles of 1- to 3-day-old Sprague-Dawley rat hearts by an adaptation of the method of Iwaki *et al*. (24) as previously described (25). Unless stated otherwise, cells were plated in gelatin-coated Primaria culture dishes (BD Biosciences) at a density of 2×10^6 cells/35 mm dish or 4×10^6 cells/60 mm dish for 18 h in 15% (v/v) fetal calf serum. For most experiments, serum was withdrawn for the 24 h before experimentation and the cells were used 2 d after their isolation.

Extraction of RNA, cDNA synthesis and semiquantitative or quantitative polymerase chain reaction (RT-sqPCR or RT-qPCR) - RNA [for which quantity and purity was assessed from the A_{260} and the A_{260}/A_{280} ratio (1.8-2.0 deemed acceptable)] was isolated from cardiac myocytes with RNA-Bee (GE Healthcare) according to the manufacturer's instructions, cDNA synthesized with random hexamers and RT-sqPCR (see Table 1 for details of primers) measurements performed as described in detail previously (3,10). To ensure that there was no amplification of genomic DNA, primers were designed to cross exon boundaries where possible (Table 1) and controls in which the RT step was omitted were carried out. The resulting RT-sqPCR products were analysed by ethidium bromide-agarose gel electrophoresis and the bands captured under UV illumination. Sizes of PCR products were estimated by comparison with a **N**X174 RF DNA HaeIII digest DNA ladder or a 100 bp DNA ladder (Invitrogen). Densitometric analysis was performed using ImageMaster 1D Prime, version 3.0 (GE Healthcare). Values were normalized to the housekeeping gene *Gapd*. PCR cycles used (Table 1) lay within the exponential phase of amplification (confirmed by construction of amplification curves from PCR over a range of cycle numbers). Sequences of PCR amplicons were determined using an ABI 3100 Genetic Analyser by the Advanced Biotechnology Centre, Imperial College London).

For RT-qPCR, cDNA was prepared from 1 mg RNA using High Capacity Reverse Transcription Kits (Applied Biosystems). Primers for qPCR were designed with Primer Express v3.0 (http://www.appliedbiosystems.com) using deduced *Rdm2* sequences (Supplemental Material Fig. 2, Table 1). qPCR was performed using a 7500 Real-Time PCR System (Applied Biosystems). A master-mix containing (per reaction) 12.5 :l Sybr-Green Jump Start Taq Readymix (Sigma-Aldrich) and 5 :l oligonucleotides (200 mM forward and reverse primers) was dispensed into Optical 96-well reaction-plates (Applied Biosystems), and cDNA template added (7.5 :l, 1/15 dilution in water). PCR conditions for all primer pairs were 50EC for 2 min, 95EC for 10 min, followed by 40 cycles of 95EC for 15 s and 60EC for 60 s. Following qPCR, dissociation curve analysis was performed to check for aberrant amplification products. An absolute quantification protocol was used. Values were normalized to *Gapd* expression.

5´-RACE - Poly(A)$^+$ RNA was purified from total RNA (150 :g) using oligo dT-cellulose (Sigma Aldrich). Briefly, total RNA (in 600 :l deionized water) was incubated for 5 min at 65EC followed by addition of 1 vol. of 2× binding buffer [20 mM Tris.HCl pH 7.5, 1 M NaCl, 2 mM EDTA, 0.1% (w/v) SDS]. Oligo dT-cellulose (5 mg in 100 :l binding buffer) was added followed by rotation at room temperature for 15 min. The oligo dT-cellulose was washed twice with binding buffer (1 ml) and twice with washing buffer [10 mM Tris.HCl pH 7.5, 0.2 M NaCl, 1 mM EDTA, 0.05 % (w/v) SDS]. Poly(A)$^+$ RNA was recovered in 250 :l of elution buffer [10 mM Tris.HCl pH 7.5, 1 mM EDTA, 0.05 % (w/v) SDS; two incubations of 5 min at 37EC]. The purification procedure was repeated and poly(A)$^+$ RNA was precipitated with 50 :l of 4 M NaCl and two volumes of ethanol followed by centrifugation (12,000 × g, 15 min, 4EC). After washing with 70 % (v/v) ethanol and centrifugation, poly(A)$^+$ RNA was dissolved in deionized water (8 :l). Poly(A)$^+$ RNA was reverse-transcribed using a SMART RACE cDNA Amplification Kit (Clontech/BD Bioscience). Poly(A)$^+$ RNA

(0.9 :g) was mixed with oligo-dT primer (10 mM 5´-CDS primer A, 1 :l), 10 mM SMART II A oligonucleotide (10 mM, 1 :l) in a total volume of 5 :l. This was heated at 72EC for 2 min and cooled on ice. Subsequently, 5× reaction buffer (2 :l; 250 mM Tris.HCl pH 8.3, 30 mM $MgCl_2$, 375 mM KCl), 10 mM dNTP (1 :l), 20 mM DTT (1 :l) and 1 :l PowerScript™ reverse trancriptase (1 :l) were added and mixture was incubated at 42EC for 90 min. Reverse transcriptase was inactivated at 72EC for 7 min. The 5´-RACE cDNA reaction mixture (10 :l) was diluted to 250 :l with 10 mM Tricine pH 8.5, 1 mM EDTA and stored at -80EC. Sequence corresponding to 5´-end of *Rdm2* mRNA was amplified using the SMART RACE cDNA Amplification Kit UPM (complementary for the extended 5´-end of the 5´-RACE cDNA) and a reverse primer to mRNA in exon 3 of *Rdm2* exon 3 (bases 269-302, Table 1). For each PCR reaction, 5´-RACE cDNA (2.5 :l) was combined with 10× 'Advantage' Polymerase Buffer [10 :l; 40 mM Tricine-KOH pH 9.2, 15 mM KOAc, 3.5 mM $Mg(OAc)_2$], 5'-RACE 1 primer (1 :l, 10 :M), 10× UPM (5 :l), dNTP (10 mM) and Advantage 2 Polymerase Mix (1 :l). The PCR programme was as follows: 94EC, 5 min; 5 cycles of 94EC, 30 s and 72EC, 2 min; 5 cycles of 94EC, 30 s, 70EC, 30 s and 72EC, 2 min; then 25 cycles of 94EC, 30 s, 68EC, 30 s and 72EC, 2 min, and finally 72EC, 4 min. PCR products were analysed on ethidium bromide-stained 2 % agarose gels and the bands were captured under UV illumination. Products were excised from the gel and then purified using QIAquick PCR purification columns for automated sequencing (Advanced Biotechnology Centre, Imperial College London).

Soluble and nuclear protein extraction - For immunoblotting, myocytes were washed in ice-cold PBS (3×) and scraped into 100 :l (35 mm dishes), or (in the case of 60 mm dishes) into 80 :l (antisense experiments) or 150 :l (remaining experiments) of ice-cold Buffer A [20 mM $-glycerophosphate pH 7.5, 20 mM NaF, 2 mM EDTA, 0.2 mM Na_3VO_4, 10 mM benzamidine, 5 mM DTT, 1 mM PMSF, 0.2 mM leupeptin, 2 :M microcystin LR, 10 :M E64, 1% (v/v) Triton X-100]. After centrifugation (10,000 × g, 5 min, 4EC), supernatant protein concentrations were determined using the Bradford method (26), boiled with 0.33 vol SDS electrophoresis buffer [300 mM Tris-HCl pH 6.8, 10% (w/v) SDS, 13% (w/v) glycerol, 130 mM DTT, 0.2% (w/v) bromophenol blue] and stored at !20EC.

For crude nuclear extracts, PBS-washed myocytes were scraped into 150 :l ice-cold Buffer B (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.3 mM Na_3VO_4, 0.2 mM leupeptin, 10 :M E64, 5 mM DTT, 0.3 mM PMSF, 2 :M microcystin LR). Extracts were centrifuged (10,000 g, 5 min, 4EC) and the pellets resuspended in 100 :l Buffer B containing 0.1% (v/v) Nonidet P40 (NP-40). Samples were centrifuged (10,000 g, 5 min, 4EC) and the pellets resuspended in 50 :l ice-cold Buffer C (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (v/v) glycerol, 0.3 mM Na_3VO_4, 0.2 mM leupeptin, 10 :M E64, 5 mM DTT, 0.3 mM PMSF, 2 :M microcystin LR). Suspensions were incubated on ice (1 h) with mixing every 15 min, and then centrifuged (10,000 g, 5 min, 4EC). Supernatants were retained and stored at !80EC or were boiled with SDS electrophoresis buffer.

Immunoblotting - Proteins were separated by SDS-PAGE using between 8 to 12% (w/v) resolving gels as appropriate with 4 to 6% (w/v) stacking gels, and transferred to nitrocellulose membranes as described previously (27). Gels were standardized using 'rainbow' markers (GE Healthcare). Nonspecific binding sites were blocked (60 min, room temperature) with 5% (w/v) non-fat milk powder in TBST [20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% (v/v) Tween 20]. Blots were incubated overnight with primary antibodies diluted in TBST containing 5% (w/v) bovine serum albumin. Membranes were washed in TBST (3 × 5 min, room temperature) and incubated (1 h, room temperature) with horseradish peroxidase-conjugated secondary antibodies in TBST containing 1% (w/v) non-fat milk powder. After washing in TBST (3 × 5 min, room temperature), bands were detected by the enhanced chemiluminescence method (reagents from Santa Cruz Biotechnology Inc.). In some cases the sensitivity of detection was increased by using unconjugated secondary antibodies in TBST/1% (w/v) non-

fat milk powder and, after washing with TSBT, blots were incubated with horseradish peroxidase-conjugated tertiary antibodies before detection by enhanced chemiluminescence.

Antisense ODN transfection - The 20-mer antisense phosphothioate-protected ODN, 5'-TGACATCTGCTCTCACTCGG-3', was directed against *Rdm2* mRNA exon 7 bases 602-621 (Supplemental Material Fig. 2). A mixed back-bone HDM2 antisense ODN (5´-UGACACCTGTTCTCACUCAC-3´) against the corresponding region (*HDM2* mRNA bases 660-679, accession no. NM_002392.2) has been used previously to inhibit *HDM2* expression in colon cancer cells (28). A random sequence (5´-TGGATCCGACATGTCAGA-3´) was used as a control. For transfection experiments, cells in 60 mm dishes were washed 3 times with antibiotic-free DMEM before exposure to DMEM containing 200 nM ODN and 20 :g/ml lipofectin (Invitrogen). Myocytes were incubated for 24 h at 37°C, then the medium was then replaced with lipofectin-free maintenance medium containing 200 nM ODN. Cells were pre-incubated for 1 h before exposure to 0.2 mM H_2O_2 for 6 h and extracted into 80 :l Buffer A (see above).

Electrophoretic mobility shift assays (EMSAs) - Double-stranded ODN EMSA probes (see Table 2) were prepared essentially as described previously (29). Briefly, ODNs were 5'-labelled with 15 :Ci [(32P]ATP (GE Healthcare) ,10 IU T4 polynucleotide kinase (Promega), and 1.2 :l of Promega 10 × kinase buffer. The reaction mixture was incubated (37EC, 30 min) and the reaction then terminated by addition of 1 :l EDTA (13 :M). Excess [(32P]ATP was removed by centrifugation (700 g, 3 min, room temperature) through Chroma Spin columns (pore size 10) (Clontech/BD Biosciences) in 1× TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and the EMSA probes were diluted to 0.4 ng/:l. Crude nuclear extracts (10 :g) were incubated (10 min, room temperature) with 4 :l binding buffer [50 mM Tris-HCl pH 7.5, 250 mM NaCl, 5 mM EDTA, 1 mM DTT, 25% (v/v) glycerol] and 1 :g poly dI-dC (GE Healthcare), in a total volume of 20 :l. Radiolabelled EMSA probe (1 :l, 0.4 ng) was added and the mixture incubated (20 min, room temperature). Samples were subjected to electrophoresis (120 V, 3 h) on 5% (w/v) non-denaturing polyacrylamide gels formed in 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) with 0.5× TBE as running buffer. Gels were dried onto Whatman 3MM paper and exposed to X-ray film for 24 ! 48 h at !80EC. Images were analysed semi-quantitatively by densitometry (ImageMaster 1D Prime, version 3.0, GE Healthcare). For competition experiments, crude nuclear extracts were incubated (10 min) with the required amount of unlabelled competing probe in binding buffer with poly dI-dC as in the standard assay, followed by addition of the radiolabeled wild type probe and continuation of the assay in the standard manner. For supershift assays, crude nuclear extracts (3 :g protein) were pre-incubated (1 h, 4EC) with 1!2 :g of antibody.

ChIP assays - Myocytes were exposed to H_2O_2 (0.2 mM, 2 h) and formaldehyde [final concn. 1% (w/v)] was added to medium (10 min, room temperature). Cross-linking was terminated with 0.125 M glycine (5 min). Myocytes were rinsed twice in ice-cold PBS and scraped into 200 :l PBS containing 0.2 mM leupeptin, 10 :M E64, 5 mM DTT, 1 mM PMSF and 2 :M microcystin LR. Suspensions were centrifuged (4,000 g, 10 min, 4EC) and pellets resuspended in 0.8 ml ChIP lysis buffer [50 mM Tris-HCl pH 8.0, 85 mM KCl, 0.2 mM leupeptin, 10 :M E64, 5 mM DTT, 0.3 mM PMSF, 2 :M microcystin LR, 0.5% (v/v) NP-40]. Suspensions were incubated on ice (10 min), centrifuged (4,000 g, 10 min, 4EC) and the pellets resuspended in 0.8 ml sonication buffer [50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% (v/v) SDS, 0.2 mM leupeptin, 10 :M E64, 5 mM DTT, 1 mM PMSF and 2 :M microcystin LR]. Samples were sonicated [5 × 30 sec, 0EC, amplitude 30% (Sonics Vibra-Cell™ sonicator with a 2 mm probe) followed after each sonication by 2 min recovery in ice-water] to shear DNA into fragments of 200-800 bp. Sonicated samples were centrifuged (4,000 g, 10 min, 4EC) and the supernatants were retained. Samples (20 :l) for total DNA input were taken at this stage. Sonicated extracts (750 :l) were diluted with 3.65 ml ChIP buffer [20 mM Tris.HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.01% (w/v) SDS, 1% (v/v) Triton X100] and were pre-

cleared with 30 µl 50% Protein A-Sepharose slurry containing 5 µg/ml sonicated salmon sperm DNA (rotation at 4EC for 30 min). Following centrifugation (200 g, 1 min, 4EC), anti-transcription factor antibodies (5 µg) were added followed by incubation (4EC, 16 h, with mixing by rotation). Antibody-protein-DNA complexes were recovered by incubation (1 h, 4EC, with mixing by rotation) with 50% Protein A-Sepharose slurry (80 µl containing 5 µg/ml sonicated salmon sperm DNA). Protein A-Sepharose-antibody-transcription factor complexes were recovered by centrifugation (200 g, 1 min, 4EC) and pellets were washed in low salt buffer [20 mM Tris.HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.1% (w/v) SDS, 1% (v/v) Triton X100, 1 ml], high salt buffer [20 mM Tris.HCl pH 8.0, 500 mM NaCl, 2 mM EDTA, 0.1% (w/v) SDS, 1% (v/v) Triton X100, 1 ml], LiCl buffer [10 mM Tris.HCl pH 8.0, 250 mM LiCl, 1 mM EDTA, 1% (w/v) deoxycholic acid, 1% (v/v) NP-40] and then twice in 10 mM Tris.HCl pH 8.0, 1 mM EDTA. Protein-DNA complexes were recovered by incubation (15 min, 65EC) with 0.1 M $NaHCO_3$, 1% (w/v) SDS (250 µl), samples were centrifuged (200 g, 1 min, 4EC) and supernatants retained. This elution step was repeated and supernatants combined. Cross-linking was reversed by incubation (16 h, 65EC) with 0.2 M NaCl. DNA was extracted by mixing equal volumes of samples with phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0) and with separation of the phases by centrifugation (15,000 g, 10 min, 4EC). The upper aqueous phases were retained and DNA was precipitated by adding 1 vol. isopropanol for 1 h on ice. Following recovery of DNA by centrifugation (21,000 g, 10 min, 4EC), DNA was washed [70% (v/v) ethanol] and resuspended in 20 µl Milli-Q water for subsequent PCR. PCR reactions were performed in 1× Buffer IV® (AB Gene, 25 µl) containing Taq polymerase (1 U), dATP, dCTP, dGTP and dTTP (0.2 mM each) and 50 pmol primers. PCR settings were: 94EC, 4min; 35 × (94EC, 30s; 59EC, 30s; 72EC, 30s), followed by 72EC, 4 min. The resulting RT-PCR products were analysed by ethidium bromide-agarose gel electrophoresis and the bands captured under UV illumination.

Statistical analysis - Results are expressed as means ± SEM. Statistical significance was determined by either Student´s *t*-test or one-way analysis of variance (ANOVA) followed by Tukey´s multiple comparison test, as appropriate. *P* values of <0.05 were considered significant.

RESULTS

Structure of the Rdm2 gene - Mdm2 (NP_034916.1, 489 residues) and HDM2 (NP_002383.2, 497 residues) are encoded by established genes and are highly conserved, sharing precise identity in about 80% of residues (Supplemental Material Fig. 1). In contrast, *Rdm2* is a predicted gene on chromosome 7(q22) in the NCBI database (http://www.ncbi.nlm.nih.gov) whose provisional processed transcript NM_001108099.1 (21 December 2008) encodes a protein that differs significantly from Mdm2 and HDM2. A BLAST search of the mouse *Mdm2* transcript NM_010786.3 against the rat genome identified a 24233 bp sequence on the minus strand of contig NW_047774 corresponding to a 2930 nucleotide transcript from 12 exons with high homology to the *Mdm2* transcript (Supplemental Material Fig. 2). The deduced 2930 nucleotide sequence of the full-length transcript is identical to 2855 nucleotide NM_001108099.1 except that it contains an extra exon, the 75 nucleotide (protein-coding) exon 4. A BLAST search of this exon revealed 14 rat ESTs with a region of precise sequence identity within them, thus establishing that exon 4 is transcribed and is present in mature *Rdm2* mRNA. The structural relationship of the first four exons in *Rdm2* is shown in Fig. 1. The initiation codon for p90-Rdm2 lies in exon 3 whereas that for p76-Rdm2 lies in-frame in exon 4 (Fig. 1). Two short and overlapping upstream open reading frames are present in exon 1 of the deduced *Rdm2* mRNA sequence (Supplemental Material Fig. 2). The *HDM2* and *Mdm2* transcripts also each contain two (differing) upstream open reading frames (17,18). The deduced amino acid sequence of Rdm2 and the Mdm2 sequence are approximately 90% identical, though Mdm2 possesses a more extended Ser-rich region at residues 203-211 (Supplemental Material Fig. 1).

