THE ROLE OF THE P53 FAMILY PROTEINS IN HYDROXYUREA INDUCED EMBRYOTOXICITY IN THE ORGANOGENESIS STAGE MOUSE EMBRYO

by

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ABSTRACT

The embryonic stress response to DNA damage during organogenesis was examined using the model teratogen, hydroxyurea. Intraperitoneal administration of hydroxyurea (400 mg/kg or 600 mg/kg) to timed pregnant CD-1 mice on gestational day 9 significantly affected the expression of ~1300 transcripts in the embryo within three hours. Pathway analysis predicted that the P53 signaling pathway was the most activated in response to hydroxyurea. The steady-state levels of P53 and its phosphorylation increased dramatically in hydroxyurea-exposed embryos. The nuclear localization of phosphorylated P53 was increased significantly in the embryo heart and rostral and caudal neuroepithelia. Hydroxyurea also induced P53 transcriptional activity, leading to the upregulation of P53 downstream targets involved in DNA damage repair, cell cycle arrest and apoptosis.

Using a *Trp53* null transgenic mouse model, the effects of treatment with hydroxyurea on P53 family members, P63 and P73, were determined in the gestational day 9 embryo. Appreciable amounts of P63 and P73 were detected under normal conditions; hydroxyurea did not affect their steady-state or phosphorylation levels. *Trp63* and *Trp73* transcript levels were affected by hydroxyurea treatment, although only *Trp73* levels were P53-dependent. As transcription factors, neither P63 nor P73 were capable of compensating for the absence of P53 in upregulating the expression of downstream targets involved in cell cycle arrest (*Cdkn1a, Rb1*) or apoptosis (*Fas, Pmaip1*) in response to hydroxyurea exposure. Hydroxyurea induction of Caspase-3 and MST-1 cleavage, markers for apoptosis and DNA damage repair, required the presence of P53. Thus, P53 is the main member of the P53 family that responds to genotoxic stress in the organogenesis-stage embryo.

To determine whether P53 acts as a suppressor or inducer of hydroxyurea embryotoxicity, $Trp53^{+/+}$, $Trp53^{+/-}$ and $Trp53^{-/-}$ embryos were exposed *in utero* on gestational day 9 with saline or hydroxyurea (200 or 400 mg/kg). On gestational day 18, $Trp53^{-/-}$ fetuses from the saline treatment group were found to exhibit a higher rate of malformations compared to their $Trp53^{+/+}$ littermates. Hydroxyurea treatment induced a dose-dependent increase in resorptions and congenital malformations; these included hypoplastic tails and fore- and hind-limb syndactly, oligodactyly and amelia. In the 200 mg/kg treatment group, hydroxyurea exposed fetuses lacking P53 had a higher rate of malformations than their $Trp53^{+/+}$ littermates

and the rate of resorptions was elevated in these fetuses after exposure to 400 mg/kg hydroxyurea. Thus, the organogenesis stage embryo responds to hydroxyurea-induced stress by activating the P53 signaling pathway that mediates DNA damage repair, cell cycle arrest and apoptotic factors to suppress hydroxyurea embryotoxicity.

RÉSUMÉ

Ce projet consiste à étudier la réponse embryonnaire au stress oxydatif induit par les dommages à l'ADN produits par un tératogène connu, l'hydroxyurée, au cours de l'organogenèse. Dans les trois heures suivant une injection intrapéritonéale d'hydroxyurée (400 ou 600 mg/kg) chez des souris CD-1 au jour 9 de gestation, plus de 1300 transcrits embryonnaires voient leur expression affectée. L'analyse des voies de signalisation impliquées dans cette réponse prédit une activation marquée de la voie P53 : chez les embryons exposés, les niveaux d'expression de P53 et sa phosphorylation augmentent de façon drastique, et la localisation nucléaire de la forme phosphorylée de P53 révèle une expression prononcée dans le cœur et les neuro-épithéliums rostraux et caudaux des embryons. L'exposition à l'hydroxyurée stimule également l'activité transcriptionnelle de P53, conduisant à une activation de ses cibles impliquées dans la réparation des dommages à l'ADN, l'arrêt du cycle cellulaire ou l'apoptose.

Nous avons exploré les effets de l'hydroxyurée au jour 9 de gestation sur d'autres membres de la famille P53, les protéines P63 et P73, grâce au modèle de souris transgénique knockout *Trp53*. Dans des conditions normales, des niveaux non négligeables de P63 et P73 sont détectés ; l'hydroxyurée n'affecte ni leur production ni leur phosphorylation. En revanche, les niveaux de transcription de *Trp63* et *Trp73* sont affectés par l'exposition à l'hydroxyurée, bien que seuls les niveaux de *Trp73* ne soient affectés par l'absence de P53. Par ailleurs, ni P63 ni P73 ne peuvent compenser l'absence de P53 dans la stimulation de l'arrêt du cycle cellulaire (*Cdkn1a, Rb1*) ou de l'apoptose (*Fas, Pmaip1*). Le clivage de la Caspase-3 et de MST-1, marqueurs de l'apoptose et de la réparation des dommages à l'ADN, requièrent également la présence de P53. Ainsi, P53 est l'acteur principal de la réponse au stress génotoxique au cours de l'organogénèse chez l'embryon.

Afin de déterminer si P53 agit comme un suppresseur ou un inducteur de l'embryotoxicité de l'hydroxyurée, nous avons exposé des embryons $Trp53^{+/+}$, $Trp53^{+/-}$ et $Trp53^{-/-}$ au jour de gestation 9 à une solution saline ou à 200 ou 400 mg/kg d'hydroxyurée. Au jour 18 de gestation, les fœtus $Trp53^{-/-}$ ayant été exposés à la solution saline présentent plus de malformations que les fœtus $Trp53^{+/+}$. Les fœtus exposés à l'hydroxyurée présentent quant à eux une augmentation dose-dépendante du nombre de résorptions et de malformations congénitales (queue hypoplasique, syndactylie des membres antérieurs et postérieurs, oligodactylie et

dysmélie). Après une exposition à 200 mg/kg d'hydroxyurée, les fœtus présentant une déficience en P53 présentent plus de malformations que les fœtus *Trp53*$^{+/+}$. Enfin, après une exposition à la dose la plus élevée d'hydroxyurée, le taux de résorptions est plus important chez les fœtus *Trp53*$^{-/-}$. Ainsi, l'embryon en cours d'organogénèse répond au stress induit par une exposition à l'hydroxyurée par une activation de la voie de signalisation P53, qui contrôle la mise en place de mécanismes de réparation de l'ADN, d'arrêt du cycle cellulaire et d'apoptose pour contrecarrer ses effets.

