The Roles of P53 and Its Family Proteins, P63 and P73, in the DNA Damage Stress Response in Organogenesis-Stage Mouse Embryos

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ABSTRACT

Members of the P53 transcription factor family, P53, P63, and P73, play important roles in normal development and in regulating the expression of genes that control apoptosis and cell cycle progression in response to genotoxic stress. P53 is involved in the DNA damage response pathway that is activated by hydroxyurea in organogenesis-stage murine embryos. The extent to which P63 and P73 contribute to this stress response is not known. To address this question, we examined the roles of P53, P63, and P73 in mediating the response of *Trp53*-positive and *Trp53*-deficient murine embryos to a single dose of hydroxyurea (400 mg/kg) on gestational day 9. Hydroxyurea treatment downregulated the expression of *Trp63* and upregulated *Trp73* in the absence of effects on the levels of *Trp53* transcripts; *Trp73* upregulation was P53-dependent. At the protein level, hydroxyurea treatment increased the levels and phosphorylation of P53 in the absence of effects on P63 and P73. Upregulation of the expression of genes that regulate cell cycle arrest and apoptosis, *Cdkn1a, Rb1, Fas, Trp53inp1*, and *Pmaip1*, was P53-dependent in hydroxyurea-treated embryos. The increase in cleaved caspase-3 and cleaved mammalian sterile-20-like-1 kinase levels induced by hydroxyurea was also P53-dependent; in contrast, the increase in phosphorylated H2AX, a marker of DNA double-strand breaks, in response to hydroxyurea treatment was only partially P53-dependent. Together, our data show that P53 is the principal P53 family member that is activated in the embryonic DNA damage response.

Key words: P53 family proteins; hydroxyurea; teratogen; embryonic stress; developmental toxicity; birth defects.

Hydroxyurea (HU), a drug used to treat sickle cell anemia and cancer, inhibits ribonucleotide reductase, causing DNA-strand breaks as a result of replication fork stalling, leading to cell cycle arrest and apoptosis (Kovacic, 2011). In mice, exposure to HU during organogenesis causes DNA damage (Banh and Hales, 2013) and leads to gross and skeletal malformations in the fore- and hind-limbs, tail and craniofacial regions (Yan and Hales, 2005). Doses of HU that are teratogenic in organogenesis-stage murine embryos increase P53 phosphorylation and activate the P53 signaling pathway (El Husseini et al., 2016); using microarray and pathway analysis, we demonstrated that genes that control DNA damage repair, cell cycle arrest and apoptosis are upregulated. Fitting to its proposed role as a "Guardian of the Genome," previous studies have reported that P53 activation protects the developing conceptus against congenital

malformations after exposure to DNA damaging agents such as benzo[a]pyrene, cyclophosphamide, or radiation (Mikheeva et al., 2004; Nicol et al., 1995; Norimura et al., 1996). Nevertheless, the ocular teratogenic effects of 2-chloro-2′-deoxyadenosine were reduced in P53 null transgenic mice (Wubah et al., 1996). It is clear that the role of P53 in mediating how embryos respond to a stressor is complex.

P53 is a member of a family of proteins that includes the homologs P63 and P73 (Levrero et al., 2000). Each of the P53 family proteins has unique functions during embryonic development. Under normal conditions, P53 expression is widespread until gestation day (GD) 11 in organogenesis-stage mouse embryos but it is largely transcriptionally inactive; at later stages of development, P53 expression is restricted to differentiating tissues, such as specific regions of the brain

(Choi and Donehower, 1999; Molchadsky et al., 2010). P63 is essential for proper limb and epithelial development, while P73 is involved in the development of the nervous and immune systems (Yang et al., 1999, 2000). Both P63 and P73 have numerous isoforms which have been reported to have divergent and even antagonistic functions (Levrero et al., 2000). P63 and P73 each have 2 transcription start sites which produce protein isoforms with transactivation domains (TAs), or with truncated N-termini (ΔN) that lack the TA domain (Murray-Zmijewski et al., 2006). Each of these isoforms also has several splice variants (P63: α-γ, P73: α-ζ) (Levrero et al., 2000); the functions of these isoforms during development are not well-understood. The TA isoforms of P63 and P73 exhibit significant functional homology to P53 in response to stress stimuli, including DNA damage (Levrero et al., 1999; Yang et al., 2002).

Because the DNA binding domains of P63 and P73 share considerable homology to that of P53 they bind to many P53 DNA binding sites and upregulate a number of the same downstream target genes (Riley et al., 2008). All 3 P53 family proteins are activated in response to various stress stimuli and act as transcription factors, translocating from the cytoplasm to the nucleus and upregulating the expression of transcripts that are involved in regulating cell cycle arrest (eg, Cdkn1a, Rb1) and apoptosis (eg, Fas, Pmaip1, Trp53inp1) (Costanzo et al., 2002; Kruse and Gu, 2009; Petitjean et al., 2008). Furthermore, under certain stress conditions, the ability of P53 to promote apoptosis appears to rely on the activation of P63 and P73 (Fatt et al., 2014; Flores et al., 2002). However, there are some gene targets that are unique to each family member (Fontemaggi et al., 2002; Wu et al., 2003; Yang et al., 2010). Trp53inp1, a direct downstream transcriptional target of P53, is involved in mediating apoptosis and the response to oxidative stress (Seillier et al., 2012). Although they are not direct targets, the expression levels of Pax9 and Tbx5, which are involved in limb and skeletal development, have been negatively correlated with the upregulation of P63 and P73, respectively (Holembowski et al., 2014; Wang et al., 2011). In addition, Jag2 expression is upregulated directly by P63 and P73 but is either mildly upregulated or not upregulated at all by P53 (Sasaki et al., 2002; Wu et al., 2003).