Expression of Rdm2 mRNA and protein in response to H_2O_2 - We examined the effects of H_2O_2 on *Rdm2* expression by RT-sqPCR and RT-qPCR. Up-regulation of *Rdm2* mRNA was rapid being detectable at 1 h (Fig. 2, *A*-*C*), maximal at 2 h and maintained for at least 6 - 24 h. The absolute extent of maximal upregulation depended on the technique used with sqPCR giving 3- to 4-fold (Fig. 2*A*) but with qPCR giving 20- to 30-fold (Fig. 2, *B* and *C*). This probably reflects differences in primer design and the semi-quantitative nature of densitometric analysis. At 1 h, *Rdm2* mRNA was detectably-induced with 0.1 mM H_2O_2 and maximally-induced with 0.2-0.3 mM H_2O_2 (Fig. 2, *D* and *E*). At higher concentrations of H_2O_2, *Rdm2* mRNA abundances remained at control values (Fig. 2, *D* and *E*). At 2 h of exposure, the maximal response was greater and was shifted to higher H_2O_2 concentrations (Fig. 2*E*). These 'bell-shaped' concentration dependencies are typical of a number of responses of the cardiac myocyte to H_2O_2. The stimulation of *Rdm2* expression by H_2O_2 at 1 h was prevented by CHX (Fig. 3, *A* and *B*), indicating that *Rdm2* is not an immediate early gene (i.e. the stimulation of its transcription by H_2O_2 is dependent on preceding *de novo* protein synthesis).

Since H_2O_2 can inhibit overall protein synthesis in cardiac myocytes (3), we investigated whether the increased abundance of *Rdm2* transcripts was reflected in their translation into protein (Fig. 2, *F* and *G*). Rdm2 migrated at about 90 kDa on SDS-PAGE and protein abundance increased about 5-fold over 45 min -1.5 h (Fig. 2*F*). In some blots, multiple bands were observed (Fig. 2, *F* and *G*). Whilst one of these may represent p76-Rdm2, they may be degradation products or cross-reacting species. Abundance of Rdm2 protein was maximal at 1 to 2 h and declined gradually thereafter (Fig. 2*G*), but remained elevated over control values (about 2- to 3-fold greater) for up to 8 h.

Abundance of any individual mRNA species is regulated by the rate of its transcription and the rate of its degradation. The 3' untranslated region of *Rdm2* mRNA contains an AU-rich instability element [probably a class I ARE consisting of overlapping or adjacent AUUUA pentamers (30), bases 2072-2085, Supplemental Material Fig. 2] which is orthologous with a putative *Mdm2* mRNA ARE [see the Organism ARED (30) at http://brp.kfshrc.edu.sa/ARED/ and Reference (23)], though the Mdm2 ARE may be non-functional (23). We therefore examined the half-life of *Rdm2* transcripts when transcription was inhibited (by actinomycin D) in the absence or presence of 0.2 mM H_2O_2 (Fig. 3*C*). The data sets (n = 4 for each condition) were treated as monophasic exponential decays. *Rdm2* mRNA was degraded with a half-life of 1.16 h ($k = 0.596 \pm 0.140$ h^{-1}) in the absence of H_2O_2, whereas it was 1.82 h ($k = 0.381 \pm 0.141$ h^{-1}) in the presence of H_2O_2. There was thus no significant difference in the stability of the transcripts under the two conditions and thus upregulation of *Rdm2* expression results primarily from increased transcription.

Alternative promoter usage in Rdm2 transcription - Transcription of *Mdm2* and *HDM2* is regulated by two alternative promoters, P1 or P2, lying 5' to exon 1 or exon 2, respectively (16). To assess alternative promoter usage for *Rdm2*, we used forward primers in exon 1 (F1) or in exon 2 (F2) and a reverse primer in exon 4 (Fig. 1, Supplemental Material Fig. 2) to produce amplicons of 333 bp from P1 (long mRNA) or 220 bp from either P1- or P2 (long + short mRNAs). In unstimulated cardiac myocytes, mRNA was detectable at 333 bp (Fig. 4*A*) using primer F1 and this would contribute to the 220 bp band seen with primer F2 to an unassessable extent. Following exposure to H_2O_2 (0.2 mM, 2 h), there were no changes in the amplicon abundances using primer F1, but primer F2 amplicon abundances increased about 4- to 5-fold. The sequences of the 220 and 333 bp PCR products were confirmed, indicating that H_2O_2-inducible transcription was regulated by the P2 promoter.

To confirm increased P2 promoter usage, we conducted 5'-RACE. The theoretical lengths of the amplicons were 332 bp for a transcript expressed from the P1 promoter and 162 bp for a transcript expressed from the P2 promoter (Fig. 1, Supplemental Material Fig. 2). Under control conditions, the major product (amplicon A) was 300 ! 400 bp in length with a weaker band (amplicon B) at 100 ! 200 bp (Fig. 4*B*). Following exposure to H_2O_2 (0.2 mM, 2 h), the product corresponded

essentially completely to the shorter amplicon B. DNA sequencing confirmed that amplicon A corresponded to exons 1, 2 and 3 (up to the reverse primer site) whereas amplicon B corresponded to exons 2 and 3. These results confirm that transcripts originate from P1 with some expression from P2 in the control, whereas promoter usage shifts primarily to P2 following exposure to H_2O_2.

Transcription factor-response elements in intron 1 of the Rdm2 gene - We identified potential regulatory elements in *Rdm2* intron 1by analogy with published data for intron 1 of the *Mdm2* and *HDM2* genes and by using TFSEARCH (www.cbrc.jp/research/db/TFSEARCH.html). A region in intron 1 of the *Mdm2* (402-523), *Rdm2* (374-494, Fig. 1) and *HDM2* (406-524) genes is highly conserved with ~75% identity (Supplemental Material Fig. 3). This contains the two p53-response elements (31,32) and a composite AP-1-Ets element [$Ets^{(AP-1)}$] (33,34). An upstream Ets [$Ets^{(upstream)}$] element in *Rdm2* and *Mdm2* lies outside the conserved region (Supplemental Material Fig. 3, Fig. 1). High-stringency searching also reveals an NF-E2-like site (35) in *Rdm2* which overlaps with the AP-1 site and which is conserved between *Mdm2* and *HDM2*. *Rdm2* and *Mdm2* also contain a sequence reminiscent of an MZF-1 element (36) that is not conserved in *HDM2*, though *HDM2* contains an alternative MZF-1 site (Supplemental Material Fig. 3). However, neither NF-E2 or MZF-1 are thought to be significantly expressed outside hematopoietic cells (35,37), and they were not studied further.

p53 - Induction of *p53* is frequently a response to mutational or cytotoxic interventions and is of established importance in the regulation of inducible expression of *Mdm2* (16). We examined whether *p53* mRNA expression increased in response to H_2O_2. Though an amplicon of the appropriate size for *p53* was detectable by RT-sqPCR, exposure of myocytes to 0.2 mM H_2O_2 for up to 24 h did not alter its expression (results not shown).

The matter of whether p53 protein in induced by ROS is contentious and doubts have been raised about the reliability of antibodies obtained from Santa Cruz Biotechnology Inc. (see Discussion). We characterized six different antibodies using lysates of 293T cells with (positive control) or without (negative control) transfection with mouse *p53* (Fig. 2). Three of these reliably detected p53 by immunoblotting in our hands [FL-393 (Fig. 5*A*), Pab 240 (Fig. 5*B*) and 2B2.71 (Fig. 5*C*)]. In all cases, p53 migrated slightly more rapidly than a 52 kDa marker (GE Healthcare, RPN800E). With FL-393 (Fig. 5*A*) and 2B2.71 (Fig. 5*C*), cross-reacting proteins were detectable in whole cell extracts of 293T cells, one of which migrated slightly more slowly than p53 at ~52 kDa. We confirmed that p53 in crude nuclear extracts of rat cardiac myocytes which had been exposed to the anthracycline, daunorubicin, [anthracyclines induce p53 in these cells (38)] co-migrated with murine p53 in the positive-control 293T cells (Fig. 5*D*).

Following exposure of cardiac myocytes to 0.2 mM H_2O_2, *Rdm2* transcript abundances reach their maximum levels in crude nuclear extracts after about 2 h (Fig. 2, *A-C*). If *de novo* induction of transcription factor proteins is a prerequisite for this event, it should occur over within this period. We assessed whether p53 protein was induced over a period of up to 8 h in crude nuclear extracts of cardiac myocytes exposed to 0.2 mM H_2O_2 using antibodies FL-393 (Fig. 6*A*), 2B2.71 (Fig. 6, *B* and *C*) and Pab 240 (Fig. 6*D*). We also assessed whether increasing the H_2O_2 concentration to 0.5 mM had any additional effect (Fig. 6*C*). In all cases, a positive control for p53 protein induction was included using crude nuclear extracts of cardiac myocytes exposed to 0.5 **:**M daunorubicin for 6.5 - 7.5 h. In no instance could we detect induction of p53 by H_2O_2 (Fig. 6, *A-D*) although p53 protein was reliably detected following treatment with daunorubicin (0.5 **:**M for 6.5-7.5h).

To confirm the immunoblotting results (Fig. 6, *A-D*), we assessed whether any p53 DNA binding activity could be detected in crude nuclear extracts of myocytes exposed to H_2O_2 using EMSAs. Although daunorubicin increased protein binding to a p53 consensus response element consensus sequence, 0.2 mM H_2O_2 did not induce any detectable binding (Fig. 6*E*). DO-1 antibodies to p53 produced a supershifted band on EMSAs with daunorubicin whereas FL-393 antibodies prevented binding to the probe (Fig. 6*F*), thus

confirming that the band detected on EMSAs with daunorubicin was indeed p53. An irrelevant antibody (to JunD) did not supershift the complex (Fig. 6*F*). Thus, using two independent methodologies and a variety of antibodies, we were unable to find evidence for p53 induction by H_2O_2. We conclude that it is unlikely that p53 is responsible for the increased *Rdm2* P2 promoter activity induced by H_2O_2.

Ets - We examined whether Ets proteins may regulate *Rdm2* expression. An EMSA probe corresponding exactly to the $Ets^{(upstream)}$ region (Table 2, Fig. 1) detected three complexes (1, 2 and 3) in crude nuclear extracts of myocytes exposed to 0.2 mM H_2O_2 for zero or 30 min, with complex 2 showing evidence of inducibility (Fig. 7*A*). Unlabelled wild type probe competed effectively for binding to complexes 1 and 2 (but not to complex 3) even at an equimolar concentration, whereas an unlabelled probe with two mutated bases did not completely compete for binding even when in an 100-fold excess (Fig. 7*A*). Thus complex 3 represents non-specific binding. Binding of complex 1 was 'constitutive' and remained constant over 2 h (Fig. 7*B*). Nevertheless, complex 1 may still be important in regulation of the P2 promoter as its transactivating activity (as opposed to DNA binding activity) could be subject to regulation [e.g. phosphorylation (39)]. Complex 2 was transiently induced to a maximum of about 8-fold after 30 min (Fig. 7*B*). By 1 h, approximately 60% of the induction had been lost and abundances had returned to baseline by 2 h (Fig. 7*B*), i.e. its peak induction significantly preceded *Rdm2* induction. We also examined the $Ets^{(AP-1)}$ element using EMSAs with a probe corresponding exactly to the $Ets^{(AP-1)}$ region (Table 2, Fig. 1). Although a complex was detectable, its abundance was not altered by exposure of myocytes to 0.2 mM H_2O_2 for up to 8 h (results not shown). Furthermore, unlabeled probe for the $Ets^{(AP-1)}$ region did not effectively compete until present at a 50- to 100-fold excess (results not shown). Overall, therefore, any binding of proteins to the Ets sequence EMSA probes is either not inducible, not specific, or does not follow a time course appropriate for induction of *Rdm2* expression.

AP-1- The AP-1 element is situated immediately 5' to the $Ets^{(AP-1)}$ element in the *Rdm2* P2 promoter (Fig. 1). An EMSA probe corresponding exactly to this AP-1 sequence (Table 2, Fig. 1) detected a complex in crude nuclear extracts of myocytes that was competed by unlabeled wild type, but not mutant, probe (Fig. 7*C*). Levels of this complex were increased by H_2O_2 to a maximum at 1!2 h (Fig. 7*D*). Supershift EMSAs were used to identify the AP-1 proteins (the Jun and Fos family members) binding to the AP-1 probe. In the absence of stimulation, the complex was partially supershifted by antibodies to JunB or JunD, but not by antibodies to c-Jun (Fig. 8*A*). Following exposure to H_2O_2 (0.2 mM 1.5 h), antibodies to JunB, JunD, and, additionally, c-Jun partially supershifted the complex (Fig. 8*B*). Antibodies to c-Fos, FosB, and Fra-1 also supershifted this complex but antibodies to Fra-2 or ATF2 were ineffective (Fig. 8*C*). When a mixture of all supershifting antibodies (c-Jun, JunB, JunD, c-Fos, FosB and Fra-1) was used, supershifting of the complex was essentially complete (Fig. 8*D*). For c-Jun and JunB, ChIP assays confirmed that binding to an *Rdm2* P2 sequence containing the AP-1 site (Fig. 1, Table 1) increased following exposure of cardiac myocytes to H_2O_2 (0.2 mM, 1.5 h) (Fig. 8*E*). These results indicate that Fos and/or Jun family transcription factors bind to the *Rdm2* P2 AP-1 sequence and this is increased by H_2O_2.

Expression of Jun and Fos family members - We examined the time courses of expression of *c-Jun*, *JunB* and *JunD* transcripts and proteins in response to 0.2 mM H_2O_2. Transcripts for both c-Jun (Fig. 9*A*) and JunB (Fig. 9*B*) were increased maximally (about 8- and 4-fold for c-Jun and JunB, respectively) by H_2O_2 after about 1 h, but had declined to control values by 4 h. Protein abundances followed time courses essentially similar to those for transcripts with c-Jun showing a 7- to 8-fold increase (Fig. 9, *C* and *D*), and JunB showing a 2.0- to 2.5-fold increase (Fig. 9, *E* and *F*). There were no changes detectable in JunD transcript or protein abundances (results not shown).

A similar pattern of mRNA expression was observed for the Fos family members implicated by EMSAs in the binding to the *Rdm2* P2 AP-1 sequence (Fig. 10, *A-D*). Transcript abundances increased by approximately 20-fold for c-Fos (Fig. 10*A*),

15-fold for FosB (Fig. 10*B*) and 10-fold for Fra-1 (Fig. 10*C*) over 1 to 2 h before subsequently declining. Abundances of Fra-2 did not change significantly (results not shown) in accord with the results of supershift EMSAs (Fig. 8*C*). For c-Fos protein, abundance increased by approximately 15-fold over the same time period before declining (Fig. 10*D*). Antibodies against FosB and Fra-1 were not satisfactory for unequivocal use in immunoblotting in our hands. These results suggest that one factor in the increase in binding of AP-1 proteins to the *Rdm2* P2 sequence is an increase in their abundances.

Effects of Rdm2 on caspase 3 cleavage in H_2O_2-treated cardiac myocytes - Mdm2 and its orthologues are generally considered to be antiapoptotic. We examined whether *Rdm2* expression delayed the pro-apoptotic effects of oxidative stress in cardiac myocytes using an antisense ODN to a region of *Rdm2* exon 7 (Supplemental Material Fig. 2). This reduced H_2O_2-stimulated expression of Rdm2 protein by approximately 75% (Fig. 11*A*, *top* panel and Fig 11*B*). Apoptosis was assessed by formation of the 17 kDa active (cleaved) form of caspase 3. H_2O_2 increased the abundance of 17 kDa caspase 3 in the absence or presence of lipofectin by about 2.5- to 4-fold (Fig. 11*A*, *middle* panel, Fig. 11*C*), thus demonstrating that, under the conditions used, H_2O_2 was pro-apoptotic. The *Rdm2* antisense ODN (but not a scrambled ODN) further increased the H_2O_2-mediated stimulation of 17 kDa caspase 3 formation by some 2- to 3.5-fold [depending on the value of 17 kDa caspase 3 formation (lipofectin, no H_2O_2, or nonsense oligodeoxynucleotide + H_2O_2) used as denominator] (Fig. 11*A*, *middle* panel, Fig. 11*C*). As judged by immunoblotting for **"**-actin, the adherence of the myocytes to the culture dishes was unaffected by any treatment (Fig. 11*A*, bottom panel). These results suggest that one role of increased *Rdm2* expression in myocytes exposed to oxidative stress is to reduce or delay apoptosis.

DISCUSSION

The effects of H_2O_2 on Mdm2 expression in the heart - The effects of ROS generally and H_2O_2 in particular on *Mdm2* expression in cardiac and non-cardiac cells have not been extensively examined previously. In non-cardiac cells, H_2O_2 upregulates *Mdm2* mRNA and protein expression in concert with upregulation of *p53* and the assumption is that induction of p53 protein is responsible for upregulation of *Mdm2* (40-42). However, this may not be universal (43). Of relevance to cardiac myocytes, in the H9c2 rat ventricular myoblast line, H_2O_2 (0.4 mM, 24 h) increases Rdm2 protein abundance (44) whereas, in contrast to our microarray data (3,10), *Rdm2* transcript abundance was reported to decrease in cardiac myocytes exposed to H_2O_2 (0.1 mM, 1 h) (45). To study Rdm2 in more detail, we first needed to characterize the *Rdm2* gene and its transcript, and Rdm2 protein using bioinformatics approaches (Fig. 1, Supplemental Material Figs. 1-3). The current details for *Rdm2* mRNA in the NCBI database (http://www.ncbi.nlm.nih.gov) are misleading as the 75 bp exon 4 has been omitted. It could be argued that this shorter is specific to the rat and could arise, for example, by altenative splicing. However, in addition to identifying exon 4 in rat ESTs, the reverse primer (Table 1) used in the experiments described in Fig. 3*A* lies within exon 4 and products of the predicted sizes were generated, thus establishing that exon 4 is expressed in mature *Rdm2* mRNA.

We showed that Rdm2 transcripts and protein were rapidly induced (within 1 - 2 h) by H_2O_2 and that this occurs over the 0.1 - 1 mM range (Fig. 2, *A*-*E*). At higher concentrations, expression declines (Fig. 2, *D* and *E*). *Rdm2* was not regulated as an immediate early gene (Fig. 3, *A* and *B*) and the upregulation of the transcript did not involve transcript stabilization (Fig. 3*C*). As in other cells (16), constitutive *Rdm2* expression involved the 5'-P1 promoter, whereas H_2O_2-inducible expression was largely regulated through the P2 promoter within intron 1 (Fig. 4, *A* and *B*). Intron 1 contains several potential transcription factor binding sites including sites for p53, AP-1 and Ets (Fig. 1), any or all of which may be important in inducible expression of *Rdm2*.