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ABBREVIATIONS

µg, microgram
2-CdA, 2-chloro-2'-deoxyadenosine
4-HNE, 4-hydroxynonenol
4OOH-CPA, 4-hydroperoxycyclophosphamide
ADULT, acro-dermato-ungual- lacrimal-tooth syndrome
AER, apical ectodermal ridge
Aldh4a1, aldehyde dehydrogenase 4 family member A1
Anxa8, annexin A8
AP-1, activator protein 1
APAF, apoptotic peptidase activating factor
ATP, Adenosine triphosphate
BAK, BCL2 Antagonist/Killer
BAX, BCL2 Associated X, Apoptosis Regulator
BMP, bone morphogenetic protein
BSA, bovine serum albumin
bw, body weight
Cat, catalase
CDK, cyclin-dependent kinase
Cdkn1a, Cyclin Dependent Kinase Inhibitor 1A
CHARGE, coloboma, heart defects, atresia choanae, growth retardation, genital abnormalities, and ear abnormalities syndrome
CNE, caudal neuroepithelium
CO2, carbon dioxide
DAPI, 4',6-diamidino-2-phenylindole
DBD, DNA binding domain
Ddit4l, DNA Damage Inducible Transcript 4 Like
DNA, deoxyribonucleic acid
dNTP, deoxyribonucleoside triphosphate
EDTA, Ethylenediaminetetraacetic acid
EEC, ectrodactyly, ectodermal dysplasia, clefting syndrome
EGF, epidermal growth factor
ERK, extracellular signal regulated kinase
ESC, embryonic stem cell
Fas, Fas Cell Surface Death Receptor
FGF, fibroblast growth factor
g, gram
GD, gestational day
GIT, gastrointestinal tract
GLS2, Glutaminase liver isoform, mitochondrial
GLUT1, glucose transporter 1
Gpx1, glutathione peroxidase 1
GSH, reduced glutathione
H, heart
h, hour
HIF, hypoxia inducible factor
Hox, homeobox
HU, hydroxyurea
ICM, inner cell mass

IPA™, Ingenuity Pathway Analysis
Jag2, Jagged 2
JNK, c-Jun N-terminal kinase
kg, kilogram
LMS, limb-mammary syndrome
MAPK, mitogen-activated protein kinase
mg, milligram
min, minute
MST-1, mammalian sterile 20-like-1
mTOR, mechanistic target of rapamycin
NF- κB, nuclear factor κB
NRF, nuclear factor-E2 related factor
OD, oligomerization domain
P53INP1, tumor protein P53 inducible nuclear protein 1
PAGE, polyacrylamide gel electrophoresis
Pax9, paired box 9
PBS, phosphate-buffered saline
PCA, principal component analysis
PCBP2, Poly(RC) Binding Protein 2
Phlda3, pleckstrin homology like domain family A member 3
PITX1, paired-like homeodomain 1
Pmaip1, phorbol-12-myristate-13-acetate-induced protein 1
PVDF, Polyvinylidene fluoride
qRT-PCR, quantitative reverse transcription polymerase chain reaction
Rb1, retinoblastoma 1
Ref-1, redox factor 1
RNA, ribonucleic acid
RNE, rostral neuroepithelium
RNOS, reactive nitrogen and oxygen species
RNR, ribonucleotide reductase
RUNX2, runt-related transcription factor 2
s, second
SAL, saline
SAM, sterile alpha motif
SDS, sodium dodecyl sulfate
Sesn1, sestrin 1
SHFM, nonsyndromic split hand/foot malformation
SHH, sonic hedgehog pathway
SOD, superoxide dismutase
SOX9, sex-determining region Y box 9 protein
TA, transactivation
TAD, transactivation domain
TBS, tris-buffered saline
TBS-T, tris-buffered saline, Tween
Tbx5, T-box transcription factor 5
TRX, thioredoxin
UV, ultraviolet
VEGF, vascular endothelial growth factor
WNT, wingless-related integration site
Zfp365, zinf finger protein 365
ZPA, zone of polarizing activity
ΔN, lacking the transactivation N-terminal domain
µl, microliter
µm, micrometer

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smile instantly resolves any regrets or difficulties I may have encountered. I eagerly look forward to the adventures of my next chapter in life with her by my side. I thank God day and night for such a blessing.

PREFACE

Format of the Thesis

This is a manuscript-based thesis in accordance with the Thesis Preparation and Submission Guidelines outlined by the Faculty of Graduate and Postdoctoral Studies at McGill University. This thesis entails five chapters. Chapter I provides an introduction to the subject matter of the three studies presented in this dissertation. An overview of embryonic development is briefly discussed, along with a review of the current understanding of how embryos respond to genotoxic and oxidative stress during organogenesis. A description of the model teratogen, hydroxyurea, is given, with a focus on its ability to cause DNA damage and oxidative stress. A detailed description of the P53 family proteins, P53, P63 and P73, is also presented, with a focus on their regulation and function during development and in stress response.

Chapters II, III and IV show the data from the three studies performed for this dissertation. Chapters II and III are published in Toxicological Sciences (Chp. II: 152, 297-308, 2016; Chp. III: 162, 439-449, 2017). Chapter IV has been submitted for publication.

Chapter V discusses the conclusions that can be surmised from the findings presented in the data chapters and their relevance to the fields of developmental toxicology and molecular biology. Lastly, lists of original contributions and references are provided.

Contribution of Authors

All the experiments and analyses in this thesis were performed by the candidate with the exception of the microarray experiment described in Chapter II, which was performed by Dr. Ava Schlisser.

CHAPTER I

Introduction

1.1 Statement of the problem and purpose of the investigation

Our understanding of the biology of mammalian development has improved dramatically over the past few decades; the early discovery of the process of fertilization and zygote formation, as well as the molecular genetic and epigenetic underpinnings that can influence progeny outcome, show us how miraculous, and perilous, the process of mammalian development is. However, with the ever-changing environment of the modern world, our current understanding of the ways by which various external factors can influence the normal progression of embryonic and fetal development is limited. Around 3-6% of babies born worldwide suffer from a congenital defect, most of which are idiopathic in nature (Turnpenny *et al.*, 2005; Yoon *et al.*, 1997). In addition, the incidence of specific congenital defects has been increasing over time (Correa *et al.*, 2007; Parker *et al.*, 2010). It is possible that the cause of many of these defects is due to xenobiotic agents that include environmental, chemical, biological, physical or nutritional factors that negatively affect mammalian development (Saffran, 1996).

To better equip ourselves with the necessary means to protect current and future generations from the effects of teratogens during gestation, we first need to better understand the mechanisms by which the embryo responds to such detrimental influences. Since mammalian development is most vulnerable to teratogenic insult during the stage of organ formation, known as organogenesis, it is our aim to study and identify the embryonic response mechanisms to stressful teratogenic stimuli at this sensitive stage of development.