One of the main mechanisms by which P53 family proteins induce apoptosis is by activation of the caspase cascade. All 3 transcription factors are capable of inducing proapoptotic factors (eg, Pmaip1, Puma, Bax, Bak) which interact with the mitochondrial membrane to release cytochrome c into the cytoplasm and activate the caspase cascade (Borrelli et al., 2009; Chipuk et al., 2004; Gong et al., 1999; Gressner et al., 2005; Jones et al., 2007; Schuler et al., 2000). This involves the promotion of procaspase-3 cleavage into its proteolytic form, cleaved caspase-3, the effector caspase that drives the process of apoptotic cell death by the specific cleavage of key cellular proteins (Porter and Janicke, 1999). One of the targets of caspase-3 is the mammalian sterile-20-like-1 (MST-1) kinase (Lee et al., 2001). Mammalian sterile-20-like-1 is a serine/threonine kinase that is involved in promoting DNA damage repair and apoptosis (Pefani and O'Neill, 2016); after cleavage by caspase-3, MST-1 translocates to the nucleus where it phosphorylates the tail of the H2AX histone variant on Ser139, contributing to the formation of γH2AX foci (Teraishi et al., 2006). These foci are important for the modification of chromatin structure and the recruitment of the DNA damage repair machinery (eg, the MRE11/RAD50/NBS1 complex) required to repair DNA double-strand breaks (Furuta et al., 2003; Rogakou et al., 2000). Furthermore, γH2AX foci are essential for nuclear condensation and DNA fragmentation that are hallmarks of the commitment of a cell to apoptosis (Wen et al., 2010). Previously,

it has been shown that HU exposure increases the levels of γH2AX foci in the caudal regions of the organogenesis-stage embryo (Banh and Hales, 2013). The contributions of P53, P63, and P73 to the activation of caspase-3 and MST-1 and the increase in γH2AX foci that is triggered in the organogenesis-stage embryo by DNA damage remain to be determined.

Although genotoxic agents and oxidative stress have been reported to increase the protein levels of both P63 and P73 in in vivo and in vitro models (Flores et al., 2002; Gonfloni et al., 2009; Klein et al., 2011; Petitjean et al., 2008), the contributions of these proteins in the response of embryos to DNA damage is not known. Here, we elucidated the impact of exposure to HU on the activation of P63 and P73 in early embryos in the presence and absence of P53.

MATERIALS AND METHODS

Experimental animals. Trp53 transgenic (B6.129S2-Trp53tm1Tyj/J) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and housed in the McIntyre Animal Resource Centre (Montreal, QC, Canada). Animal treatments were conducted in accordance with the guidelines outlined in the Canadian Guide to the Care and Use of Experimental Animals. To produce timed pregnant mice heterozygote females were mated with heterozygote males overnight; the mice were separated at 10 AM the following day (designated as GD 0). Between 8 and 10 AM on GD 9 pregnant dams were treated with either saline (control) or 400 mg/kg HU (Aldrich Chemical Co, Madison, Wisconsin) by intraperitoneal injection.

All dams were euthanized by CO_2 asphyxiation and cervical dislocation 3 h after HU treatment. Tail tips were taken for DNA extraction and genotyping. The uteri were removed and embryos were explanted in Hanks' balanced salt solution (Invitrogen Canada, Inc, ON, Canada). At the time of collection, single whole embryos representing each genotype from every litter were separated for future sample processing for genotyping, Western blot analysis, and real-time Quantitative Reverse Transcriptase (qRT)-PCR experiments.

DNA, RNA, and protein extraction from single embryos. Embryos were placed in RNAlater Stabilization Reagent (Qiagen, Mississauga, ON, Canada) at the time of collection and stored at −80°C. RNA, DNA, and protein were extracted from single embryos using the RNeasy Plus Mini Kit and the DNeasy Blood and Tissue Kit with a modified protocol (Qiagen). RNA and DNA were extracted using the manufacturer's protocol and the concentration and purity of each sample were assessed by spectrophotometry using a NanoDrop1000 spectrophotometer (Fisher Scientific, Wilmington, Delaware). Proteins were precipitated from the remaining embryonic lysate using ice-cold 100% acetone and dissolved in protein lysis buffer (8 M urea, 2 M thiourea, 0.5% [3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate]). Total protein content from each sample was quantified using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Ltd, Mississauga, ON, Canada).

Genotyping by high resolution melt-curve analysis. DNA from individual embryos was extracted as described earlier for genotyping. Tail tip samples obtained from adult male and female mice were used to extract DNA using a modified version of the ethanol precipitation method (Zeugin, 1985). Briefly, tissue samples were kept overnight at 55°C in 250 μl of DNA extraction buffer (50 mM Tris-HCL, 10 mM EDTA, 100 mM NaCl, 1% SDS) and 0.4 mg/ml proteinase K (Sigma-Aldrich, St Louis, Missouri). Samples were then centrifuged at 20 000 × g for 10 min to

remove fur and debris, followed by the addition of 250 µl 100% ethanol to the supernatant. Lastly, the samples were centrifuged to isolate the precipitated DNA, the ethanol was discarded and the pellet left to air dry. The DNA was then dissolved in distilled water and the concentration was determined using a NanoDrop1000 spectrophotometer (Fisher Scientific).

The Power SYBR Green RNA-to-C_T 1-Step Kit (Applied Biosystems, Foster City, California) and the StepOnePlus Real-Time PCR System (Applied Biosystems) were used to do high resolution melt-curve analysis to determine the zygosity of adult and embryonic DNA samples (Pryor and Wittwer, 2006). Each reaction was composed of 5 µl of 25 ng/µl DNA mixed with 10 µl SYBR Green Master Mix, 0.3 µl of each of the 10 µM forward and reverse primers, and completed to a total reaction volume of 20 µl with DNase and RNase free water. Samples were run in single-plex for each allele. Temperature cycling was determined per the supplier's recommended protocol: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Subsequently, the melt-curve stage was as follows: 95°C for 15 s, 60°C for 1 min, followed by incremental increases of 0.3°C up to 95°C. The peak melting temperatures for the mutant and wild-type amplicons matched those of the supplier (approximately 78°C and 84°C, respectively). The sequences of the forward and reverse primers for the wild type and mutant alleles of *Trp53* were provided by the supplier (wild type forward: AGGCTTAGA GGTGCAAGCTG; mutant forward: CAGCCTCTGTT CCACATACACT; common reverse: TGGATGGTGGTATACT CAGAGC) and were synthesized by Alpha DNA (Montreal, QC, Canada).