Induction of p53 by H_2O_2 in cardiac myocytes - At least two studies report that H_2O_2 induced expression of p53 transcripts and protein in neonatal rat cardiac myocytes in culture (2,45). Because our original data contradicted these studies, we have been

encouraged to go to great lengths in order to prove that our data are correct. In addition to the antibody (FL-393) we originally used, we used two further antibodies (Pab 240 and 2B2.71) for immunoblotting. We first confirmed the selectivity and specificity of these antibodies for p53 by immunoblotting commercially-available whole cell extracts of 293T cells transfected with mouse p53 (Fig. 5, *A*-*C*). All three antibodies detected p53 as a band that migrated slightly more rapidly that the 52 kDa marker (Fig. 5, *A*-*C*). This band was absent from the negative control cell line, though it should be noted that cross-reacting proteins of different apparent molecular masses could be detected with two of the antibodies including one which migrated with the 52 kDa marker (Fig. 5, *A, C* and *D*). Furthermore, rat p53 (induced by treatment of cardiac myocytes with daunorubicin) co-migrated with murine p53 in the positive control cell line (Fig. 5*D*). None of the three antibodies detected any increase in p53 expression in cardiac myocytes following exposure to H_2O_2, despite a clear induction with daunorubicin (Fig. 6, *A*-*D*). Furthermore, we could not detect any induction of p53 by H_2O_2 using EMSAs despite the clear induction by daunorubicin of a band which could be supershifted with p53 antibodies (Fig. 6, *E* and *F*). Thus, we are confident that 0.2 mM H_2O_2 does not induce expression of p53 in cardiac myocytes and this probably excludes any role for p53 in H_2O_2-induced *Rdm2* expression.

It is not entirely clear why others have detected p53 induction by H_2O_2 (2,45), but there are at least four potential areas of concern: (i) the antibody used, (ii) the cell extracts used, (iii) the conditions used, and (iv) the absence of positive controls for p53. First, both von Harsdorf *et al*. (2) and Long *et al*. (45) used a monoclonal antibody from Calbiochem of which there are currently six currently listed (http://www.emdbiosciences.com or http://www.merckbiosciences.co.uk). Not surprisingly given the time elapsed, none of the three authors of Reference (2) could recall which antibody was used, and indeed the choice may have been limited or nomenclature not specified by the supplier at this stage. In the report by Long *et al*. (45), Dr. José Marín-García informed us that they had used the Calbiochem Ab-4 antibody (cat. no. OP32) from the Pab 246 clone, but he now purchases this from Santa Cruz Biotechnology Inc. (cat no. sc-100). We found that Pab 246 cross-reacted with several proteins in the 52 kDa region in 293T whole cell extracts (results not shown) and we did not use this antibody . Secondly, in both reports, whole cell extracts of cardiac myocytes were used rather than crude nuclear extracts. Generally, we find that whole cell extracts are unsatisfactory for detection of nuclear transcription factors by immunoblottine, mainly because of cross-reaction with other proteins. Indeed, two of the three antibodies showed cross-reactivity with a band at 52 kDa in 293T whole cell extracts (Fig. 5, *A, C* and *D*) which was not detectable in nuclear extracts from rat cardiac myocytes (Fig. 5*D* and Fig. 6, *A*-*D*) but which, in the absence of a positive control, could be mistaken for p53. Thirdly, although the conditions used by Long *et al*. (45) (0.1 mM H_2O_2 for 30 min, 1 h or 24 h) resembled our own, von Harsdorf *et al*. (2) used a potentially more severe oxidative stress (0.1 mM H_2O_2/0.1 mM Fe^{2+} for 1 h, followed by further culture in H_2O_2/Fe^{2+}-free medium for up to 23 h). The combination of H_2O_2 and Fe^{2+} leads to a catalytic disproportionation of H_2O_2 into the highly-reactive hydroxyl radical (which is toxic and induces a severe oxidative stress), and a peroxide radical, and is commonly known as the Fenton reaction ($Fe^{2+} + H_2O_2$ **6** $Fe^{3+} + OH^{c} + OH^{!}$; $Fe^{3+} + H_2O_2$ **6** $Fe^{2+} + OOH^{c} + H^{+}$). Fourthly, for both of the published studies (2,45), there were no positive controls for p53, nor were the positions of molecular mass markers included.

Regulation of inducible Mdm2 by p53-independent mechanisms - Expression of the *Mdm2* gene can also be regulated by p53-independent mechanisms (19-21). Thus, point mutations in any one of the AP-1, $Ets^{(AP-1)}$ or $Ets^{(upstream)}$ elements reduce induction of *Mdm2* expression by oncogenic Ras or Raf in NIH-3T3 cells (19). Furthermore, elevated *HDM2* expression in a breast cancer cell line is partly dependent on the composite AP-1!$Ets^{(AP-1)}$ element (20). In contrast, up-regulation of *Mdm2* in certain mouse erythroleukaemia lines is partly attributable to increased expression of Fli-1 (an Ets family transcription factor) and may involve the $Ets^{(upstream)}$ element (21).

Ets family transcription factors number >20 members (39) that require a core GGA motif for response element recognition with slightly varying preferences in the flanking sequences (34). In cardiac myocytes exposed to H_2O_2, constitutive (complex 1) and transiently-inducible (complex 2) protein binding to the $Ets^{(upstream)}$ element was detected by EMSAs (Fig. 7, *A* and *B*). We attempted to supershift the $Ets^{(upstream)}$ complexes with antibodies to the biologically activated form of Elk-1 [phospho-Elk-1(Ser-383)] (the orthologous residue in rat Elk-1 is in fact Ser-382) and to Fli-1but were unsuccessful (results not shown). We consider it unlikely that the $Ets^{(upstream)}$ element is significant in *Rdm2* upregulation because (i) the level of complex 1 does not change and (ii) levels of complex 2 were returning to zero-time values before any significant induction of *Rdm2* transcripts or Rdm2 protein was detectable (Fig. 2, *A-C*, and *F*, Fig. 7*B*). Equally, we could not obtain any convincing evidence that the $Ets^{(AP-1)}$ element might be involved. The EMSA band with the $Ets^{(AP-1)}$ probe was not inducible, and was not competed by unlabeled probe.

We turned our attention to the AP-1 site. AP-1 is a heterodimer of Fos and Jun family members or possibly a homodimer of Jun family members, and it controls the transcription of numerous genes (46). The AP-1 complex can either activate or inhibit gene expression and its two subunits do not necessarily act in concert. Thus, whereas c-Fos and FosB act in concert with c-Jun (the most powerful transcriptional regulator), c-Jun may be antagonized by JunB, and by Fra-1 and Fra-2 which do not contain transactivation domains. Furthermore, the transactivating activities of AP-1 proteins can be influenced by post-translational modification (e.g. phosphorylation). As with Ets transcription factors, there is evidence that AP-1 may participate in regulation of *Mdm2* expression (19). In our study, EMSAs showed specific binding of proteins to the *Rdm2* AP-1 element (Fig. 7*C*) with a time-course consistent with induction of *Rdm2* expression (Fig. 2, *A-C*, and Fig. 6*D*). c-Jun and JunD bound to the probe in unstimulated cells (Fig. 8*A*). In cells exposed to H_2O_2, JunB (Fig. 8*B*) and Fos family members (c-Fos, FosB, Fra-1, but not Fra-2) were also bound (Fig. 8*C*). The binding of Fra-1 is a little surprising given that expression of Rdm2 is increased but Fra-1 is supposed to reduce AP-1-dependent stimulation of gene expression (46), and we do not have any explanation for this at present. A cocktail of antibodies to c-Jun, JunB, JunD, c-Fos, FosB and Fra-1 resulted in essentially a complete supershift of the complex (Fig. 8*D*). Of the Jun and Fos members mentioned but with the exceptions of JunD and Fra-2, transcripts (and usually proteins) were induced by H_2O_2 (Figs. 9 and 10) within the time-period chosen for supershifting (1.5 h). Using ChIP assays, we demonstrated increased binding of c-Jun and JunB to an *Rdm2* P2 promoter region encompassing the AP-1 site (Fig. 8*E*). ChIP assays using antibodies to the remaining Fos/Jun proteins were less successful, presumably because of their inadequacy in immunoprecipitation protocols. These results strongly suggest that the *Rdm2* AP-1 element is of significance in the upregulation of *Rdm2* expression by H_2O_2, and that increased abundances of AP-1 transcription factors [several of which have been confirmed as immediate early genes in cardiac myocytes in the context of endothelin stimulation (47)] participate in *Rdm2* mRNA upregulation.

H_2O_2 and ROS are established regulators of AP-1 (48). All of the three major MAPK cascades (JNK, ERK1/2, p38-MAPK) are activated by H_2O_2 in cardiac myocytes (49) and may influence AP-1 expression and activity. JNKs phosphorylate c-Jun in its transactivation domain and increase its transactivating activity (46). In addition, *c-Jun* mRNA and protein is upregulated by a combination of activation of ERK1/2 (to increase transcription of *c-Jun*) and JNKs (to increase protein stability) (50). Thus, H_2O_2-mediated stimulation of MAPK cascades might be a factor in the upregulation of AP-1 transcription factor levels and activities in our study.

Cardioprotective effects of Mdm2 - p90-Mdm2 is present in whole murine heart in greater abundance than p76-Mdm2 (though both at much lower abundances than in other tissues such as testis) (23). Cardioselective deletion of *Mdm2* is embryonically-lethal and this lethality can be rescued by deletion of *p53* (51), thus emphasizing the importance of Mdm2 in limiting p53 expression during cardiac development. In the developed heart,

the role of Mdm2 in cardioprotection is implicit from a number of studies. Increasing Mdm2 abundance by overexpression of the transcriptional coactivator p300 protects against apoptosis induced by the cardiotoxic anthracycline doxorubicin in mice *in vivo* (52). Cardiospecific overexpression of insulin-like growth factor 1 increases the association between p53 and Mdm2 thus reducing the binding of p53 to the promoter of the proapototic *bax* gene (53). The growth factor also reduces mechanical strain-induced apoptosis in isolated cardiac myocytes, one possible mechanism involving an Rdm2-mediated reduction in p53 abundance (54). Overexpression of Mdm2 also protects against the pro-apoptotic effects of hypoxia and inhibition of the p53-Rdm2 interaction increases the sensitivity to hypoxia (55). Furthermore, reduced expression of *Mdm2* increases the susceptibility of the isolated heart to injury induced by ischemia and reperfusion (55).

In general agreement with these earlier studies, our studies here suggest that the induction of *Rdm2* expression by H_2O_2 represents a cytoprotective defence against oxidative stress in the cardiac myocyte (Fig. 11). Thus, diminished H_2O_2-mediated induction of *Rdm2* using an antisense ODN to *Rdm2* promoted caspase 3 activation (Fig. 11, *A* and *C*). It is pertinent to ask how Rdm2 can decrease apoptosis, given that H_2O_2 did not induce p53 expression (Fig. 6). One explanation may be that other pro-apoptotic proteins expressed in myocytes are inhibited or downregulated by Rdm2. Precedents for this exist. For example, treatment of p53-null neuroblastoma cells with the HDM2-protein interaction inhibitor, nutlin-3, stimulates apoptosis by inhibiting the interaction with the p53-related protein, p73 (56). It should perhaps also be noted that inhibition of HDM2 is currently under intensive investigation as a chemotherapeutic intervention (57). From the results of our own and other studies, such therapies may decrease cardiac myocyte survival, particularly under conditions of oxidative stress which probably occur during myocardial ischemia and heart failure.

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ABBREVIATIONS

[2] 5'-RACE, 5'-rapid amplification of cDNA ends; ARE, AU-rich instability region; ChIP, chromatin immunoprecipitation; CHX, cycloheximide; DCM, dilated cardiomyopathy; DTT, dithiothreitol; E64, trans-epoxy-succinyl-l-leucylamido-(4-guanidino)-butane; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; Gapd, glyceraldehyde 3-phosphate dehydrogenase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; Mdm2, mouse double minute 2 (HDM2 and Rdm2, *Homo sapiens* and rat orthologues of Mdm2, respectively); MZF-1, myeloid zinc finger protein-1; ODN, oligodeoxynucleotide; PBS, phosphate-buffered saline; PMSF, phenyl methyl sulfonyl fluoride; ROS, reactive oxygen species; RT-qPCR and RT-sqPCR, reverse transcriptase-quantitative polymerase chain reaction and semiquantitative PCR; SDS-PAGE, sodium dodecyl sulfate - polyacrylamide gel electrophoresis; TBE, Tris-borate-EDTA; TBST, Tris-buffered saline with Tween 20; UPM, universal primer A mix.

[3]Rdm2 and HDM2 refer specifically to rat and *Homo sapiens*, respectively, whereas Mdm2 will refer specifically to the mouse or to the three orthologues generally.

CONFLICT OF INTERESTS

There are no conflicts of interest to report.

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FIGURE LEGENDS

FIG. 1. The deduced structure of the 5' region of the *Rdm2* gene. Further details are provided in the Results section. The complete deduced sequence of mature *Rdm2* mRNA is shown in Supplemental Material Fig. 2. The numbering refers to the positions of the nucleotides in the gene sequence. The positions of the primers used to determine P1/P2 promoter usage in exons 1, 2 (forward primers F1 and F2, respectively) and in exon 4 are indicated, as are the two alternative translational initiation codons [ATG(1) for p90-Rdm2, ATG(2) for p76-Rdm2]. The consensus sequences for the transcription factor response elements are: MZF-1, AGTGGGGA or the more-extended CGGGNGAGGGGGAA (36); Ets, C(A/C)GGA(A/T)G(C/T) (34); AP-1, TGA(C/G)TCA (33); NF-E2, (C/T)TGCTGA(C/G)TCA(C/T) (35); p53, two (A/G)(A/G)(A/G)C(A/T)(A/T)G(C/T)(C/T)(C/T) elements separated by 0 to 13 nucleotides (31). The positions of the response elements and the 5' to 3' directions applicable are marked by arrows. The

positions of the primers used in the chromatin immunoprecipitation studies of the AP-1 response element are indicated by underlying asterisks (see also Table 1).

FIG. 2. Expression of *Rdm2* transcripts and protein in cardiac myocytes exposed to H_2O_2. RNA or protein was extracted and *Rdm2* mRNA or protein abundances were determined by RT-sqPCR, RT-qPCR or immunoblotting as described under 'Experimental Procedures'. (*A,B,C*) Myocytes were exposed to 0.2 mM (*A,B*) or 1 mM (*C*) H_2O_2 for the times indicated and Rdm2 transcripts measured by RT-sqPCR (*A*) or RT-qPCR (*B,C*). (*D,E*) Dependence of *Rdm2* mRNA expression at 2 h [(*D*), RT-sqPCR; (*E*), RT-qPCR] or 1 h (*E*, RT-qPCR) on H_2O_2 concentration. No RT: as a control for genomic contamination, the RT step was omitted. (*F,G*) Rdm2 protein abundances were determined by immunoblotting of whole cells extracts at the times indicated. Primary antibody dilution: both at 1/750. Representative primary data are shown and the quantified results are expressed as means ± SEM for at least 3 independent observations on separate preparations of myocytes.

FIG. 3. Rdm2 is not and immediate early gene, nor is Rdm2 mRNA stabilized by H_2O_2. (*A,B*) Cardiac myocytes were pretreated with cycloheximide (CHX, 20 **:**M) for 30 min, then exposed additionally to 0.2 mM H_2O_2 for 2 h (*A*, RT-sqPCR) or 1h (*B*, RT-qPCR). (*C*) H_2O_2 (0.2 mM) exposure does not affect *Rdm2* transcript stability. Myocytes were exposed (●) or not exposed ( ) to 0.2 mM H_2O_2 for 2 h, then to 4 **:**M actinomycin D for the times indicated, followed by RT-sqPCR. Results were fitted to monophasic exponential decay curves. Representative primary data are shown and the quantified results are expressed as means ± SEM for 3-4 independent observations on separate preparations of myocytes. Statistical significance: *, $P<0.001$ by one-way ANOVA with Tukey's multiple comparison test.

FIG. 4. Promoter usage for *Rdm2* expression. (*A*) Cardiac myocytes were exposed to or not treated with 0.2 mM H_2O_2 for 2 h. RNA was extracted and RT-sqPCR was used with a reverse primer in exon 4 and forward primers lying in exon 1 (primer F1) to assess P1 promoter usage (long product, 333 bp) or exon 2 (primer F2) to assess combined P1 and P2 promoter usage (short product, 220 bp) (Fig. 1 and Table 1). Representative primary data are shown and the quantified results are expressed as means ± SEM for 6 independent observations on separate preparations of myocytes. Statistical significance versus control: *, $P<0.01$ by one-way ANOVA with Tukey's multiple comparison test. (*B*) 5' RACE for *Rdm2* transcripts in myocytes exposed or not exposed to 0.2 mM H_2O_2 for 2 h. The theoretical lengths of amplicon A (from P1 promoter use) and amplicon B (from P2 promoter use) are 332 and 162 bp, respectively.

FIG. 5. Characterization of p53 antibodies. Extracts of 293T cells that had either been transfected with mouse *p53* (positive control) or not transfected (negative control) were examined by SDS-PAGE and immunoblotting with (*A*) FL-393 antibody, (*B*) Pab 240 antibody, or (*C* and *D*) 2B2.71 antibody, all at 1/1000 dilution. In (*D*), a crude nuclear extract from cardiac myocytes exposed to 0.5 **:**M daunomycin for 6.5 h was included. The position of the 52 kDa molecular mass marker is indicated.

FIG. 6. H_2O_2 does not induce expression of p53 in cardiac myocytes. Myocytes were exposed to 0.2 mM (*A-F*) or 0.5 mM (C) H_2O_2 for the times indicated or to daunorubicin. (*A-D*) Immunoblots of crude nuclear extracts from myocytes. Blots were probed with the p53(FL-393) antibody (*A*) at a dilution of 1/1200, with the 2B2.71 antibody (*B,C*) at a dilution of 1/1000, or with the Pab 240 antibody (*D*) at a dilution of 1/1000. For 2B2.71 and Pab 240, an amplification step with an rabbit anti-mouse antibody and a goat anti-rabbit horseradish peroxidase-conjugated tertiary antibody was used. (*E*) EMSAs of crude nuclear extracts were carried out with a p53 consensus binding sequence probe. (*F*) Myocytes were exposed to 0.5 **:**M daunorubicin for 7.5 h and supershift EMSAs were carried out with the antibodies indicated. Representative primary data are shown for 3 independent observations on separate preparations of myocytes.

FIG. 7. Binding of proteins to the Ets$^{(upstream)}$ and AP-1 response elements in the *Rdm2* P2 promoter as assessed by EMSAs. Cardiac myocytes were exposed to 0.2 mM H_2O_2 as indicated and crude nuclear extracts prepared. Sequences of probes are given in Table 2. (*A*,*C*) EMSAs using the Ets$^{(upstream)}$ probe (*A*) or the AP-1 probe (*C*). Binding specificities were assessed using unlabelled probe. Two specific complexes (1 and 2) were detected with the Ets$^{(upstream)}$ probe (*A*) and one was detected for the AP-1 probe (*C*). (*B*,*D*) Time courses of binding to the Ets$^{(upstream)}$ probe (*B*) or the AP-1 probe (*D*). Representative primary data are shown and the quantified results are expressed as means ± SEM for 3-5 independent observations on separate preparations of myocytes.