To this effect, we aim to examine the organogenesis-stage murine embryo's *in vivo* response to the genotoxic and oxidative effects of a known teratogen, hydroxyurea. Previously it was shown that hydroxyurea activates several stress response signaling pathways, including the DNA damage response pathway and the MAPK signaling pathway (Banh and Hales, 2013). These pathways are known to activate a downstream mediator that acts as a central regulator of the cell's response to stress (Kruse and Gu, 2009). We propose that this mediator is the P53 protein and that its signaling pathway is involved in the early embryonic stress response to hydroxyurea. P53 is a well-known DNA damage and oxidative stress response protein and has been shown to have important roles in development (Armstrong *et al.*, 1995; Choi and Donehower, 1999). It is known to be activated in the organogenesis-stage embryo in response to a variety of known teratogens, but whether it functions as a suppressor or inducer of the

embryotoxicity of such agents is unclear (Torchinsky and Toder, 2010). In addition, P53 is one member of a larger family of similar proteins that include P63 and P73, which are essential for proper embryonic development and have many similar functions to P53 (Moll and Slade, 2004). Whether these proteins are involved in the embryo's response to teratogenic stress is unknown.

To learn more about how the embryo copes with teratogenic insult, we aim to determine if indeed P53 and its homologs are involved in the early embryonic stress response to hydroxyurea and whether they function to suppress or induce its embryotoxicity.

1.2 Embryogenesis

Mammalian embryogenesis is a fascinating and complex process. Amazingly, the fundamental aspects of this process do not differ greatly between different species of vertebrates; the early embryos of humans and mice, for example, undergo the same basic steps of fertilization, cleavage, gastrulation and organogenesis. Indeed, as Karl Ernst von Baer observed in 1828 , in what was later canonized as the "von Baer's laws", it is only after the gastrulation stage that the distinct morphological features that set humans and mice apart can start to be observed, and even then the mechanisms by which most vertebrate organ systems develop share remarkable characteristics (Abzhanov, 2013). Consequentially, the study of mammalian embryogenesis relies greatly on the mouse as a model organism, which has proven to be an astounding and versatile means of understanding not only how normal development takes place, but also how teratogens and genetic mutations can disrupt this fine-tuned process.

The process of embryogenesis in mice and humans shares a common beginning with the successful fertilization of an ovum by a spermatozoon, which produces a diploid cell called the zygote (Gilbert, 2010). This occurs in the oviduct of the pregnant mother, where the zygote undergoes a process of holoblastic cleavage, in which several mitotic divisions of the whole zygote result in the formation of small cells called blastomeres that eventually form a sphere known as the blastula. At this stage, the blastula transforms into a blastocyst that contains two main layers; the inner cell mass (ICM) (which is the precursor for embryonic tissue, the amnion and yolk sac) and the trophoblast (which gives rise to the extraembryonic tissue). At this point, which is around gestational day (GD) 5 in mice and GD 6-13 in humans, embryos can attach to the uterine endometrium, thus forming an implantation site (Hood, 2016). This is an essential feature of mice and humans, as throughout most of their gestation embryos obtain nutrients

directly from the mother and not from the yolk sac, unlike other species. They can achieve this by forming the placenta, an organ composed of the embryo's chorion (which originates from the trophoblast) and the mother's decidua (which originates from the uterine wall) (Hood, 2016).

While the trophoblast forms the basis for the chorion, the ICM divides into two further layers: the lower hypoblast and the upper epiblast. The hypoblast surrounds the embryo and gives rise to the extraembryonic endoderm, which in many species later forms the yolk sac. However, the epiblast undergoes another segregation, forming an extraembryonic ectoderm (which is the basis for the amnion) and the embryonic epiblast (Chazaud *et al.*, 2006). The cells in the embryonic epiblast rearrange themselves relative to one another to form the germ layers. At this point, around GD 6 in the mouse and GD 10-13 in humans, the embryo is in the gastrulation phase and the germ layers develop into three distinct entities: the ectoderm, the mesoderm and the endoderm (Gilbert, 2010; Hood, 2016). Each of these layers gives rise to specific body tissues and organs, which become defined during the organogenesis stage.

1.2.1 Organogenesis

Following specific axial and differentiation cues, the three germ layers go through a series of migrations and structural changes that build and shape the embryo and then continue to grow and differentiate into specialized tissues and organs throughout the period of organogenesis. The phase of organogenesis overall lasts from GD 9 in the mouse and GD 21 in humans up to the end of gestation (and in some systems even postnatally); thus the process of organogenesis occurs relatively early in development and lasts for a long period (Hood, 2016). It is during organogenesis that the embryo is most vulnerable to insult; the disruption of any of the fine processes that govern the formation of various organs may result in detrimental and catastrophic events that lead to congenital malformations and death (Wilson, 1965).

During gastrulation, the cells of the embryonic epiblast form the endoderm (which eventually forms the inner lining of the gut and the lungs), the ectoderm (which forms the surface epidermal layer of the skin and forms the brain and nervous system) and the mesoderm (which is between the endoderm and ectoderm and forms the blood, heart, kidney, gonads, bones, muscles and connective tissues) (Gilbert, 2010). This occurs by the movement of epiblastic cells of the mesoderm and endoderm through the primitive streak, a central structure within the embryo that forms by the thickening of cells at the posterior marginal zone. A process

of intercalation and convergent extension causes the streak to progress lengthwise, and ultimately serves to define the anterior-posterior axis of the embryo and to separate its left and right portions. Concomitantly, as cells form the primitive streak, a depression is formed that is called the primitive groove. This allows cells to pass through and migrate into the deeper layers of the embryo, setting the stage for the division of the three germ layers.

During this phase of gastrulation, as the primitive streak elongates, cells entering through it are transformed from epithelial to mesenchymal in nature. Cells that are going through the posterior end of the streak give rise to the majority of the mesodermal tissues. Those that are in the anterior end of the streak form the endoderm, head mesoderm and the notochord. The notochord is a structure that is found in all chordates; in mammals, it serves as a foundation for the eventual formation of the neural tube (Gilbert, 2010).

The neural tube is the basis for the formation of the central nervous system. It is derived from the dorsal ectoderm that gives rise to a region called the neural plate. The neural plate goes through a process of primary and secondary neurulation that invaginates and hollows out tissues to form two tubes that eventually fuse together, forming the neural tube (Harrington *et al.*, 2009). As the embryo goes through the stages of organogenesis and the neural tube develops in the brain and spinal cord, the rostral neuropore closes first (around GD 9 in the mouse embryo) while the caudal neuropore closes later (around GD 10 in the mouse embryo) (Kaufman, 1992).