Western blotting using SDS-PAGE and Phos-tag gels. Single embryos from across all treatment groups and genotypes were used for the detection and quantification of proteins by Western blot analysis. The phosphorylated forms of proteins were detected using Phos-tag acrylamide gel electrophoresis (Waco Chemicals USA Inc, Richmond, Virginia). Proteins (10 µg) were loaded and separated on 10% SDS-PAGE gels. Phos-tag gels were prepared in the same manner as for regular acrylamide gels but with the addition of 20 µM Phos-tag and 20 µM $MgCl_2$. A negative control for the Phos-tag gel was done by incubating 20 µM Phos-tag and 20 µM $MgCl_2$ with 10 mM EDTA before preparing the acrylamide gel. After gel electrophoresis, Phos-tag gels were washed twice in 10 mM EDTA for 5 min, and twice in transfer buffer. Proteins from regular and Phos-tag gels were then transferred to PVDF membranes at 14 volts overnight at 4°C (Bio-Rad Laboratories, Ltd). Afterwards, membranes were blocked with 5% milk in 1X tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature. Membranes were incubated overnight, at 4°C, with primary antibodies against P53 (1:1000, cs2524, Cell Signaling Technology, Inc, Danvers, Massachusetts), P63 (1:1000, PA121739, Thermo Fisher Scientific, Waltham, Massachusetts), P73 (1:1000, ab189896, Abcam), cleaved caspase-3 (1:1000, cs9661, Cell Signaling Technology, Inc), γH2AX (1:1000, cs9718, Cell Signaling Technology, Inc), MST-1 (1:1000, cs3682, Cell Signaling Technology, Inc), or actin (1:1000, sc-1616, Santa Cruz Biotechnology, Texas). Membranes were washed 3 times for 5 min intervals with 1X TBS-T and then incubated for 1 h at room temperature with horseradish peroxidase conjugated anti-rabbit (1:5000, cs7074, Cell Signaling Technology, Inc), anti-mouse (1:5000, cs7076, Cell Signaling Technology, Inc), or anti-goat (1:5000, sc-2020, Santa Cruz Biotechnology) secondary antibodies.

Membranes were washed and proteins were detected using the enhanced chemiluminescence technique (ECL Prime, RPN2236, GE Healthcare). Protein bands were visualized using the Amersham Imager 600 (General Electric Healthcare, Mississauga, ON, Canada) and quantified using ImageJ software (National Institutes of Health, Maryland) where the area under the curve represents band intensity. All intensities were normalized to that of the actin loading control. An internal control was run on each blot to account for interblot variation.

Real-time qRT-PCR. RNA extracted from individual embryos was diluted to a working solution of 4 ng RNA/µl and transcripts were quantified using the Power SYBR Green RNA-to-C_T 1-Step Kit (Applied Biosystems) and the StepOnePlus Real-Time PCR System (Applied Biosystems). Each reaction was composed of 10 µl SYBR Green Master Mix, 1–2 µl forward/reverse primer, 0.16 µl Reverse Transcriptase mix, 5 µl sample and completed to 20 µl with RNase-DNase-free water. The PCR reactions were conducted under the following conditions: 48°C for 30 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The following primer sets were purchased from Qiagen: transformation related protein 53 (*Trp53*, QT00101906); transformation related protein 63 (*Trp63*, QT00197904); transformation related protein 73 (*Trp73*, QT00123487); cyclin-dependent kinase inhibitor 1A (*Cdkn1a*, QT00137053); Fas cell surface death receptor (*Fas*, QT00095333); phorbol-12-myristate-13-acetate-induced protein 1 (*Pmaip1*, QT00163506); retinoblastoma 1 (*Rb1*, QT00164255); transformation related protein 53 inducible nuclear protein 1 (*Trp53inp1*, QT00112910); jagged 2 (*Jag2*, QT01043819); paired box 9 (*Pax9*, QT00110040); T-box transcription factor 5 (*Tbx5*, QT00124362); and hypoxanthine phosphoribosyltransferase 1 (*Hprt1*, QT00166768). Serial dilutions of embryonic RNA, pooled from all treatment groups and genotypes, were used as an internal reference and to create a standard curve for optimizing primer efficiency and concentration. Each reaction was done in triplicate, and was averaged and normalized to the amounts of *Hprt1* RNA transcripts. The levels of *Hprt1* were found to be stable in all treatment groups. The relative quantity of each transcript was determined using the StepOnePlus Software (version 2.3).

Statistical analyses. All data were analyzed using GraphPad Prism Software (version 5, Graph Pad Software Inc, La Jolla, California). Data were tested by 2-way ANOVA, followed by a Bonferroni *post hoc* multiple comparison correction, to detect statistical differences between the control and HU-treated groups and the different genotypes. Three embryos were evaluated per genotype treatment group in all experiments. The litter was used to define the statistical unit (*n*). The level of significance for all statistical tests was set to $P < .05$.

RESULTS

Hydroxyurea Exposure Affects the Transcript Levels of Trp63 and Trp73 but not Trp53