FIG. 8. Binding of AP-1 proteins to the AP-1 response element in the *Rdm2* P2 promoter as assessed by supershift EMSAs or ChIP assays. Cardiac myocytes were exposed to 0.2 mM H_2O_2 as indicated and crude nuclear extracts prepared. (*A*-*D*) Supershift EMSAs were performed using the antibodies indicated. SS: supershifted complex. (*E*) ChIP assays using c-Jun or JunB antibodies, as indicated. Representative primary data are shown and the quantified results are expressed as means ± SEM for 3 independent observations on separate preparations of myocytes. Statistical significance versus control: *, $P<0.05$ by an unpaired two-way Student's *t* test.

FIG. 9. Expression of c-Jun and JunB transcripts and proteins. Cardiac myocytes were exposed to 0.2 mM H_2O_2 as indicated. mRNA abundance was determined by RT-sqPCR, and protein abundance in crude nuclear extracts was assessed by immunoblotting. (*A*,*B*) Expression of *c-Jun* (*A*) and *JunB* (*B*) transcripts. No RT: no reverse transcriptase step. (*C*-*F*) Expression of c-Jun (*C*,*D*) and JunB (*E*,*F*) proteins. Antibody dilutions: c-Jun, 1/750; JunB, 1/5000. Representative primary data are shown and the quantified results are expressed as means ± SEM for 3-4 independent observations on separate preparations of myocytes.

FIG. 10. Expression of *c-Fos*, *Fos-B* and *Fra-1* transcripts and c-Fos protein. Cardiac myocytes were exposed to 0.2 mM H_2O_2 as indicated. mRNA abundance was determined by RT-sqPCR, and protein abundance in crude nuclear extracts was assessed by immunoblotting. (*A*-*C*) Expression of *c-Fos* (*A*), *FosB* (*B*) and (*C*) *Fra-1* transcripts assessed by RT-sqPCR. No RT: no reverse transcriptase step. (*D*) Expression of c-Fos protein. Antibody dilution for c-Fos: 1/20,000. Representative primary data are shown and the quantified results are expressed as means ± SEM for 3-4 independent observations on separate preparations of myocytes.

FIG. 11. 'Knock-down' of Rdm2 protein in cardiac myocytes with an antisense ODN to the *Rdm2* transcript increases H_2O_2-induced activation of caspase 3. AS, antisense ODN; NS, scrambled ODN. (*A*) Abundances of Rdm2 (top panel), active caspase 3 (middle panel) and sarcomeric **"**-actin (bottom panel) in whole cell extracts were assessed by immunoblotting. (*B*,*C*) Quantification of (*B*) Rdm2 or (*C*) active caspase 3 abundances. Antibody dilutions: Rdm2, 1/500; caspase 3, 1/1000; **"**-actin, 1/2000. Representative primary data are shown and the quantified results are expressed as means ± SEM for 4 independent observations on separate preparations of myocytes. Statistical significance versus control: *, $P<0.05$; **, $P<0.001$ by one-way ANOVA with Tukey's multiple comparison tes

TABLE 1. Details of RT-sqPCR/qPCR primers used. For the *Rdm2* transcript, the numbering used refers to the deduced sequence shown in Supplemental Material Fig. 2. For *Rdm2*, primers for *Rdm2*(A) were used for sqPCR analysis of general *Rdm2* expression, primers for *Rdm2*(B) were used for qPCR analysis of general *Rdm2* expression, primers for *Rdm2*(C) and *Rdm2*(D) were used to investigate P1/P2 promoter usage (sqPCR), primers for *Rdm2*(E) were used for 5' RACE (sqPCR), and primers for *Rdm2*(F) were used for ChIP analysis (sqPCR). The sequence of *Rdm2* intron 1, in which the primers used for chromatin immunoprecipitation lie, is shown in Fig. 1. The predicted rat *FosB* transcript sequence (NM_001013146.1) is truncated at its 3' end. The rat *FosB* gene is located on chromosome 1 at 78668523..78673784 (RGSC genome assembly v3.4). The RT-sqPCR product corresponds to 78667636..78667773, sequence:

ACCCCTGTGCAGTATTATGCCATGTCCCTCTCACCCCCACGGGCAACCCAGGCGCCCTTGGCCGTCCTCGTTGGGCCTTTCTGGT

TTTGGGCAGCAGGGGGCGCTGCGACGCCCGTCT**TGCTGGAGCGCTTTATACTG** (primers underlined and emboldened). In contrast, the mouse *FosB* mRNA sequence (NM_008036.2) is more extended and contains the PCR sequence described. The PCR sequence identifies two rat ESTs (C06882.1, CB747983.1) and these lie 3' to the predicted rat *FosB* transcript. In addition, the two rat ESTs correspond to the 3' untranslated region of the mouse *FosB* mRNA.

Transcript for	Forward primer (5'→3')	Exon	Reverse primer (5'→3')	Exon	Amplicon identity or transcript accession no.	Position of primers in mRNA	Product length (bp)	No. of cycles used
Rdm2 (A)	ATCCAGCTTCTCTCTGAACG	12	AGACTAAGACAATGCTCCGG	12	Supplemental Material Fig. 1	1504-1802	299	27
Rdm2 (B)	TCCGACCACCGTGCTTCT	2	TCGGTAGACACAGACATGTTGGTA	3	Supplemental Material Fig. 1	211-279	69	---
Rdm2 (C)	TCGCTTCGCTCGACCTCC	1	CTCTTTCATAGTGTAAATATCTTT	4	Fig. 1	71-403	333	27
Rdm2 (D)	AGCGGAGACGGACACACC	2	CTCTTTCATAGTGTAAATATCTTT	4	Fig. 1	184 ! 403	220	27
Rdm2 (E)	AAGCAGTGGTATCAACGCAGA GTACGCGGG	N/A	TCTGTGAGGTGCCTGCAGCACCCTCG GTAGACAC	3	5' RACE tag	N/A-302	332 (P1) 162 (P2)	35
Rdm2 (F)	CAGGGGTCGGGGAGGTAA	Intron 1	TTAGCTCCTCCAACAGCCAA	Intron 1	Fig. 1	Intron 1	144	35
Gapd	ACCACAGTCCATGCCATCAC	6	TCCACCACCCTGTTGCTGTA	8	NM_17008.2 NM_17008.3	1369-1820 596-1047	452	20
p53	TTGAGGTTCGTGTTTGTGCC	7	CACGGGCATCCTTTAATTCC	9	NM_030989.3	934-1188	255	
c-Jun	ATGACTGCAAAGATGGAAACG	N/A	TATTCTGGCTATGCAGTTCAG	N/A*	NM_021835	859-1234	376	24
JunB	ATCACGACGACTCATACGCA	N/A	TGGAGGCTAGCTTCAGAGAT	N/A*	NM_021836	299-523	225	25
JunD	ATGGAAACGCCCTTCTATGG	N/A	CTTTCTTCAGCATGCTGCTC	N/A*	NM_138875	264-420	157	28
c-Fos	AGTGGTGAAGACCATGTCAG	2	AATGTTCTTGACCGGCTCCA	4	NM_22197.1 NM_22197.2	464-911 482-929	448	25
FosB	ACCCCTGTGCAGTATTATGC		CAGTATAAAGCGCTCCAGCA	See Table 1 Legend			138	27
Fra-1	AATTGCAGTGGATGGTGCAG	2	ACTTAGCAGCTGCTAGCTTG	3	NM_012953	377-595	219	26
Fra-2	AATCAACGCCATCACCACCA	2	GGTGGTACCAATGGTCTTGA	2	NM_012954	150-330	181	26

TABLE 2. Sequences of EMSA probes. See Fig. 1 for details of position in the *Rdm2* gene and its intron 1. p53 EMSAs, a consensus p53 probe was used.

EMSA Probe for	EMSA Probe (5'→3')	Position in gene	Position in intron 1	Mutant EMSA Probe (5'→3')
Rdm2 AP-1	GCCTAGTTGACTCAGCTTTTC	539 ! 559	369-389	GCCTAGTTGACT***TG***GCTTTTC
Rdm2 Ets$^{(upstream)}$	GGACGTGGCTTCCGGGACGGGT	504 ! 525	334-355	GGACGTGG***A***TTCC***A***GGACGGGT
Rdm2 Ets$^{(AP-1)}$	GACTCAGCTTTTCCTGTGGGGCTGG	547 ! 571	277-301	GACTCAGCTTTTCC***C***G***G***GGGGCTGG
Rdm2 MZF-1	ATGGAGGGAGGGGAGAGGAG	384-403	214-233	ATGGAGGGA***A***GG***T***AGAGGAG
p53 response element	TACAGAACATGTCTAAGCATGCTGGGGACT	N/A	N/A	N/A

Pikkarainen et al. Figure 1

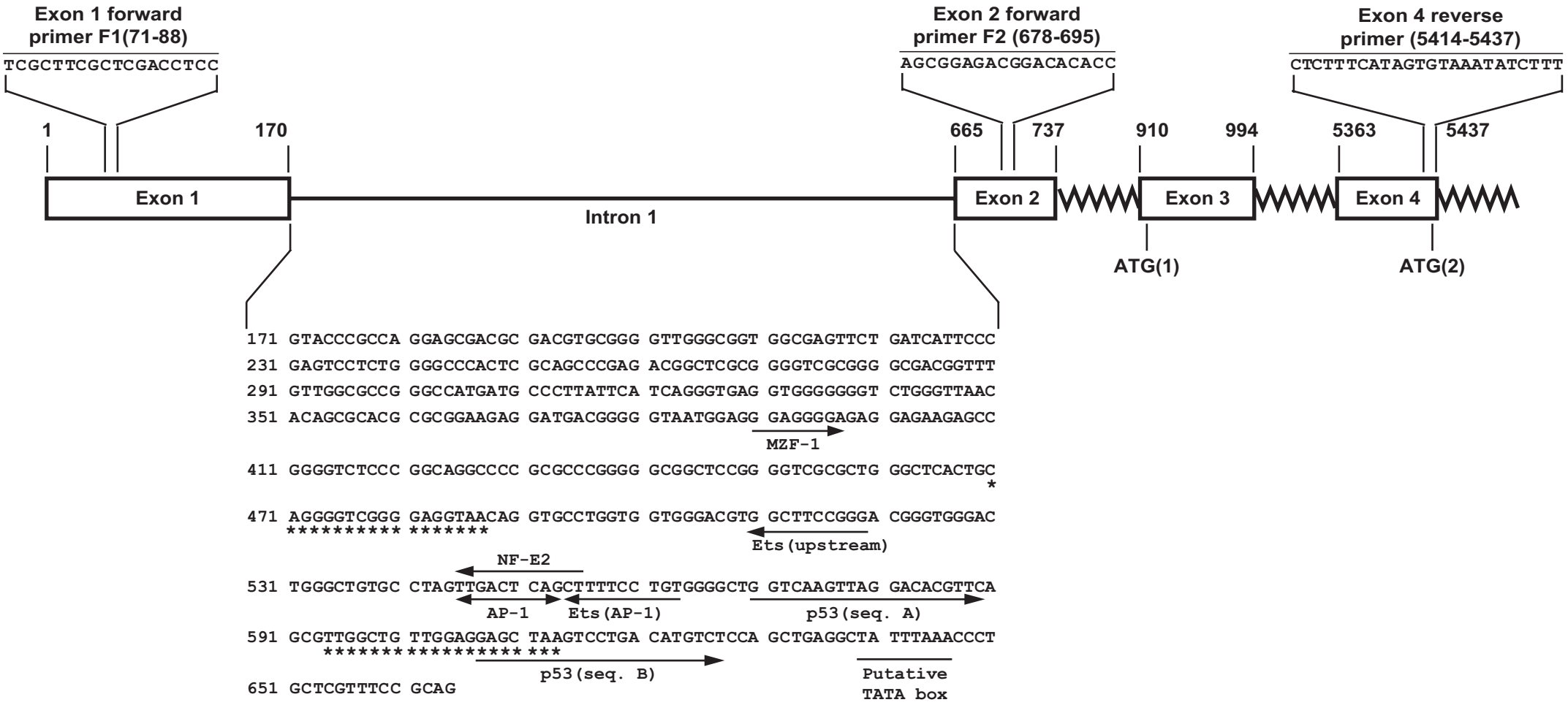

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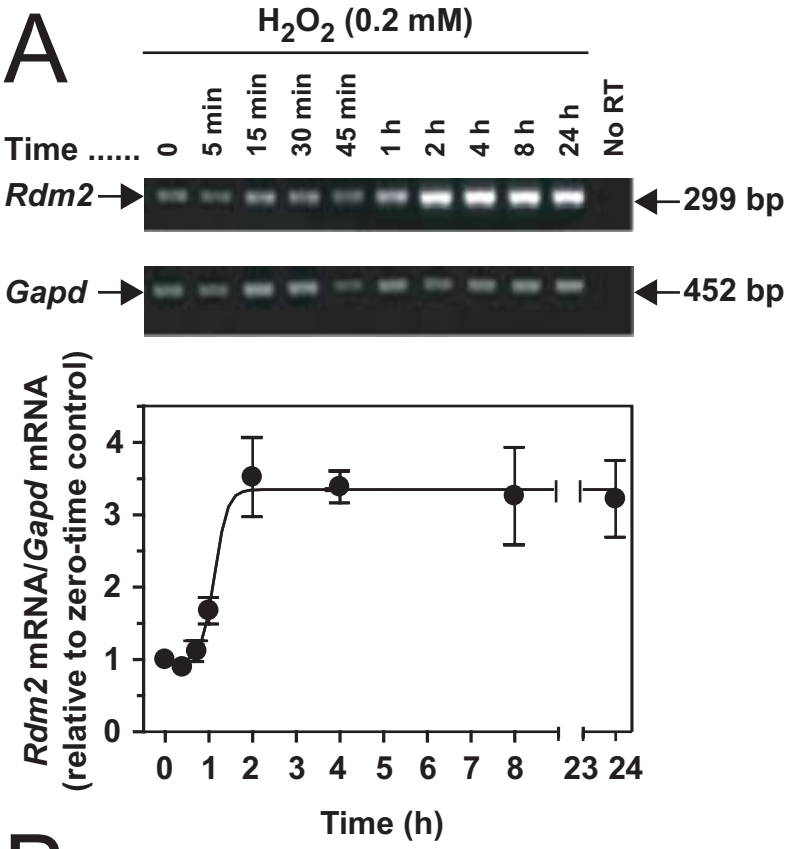
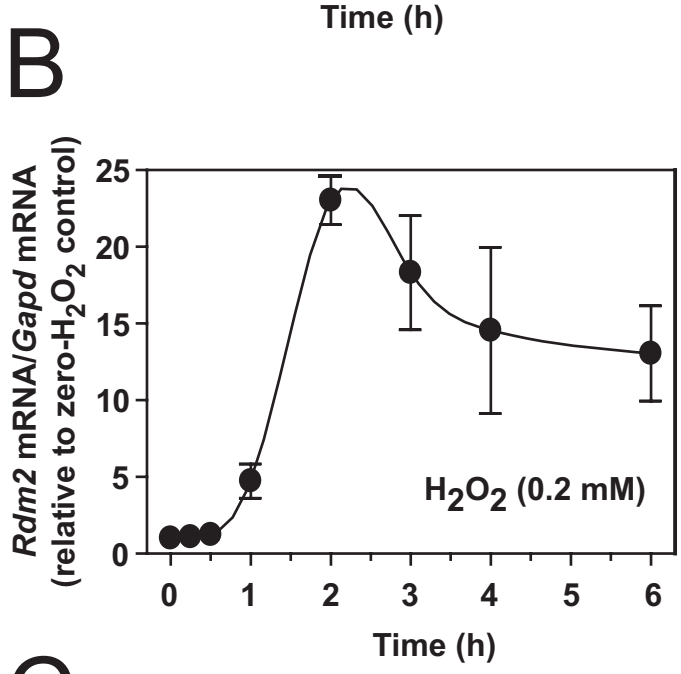
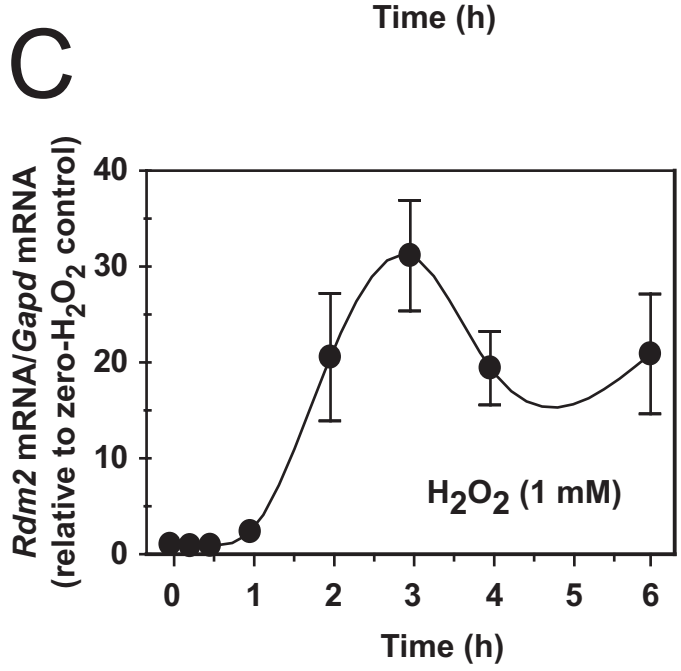
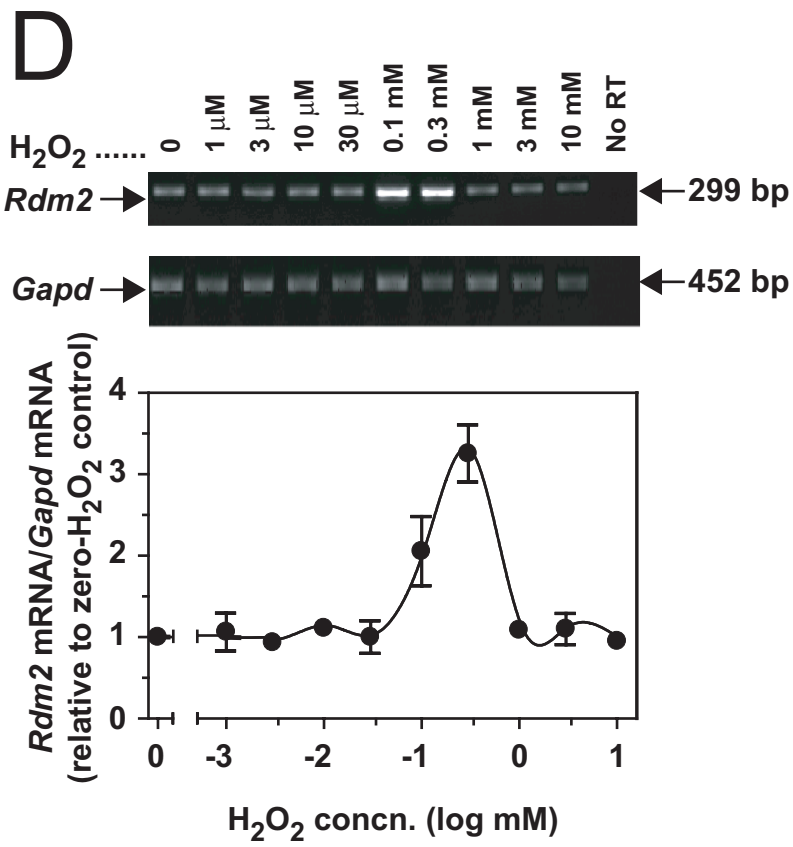
Figure 2, A - C