Along the neural tube multiple structures termed “somites” form in pairs; originating from the mesoderm, somites are made up of several compartments, including the sclerotome – which forms the vertebrae that surround and protect the spinal cord – and the myotome – which forms the muscles of the back, rib cage and ventral body wall. In addition, they form the tendons, vertebral joints and the proximal ends of the ribs (Christ *et al.*, 2007; Gilbert, 2010; Mittapalli *et al.*, 2005). An interesting feature of somites is the periodicity of their formation; they appear at exactly the same time on either side of the embryo, appear at set intervals (although this varies slightly in the mouse embryo), and their number is set from the initial stages of mesoderm formation (Tam, 1981). Actually, this is what allows for the identification of the stages through which the embryo progresses during organogenesis (from 13-20 pairs at the start of organogenesis on GD 9 in the mouse embryo, to 60 somites by the end, on GD 15) (Gilbert, 2010; Kaufman, 1992).

By GD 9, the mouse embryo has just completed "turning", a unique phenomenon that does not occur in human embryos. This step orients the embryo so that the head is facing the ventral side of the tail. At this stage, the rostral end of the neural tube closes while the caudal end is still in the process of closing, showing that the neural tube is still in its elementary stages of development and that there is a developmental lag between the rostral and caudal ends of the embryo. In addition, the heart starts to beat regularly, coinciding with the initial circulation of red blood and a significant increase in the number of nucleated red blood cells. Another feature of development at this stage is the appearance of the forelimb bud, which rises from the mid-trunk region around the level of somites 8-12 (Kaufman, 1992).

While many organs and systems are formed throughout organogenesis, we will be focusing on the processes occurring in early organogenesis in the mouse embryo (~GD 9), with an emphasis on skeletal and limb development, as they relate the most to this thesis.

1.2.1.1 Skeletal development

As mentioned earlier, the somites are involved in the development of the skeleton. However, this is only part of the picture; somites generate the vertebral skeleton, but the lateral plate mesoderm generates the limb skeleton, and the pharyngeal arch, craniofacial bones and cartilage develop from the neural crest region. Taken together, the process of skeletal development is known as osteogenesis, and involves two main processes: intramembranous ossification, whereby mesenchymal tissue is directly converted into bone, and endochondral ossification, whereby mesenchymal tissue is first converted to cartilage that is later replaced by bone (Gilbert, 2010). It is the latter process that occurs predominantly in limb formation, and so we will mainly focus our discussion on this type of osteogenesis.

The process of endochondral ossification can be divided into five main stages, starting with the commitment of mesenchymal cells to becoming cartilage cells. This is governed by the Sonic hedgehog pathway (SHH) which acts in a paracrine fashion on nearby cells to stimulate commitment (Sosic *et al.*, 1997). This then leads to the next stage, when the committed mesenchymal cells start to form condensed, compact nodules and differentiate into a type of cartilage-producing cells known as chondrocytes. At this stage an important transcription factor, the sex-determining region Y box 9 protein (SOX9), begins to be produced to activate transcription factors that induce the production of collagen, an essential component of cartilage

(Hall and Miyake, 1995). In the third stage, the chondrocytes divide rapidly and release an extracellular matrix that gives the cartilage its form. In the fourth stage, the chondrocytes switch from a rapidly proliferating phase to a more quiescent one, where they grow in size and volume and become hypertrophic. This is regulated by another transcription factor, runt-related transcription factor 2 (RUNX2), and requires a shift from aerobic to anaerobic conditions (Shapiro *et al.*, 1982). These hypertrophic chondrocytes release new types of collagen and fibrous proteins (i.e. collagen X and fibronectin) which induce calcification. In addition, they produce vascular endothelial growth factor (VEGF), an angiogenesis factor that promotes blood vessel formation (Gerber *et al.*, 1999). As the blood vessels enter and spread through the cartilage matrix, the fifth and final phase of osteogenesis occurs. The hypertrophic chondrocytes die while cells that border the cartilage matrix become osteoblasts, so that chondrocytes become replaced by bone cells that begin to form the bone matrix. This process is also governed by RUNX2, however new factors are now also involved, including the transcription factor OSTERIX that is regulated by the wingless-related integration site (WNT) signaling pathway (Hu *et al.*, 2005; Nakashima *et al.*, 2002). By this stage the process of endochondral ossification is complete and the bones continue to grow in size or stall, based on external environmental and signaling factors (Gilbert, 2010). Thus, the process of bone formation relies on the precise timing and intensity of signaling molecules, most of which are part of canonical signaling pathways (e.g. SHH, WNT) that act in a paracrine fashion to stimulate the production of transcription factors and extracellular matrix ingredients. Therefore, any agent that disrupts these pathways could ultimately affect bone formation and growth, leading to structural anomalies and the failure of organ formation. Indeed, teratogenic agents, such as thalidomide and valproic acid, cause severe birth defects of the limbs and skeletal system by directly or indirectly disrupting the pathways that govern osteogenesis (Knobloch and Ruther, 2008; Paradis and Hales, 2013).

1.2.1.2 Limb development

Limb development begins early in the organogenesis phase and originates from mesodermal cells. It is a tightly controlled process that involves a variety of signaling pathways that govern three main dimensions to ensure a properly developed limb: i) the proximal-distal axis, which determines the positions of bones in the stylopod (most proximal), zeugopod, and autopod (most distal); ii) the anterior-posterior axis, which determines the position and identity

of each digit; iii) the dorsal-ventral axis, which distinguishes the knuckle-side from the palm-side of the limb (Gilbert, 2010).

In the mouse embryo, the forelimb buds are first observed on GD 9, followed by the appearance of the hindlimb buds on GD 10. Limb buds originate from a region known as the limb field; mesenchymal cells from the somatic layer of the mesodermal plate of the limb field, as well as cells from the somites, proliferate and accumulate to form the beginning stages of the limb bud (Gilbert, 2010; Kaufman, 1992). The growth of buds of the fore- and hindlimbs is determined by a variety of signaling and transcription factors, including fibroblast growth factor 10 (FGF10), paired-like homeodomain 1 (PITX1), and T-box transcription factors 4 and 5 (TBX4 and TBX5). These factors determine the site of limb bud outgrowth from the mesodermal plate, and distinguish forelimbs from hindlimbs (Chapman *et al.*, 1996; DeLaurier *et al.*, 2006; Ohuchi *et al.*, 1997).

The apical ectodermal ridge (AER) is an essential structure that develops at the distal part of the limb bud, at the boundary of the dorsal and ventral ectoderm; it determines and maintains the three main dimensions of limb development (Saunders and Reuss, 1974). It does this by keeping the mesenchymal cells right underneath it in a continuously proliferating state to promote growth along the proximal-distal axis; it maintains the expression of signaling molecules that specify the anterior-posterior and dorsal-ventral axes (Gilbert, 2010). An example of these signaling molecules is SHH which, as mentioned earlier, is part of the SHH signaling pathway and is also involved in osteogenesis. In the limb bud, SHH controls the anterior-posterior axis from its site of production in the zone of polarizing activity (ZPA), a region found in the posterior portion of the limb bud. The gradient expression of SHH across the anterior-posterior axis right underneath the AER eventually determines the position and identity of the various digits that form (Riddle *et al.*, 1993). Naturally, osteogenesis is a central component to this process and thus SHH serves a dual role; it maintains the anterior-posterior axis and induces mesenchymal cells to commit to becoming the cartilage-producing chondrocytes. As such, the bones of the limbs (e.g. digits, carpels, tibia, etc.) are formed by the same basic process of endochondral osteogenesis (Gilbert, 2010).