Hydroxyurea treatment did not affect the transcript levels of *Trp53* in *Trp53*$^{+/+}$ embryos (Figure 1A). As expected, the levels of *Trp53* were 50% lower in the heterozygote embryos (*Trp53*$^{+/-}$) compared with *Trp53*$^{+/+}$ and *Trp53* was not detected in the homozygote knockout (*Trp53*$^{-/-}$) embryos in either the control or HU-treated groups. Transcript levels of *Trp63* were significantly reduced in all drug-treated embryos (Figure 1B). This effect was independent of the *Trp53* gene as the levels of *Trp63* were not statistically different between HU-treated embryos that expressed or lacked *Trp53*. The transcript levels of *Trp73* were
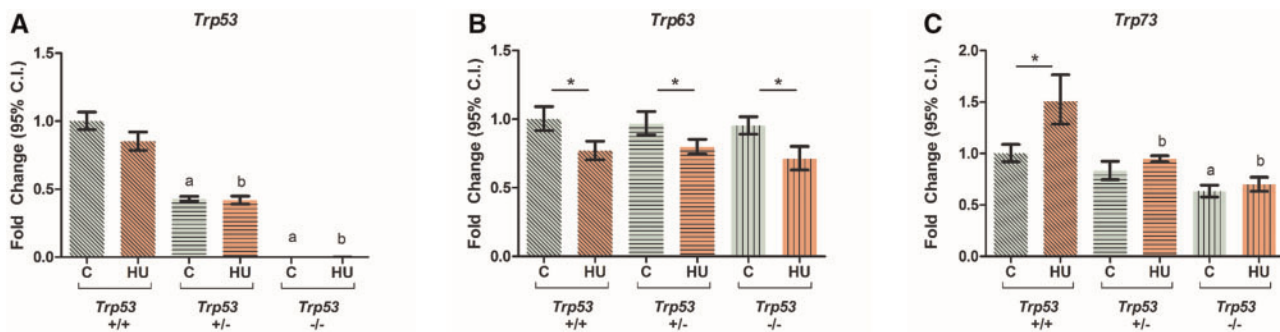
Figure 1. Effects of HU on the relative expression of *Trp53*, *Trp63*, and *Trp73* in embryos. A–C, Relative expression levels of *Trp53*, *Trp63*, and *Trp73*. Each bar represents the fold change of the mean quantity of the transcript relative to *Hprt*. C, control; HU, hydroxyurea. $P < .05$, 2-way ANOVA followed by a Bonferroni post hoc test, $n = 3$. (*) denotes significant change compared with control group with the same genotype, (a) denotes significant change compared with the $Trp53^{+/+}$ control group, and (b) denotes significant change compared with the $Trp53^{+/+}$ HU-treated group.
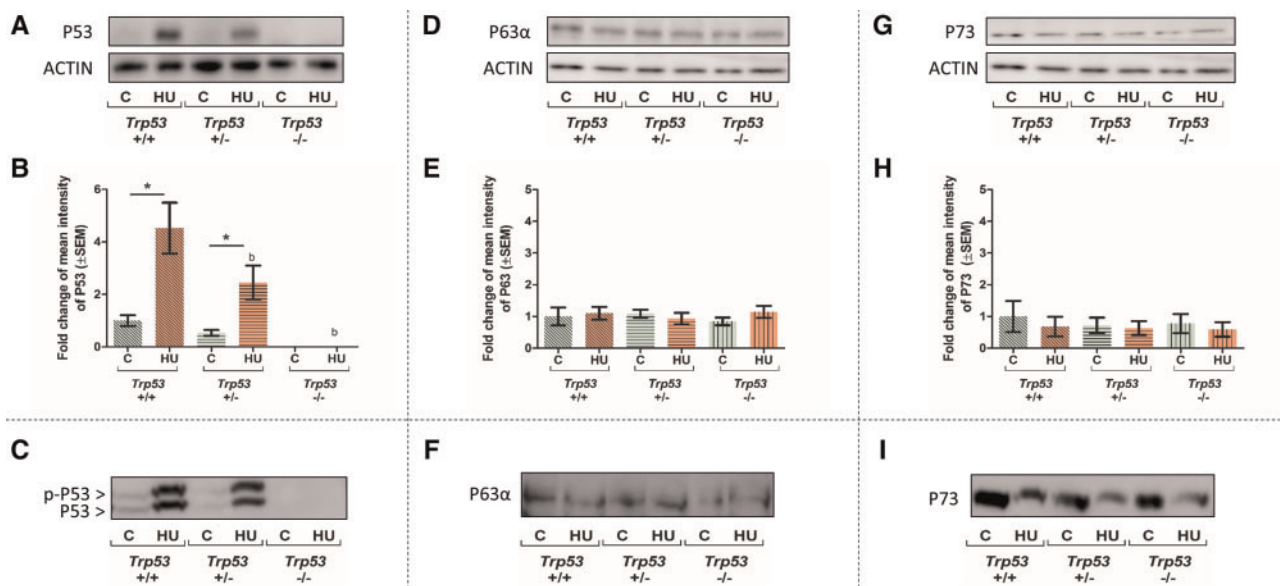

Figure 2. Effects of HU treatment on P53, P63, and P73 protein expression and phosphorylation status in embryos. *Top*: Representative Western blots and quantification of P53 (A and B), P63 (D and E), and P73 (G and H) immunoreactivity, normalized to the loading control, actin, across all treatment groups, and *Trp53* genotypes. Each bar represents the fold change of the mean quantity of the protein relative to actin. *Bottom*: Representative Phos-tag blots of P53 (C), P63 (F), P73 (I). Only P53 showed a higher molecular weight band, representing phosphorylated P53, p-P53. C, control; HU, hydroxyurea. $P < .05$, 2-way ANOVA followed by a Bonferroni post hoc test, $n = 3$. (*) denotes significant change compared with control group with the same genotype and (b) denotes significant change compared with the $Trp53^{+/+}$ HU-treated group.

significantly increased by HU exposure in $Trp53^{+/+}$ embryos (Figure 1C). Interestingly, HU-increased *Trp73* expression was dependent on the gene dosage of *Trp53*; the induction of *Trp73* expression by HU was ablated in both $Trp53^{+/-}$ and $Trp53^{-/-}$ embryos. In addition, the levels of *Trp73* in saline-treated $Trp53^{-/-}$ embryos were significantly less than in their $Trp53^{+/+}$ littermates. Thus, the regulation of *Trp73* transcription in the organogenesis-stage embryo relies on P53.

Hydroxyurea Exposure Increases the Protein Levels and Phosphorylation of P53 but not Those of P63 and P73

Low or no P53 immunoreactivity was detected in control GD 9 embryos from all genotypes (Figure 2A). Hydroxyurea treatment of $Trp53^{+/+}$ embryos induced a significant increase in P53 protein levels (Figs. 2A and 2B). The amounts of P53 detected in HU-exposed embryos reflected the *Trp53* gene dosage; P53 decreased by 50% in $Trp53^{+/-}$ embryos and was not detected in $Trp53^{-/-}$ embryos. Because an essential component of P53 stabilization is its posttranslational phosphorylation, Phos-tag gels

were used to determine if P53 was phosphorylated. Phos-tag causes phosphorylated proteins to migrate at a slower rate during electrophoresis, due to their relatively higher molecular weights and altered electric charge (Kosako *et al.*, 2009). In HU-treated $Trp53^{+/+}$ and $Trp53^{+/-}$ embryos a higher molecular weight band (approximately 55 kDa), representing phosphorylated P53, appeared above the P53 band (approximately 53 kDa) (Figure 2C). As expected, no detectable levels of phosphorylated P53 were found in $Trp53^{-/-}$ embryos.

TAP63α, the P63 full-length isoform, is shown in Figure 2D. In contrast to P53, P63 was readily detected in control embryos from all genotypes. Furthermore, HU treatment did not significantly alter the expression of TAP63α (Figure 2E). Moreover, no higher molecular weight TAP63α band was observed in Phos-tag gels, suggesting that HU treatment did not induce P63 phosphorylation (Figure 2F).

P73 protein immunoreactivity was detected in control embryos (Figure 2G); HU treatment did not alter P73 protein concentrations in embryos with or without *Trp53* (Figure 2H).