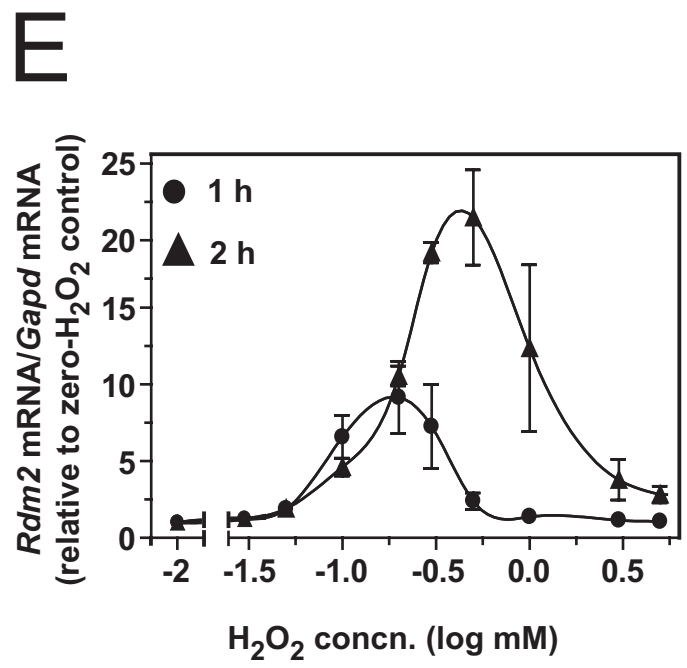




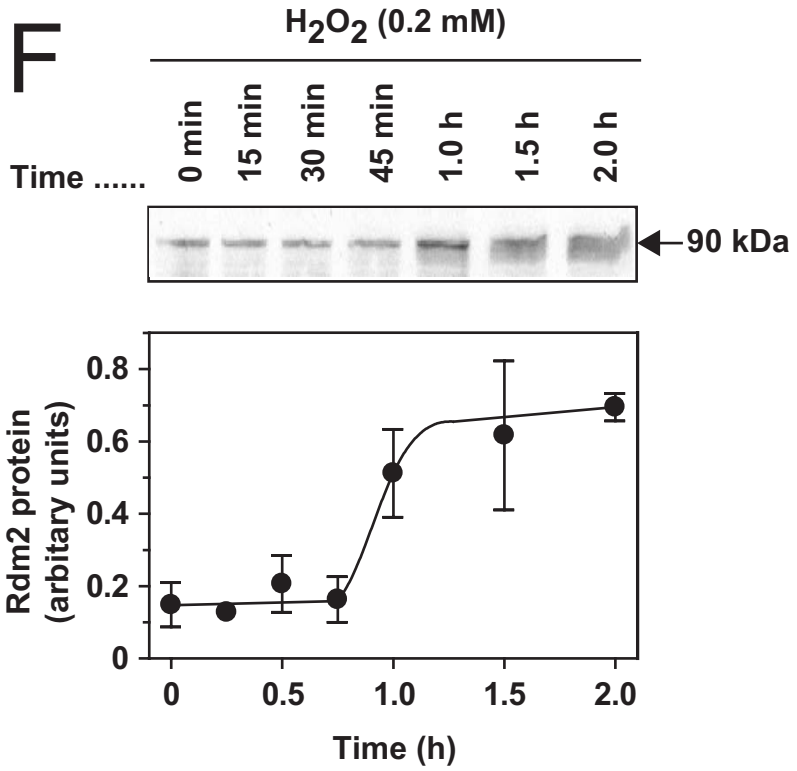


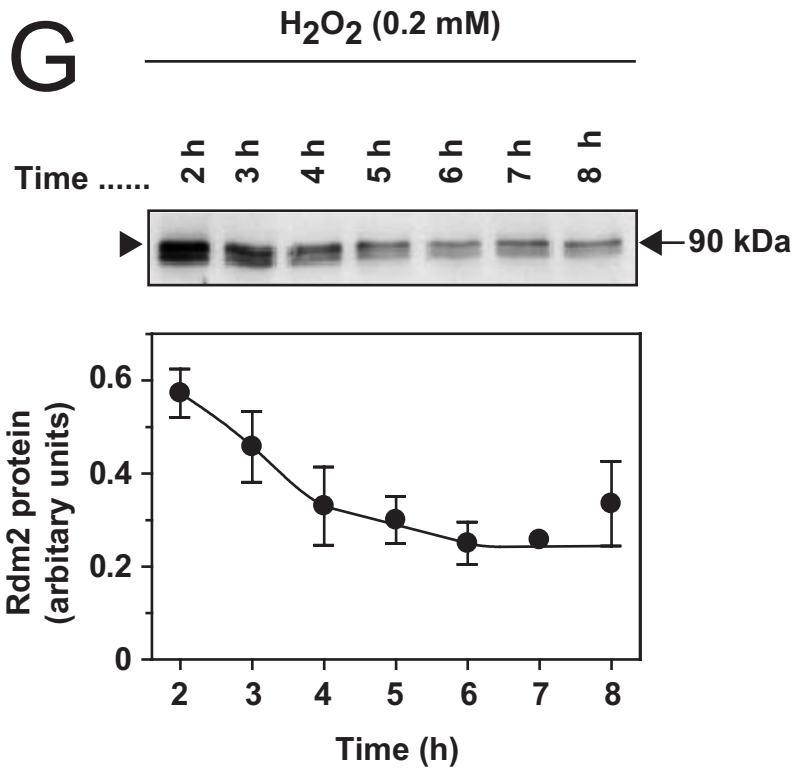
Pikkarainen et al. Figure 2, D and E



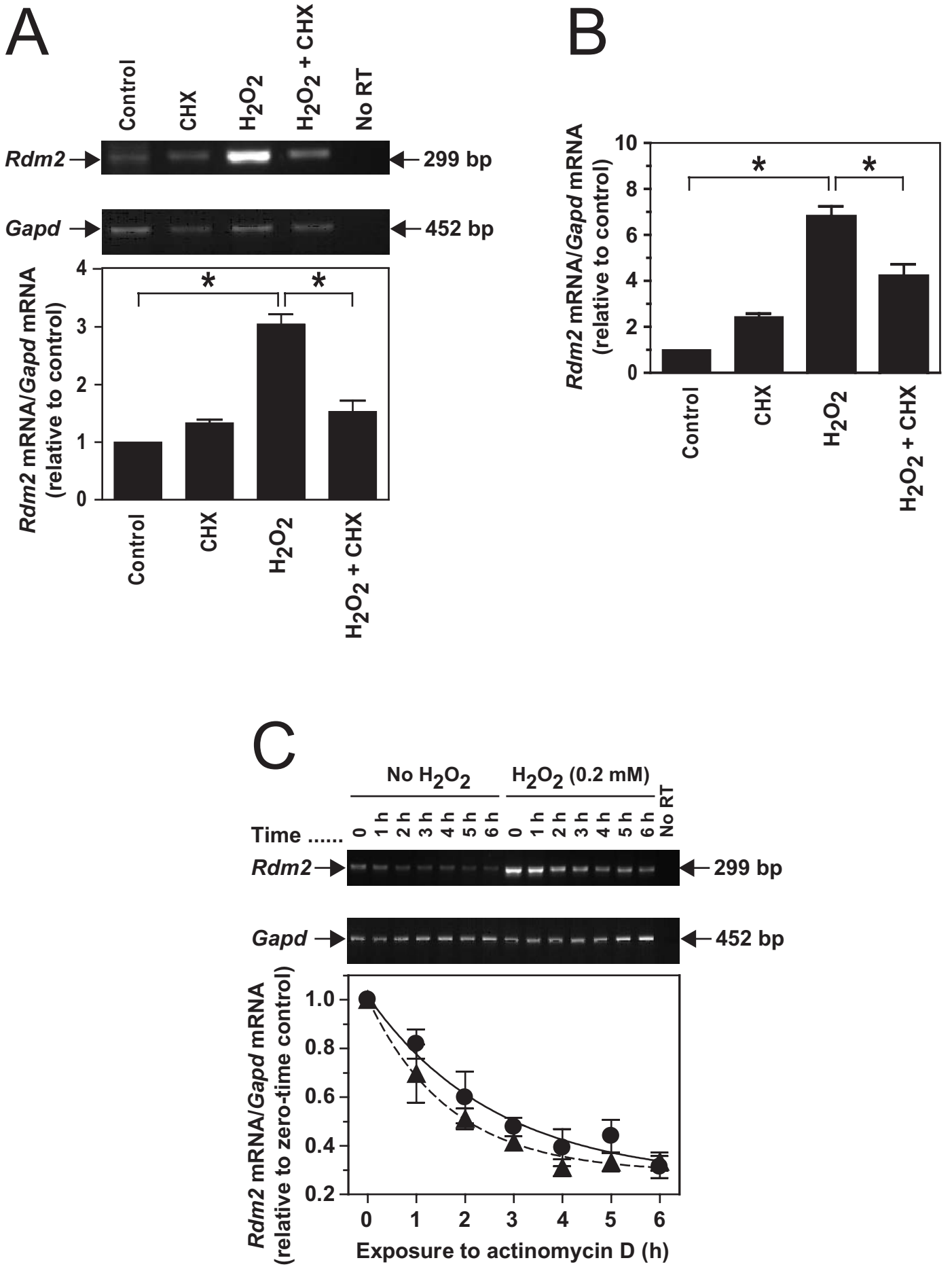


Pikkarainen et al. Figure 2, F and G

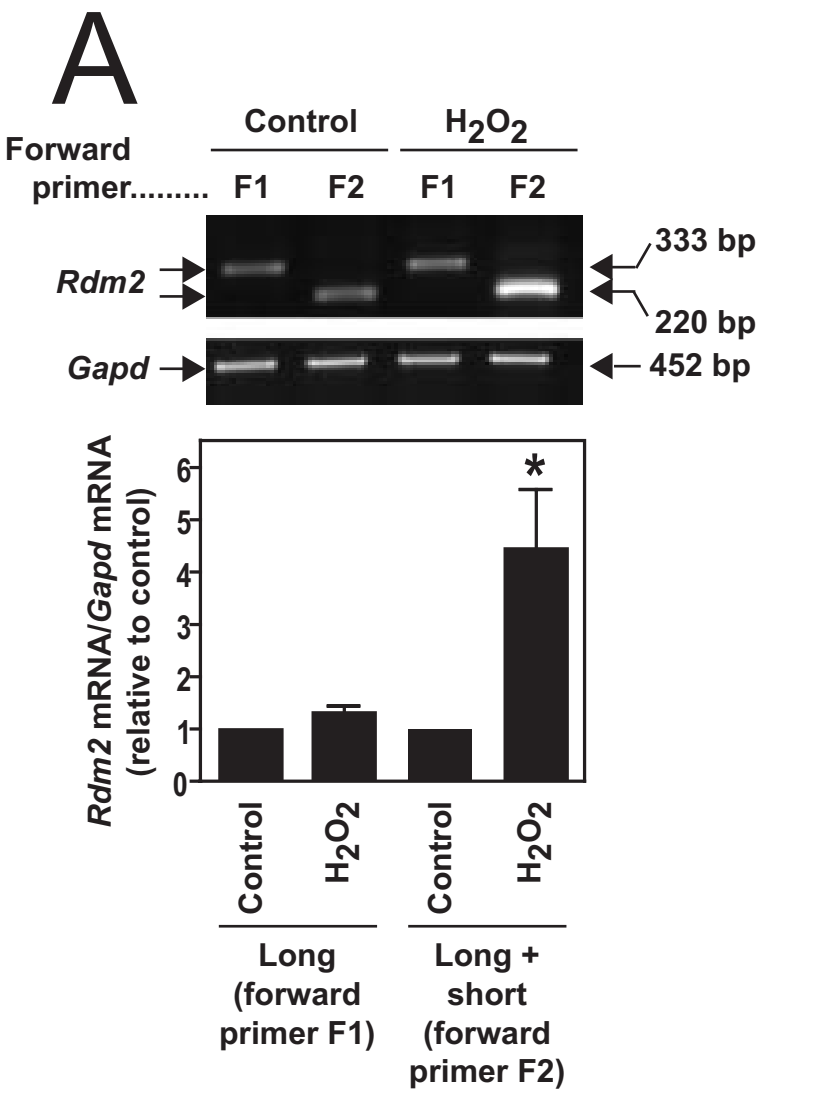


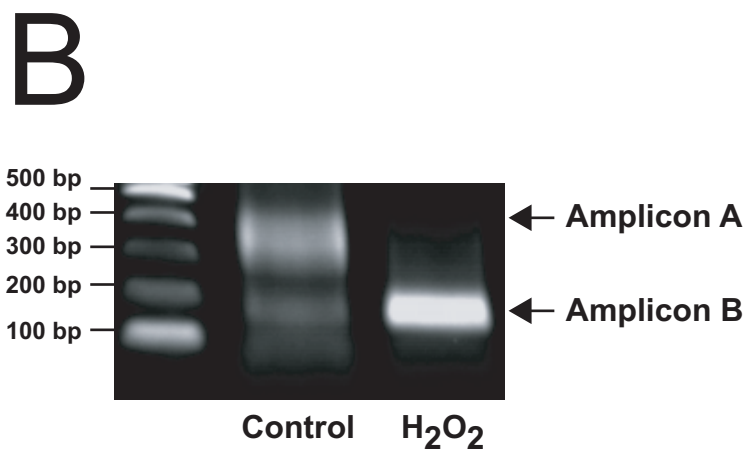


Pikkarainen et al. Figure 3

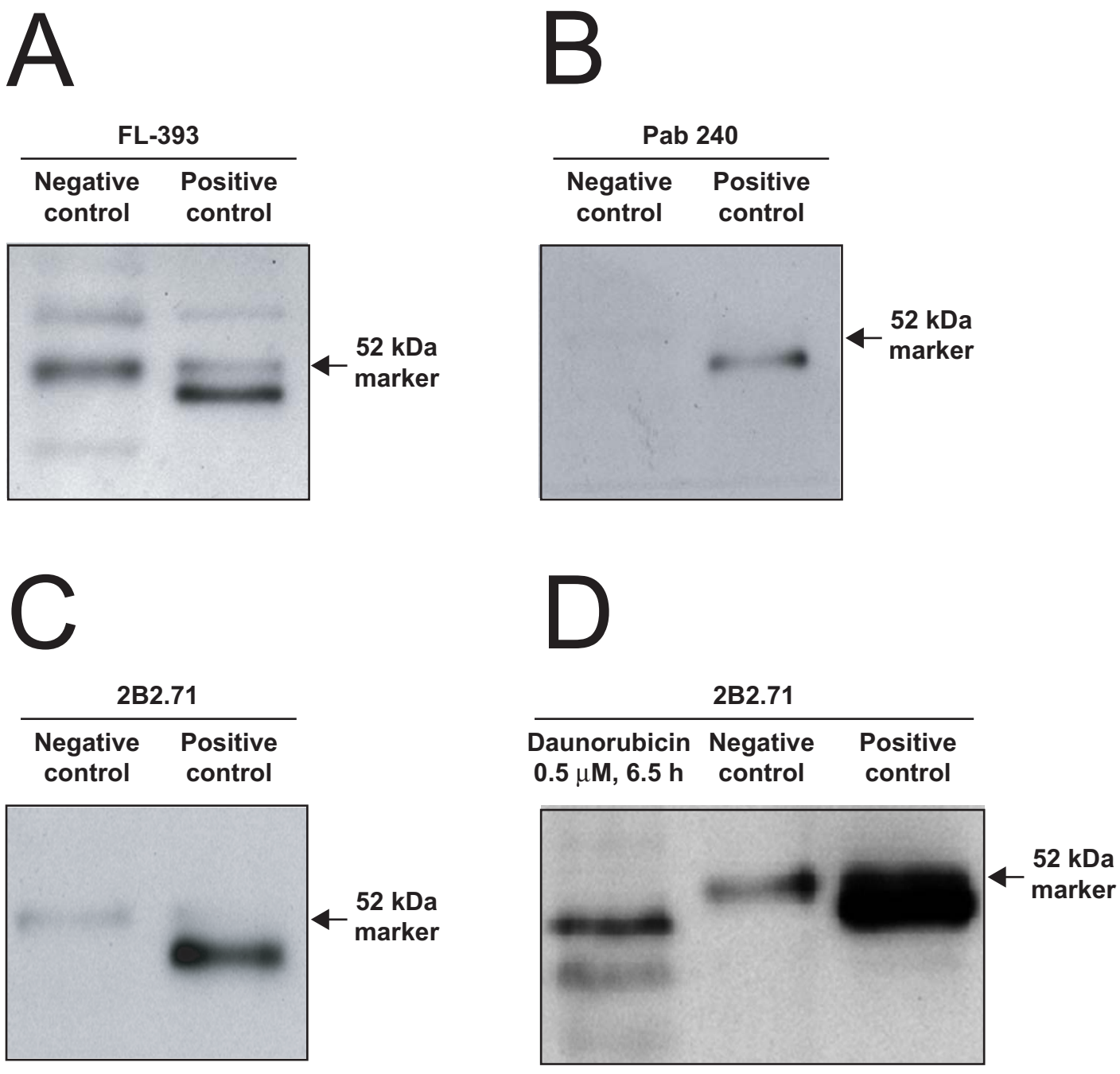


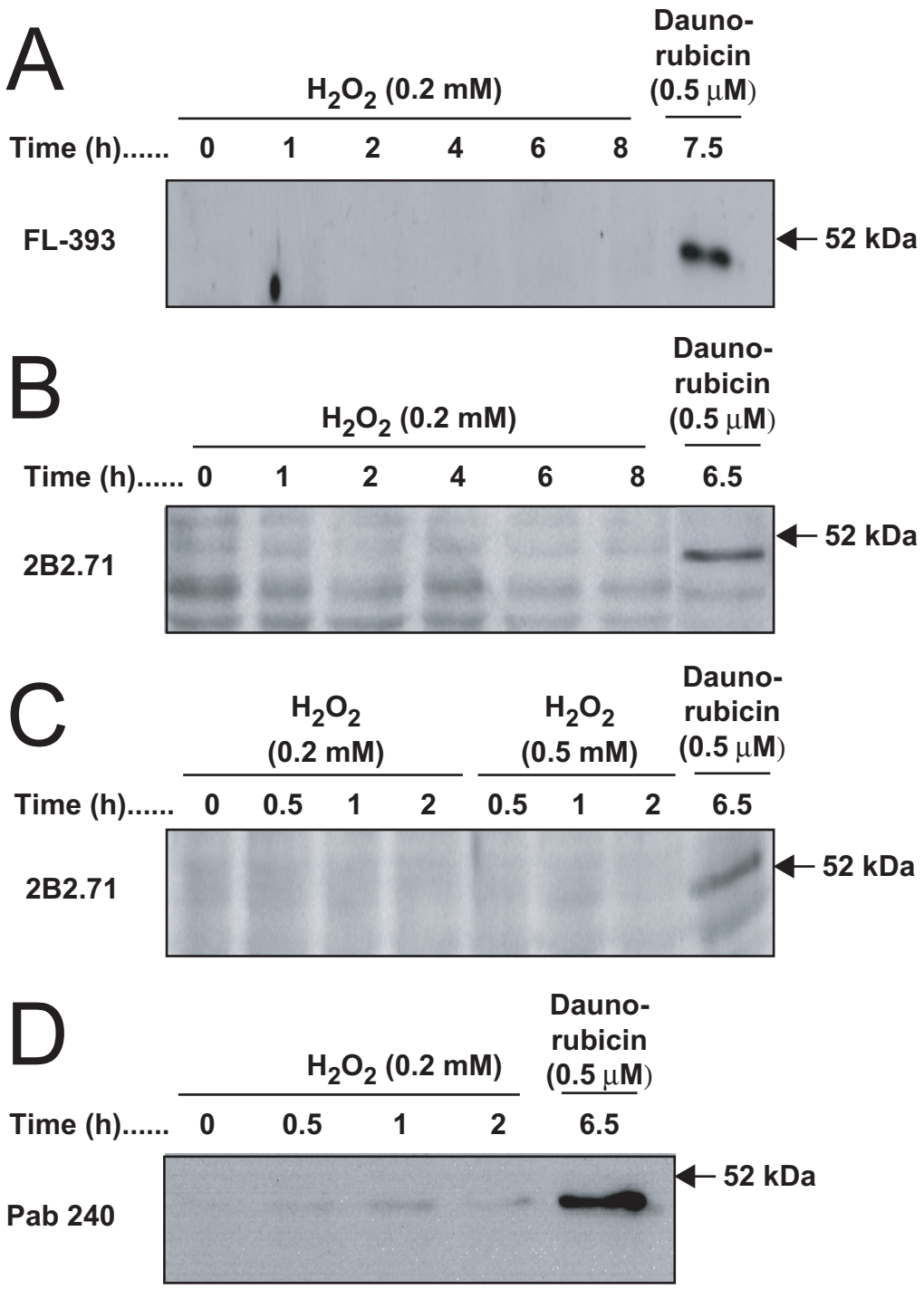
Pikkarainen et al. Figure 4

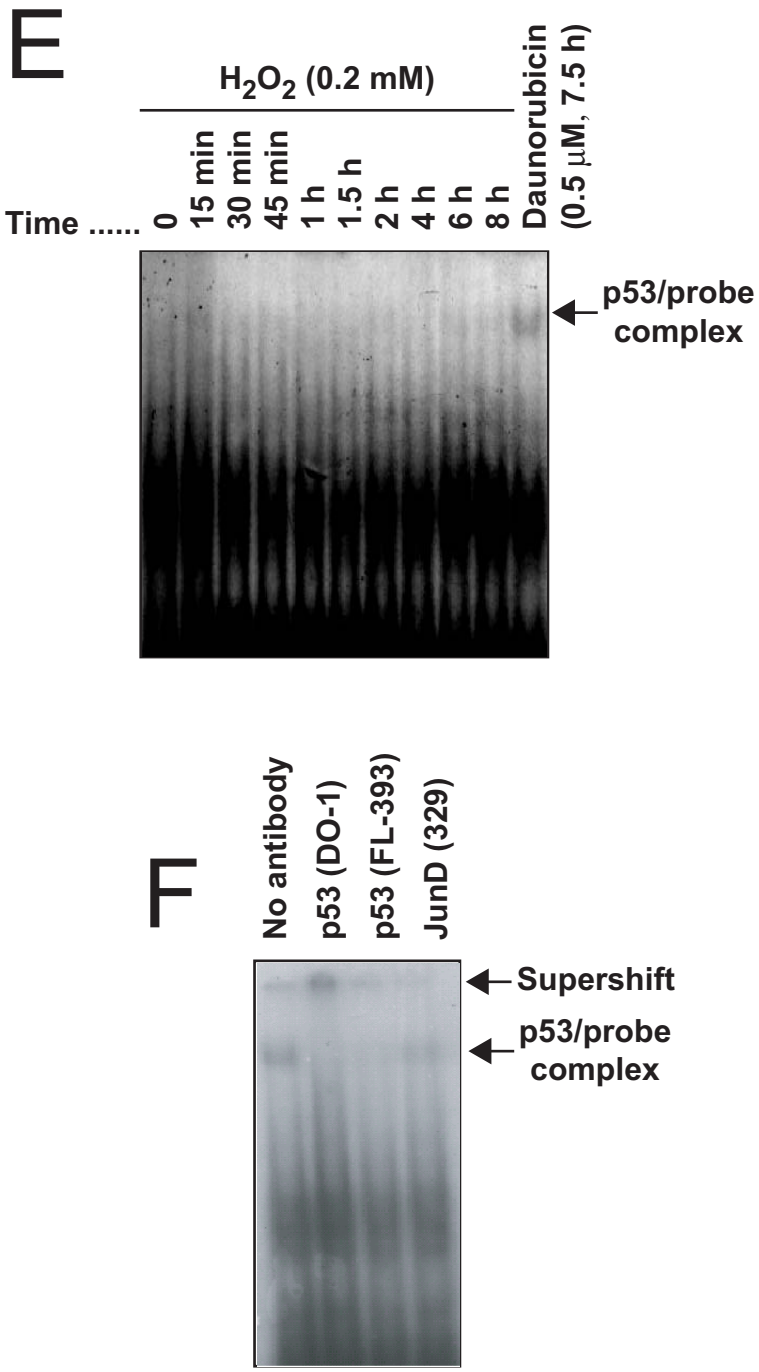




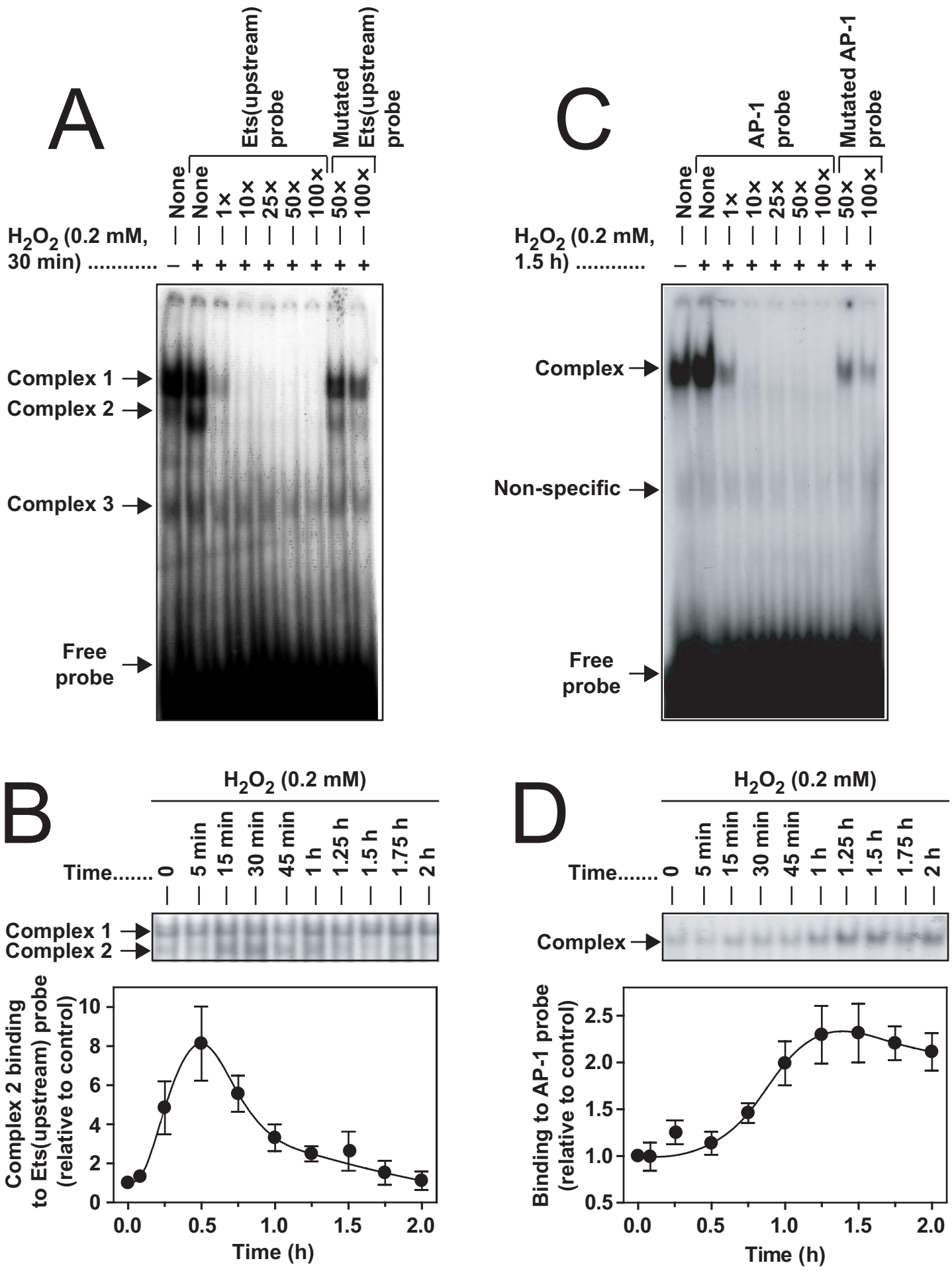
Pikkarainen et al. Figure 5