After the digits are formed and the three-dimensional pattern of the autopod has been well-defined, another important process takes place to give the limb its final shape. This process is known as “sculpting” and involves the death of cells within specific regions of the autopod.

These regions include the interdigital necrotic zone (which is located between the digits), the interior necrotic zone (which separates the ulna and radius within the zeugopod), and the anterior and posterior necrotic zones (which shape the ends of the limb) (Saunders and Gasseling, 1962). The process of cell death occurs by apoptosis (and not necrosis, as the earlier terms suggest); this is a type of programmed cell death that is associated with the fragmentation of DNA and the activation of the proteolytic caspase enzyme cascade (Mori *et al.*, 1995). The bone morphogenetic proteins (BMPs) are thought to be the main signaling molecules that prime a cell in the necrotic zones to undergo apoptosis (Yokouchi *et al.*, 1996). Interestingly, these molecules are expressed throughout the mesenchyme underlying the AER, so to limit the regions where cell death takes place, another molecule. Noggin, is co-expressed to suppress BMP's pro-apoptotic signal in regions other than the necrotic zones (Merino *et al.*, 1998). Once primed for apoptosis, cells in the necrotic zones activate signaling pathways that regulate cell survival, including the "guardian of the genome", the P53 transcription factor (see Section 1.4.1). Disruptions in the signaling pathways that govern the growth of the limb bud, the determination of the three axes, as well as the death of cells in the necrotic zones, could lead to a variety of limb defects, such as adactyly (when the fetus is born without digits), ectrodactyly (a deficiency or absence of one or more digits in the hand or foot, also known as split hand/split feet malformation), syndactyly (where two or more digits are fused), and phocomelia (when the bones of one or more limbs are stunted or fail to develop), among others.

1.2.1.3 Stem cells

An important feature of embryonic cells that allows them to undergo organogenesis is "stemness". Embryonic cells are initially stem cells, undifferentiated cells that have the ability to give rise to all specialized tissue types. Interestingly, only the zygote has totipotent stem cells which can produce both embryonic and extraembryonic tissue. In contrast, embryonic stem cells derived from the ICM are pluripotent and can give rise to all types of tissue except the trophoblast. Stem cells are unique in that they are able regenerate; once they divide by mitosis one daughter cell retains its undifferentiated characteristic while the other commits to one or more paths of differentiation (Gilbert, 2010). How each daughter cell decides whether to remain undifferentiated or not depends on several factors, including the niche environment and signaling cues from the surrounding tissue. Indeed, stem cells persist even into adulthood, albeit with

much limited potency compared to embryonic stem cells; adult stem cells are multipotent, are restricted to certain niches and can give rise to a subset of related specialized cells rather than all tissue types (e.g. hematopoietic stem cells). As mentioned earlier, the three germ layers of the embryonic epiblast have the potential to generate the tissues of all three germ layers, thus the regulation and preservation of stem cells is a crucial factor for the proper development of the embryo. Some of the factors involved in maintaining stem cells during the embryonic and adult stages of life include the P53 family of proteins (as is discussed later), showing that agents that can disrupt their activity or expression may lead to congenital malformations by affecting the integrity of stem cell populations in the embryo.

1.2.2 Susceptibility to genotoxic and oxidative stress-inducing agents

During organogenesis, embryonic cells undergo a tremendous increase in proliferation and later differentiation to lay down the foundation for the development and growth of various organs and systems. As such, these cells experience increased aberrant DNA damage that occurs from the inborn errors that arise naturally during replication, as well as from DNA oxidizing agents that are produced from the cell as a by-product to cellular growth and metabolism (i.e.. reactive nitrogen and oxygen species [RNOS]) (Ames and Shigenaga, 1992; Burcham, 1999).

1.2.2.1 The DNA damage stress response

Since the sustention of damaged DNA can jeopardize the function and survival of the cell and its daughters, an intrinsic component that enables them to maintain their viability is the DNA damage repair machinery. This conglomeration of various enzymes and signaling proteins acts in response to various types of DNA damage to maintain the native structure of the DNA, both at the nucleotide and the macromolecular levels. Several types of repair mechanisms that can be triggered by specific forms of DNA damage (e.g. nucleotide excision repair, base excision repair, mismatch repair and recombination repair) have been adapted by mammalian cells (Norbury and Hickson, 2001; Wood, 1996). The embryo is no exception to this; typical DNA damage response markers (e.g. γH2AX foci, P53BP1, cleaved PARP) can be detected as early as the cleavage stage in the rodent embryo and almost all of the genes for the various DNA repair proteins are expressed throughout embryogenesis (Grenier *et al.*, 2012; Vinson and Hales, 2002a). Interestingly, the type and level of expression of different DNA repair proteins varies

depending on the stage of development; in the rodent embryo, the expression levels of *Atm* (upstream regulator of the DNA damage response pathway), *Ercc1* (involved in nucleotide excision repair), *Ung1* and *Ung2* (both involved in base excision repair), among others, are much higher in mid-organogenesis (i.e. GD 12) than in earlier stages (i.e. GD 9 and 10). In addition, their levels differ between various parts of the conceptus; while both the yolk sac and embryo proper have equal levels of expression in early organogenesis, the levels increase dramatically in the embryo (relative to the yolk sac) by mid-organogenesis (Vinson and Hales, 2002b; Vinson and Hales, 2001a; Vinson and Hales, 2001b). These findings suggest that the embryo has the capacity to repair damaged DNA, yet the extent of this ability changes over time, rendering it potentially more susceptible to DNA damage in early organogenesis.

While endogenous sources of DNA damage occur naturally throughout embryogenesis, and can be successfully countered by the nascent repair machinery, several extrinsic genotoxic agents have also been shown to trigger the embryonic cell's defenses. Xenobiotic genotoxicants can interact directly with DNA, causing physical and structural damage, or they can act indirectly by disrupting DNA segregation, affecting chromatin structure or deregulating the DNA replication process.

Regardless of the mechanism of inducing damage, the cellular pathways that respond to DNA damage generally share a uniform objective: either to repair the damage or, failing that, to instigate cellular death to avoid transferring the damaged DNA to daughter cells. If this mechanism fails during organogenesis, an excess of cells with irreparable DNA or aberrant cell death would lead to the disruption of organ formation, yielding structural or functional birth defects. Since the embryo's ability to respond to and repair damage inflicted by genotoxicants varies throughout development, there are certain "windows" of susceptibility in which it can be especially vulnerable to genotoxic damage. In addition, the embryonic cell's defense systems are not as resilient as those of adult cells, therefore embryos are more sensitive to low doses of genotoxicants (Wells *et al.*, 2010). As such, many xenobiotic genotoxicants are considered teratogenic as they can disrupt the fine-tuned developmental processes that are essential for organ formation at doses that apparently do not affect the health of the mother. Examples of such agents include pharmaceuticals (e.g. cyclophosphamide, hydroxyurea), physical agents (e.g. ultraviolet and ionizing radiation), and environmental chemicals (e.g. benzo[a]pyrene, toluene) (Wells *et al.*, 2009).