Table 1. Prediction Analysis of Unique and Common Markers of P53, P63, and P73 Transcriptional Activity

Protein	Gene	Prediction	References
P53	Trp53inp1	↑	Kenzelmann Broz *et al.* (2013); Okamura *et al.* (2001)
	Cdkn1a	↑	Kenzelmann Broz *et al.* (2013); Nayak and Das (2002)
	Fas	↑	Lima *et al.* (2011); Munsch *et al.* (2000); Thiery *et al.* (2005)
	Pmaip1	↑	Hamard *et al.* (2013); Zhu *et al.* (2010)
	Rb1	↑	Hammond *et al.* (2006); Kenzelmann Broz *et al.* (2013); Porrello *et al.* (2000)
P63	Pax9	↓	Wang *et al.* (2011)
	Cdkn1a	↑	Helton *et al.* (2008); Petitjean *et al.* (2008); Sen *et al.* (2011)
	Fas	↑	Gressner *et al.* (2005); Petitjean *et al.* (2008)
	Pmaip1	↑	Kerr *et al.* (2012)
	Jag2	↑	Candi *et al.* (2007); Sasaki *et al.* (2002); Wu *et al.* (2003)
P73	Tbx5	↓	Holembowski *et al.* (2014)
	Cdkn1a	↑	Jung *et al.* (2001); Nozell and Chen (2002); Ozaki *et al.* (1999)
	Fas	↑	Dicker *et al.* (2006); Terrasson *et al.* (2005); Wei *et al.* (2008)
	Rb1	↑	De Laurenzi *et al.* (2000)
	Jag2	↑	Fontemaggi *et al.* (2002); Sasaki *et al.* (2002)

Genes associated with the transcriptional activity of each of the P53 family proteins. Arrows indicate the predicted changes in expression in response to HU if each of the P53 proteins is activated; upregulation (upward arrow) and downregulation (downward arrow). Prediction analysis is based on Ingenuity Pathway Analysis (IPA) software using microarray data from El Husseini *et al.* (2016).

In addition, no higher molecular weight form of P73 was observed in Phos-tag gels (Figure 2I). Thus, both P63 and P73 proteins are present during organogenesis; however, they are not responsive to HU treatment and their expression is not affected by the absence of P53.

P53 Family Proteins and Regulation of the Expression of Genes Involved in Skeletal and Limb Development or Cell Cycle Arrest and Apoptosis in Response to HU-Induced Stress in the Organogenesis-Stage Embryo

In our previous study, we identified a number of transcripts with altered expression in HU-treated embryos that are either directly or indirectly associated with the transcriptional activity of one or more of the P53 family proteins (El Husseini *et al.*, 2016). These transcripts are involved in cell cycle regulation (*Cdkn1a, Rb1*), apoptosis (*Fas, Pmaip1, Tp53inp1*), or skeletal and limb development (*Pax9, Tbx5, Jag2*) (Table 1).

Using qRT-PCR, we assessed the levels of transcripts that are specifically associated with the transcriptional activity of P53 (*Trp53inp1*), P63 (*Pax9*), P73 (*Tbx5*), or both P63 and P73 (*Jag2*) (Figure 3A), under control and HU-treated conditions, in the presence and absence of *Trp53*. *Trp53inp1* transcript levels were significantly upregulated by HU in $Trp53^{+/+}$ embryos; this effect was abolished in *Trp53* null embryos (Figure 3B). In contrast, the transcript levels of *Pax9* and *Tbx5* decreased in response to HU treatment and were not affected by the presence or absence of *Trp53* (Figs. 3C and 3D). Lastly, HU treatment-induced *Jag2* transcript levels in a *Trp53*-dependent manner (Figure 3E).

We then assessed the level of transcripts that are commonly regulated by all 3 P53 family members (*Cdkn1a, Fas*), by P53 and P63 (*Pmaip1*) or by P53 and P73 (*Rb1*) (Figure 4A). For each of these downstream target genes, transcript levels were significantly increased in embryos exposed to HU; this induction was ablated by the deletion of *Trp53* (Figs. 4B–E). In addition, the levels of each of these transcripts were significantly lower in the saline-treated $Trp53^{-/-}$ embryos than in their $Trp53^{+/+}$ littermates. Thus, the induction of these downstream markers showed a strong dependence on P53 transcriptional activity, rather than on P63 or P73.

Together, P53 transcriptional activity has a dominant impact on gene expression in embryos in response to HU treatment.

Conversely, it appears that neither P63 nor P73 have active roles as transcription factors in regulating the expression of P53 family signaling pathway genes in the organogenesis-stage embryo exposed to a DNA damaging agent.

Hydroxyurea Treatment Induces P53-Dependent Caspase-3 and MST-1 Cleavage and H2AX Phosphorylation in Organogenesis-Stage Embryos

The cleavage of caspase-3 was investigated in control and HU-treated embryos as a marker of apoptosis. Cleaved caspase-3 was not detected in control embryos from any genotype (Figure 5A). A significant increase in cleaved caspase-3 was observed in HU-treated $Trp53^{+/+}$ embryos and $Trp53^{+/-}$ embryos (Figure 5B). However, no appreciable cleaved caspase-3 was observed in HU-treated $Trp53^{-/-}$ embryos (Figure 5A), indicating that HU-induced caspase-3 cleavage is dependent on the presence of P53.

To determine if activation of caspase-3 by P53 leads to the cleavage of MST-1 and the subsequent phosphorylation of H2AX, cleaved MST-1 and γH2AX were examined using Western blots. A significant increase in cleaved MST-1 occurred in HU-treated $Trp53^{+/+}$ embryos; the levels of cleaved MST-1 decreased in a dose-dependent manner relative to *Trp53* concentration (Figs. 5C and 5D), reflecting the trend observed with cleaved caspase-3. In contrast, all HU-treated embryos, including the $Trp53^{+/-}$ and $Trp53^{-/-}$ embryos, exhibited strong upregulation of γH2AX levels compared with controls (Figs. 5E and 5F). However, after HU treatment, γH2AX levels were significantly lower in $Trp53^{-/-}$ embryos compared with their $Trp53^{+/+}$ littermates (Figure 5F).