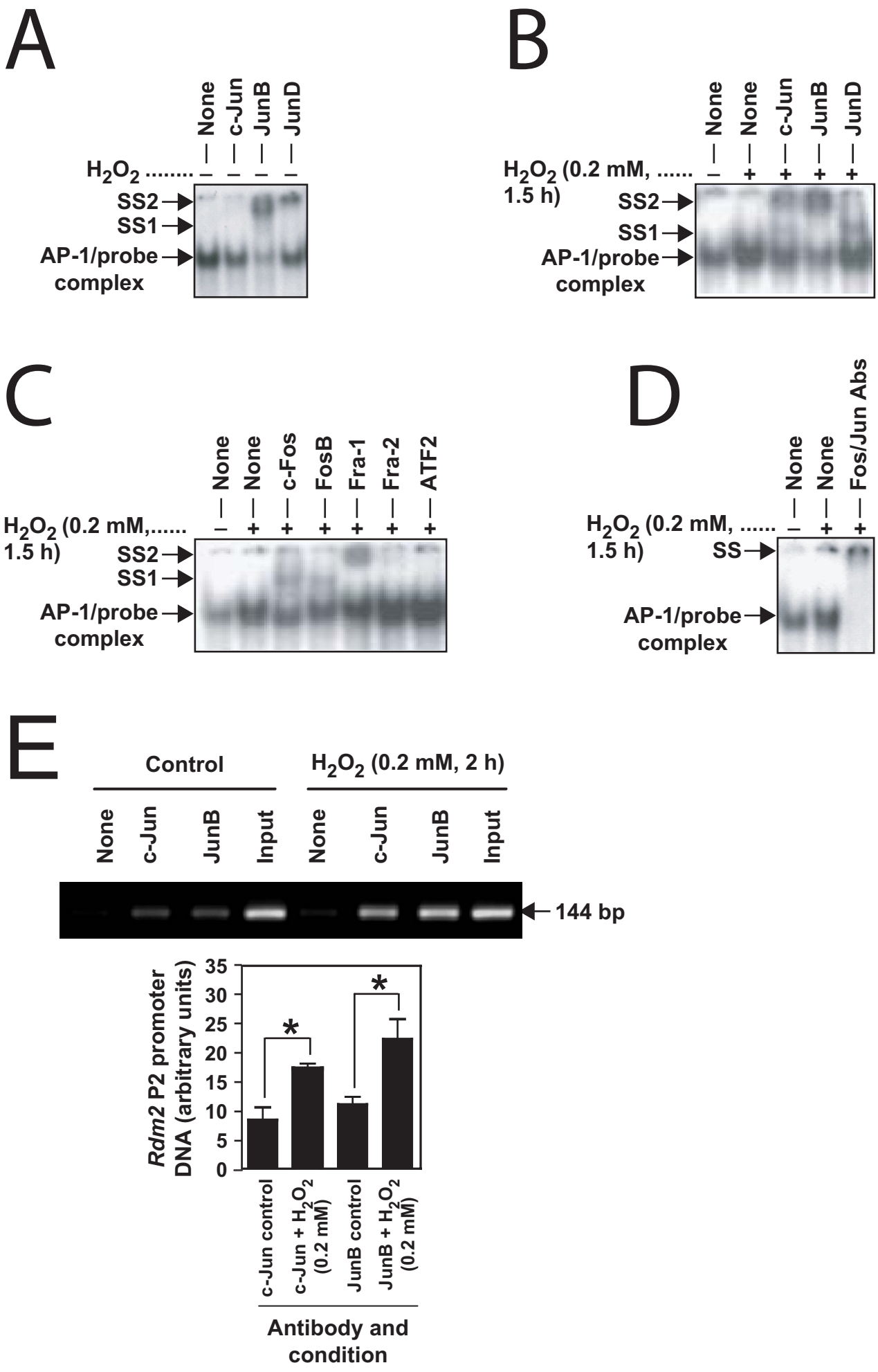

A
H_2O_2 (0.2 mM)
Dauno-rubicin (0.5 μM)
Time (h)..... 0 1 2 4 6 8 7.5
FL-393
52 kDa
B
H_2O_2 (0.2 mM)
Dauno-rubicin (0.5 μM)
Time (h)..... 0 1 2 4 6 8 6.5
2B2.71
52 kDa
C
H_2O_2 (0.2 mM)
H_2O_2 (0.5 mM)
Dauno-rubicin (0.5 μM)
Time (h)..... 0 0.5 1 2 0.5 1 2 6.5
2B2.71
52 kDa
D
H_2O_2 (0.2 mM)
Dauno-rubicin (0.5 μM)
Time (h)..... 0 0.5 1 2 6.5
Pab 240
52 kDa


E
H_2O_2 (0.2 mM)
Daunorubicin (0.5 μM, 7.5 h)
Time ..... 0 15 min 30 min 45 min 1 h 1.5 h 2 h 4 h 6 h 8 h
p53/probe complex
F
No antibody
p53 (DO-1)
p53 (FL-393)
JunD (329)
Supershift
p53/probe complex