1.2.2.2 The oxidative stress response

A mechanism of DNA damage that elicits an additional embryonic damage response is oxidative stress. This form of stress occurs when the redox homeostasis of the cell, i.e. the various mechanisms that regulate the levels of RNOS, is disrupted such that the level of RNOS is beyond what the cell's intrinsic control mechanisms can handle. An excessive increase in RNOS results in aberrant oxidation and nitration of macromolecules, including DNA, protein and lipids, which affects their function and stability (Cadenas and Sies, 1985; Dennery, 2007).

Normally, endogenous RNOS are released from the mitochondria, where the electron transport chain, which produces energy in the form of ATP leaks RNOS as a by-product (Dennery, 2007), and from the activity of endogenous enzymes (e.g. nitric oxide synthases) (Förstermann and Sessa, 2012). In addition, they may be produced from other primary messengers, such as epidermal growth factor (EGF-1) (Sundaresan *et al.*, 1996). The embryonic cell has many proteins and molecules that maintain a healthy redox balance and provide it with the adequate levels of RNOS required to perform some of its physiological functions. Indeed, the level of RNOS can promote or suppress cell growth, survival, proliferation and differentiation, all of which are hallmarks of embryogenesis and organogenesis (Schafer and Buettner, 2001). In addition, RNOS can act as important signaling messengers, activating transcription factors that are necessary for embryonic development, such as hypoxia inducible factor (HIF-1α), nuclear factor κB (NF- κB), wingless and integration site (WNT), activator protein 1 (AP-1) and nuclear factor-E2 related factor 1 (NRF-1) (Chen *et al.*, 2003; Forsythe *et al.*, 1996; Gomez del Arco *et al.*, 1997; Shin *et al.*, 2004; Yang *et al.*, 2004).

Since proper embryonic development relies heavily on the precise timing of activation or inactivation of these redox-sensitive factors the embryo's redox status varies throughout embryogenesis. At the early post-implantation stage, oxygen levels are relatively low and the embryo's environment is hypoxic; this is desired since low oxygen levels promote proliferation at this stage. However, differentiation requires higher levels of RNOS which means that in early organogenesis, when the placenta begins to mature (i.e. around GD 9 of the mouse embryo), the embryo is first exposed to a high oxygen concentration (Chen *et al.*, 1999; Kaufman, 1992; Lee *et al.*, 2006; New and Coppola, 1970; Schafer and Buettner, 2001; Takahashi and Zeydel, 1982). This makes the stage of early organogenesis particularly sensitive to oxidative stress as

the redox switch is made from anaerobic to aerobic conditions. As the embryo develops further it acquires a stronger anti-oxidant defense system to cope with the higher oxygen levels and so it is able to withstand insult from stronger environmental oxidants (Hansen, 2006).

The efficacy and resilience of this antioxidant system change throughout development and can be overwhelmed by exogenous oxidizing agents, in a manner similar to that of the DNA damage repair machinery. In addition, the expression of various antioxidant elements is heterogeneous in the developing embryo, with certain tissues having more or less expression depending on their function and stage of development, rendering certain regions more susceptible to oxidative stress (and hence the development of possible malformations) than others (Gnanabakthan and Hales, 2009; Hansen, 2006; Yan and Hales, 2006). Typical antioxidant levels and enzyme activities, such as the glutathione regulating enzymes, glutathione peroxidase and glutathione reductase, increase remarkably between GD 10 and GD 11 in the yolk sac of the rat embryo (equivalent to GD 9 and GD 10 in the mouse) and continue to increase up until late organogenesis, while remaining at very low and constant levels in the embryo proper. The level of superoxide dismutase (SOD), which catalyzes the neutralization of the hyper-reactive superoxide anion, is slightly higher in the embryo compared to the yolk sac in early organogenesis but it is still quite low relative to later stages of development (Choe *et al.*, 2001). In addition, the expression of thioredoxin (another endogenous antioxidant) is detected on GD 10, with levels being very low or almost undetectable before then; it increases gradually from GD 10 to 16 (Kobayashi *et al.*, 2000). Moreover, the caudal regions of the embryo at GD 9 are more susceptible to lipid and protein 4-hydroxynonenol (4-HNE) adducts, as well as other alterations produced from oxidative stress, most likely due to the lower levels of antioxidants in this region of the embryo, compared to the rostral regions, at that stage of development (Gnanabakthan and Hales, 2009; Yan and Hales, 2006). Strategies that aim at bolstering the embryo's antioxidant defenses, including transgenic overexpression of antioxidant proteins or supplementation with free radical scavengers, have proven effective in protecting the embryo against oxidative insult (Harris and Hansen, 2012; Larouche and Hales, 2009). Thus, while the embryo does possess antioxidant mechanisms, their relatively low abundance and activity in early organogenesis, coupled with increasing levels of oxygen exposure and RNOS production, render the organogenesis-stage embryo vulnerable to damage from oxidative stress-inducing chemicals.

Many pharmaceutical and environmental chemicals can cause oxidative stress, including some well-known teratogens (Kovacic and Somanathan, 2006). Although recent publications show that the embryotoxic mechanism of action of the most infamous teratogen, thalidomide, involves the binding of the E3 ubiquitin ligase, cereblon (which prevents it from degrading endogenous proteins and hence leading to cellular dysregulation and maldevelopment), thalidomide is also capable of releasing hydroxyl radicals and depleting endogenous glutathione, leading to oxidative DNA damage, a key event in the embryotoxicity of several known teratogens (Hansen *et al.*, 2002; Liu *et al.*, 2015; Parman *et al.*, 1999). Hydroxyurea, an anticancer drug, inhibits the activity of ribonucleotide reductase (RNR) by releasing nitric oxide radicals, eventually causing cell cycle arrest and apoptosis (Jiang *et al.*, 1997); discussed in section 1.3.1). Many inorganic metals, such as chromium, can also induce oxidative stress-induced DNA damage, causing reproductive defects in mice (Acharya *et al.*, 2006; Yang *et al.*, 2005).