DISCUSSION

The exposure of organogenesis-stage embryos to a DNA damaging agent, HU, induces a stress response that is complex. Hydroxyurea treatment does not affect steady state concentrations of the *Trp53* transcript; in contrast, *Trp63* and *Trp73* expression profiles are differentially affected, suggesting that their transcriptional regulation differs in response to genotoxic stress during organogenesis. Although *Trp63* levels decrease with HU treatment, *Trp73* levels are significantly increased. In addition, the levels of *Trp73*, but not *Trp63*, are significantly affected by the absence of *Trp53*, suggesting a dependence of
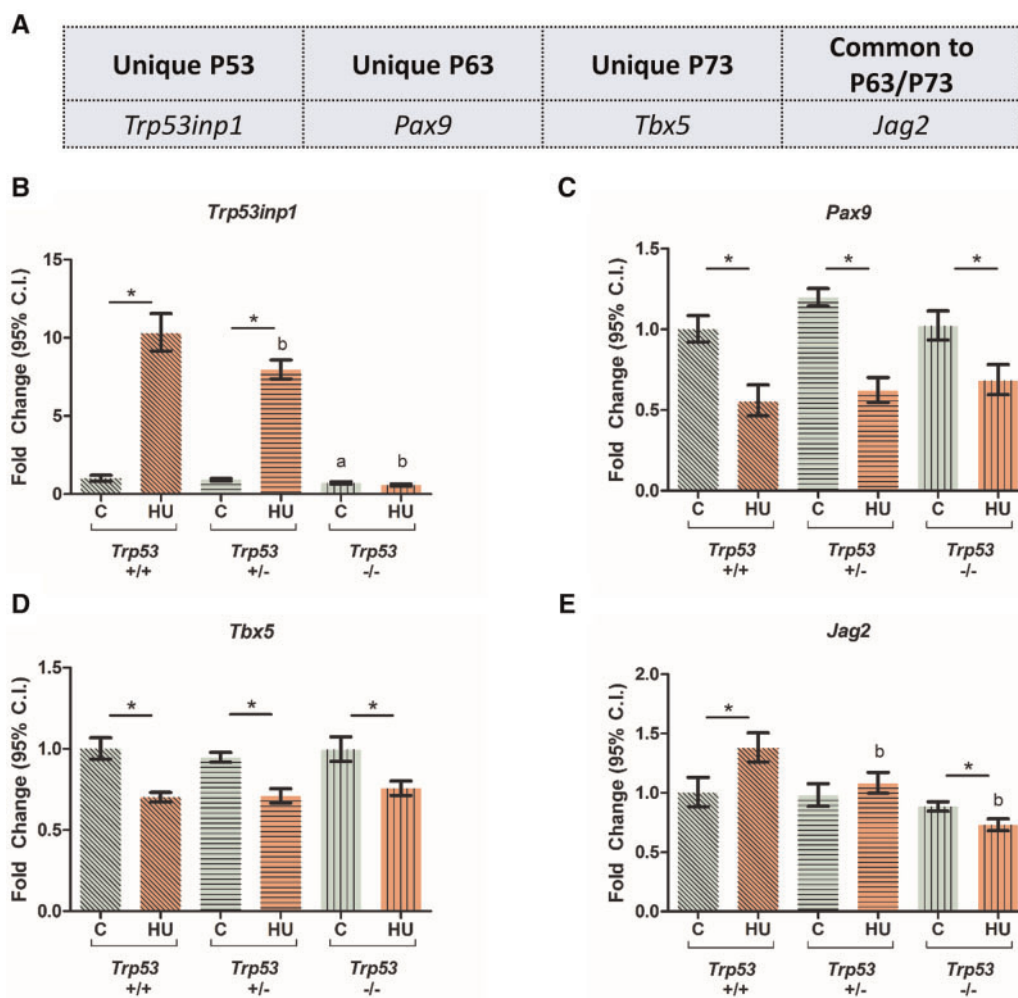
Figure 3. Effects of HU exposure in embryos on the relative expression of genes that are downstream targets of unique P53 family proteins. A, Selected candidates of unique downstream targets of P53, P63, and P73. B–E, Relative expression levels of *Trp53inp1, Pax9, Tbx5, and Jag2*. Each bar represents the fold change of the mean quantity of the transcript relative to *Hprt*. C, control; HU, hydroxyurea. $P < .05$, 2-way ANOVA followed by a Bonferroni post hoc test, $n = 3$. (*) denotes significant change compared with control group with the same genotype and (b) denotes significant change compared with the $Trp53^{+/+}$ HU-treated group.

Trp73 transcriptional regulation on P53. Indeed, it has been shown that P53 binds to a promoter site that is upstream of the *Trp73* gene and can upregulate *Trp73* in response to stress (Daily et al., 2011; Wang et al., 2007).

At the protein level, P53 is not detected in control embryos; in contrast, both P63 and P73 proteins are easily detected. P53 is minimally expressed under normal conditions due to its constant ubiquitination by MDM2, an E3 ubiquitin ligase which targets P53 for proteasomal degradation (Kruse and Gu, 2009). The mechanisms by which P63 and P73 are regulated are less well-understood, although they do seem to share some mechanisms of regulation with P53. Under normal conditions, both have been reported to be targeted for proteasomal degradation in certain cells (Rossi et al., 2006; Satija and Das, 2016). However, unlike P53, appreciable amounts of P63 and P73 are found in organogenesis-stage embryos. It is not surprising that the regulation of these 2 proteins during organogenesis differs from that of P53 because P63 and P73 are essential for the maintenance of normal embryo development (Yang et al., 1999, 2000).

Hydroxyurea treatment increases the steady state concentrations and phosphorylation of P53 in the embryo, in the absence of effects on the levels or phosphorylation of P63 or P73. Under stress conditions, P53, P63, and P73 are all phosphorylated by a

variety of upstream kinases; phosphorylation promotes their stabilization and transactivation in certain cell types, leading to the activation of cell cycle arrest and cell death pathways (Agami et al., 1999; Gonfloni et al., 2009; Jones et al., 2007; Petitjean et al., 2008; Sanchez-Prieto et al., 2002; Satija and Das, 2016; Shi and Gu, 2012). How P53 family proteins respond to a DNA damaging agent may be determined by the stressor (Levrero et al., 1999, 2000); indeed, various genotoxicants have been reported to have different effects on the protein concentrations and activation of individual P53 family members, resulting in altered cellular stress responses (Kaghad et al., 1997; Liu et al., 1996; Mirkes et al., 2000; Yang et al., 2002). In the embryo, P53 may be more sensitive or more responsive to a wider range of stress stimuli than P63 or P73 (Appella and Anderson, 2001; Gottlieb et al., 1997; Kruse and Gu, 2009; Moallem and Hales, 1998).