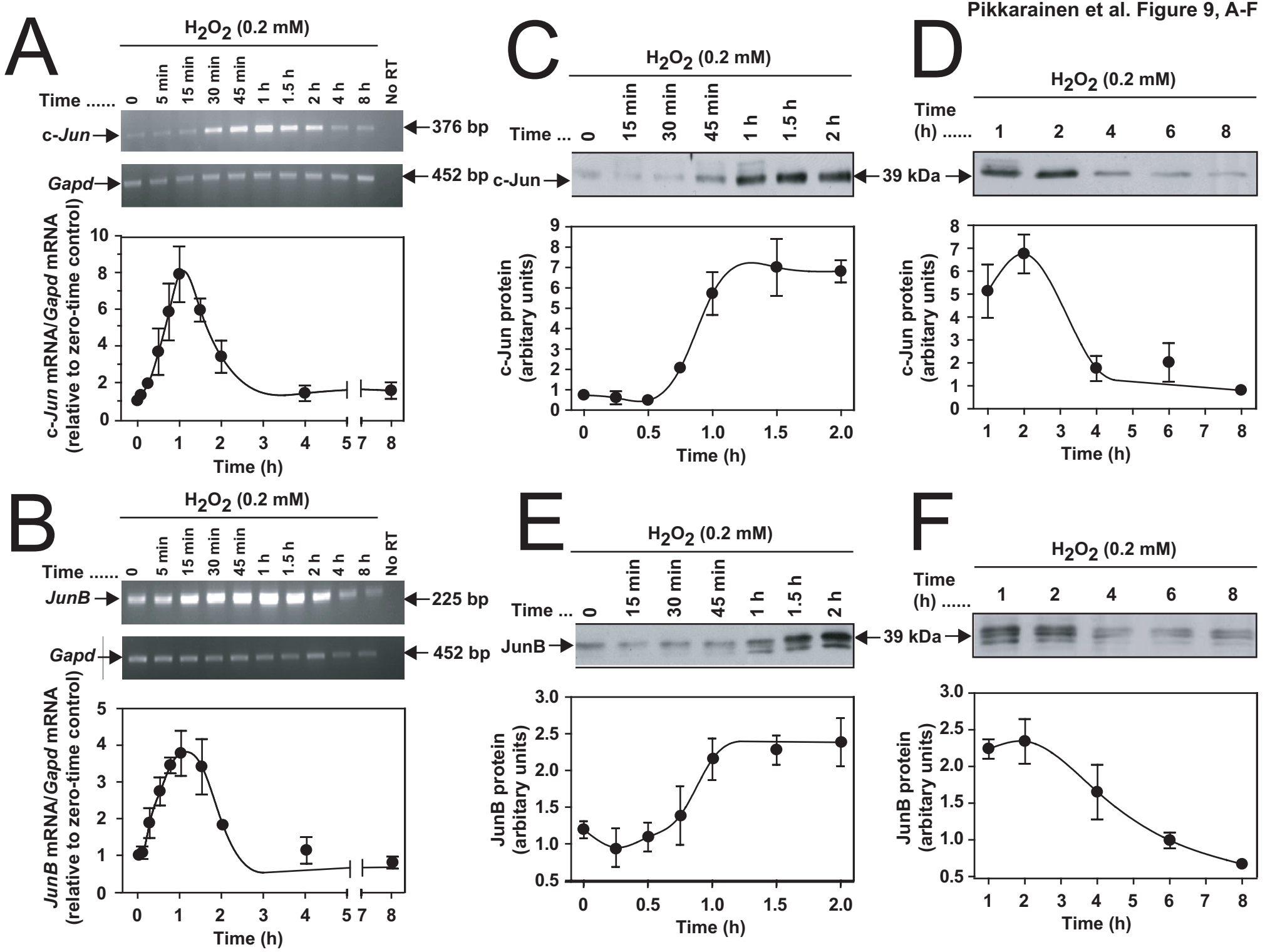
Pikkarainen et al. Figure 7



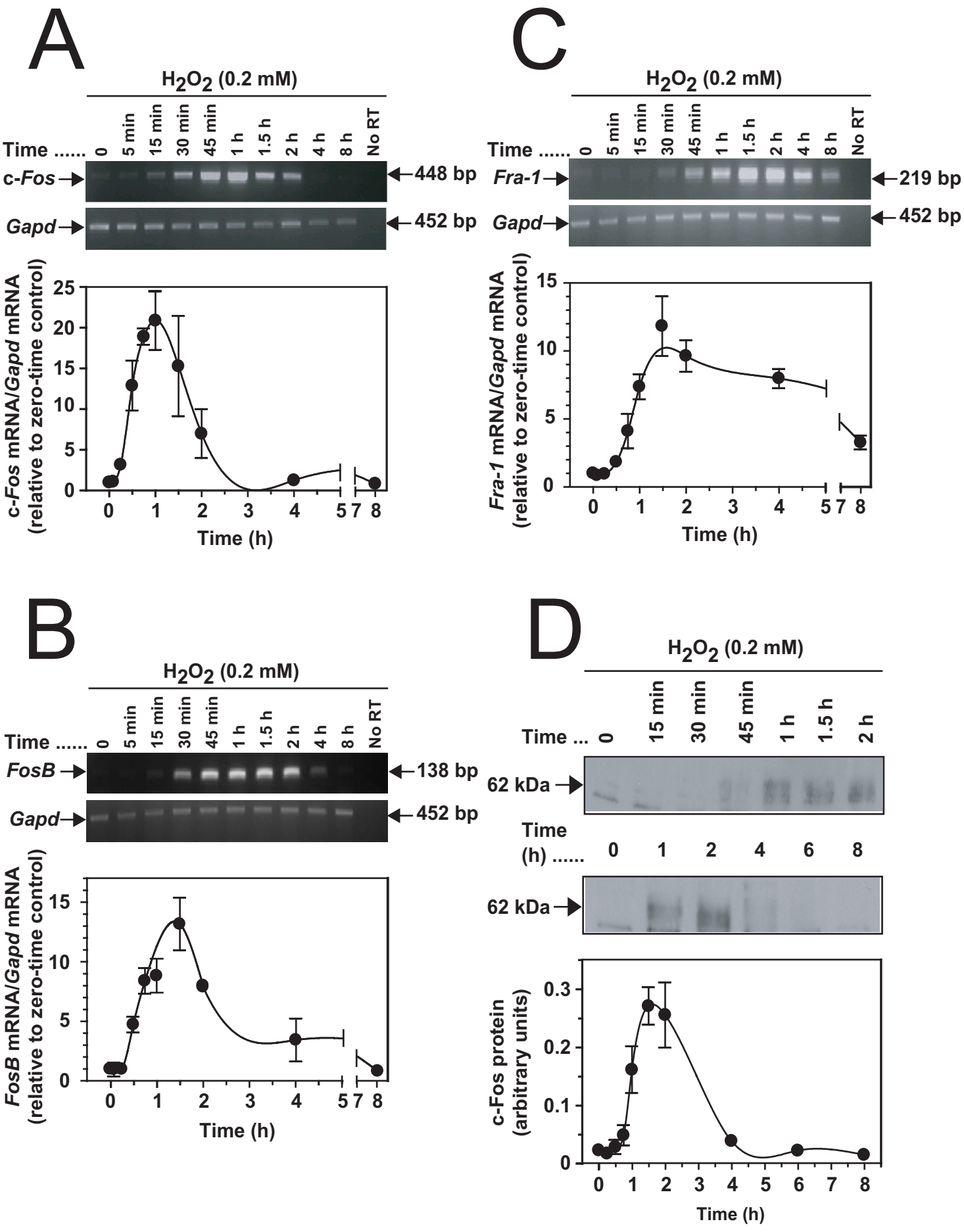
Pikkarainen et al. Figure 8



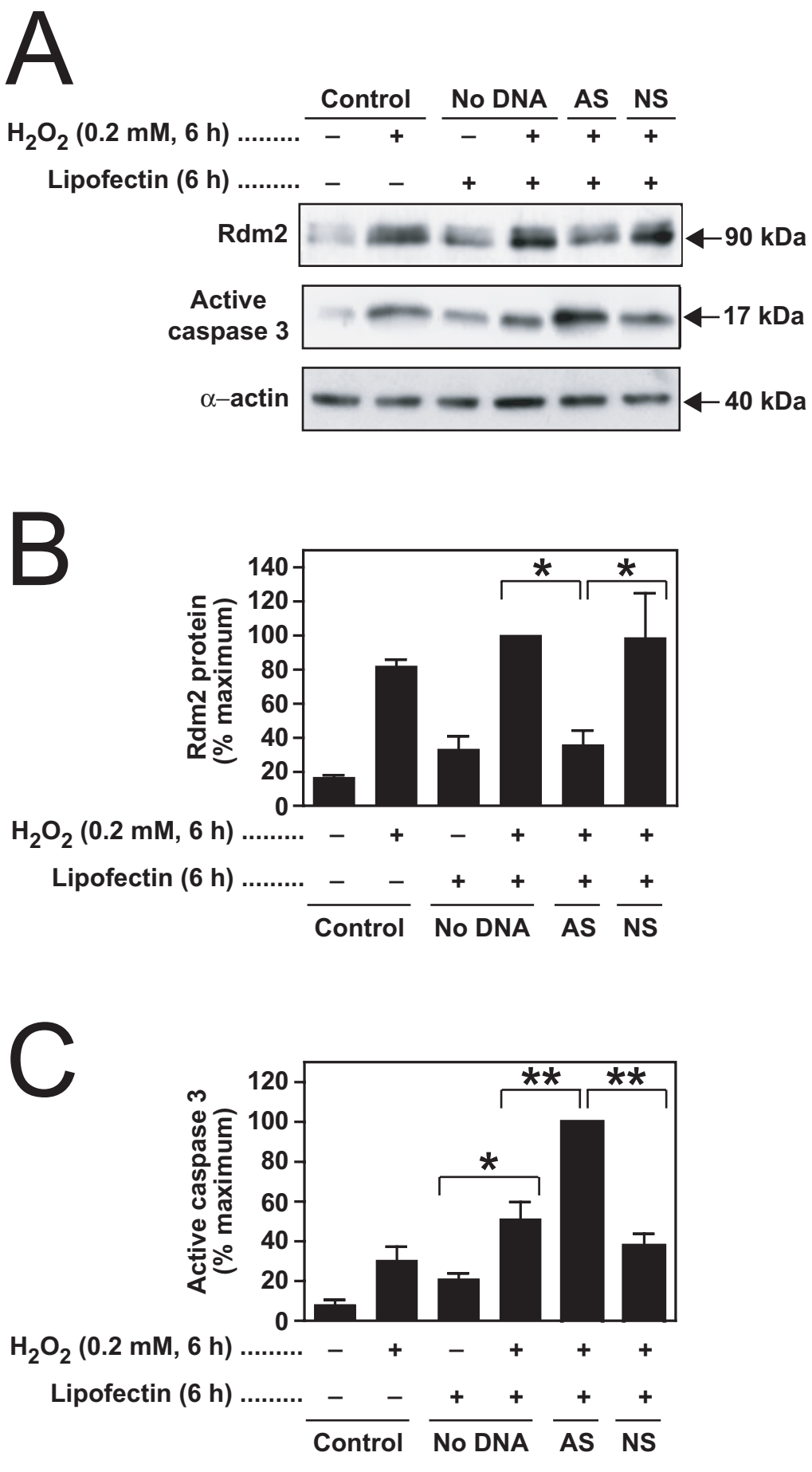
Pikkarainen et al. Figure 9, A-F



Pikkarainen et al. Figure 10



Pikkarainen et al. Figure 11



SUPPLEMENTAL MATERIAL

Supplemental Material FIG. 1. Alignment (ClustalW2) of the amino acid sequences of Mdm2 (NP_034916.1) and its human orthologue HDM2 (NP_002383.2) and the deduced amino acid sequence of the rat orthologue of Mdm2 (Rdm2) obtained from the predicted nucleotide sequence of the Rdm2 transcript (see Supplemental Material Fig. 2). The non-conserved N-terminal hexapeptide extension in HDM2, which is often ignored for the purposes of numbering HDM2 residues, is underlined.

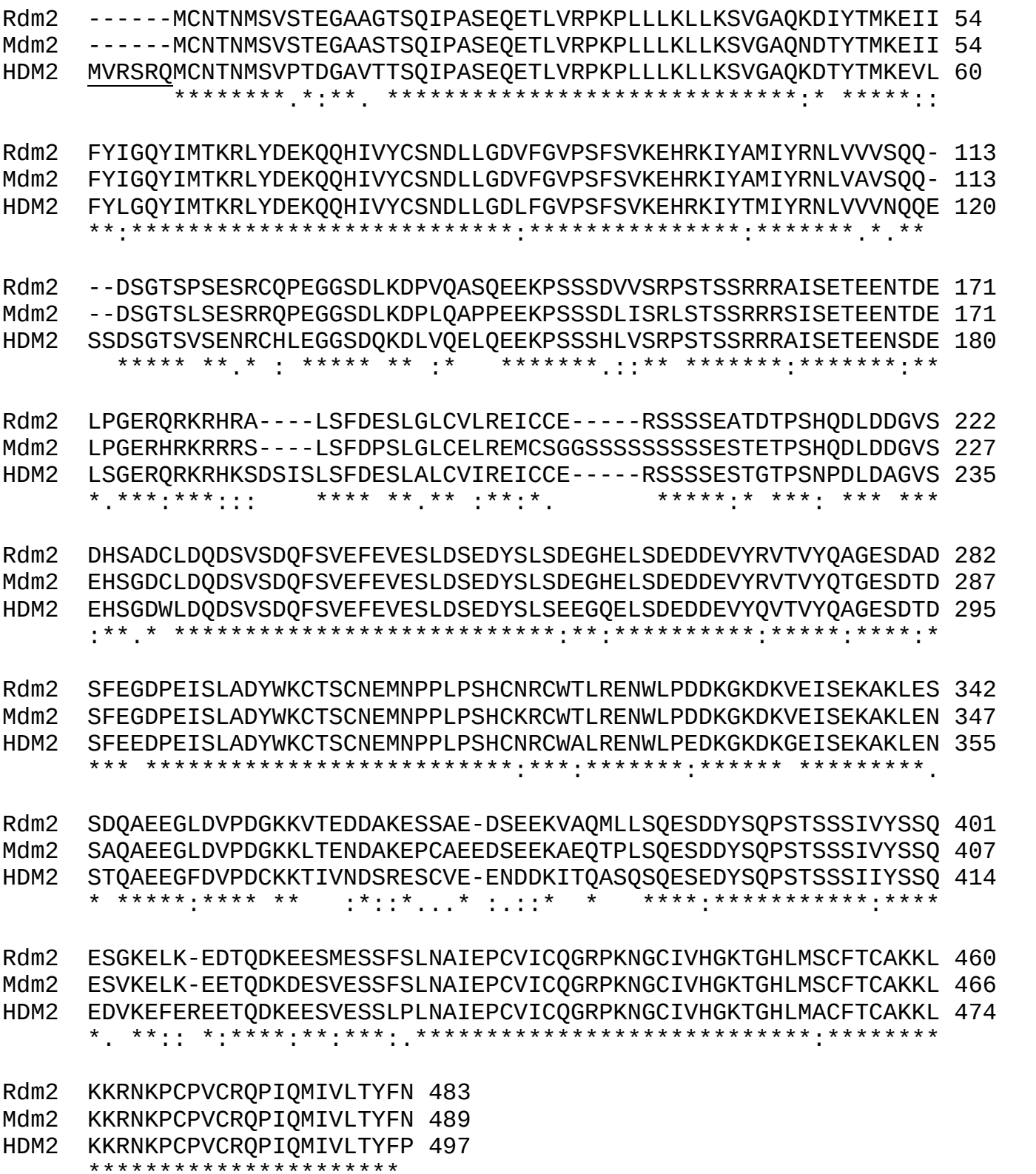
Supplemental Material FIG. 2. Alignment (ClustalW2) of the *Mdm2* transcript NM_010786.3 and the deduced sequence of the 2930 nucleotide *Rdm2* transcript. The genomic sequence (T in place of U) is shown. The transcript is encoded by 12 exons as follows: exon1, nucleotides 1-170; exon 2, nucleotides 171-243; exon 3, nucleotides 244-328; exon 4, nucleotides 329-403; exon 5, nucleotides 404-537; exon 6, nucleotides 538-587; exon 7, nucleotides 588-646; exon 8, nucleotides 647-743; exon 9; nucleotides 744-892; exon 10, nucleotides 893-1048; exon 11, nucleotides 1049-1126; exon 12, nucleotides 1127-2930. Exon boundaries are indicated by highlighting in light blue (start) and yellow (end). Primer sequences are annotated as given in Table 1. Other features are highlighted and annotated. The translational initial codon for p90-Rdm2 open reading frame (ORF) is at nucleotides 248-250 (in exon 3) and that for the p76-Rdm2 is at nucleotides 395-397 (in exon 4). In addition, there are two short upstream ORFs (uORFs) in exon 1. These are out-of-phase with the initiation codon of uORF2 overlapping with the termination codon of uORF1 in an ATGA sequence at nucleotides 138 to 141. The *Rdm2* termination codon is at nucleotides 1697-1699. There is a putative AU-rich instability signal at nucleotides 2072-2085 and a putative polyadenylation signal at nucleotides 2906-2911. ODN, oligodeoxynucleotide.

Supplemental Material FIG. 3. Alignment of intron 1 sequences from *Mdm2, Rdm2* and *HDM2*. Features are highlighted and annotated. The four response elements highly conserved in intron 1 of the *Rdm2*, *Mdm2* and *HDM2* genes (the two p53 response elements and the composite AP-1/Ets sequence) are encompassed by boxes. The features of the intron 1 regions are described in more detail in the full text. In addition to these, the *HDM2* intron 1 contains five non-conserved GGGGC repeats at bases 112-144 immediately upstream of an E-box (ACGTG) which are important in constitutive expression from the P2 promoter under some circumstances (1). RE, response element.