As discussed previously, alteration of the embryo's capacity to differentiate or proliferate could very likely result in structural and functional birth defects. While the mechanisms by which various teratogens induce oxidative stress may differ, the common outcome is an increase in cellular oxidation, either by the depletion of the cell's antioxidant capacity or the boosting of RNOS production. If cells of the early organogenesis embryo experience increased oxidation of DNA, proteins and lipids that render them dysfunctional and irreparable, they will either undergo defective proliferation and differentiation or instigate excessive cell death (Dennery, 2007). Furthermore, since not all regions of the early organogenesis stage embryo express the same levels and kinds of antioxidants, this makes certain tissues and organs more susceptible to oxidative stress-induced damage and cell death than others, leading to the development of distinct malformations (Gnanabakthan and Hales, 2009; Hansen, 2006). Thus, if not properly controlled or repaired, oxidative-stress induced damage can result in a teratogenic outcome.

1.3 Hydroxyurea

Hydroxyurea (chemical formula: $CH_4N_2O_2$) is a pharmaceutical compound first synthesized in 1869 (Dresler, 1869) and approved for medical use in the USA in 1967, where it is currently manufactured and sold by Bristol-Myers Squib (Liebelt *et al.*, 2007). It is indicated for treating melanoma, ovarian carcinoma, chronic myeloid leukemia and thalassemia.

Additionally, it is used in patients with sickle-cell anemia to reduce the frequency of painful crises and the need for blood transfusion in adults and children (Liebelt*, et al.*, 2007).

Typical doses of hydroxyurea for the treatment of sickle-cell anemia are between 15-35 mg/kg bw per day, while doses for the treatment of certain solid tumors can be as high as 80 mg/kg bw every third day. Treatment is usually chronic and may last for years (Liebelt*, et al.*, 2007). Hydroxyurea is usually formulated and administered as capsules (200, 300, 400 or 500 mg) or tablets (1000 mg). It is well absorbed from the gastrointestinal tract (GIT) after oral administration and reaches peak plasma concentrations within 1-4 hours (Bristol-Myers-Squib, 2016). It distributes in the body at an apparent volume of distribution equivalent to total body water (~42 liters) and enters the cerebrospinal fluid approximately 3 hours after oral administration (Bristol-Myers-Squib, 2016; Stevens, 1999).

In mice and other animal models, hydroxyurea is readily absorbed through the oral route, can reach peak plasma levels within 7 min after intraperitoneal (i.p) injection (Warner *et al.*, 1983), and can cross the placental barrier to reach the embryo. Half of the administered hydroxyurea is metabolized into urea, which is then mainly excreted in the urine (Adamson *et al.*, 1965). Hydroxyurea has a very short half-life ($t_{½} \leq 30$ min) in adult rats and mice, but has a much longer half-life in embryos ($t_{½} \sim 85$ min) (Wilson *et al.*, 1975). This makes hydroxyurea a potential hazard to the proper development of the conceptus during pregnancy.

1.3.1 Mechanism of action

Hydroxyurea acts as an anti-neoplastic agent; it is cytotoxic, resulting in cell cycle arrest and cell death (Philips *et al.*, 1967). The main known mechanism of action is through the inhibition of ribonucleotide reductase (RNR), an enzyme essential for the conversion of ribonucleoside triphosphates (NTPs) into deoxyribonucleoside triphosphates (dNTPs), the building blocks of DNA. By depleting the pools of dNTPs, hydroxyurea effectively stalls DNA replication forks, rendering the cell incapable of progressing through DNA replication, and instigating an arrest of the cell cycle at the S-phase, causing growth inhibition and increasing the possibility of cell death (Grallert and Boye, 2008; Koc *et al.*, 2004).

Hydroxyurea is reported to inhibit RNR by producing free radicals that scavenge a tyrosyl free radical in the M2 subunit of RNR which is necessary for its enzymatic activity (Iman *et al.*, 2016; Yarbro, 1992). Indeed, several studies have shown that hydroxyurea produces the

free radical nitric oxide (·NO), which then yields a number of harmful RNOS, such as the hydroxyl radical (·OH), the nitrogen dioxide radical ($\cdot NO_2$) and peroxynitrite ($ONOO^-$) (Burkitt and Raafat, 2006; Jiang*, et al.*, 1997; Pacelli *et al.*, 1996). These radicals are known to induce direct and indirect DNA damage, alter protein structure and activity, and elicit oxidative stress, ultimately leading to cell injury and death (Eiserich *et al.*, 1999; Jones *et al.*, 2009; Wells*, et al.*, 2009).

Interestingly, a recent study showed that hydroxyurea can induce cytostatic effects and DNA damage in a RNR-independent mechanism (Liew *et al.*, 2016). The hyperthermophilic archaeon *Sulfolobus solfataricus* contains a RNR species which is insensitive to hydroxyurea-inhibition since it does not need the tyrosyl radical in order to be enzymatically active (Jordan and Reichard, 1998; She *et al.*, 2001). When treated with hydroxyurea, these cells still undergo cell cycle arrest and have DNA strand breaks in a dose-dependent fashion. The apparent mechanism by which hydroxyurea causes this effect is through the dysregulation of specific replication initiation and cell division factors, such as primase, rather than the depletion of dNTP pools (Liew*, et al.*, 2016). Thus, it may be possible that hydroxyurea exerts its cytostatic and DNA damaging effects through various mechanisms, including oxidative stress and replication fork stalling.

The mechanisms of action of hydroxyurea in the treatment of sickle cell anemia is thought to be by indirectly promoting the synthesis of fetal hemoglobin; as hydroxyurea arrests the synthesis of erythrocytes it allows for new immature progenitors that can synthesize fetal hemoglobin to proliferate and differentiate into mature red blood cells (Halsey and Roberts, 2003; Yang and Pace, 2001). In addition, hydroxyurea induces nitric oxide concentrations, *in vivo* and *in vitro,* which can activate soluble guanylyl cyclase, leading to the upregulation of γ-globin, a necessary component for fetal hemoglobin synthesis (Cokic *et al.*, 2003; Jiang*, et al.*, 1997; Nahavandi *et al.*, 2000; Pacelli*, et al.*, 1996).

1.3.2 Hydroxyurea developmental toxicity

Hydroxyurea induces DNA replication fork stalling, DNA strand breaks and oxidative stress, rendering it a powerful cytotoxic substance; coupling these observations with hydroxyurea's ability to cross the placenta and its longer half-life in embryos makes it a potential teratogen (Liebelt*, et al.*, 2007; Wilson*, et al.*, 1975). Indeed, hydroxyurea has been shown to be

a potent teratogen in mice, rats and rabbits, producing severe gross and skeletal malformations (DeSesso and Jordan, 1977; Wilson*, et al.*, 1975; Yan and Hales, 2005). The manifestation of congenital malformations largely depends on the species under study, concentration of the drug and the time of exposure during gestation. Animal studies have shown that pregnant rats and mice treated with hydroxyurea between GD 6 and 15, with single or daily doses, produced fetuses with cleft palate, cleft lip, short and kinky tails, phocomelia, syndactyly, ectrodactyly and adactyly of the fore- and hind-limbs, hydrocephaly, and anophthalmia (Bristol-Myers-Squib, 2016; Chaube and Murphy, 1966; DeSesso *et al.*, 2000; Yan and Hales, 2005). In addition, hydroxyurea exposure has been reported to reduce bone ossification, live litter size, fetal size and weight, and cause neurobehavioral deficits (Aliverti *et al.*, 1980; DeSesso and Jordan, 1977; Vorhees *et al.*, 1979).