Several studies have shown that all P53 family proteins can translocate to the nucleus under stress conditions and act as transcription factors, upregulating the expression of downstream transcripts (Kruse and Gu, 2009; Petitjean et al., 2008; Satija and Das, 2016). We found that HU altered the expression of several genes involved in limb and skeletal development that are predicted to be regulated by one or more of the P53 family members. The dysregulation of the expression of *Pax9*, *Tbx5*, and
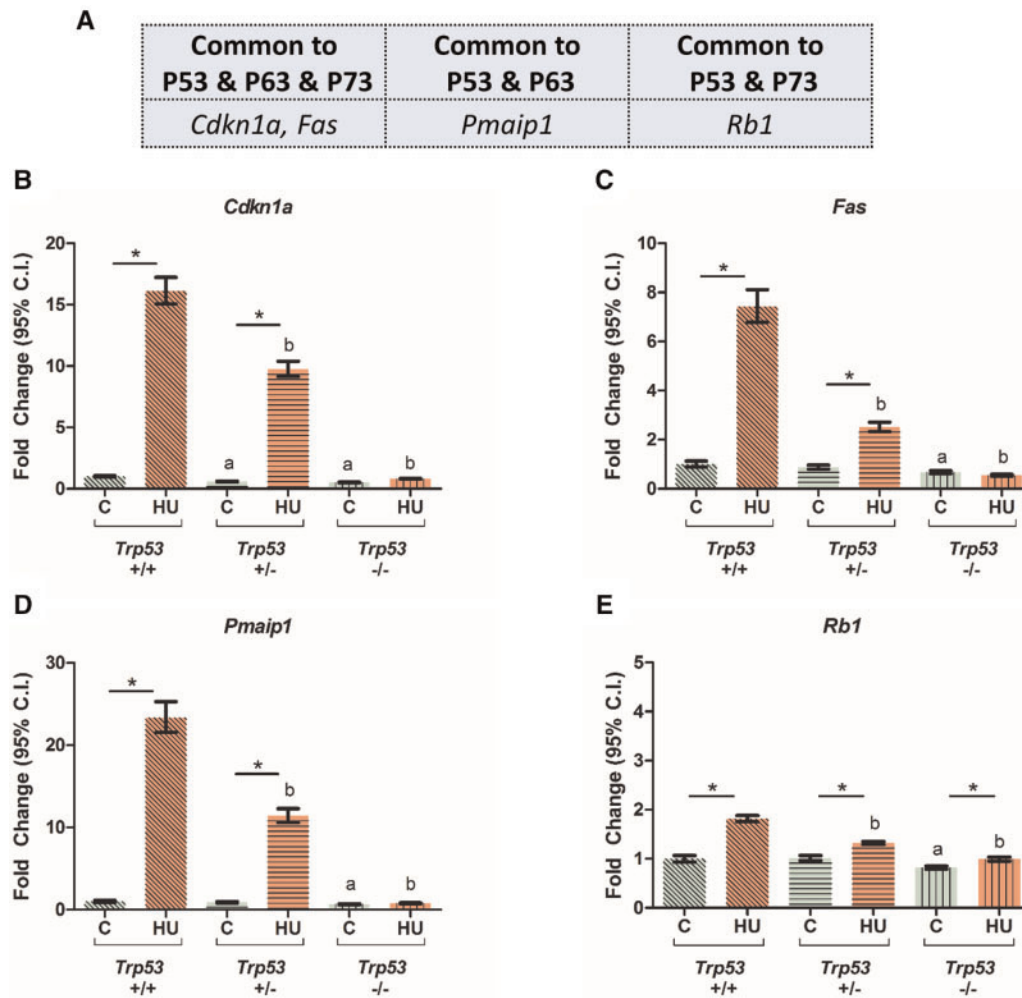
Figure 4. Effects of HU exposure in embryos on the relative expression of transcripts that control cell cycle progression and apoptosis and are regulated by P53 family proteins. A, Selected candidates of common downstream targets of P53, P63, and P73. B–E, Relative expression levels of *Cdkn1a*, *Fas*, *Pmaip1*, and *Rb1*. Each bar represents the fold change of the mean quantity of the transcript relative to *Hprt*. C, control; HU, hydroxyurea. $P < .05$, 2-way ANOVA followed by a Bonferroni post hoc test, $n = 3$. (*) denotes significant change compared with control group with the same genotype, (a) denotes significant change compared with the *Trp53*^{+/+} control group, and (b) denotes significant change compared with the *Trp53*^{+/+} HU-treated group.

Jag2 suggests that they may be partially involved in mediating the embryotoxicity of HU; *Pax9* is involved in skeletal development (McGlinn *et al.*, 2005; Peters *et al.*, 1998), *Tbx5* is essential for limb bud initiation (Agarwal *et al.*, 2003), while *Jag2* codes for an important factor of the Notch signaling pathway and is required for proper limb bud development and digit formation (Jiang *et al.*, 1998; Xu *et al.*, 2010). It is clear that *Pax9* and *Tbx5* are not affected by the absence of P53, whereas *Jag2* is. The lack of P63 and P73 upregulation in HU-exposed embryos and the concomitant decrease in *Pax9* and *Tbx5* transcript levels suggest that other upstream regulators (ie, not P63 or P73) may be involved in the transcriptional suppression of these genes. Indeed, *Pax9* transcription may be regulated by the sonic hedgehog signaling pathway (LeClair *et al.*, 1999), while that of *Tbx5* may be regulated by Hox proteins, HOXB1, HOXC4, and HOXB9, all of which play pivotal roles in embryonic development (Minguillon *et al.*, 2012).