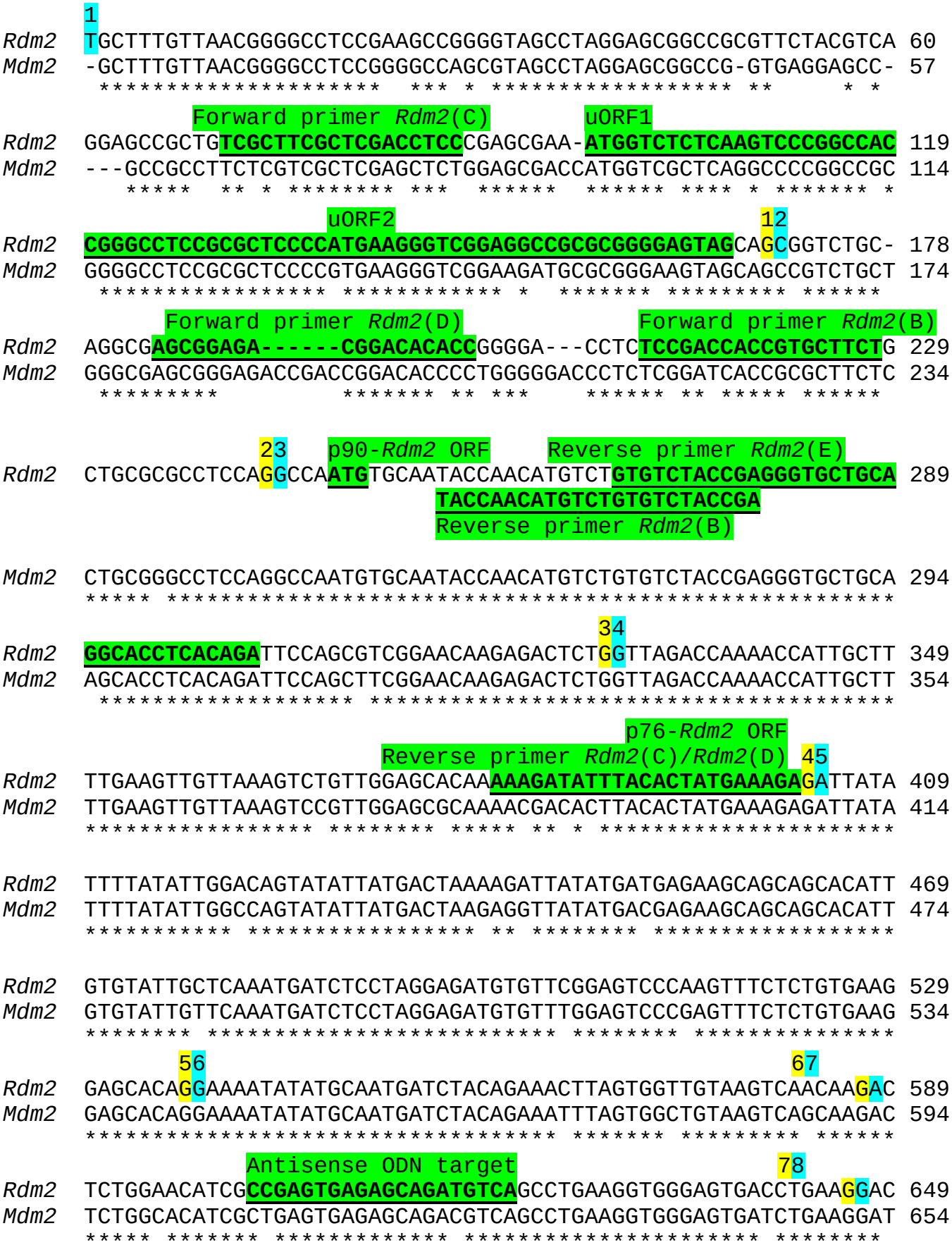
Reference

1. Phelps, M., Darley, M., Primrose, J. N., and Blaydes, J. P. (2003) *Cancer Res.* **63**, 2616-2623.

Supplemental Material Figure 1



Supplemental Material Figure 2



```
Rdm2   CCCGTGCAAGCATCACAAGAAGAGAAACCTTCATCTTCTGATGTAGTTTCTAGACCATCT 709
Mdm2   CCTTTGCAAGCGCCACCAGAAGAGAAACCTTCATCTTCTGATTTAATTTCTAGACTGTCT 714
       **  *******  *** ************************ ** *********  ***

                                          89
Rdm2   ACCTCATCTAGAAGGAGAGCAATTAGTGAAACAGAAGAGAACACAGATGAACTACCTGGG 769
Mdm2   ACCTCATCTAGAAGGAGATCCATTAGTGAGACAGAAGAGAACACAGATGAGCTACCTGGG 774
       ****************** * ******** ********************* *********

Rdm2   GAACGACAGAGGAAGCGCCACAGAGCCCTGTCCTTTGATGAGAGCCTGGGTCTGTGTGTG 829
Mdm2   GAGCGGCACCGGAAGCGCCGCAGGTCCCTGTCCTTTGATCCGAGCCTGGGTCTGTGTGAG 834
       ** ** **  ********* ***  ************** ***************** *

Rdm2   CTCAGGGAGATATGCTGTGAA---------------AGAAGCAGCAGCAGCGAGGCCACA 874
Mdm2   CTGAGGGAGATGTGCAGCGGCGGCAGCAGCAGCAGTAGCAGCAGCAGCAGCGAGTCCACA 894
       ** ******** *** * *                  ** *************** *****

                        910
Rdm2   GACACCCCCTCACATCAGGATCTTGATGATGGCGTAAGTGACCATTCTGCTGATTGCCTG 934
Mdm2   GAGACGCCCTCGCATCAGGATCTTGACGATGGCGTAAGTGAGCATTCTGGTGATTGCCTG 954
       ** ** ***** ************** ************** ******* **********

Rdm2   GATCAGGATTCAGTTTCTGATCAATTCAGTGTAGAATTTGAAGTTGAGTCTCTTGACTCA 994
Mdm2   GATCAGGATTCAGTTTCTGATCAGTTTAGCGTGGAATTTGAAGTTGAGTCTCTGGACTCG 1014
       ************************ ** ** ** ******************** *****

                                                          1011
Rdm2   GAAGATTACAGCCTGAGTGATGAAGGGCATGAGCTCTCAGATGAGGATGATGAGGTCTAT 1054
Mdm2   GAAGATTACAGCCTGAGTGACGAAGGGCACGAGCTCTCAGATGAGGATGATGAGGTCTAT 1074
       ******************** ******** ******************************

Rdm2   CGGGTCACAGTCTATCAGGCAGGAGAAAGCGATGCAGACTCTTTTGAGGGAGATCCTGAA 1114
Mdm2   CGGGTCACAGTCTATCAGACAGGAGAAAGCGATACAGACTCTTTTGAAGGAGATCCTGAG 1134
       ****************** ************** ************* ***********

             1112
Rdm2   ATTTCCTTAGCTGACTATTGGAAATGCACCTCGTGCAATGAAATGAATCCTCCCCTTCCA 1174
Mdm2   ATTTCCTTAGCTGACTATTGGAAGTGTACCTCATGCAATGAAATGAATCCTCCCCTTCCA 1194
       ********************** ** ***** ****************************

Rdm2   TCACACTGCAACAGATGTTGGACCCTTCGTGAGAACTGGCTTCCAGACGATAAGGGGAAA 1234
Mdm2   TCACACTGCAAAAGATGCTGGACCCTTCGTGAGAACTGGCTTCCAGACGATAAGGGGAAA 1254
       *********** ***** ****************************************

Rdm2   GATAAAGTGGAAATTTCTGAAAAAGCCAAACTGGAAAGCTCAGATCAGGCAGAAGAAGGC 1294
Mdm2   GATAAAGTGGAAATCTCTGAAAAAGCCAAACTGGAAAACTCAGCTCAGGCAGAAGAAGGC 1314
       ************** ********************** ***** ****************

Rdm2   TTAGATGTGCCTGATGGCAAAAAAGTGACAGAGGATGATGCTAAGGAGTCATCTGCTGAG 1354
Mdm2   TTGGATGTGCCTGATGGCAAAAAGCTGACAGAGAATGATGCTAAAGAGCCATGTGCTGAG 1374
       ** ********************  ******** ********** *** *** *******
```

```
Rdm2  GA---TAGCGAGGAAAAAGTGGCCCAGATGCTCCTGTCACAGGAGAGTGACGACTATTCC 1411
Mdm2  GAGGACAGCGAGGAGAAGGCCGAACAGACGCCCCTGTCCCAGGAGAGTGACGACTATTCC 1434
      **    ******** ** *  *  **** ** ****** **********************

Rdm2  CAGCCGTCGACTTCCAGTAGCATTGTTTACAGCAGCCAAGAAAGTGGCAAAGAGTTGAAG 1471
Mdm2  CAACCATCGACTTCCAGCAGCATTGTTTATAGCAGCCAAGAAAGCGTGAAAGAGTTGAAG 1494
      ** ** ********** ********** ************** *  ************

                                          Forward primer Rdm2(A)
Rdm2  GAGGACACACAAGACAAAGAGGAAAGTATGGAATCCAGCTTCTCTCTGAACGCCATCGAG 1531
Mdm2  GAGGAAACGCAGGACAAAGACGAGAGTGTGGAATCTAGCTTCTCCCTGAATGCCATCGAA 1554
      ***** ** ** ******** ** *** ******* ******** ***** ********
Rdm2  CCATGTGTGATTTGCCAGGGGCGGCCTAAAAATGGTTGCATTGTTCACGGCAAAACCGGA 1591
Mdm2  CCATGTGTGATCTGCCAGGGGCGGCCTAAAAATGGCTGCATTGTTCACGGCAAGACTGGA 1614
      ********** ********************** ***************** ** ***

Rdm2  CACCTGATGTCATGTTTCACGTGTGCAAAGAAGCTAAAGAAGAGGAATAAGCCCTGCCCC 1651
Mdm2  CACCTCATGTCATGTTTCACGTGTGCAAAGAAGCTAAAAAAAAGAAACAAGCCCTGCCCA 1674
      ***** ********************************** ** ** ** **********

                                                  Termination codon
Rdm2  GTGTGCAGACAGCCGATCCAAATGATTGTGCTCACGTACTTCAACTAGACGGCGGGC--- 1708
Mdm2  GTGTGCAGACAGCCAATCCAAATGATTGTGCTAACTTACTTCAACTAGCTGACCTGCTCA 1734
      ************** ***************** ** ************  * *  **

Rdm2  -AGACGCAGACCTTTACACTTCTAA-TGTATGACCCCCAAATTAGACAACATGGGTATTA 1766
Mdm2  CAAAAATAGAATTTTATATTTCTAACTATATGACCCCCAAATTAGACAACATGGGTATTA 1794
       * *   ***  **** * ****** * ********************************

                      Reverse primer Rdm2(A)
Rdm2  TTTTCATACATTAAAGCCGGAGCATTGTCTTAGTCTACATAAAGTTCATTTGTAATTTAT 1826
Mdm2  TTTTTATACATTAAAGCCAGAAAACTGTCTTAGTCCACATAAAATTCACTTATAATTTAT 1854
      **** ************ **  * ********** ******* **** ** ********

Rdm2  CCTCGAGAGTAAGAATAGTAACTGTTTTCTTCCTTTT-AGGAAAATTTCAGTTGATTATT 1885
Mdm2  CCTGGAGAGTAAATATGGTGAATATTTTCTTCCTTTTTAGGGAAATTTCACTTGTTTATT 1914
      *** ********  ** ** * * ************ *** ******** *** *****

Rdm2  TTATATTTGTGTTTTAACGTAATTTGCATTAGCTCTTTTCATTTTCCTTACGTTTTAAGT 1945
Mdm2  TTATATTTGTGTTTTAA-GTAATTTGCATTGGCTGTTTATATTTTCCTTATATTTTAAAT 1973
      ***************** ************ *** ***  **********  ****** *

Rdm2  AATCTCCACTTGGAAGGACTTTTGAAAGTATACTTTATACAGTGAGAAGTCCTCTCCATC 2005
Mdm2  AATCTCCGCTTGGAAGGACTTT-GGAAGTGTAT--------GTGAGAAGTCCTTTCCATC 2024
      ******* ************** * **** **          ************ ******

Rdm2  TCTGTCTCTTGGAGATA----------ATGATAGGCTATCGAATTGTGCTTGATTTCCTT 2055
Mdm2  TCCTGCAGATGATGGTGGACCTTCCTCATCAAGGGCTACAGAA--GTACTTGATTTCTGT 2082
      **   *   **  * *            ** *  *****  ***  ** *********  *

                        AU-rich instability element
Rdm2  TTTTTTCCTTGTTAAT------ATTTAATTTATTTAGTATCTTTCATGTAAAGAGTTAAA 2109
Mdm2  TTTTTTTGTTAATAATAAGAACATTTAATTTATTTAGTGTCTTTCATGTAAAGAGTTAAA 2142
      ******  **  ****      ********************* **********************
```

```
Rdm2  GACTATGTGAAGGATTGAGTGTATATTTAAGTTATTGAAATTCTGAACCTCCTTAGTTCT 2169
Mdm2  GACTATGTGAAGGAT----TGTATATTTAAGTTATTGAAATTCTGAAACTGTAGTAATCT 2198
      ***************    **************************** **       ***

Rdm2  CTTAAGTGGGGGTTGTGGGCTGCAGAGAAGAGTCAGCCGGTAAAGGCGCCCACTGTGTAT 2229
Mdm2  AAAATGTGTGAGTTGTGGGCTGCAGAGAAGACTCAGCCAGTAAAGGCACCTGCTGTGTAC 2258
         * *** * ******************** ****** ******** **  *******

Rdm2  GCCTAATGACACACATTTGATCCTTGCAACGCCCAGAAAGAGAGAACCAGTTCCACAAAG 2289
Mdm2  ACCCACTGACACACATTTGATCCTTGGAACCCCCAGGAAGAGTGAACCAGTTCCACAAAG 2318
       ** * ********************* *** ***** ***** ******************

Rdm2  TAGTTCTCGGATCTCCAAATGGATTCAT----------ATACACACTTTTTTTTT----- 2334
Mdm2  TTGTTCCCTGATCTTCAAATGGATGCACGCATGCACGCACACACACGCGCGCGTGCGCAC 2378
      * **** * ***** ********* **           * ******       *

Rdm2  ----------------------------------------------------TTTTTAAGGT 2344
Mdm2  ACACACACACACACACACACACACACACACACACACACACACACACACACAGTTTTAAAGGC 2438
                                                         **** ****

Rdm2  CTGAGTTGCATCTGGTGATACATAAGTGAAAACACACACCTTGTTTTCCAGC-------- 2396
Mdm2  ATGAATTGCATCTGGTGGTATGTAAGTGAAAACACACGCCTTATTTTCCAGCATTTTCAG 2498
       *** ************ **  *************** **** *********

Rdm2  -TTTTTGTCATATGGGGTGTGGCACGAGTGTTGCAGTCTGTCCCAGGTTGAAAAAGTCTG 2455
Mdm2  CTTTTTGTCATA-GGGGTGTGGCACAAGTGTTGCAGTTTGTCCCAGGTTGAAAA-GCCTG 2556
       *********** ************ *********** ***************** * ***

Rdm2  TGGCCCTTCGAAGCACCTTCATGGCCC-GCTCCATGGTTCCTGATGGCTGTTGAAGTTTC 2514
Mdm2  AGGCTGGTAGAAGCGCCTTTTTGCCTCAGCTCCGTGGTTCCTGGTGGTTGCCTATGTTTC 2616
       ***   * ***** ****  ** * * ***** ********* *** **   * *****

Rdm2  AGGCCTGTACTTAGTCTAGGTT-AGAAACCAGTCCATTCAGAAAGACTAAATCAGAGCAT 2573
Mdm2  AGGCCTGTACTTAGGCTAGGTTTAGAAACCAGCCCATTCAGAAAGACTGAATCAGAACAT 2676
      ************** ******* ********* *************** ******* ***

Rdm2  GGATGAAGTGGATCC----------------TCCAACCGTGTAGATGAGTCTCTTGATTC 2617
Mdm2  GGATAAAGTGAACTCATTCTAAGATGACTCGTCTATCCATGTAGATTAATCTCCTGGTTC 2736
      **** ***** *  *                ** * ** ******* * **** ** ***

Rdm2  ATAATAAGAATCTTCCATTTTAATTGAAGGGTCATGCCTAAGTGTAGAAAACAAGTTCCC 2677
Mdm2  ATAATAGGCCTCTTCC-CTTTGATTGAAGGGTCACGTCTAAGTATAGAAAACATAAAACT 2795
      ****** *  ******  *** ************ * ****** *********     *

Rdm2  TAACTGTATGGAGTAACCAAAGGATAGTTCTGTATTGAAGTTGACTTAAAGTATCAAAGA 2737
Mdm2  GTAAGGTA--GAGGAAGCGAAGGATAGCTTTGTATTAATGTTGCGTTAAAGCTTCAGAGA 2853
        *  ***  *** ** * ******** * ****** * ****  ******  *** ***
```

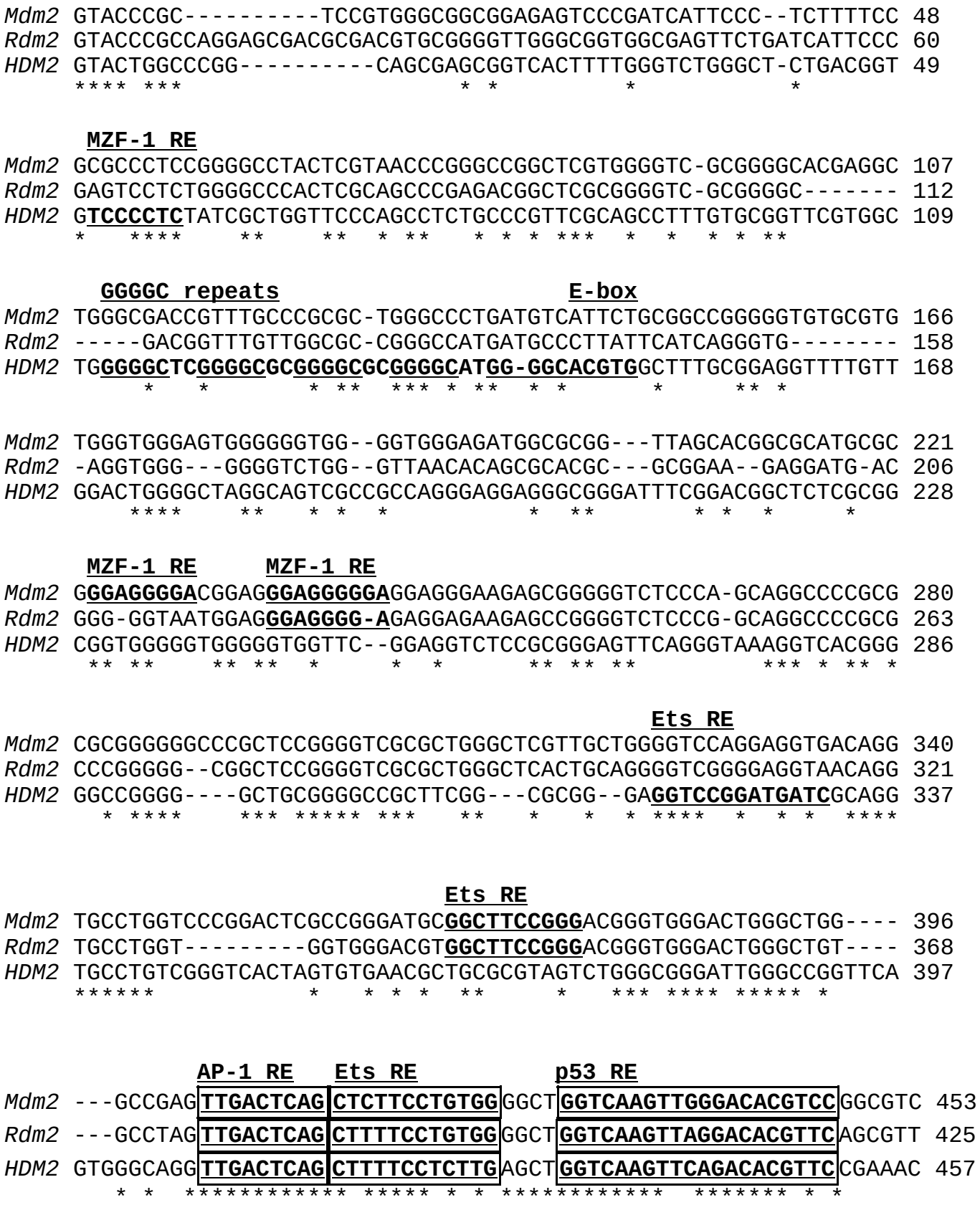
```
Rdm2  T----------CACTCCCCACATGATTTAGCAGTTAAGTCGGAGGCAGCGCCTCAGCCT 2786
Mdm2  CAAGAACAAGAACACTCCTCCCACGTGACAGCATTTGAATAGGAGGCGGTG-----GGTG 2908
                ****** * ** *    **** ** * * ****** * *     *

Rdm2  GGGCATCCTAACTCTGGACTTTGATTCCCCATTTACAATGTAATACTGAGGTTATATCCT 2846
Mdm2  CGGCAGCCTGG----GCAGCTTCAGTCCCGATTTACAATAAAGTACC----TTGT----- 2955
       **** ***      * *  ** * **** *********  * ***     ** *

Rdm2  AGTATTACTAGTTCTTAAATGTTTATTTAGGTGTGGTAGACATACATTATTTATTTGCAA 2906
Mdm2  -GTGTTATTAGTTCTTAAATGTTTATTTAGAAATGGCATTGATG--TTATTTATTTGCAA 3012
       ** *** **********************   *** *   **   **************

Polyadenylation signal      12
Rdm2  ATAAATGGTTTATTGAATTGTTTC 2930
Mdm2  ATAAATGGTTTATTG--------- 3027
      ***************
```

Supplemental Material Figure 3



```
                  p53 RE
Mdm2 GGCTGTCGGAGGAGCTAAGTCCTGACATGTCTCCAGCTGGGGTTATTTAAACGCTGCCCC 513
Rdm2 GGCTGTTGGAGGAGCTAAGTCCTGACATGTCTCCAGCTGAGGCTATTTAAACCCTGCTC- 484
HDM2 TGCAGTAAAAGGAGTTAAGTCCTGACTTGTCTCCAGCTGGGGCTATTTAAACCATGCATT 517
      ** **   ***** ********** ************ ** *********  ***

Mdm2 GTTTCCGCAG---------------------------------------------------- 523
Rdm2 GTTTCCGCAG---------------------------------------------------- 494
HDM2 --TTCC-CAGCTGTGTTCAGTGGCGATTGGAGGGTAGACCTGTGGGCACGGACGCACGCC 574
       **** ***

Mdm2 ------------------------------------------------------------
Rdm2 ------------------------------------------------------------
HDM2 ACTTTTTCTCTGCTGATCCAGGTAAGCACCGACTTGCTTGTAGCTTTAGTTTTAACTGTT 634


Mdm2 ------------------------------------------------------------
Rdm2 ------------------------------------------------------------
HDM2 GTTTATGTTCTTTATATATGATGTATTTTCCACAGATGTTTCATGATTTCCAGTTTTCAT 694


Mdm2 ----------------------
Rdm2 ----------------------
HDM2 CGTGTCTTTTTTTTCCTTGTAG 716
```