The expression of genes that regulate cell cycle arrest and apoptosis was also affected in embryos exposed to HU. Hydroxyurea strongly induced the expression of a unique P53 marker, *Trp53inp1*, as well as cell cycle arrest (*Cdkn1a*, *Rb1*) and apoptosis (*Fas*, *Pmaip1*) genes that may be activated by all 3 P53 family members. This induction was ablated in the absence of

Trp53, indicating that P53 is the main transcriptional activator in their response to HU during organogenesis; P63 and P73 are incapable of compensating for the lack of P53 in regulating these genes. This finding is consistent with the observation that in wild-type mouse embryonic fibroblasts P63 binds to the *Cdkn1a* promoter only when P53 is present; when *Trp53* is knocked out, neither P63 nor P73 can induce *Cdkn1a* transcription, suggesting that P53 is required to drive *Cdkn1a* transcriptional activity (Flores *et al.*, 2002). Taken together, we conclude that P53 is the main transcriptional activator of genes involved in cell cycle arrest and apoptosis in response to HU in the organogenesis-stage embryo. Indeed, P53 responds to HU-induced stress by mediating cell cycle arrest to allow for DNA damage repair or, failing this, to instigate apoptosis (Gatz and Wiesmuller, 2006; Vousden and Prives, 2009).

The accumulation of cells with irreparable DNA breaks instigates cell death in the form of caspase-3 mediated apoptosis. Here, we report that cleaved caspase-3 is almost undetectable in the *Trp53*^{−/−} embryos. The relationship between P53 accumulation and caspase-mediated apoptosis due to genotoxic stress is well-established (Pekar *et al.*, 2007; Schuler *et al.*, 2000). There are conflicting reports regarding the ability of P63 and P73 to induce
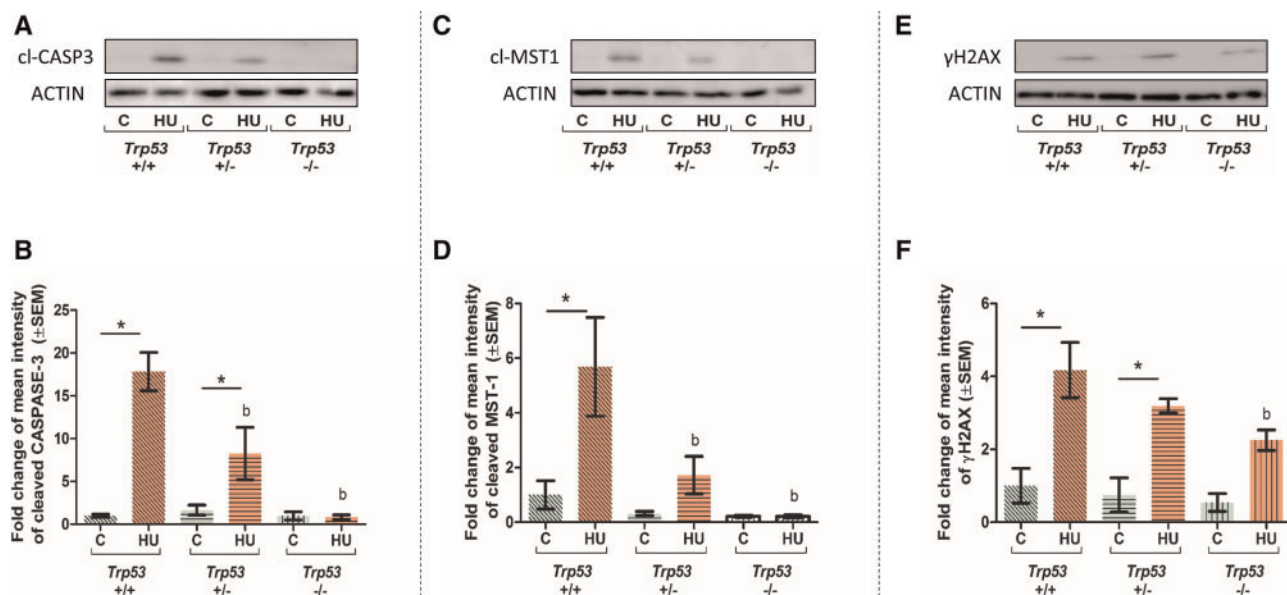
Figure 5. Effects of HU exposure on the cleavage of caspase-3 and mammalian sterile-20-like-1 (MST-1) and H2AX phosphorylation in embryos. Representative Western blots and quantification of cleaved caspase-3 (A and B), cleaved MST-1 (C and D) and γH2AX (E and F) protein expression levels, normalized to the loading control, actin. Each bar represents the fold change of the mean quantity of the protein relative to actin. C, control, HU, hydroxyurea. $P < .05$, 2-way ANOVA followed by a Bonferroni post hoc test, $n = 3$. (*) denotes significant change compared with control group with the same genotype and (b) denotes significant change compared with the $Trp53^{+/+}$ HU-treated group.

apoptosis in the absence of P53. In tissues lacking *Trp53*, but containing *Trp63* and *Trp73*, apoptosis has been reported to be either decreased or increased in response to γ-irradiation-induced DNA damage (Flores *et al.*, 2002; Senoo *et al.*, 2004). Our data suggest that P53 is the main driver of apoptosis in response to DNA damage during organogenesis. *Trp53*$^{-/-}$ embryos exposed to HU may undergo a form of "less regulated" cell death if caspase-3 dependent apoptosis is not activated; indeed Moallem and Hales (1998) reported that *Trp53*$^{-/-}$ embryonic limbs underwent necrosis, rather than apoptosis, after exposure to a preactivated metabolite of cyclophosphamide, a DNA damaging anticancer drug.

Hydroxyurea induces replication fork stalling, causing DNA-strand breaks that result in the accumulation of phosphorylated H2AX at the sites of damage (Zeman and Cimprich, 2014). An increase in γH2AX was observed in all HU-exposed embryos; however, γH2AX accumulation was significantly lower in *Trp53*$^{-/-}$ embryos treated with HU than in their *Trp53*$^{+/+}$ littermates. This decrease in γH2AX in the absence of P53 may be due to the absence of appreciable cleaved caspase-3, resulting in low levels of cleaved MST-1. Caspase cleavage activates MST-1 (Lee *et al.*, 2001; Oh *et al.*, 2009) and decreased MST-1 activation leads to less H2AX phosphorylation (Wen *et al.*, 2010). However, other kinases, including the DNA damage response proteins, Ataxia Telangiectasia Mutated and Ataxia Telangiectasia And Rad3-Related Protein, may phosphorylate H2AX in response to genotoxic stress and, at least partially, compensate for the decrease in MST-1 activation (Burma *et al.*, 2001; Ward and Chen, 2001).

Although P63 and P73 are essential for crucial developmental processes under normal conditions (Yang *et al.*, 1999, 2000), our study shows that P53 is the main member of the P53 family that drives the early embryonic stress response to DNA damage.

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