The teratogenic doses of hydroxyurea used in animal studies are higher than the recommended human therapeutic doses, but in some cases they are only 0.3 times above the maximum recommended therapeutic levels (on a mg/m^2 basis). As such, women are advised to avoid pregnancy during treatment and to discontinue the treatment if they discover that they have become pregnant or plan to do so (Bristol-Myers-Squib, 2016). While there are not many reported cases of women being exposed to hydroxyurea during pregnancy, there have been a few incidents recorded in the literature. So far, there have been no studies conducted on hydroxyurea exposure effects on pregnant women, so a causal link between hydroxyurea and birth defects in humans is unascertainable (Liebelt*, et al.*, 2007). In a case-series, 31 pregnant women were treated with hydroxyurea during either the first, second, or third trimesters, or throughout the gestation period (Thauvin-Robinet *et al.*, 2001). The pregnancy outcomes showed no major malformations and no pre/post-natal chromosomal aberrations, but a few abortions and *in utero* fetal deaths were recorded. However, it is not possible to ascertain if these abortions were correlated with hydroxyurea or the underlying medical condition for which the women were being treated (e.g. sickle cell anemia, chronic myeloid leukemia, or essential thrombocythemia) (Thauvin-Robinet*, et al.*, 2001).

The mechanism by which hydroxyurea causes malformations in animal models is still being investigated. While it has been extensively studied, several molecular processes and cellular signaling pathways are found to be affected by hydroxyurea in the developing embryo. The most prominent mechanisms of hydroxyurea teratogenicity are thought to be DNA damage

and oxidative stress that lead to cell cycle arrest and programmed cell death (i.e. apoptosis) (DeSesso*, et al.*, 2000; Torchinsky *et al.*, 2005; Yan and Hales, 2006). Hydroxyurea is most teratogenic during the stage of organogenesis (DeSesso, 2010). When the embryo switches from anaerobic to aerobic metabolism it becomes highly sensitive to the RNOS that are produced by hydroxyurea; the inhibition of cell division due to DNA replication fork stress causes developmental delays of vital organs such as the limbs, digits and skeleton (Wells*, et al.*, 2009; Yan and Hales, 2005).

In the organogenesis stage mouse embryo, hydroxyurea has been shown to disrupt the activities of several transcription factors and proteins that maintain redox homeostasis and DNA integrity. In mouse embryos treated on GD 9, hydroxyurea increases the DNA binding affinity of AP-1, a redox-sensitive transcription factor that regulates a variety of anti-oxidant, apoptotic and cell cycle control genes (Yan and Hales, 2005). In addition, hydroxyurea leads to an increase in γH2AX foci, a marker of DNA strand breaks, and activates the MAPK signaling cascade by inducing the phosphorylation of MEK3/6 and P38 (Banh and Hales, 2013). The MAPK signaling pathway is sensitive to oxidative stress and DNA damage and can control the cell's fate by the induction or prevention of cell death, by directly or indirectly inducing caspase-mediated apoptosis (Cargnello and Roux, 2011). Yet, during organogenesis, hydroxyurea has varying effects on different MAPKs; while both P38 and c-Jun N-terminal kinase (JNK) are activated, another major MAPK, extracellular signal regulated kinase (ERK), is not. Also, P38 and JNK play distinct roles in the embryo's response to hydroxyurea; inhibiting P38 induces fetal mortality rather than malformations, while inhibiting JNK produces the inverse effect (Yan and Hales, 2008). Interestingly, P38 and JNK have common downstream targets, such as AP-1 and P53 (Turjanski *et al.*, 2007). While hydroxyurea has been shown to affect AP-1 activity in the organogenesis-stage embryo, its effect on P53 remains to be determined.

1.4 The P53 protein family

The P53 protein family consists of three functionally and structurally homologous proteins: P53, P63 and P73. Since P53 is considered the evolutionary product of P63 and P73, and was the first to be discovered, there is a tendency to consider P53 as the archetypal member of this group (Lane and Crawford, 1979; Linzer and Levine, 1979; Melino *et al.*, 2002; Yang *et al.*, 2002). Yet, research has shown that both P63 and P73 play distinct, as well as similar, roles

to P53 under normal and stress conditions, including in mammalian development (Levrero *et al.*, 2000; Moll and Slade, 2004).

Like P53, both P63 and P73 generally function as tumor suppressors by inducing cell cycle arrest, DNA damage repair, senescence, autophagy or apoptosis (Table 1.1). In addition, P53, P63 and P73 all have different isoforms (Fig 1.1) that can produce the opposite effects (Grob *et al.*, 2001; Murray-Zmijewski *et al.*, 2006; Petitjean *et al.*, 2008; Wilhelm *et al.*, 2010). Moreover, there appears to be some inter-dependence between the three homologs; P53 has been shown to require P63 and P73 to exert its transcriptional activity under specific conditions, and all three proteins share considerable promoter occupancy *in vivo* (Fatt *et al.*, 2014; Flores *et al.*, 2002; Yang *et al.*, 2010). However, unlike P53, both P63 and P73 are essential for the development of mammalian organ systems, such as the epidermis, limbs and the nervous system (Donehower *et al.*, 1992; Yang *et al.*, 1999; Yang *et al.*, 2000).

These unique and overlapping functions highlight a complex relationship between the three homologs of the P53 family, yet how they interact and function during mammalian organogenesis remains unknown. Of particular interest is their response and influence on the teratogenicity of cytotoxic compounds, such as hydroxyurea, considering their ability to induce DNA damage repair, cell cycle arrest and cell death.

Table 1.1: Functions of P53/P63/P73 in development and the stress response.

	P53	P63	P73
Developmental expression	- Neural tube - Limb	- Epidermal - Limb	- Neural
Null mutation mice	- Late Cancers - Exencephaly - Limb defects	- Missing limbs - Defective Skin - Lethal	- Neurobehavioural deficits - Pheromonal defects - Growth defects
Stemness	-	+	+
Senescence	+		+
DNA Damage	+	+/-	+
Cell Cycle	+	+	+
Apoptosis	+	+	+

Simplified classification of the functions of the p53 family members. Members of this family are involved in different cellular functions, and knockout mice reveal different developmental phenotypes. For simplicity, and because current information is incomplete, this scheme does not differentiate among the TA (containing the transactivation domain) and ΔN (lacking the N-terminal transactivation domain) protein isotypes. +: indicates evidence for positive regulation by the protein. -: indicates evidence for negative regulation by the protein. Adapted from Melino et al. (2003).

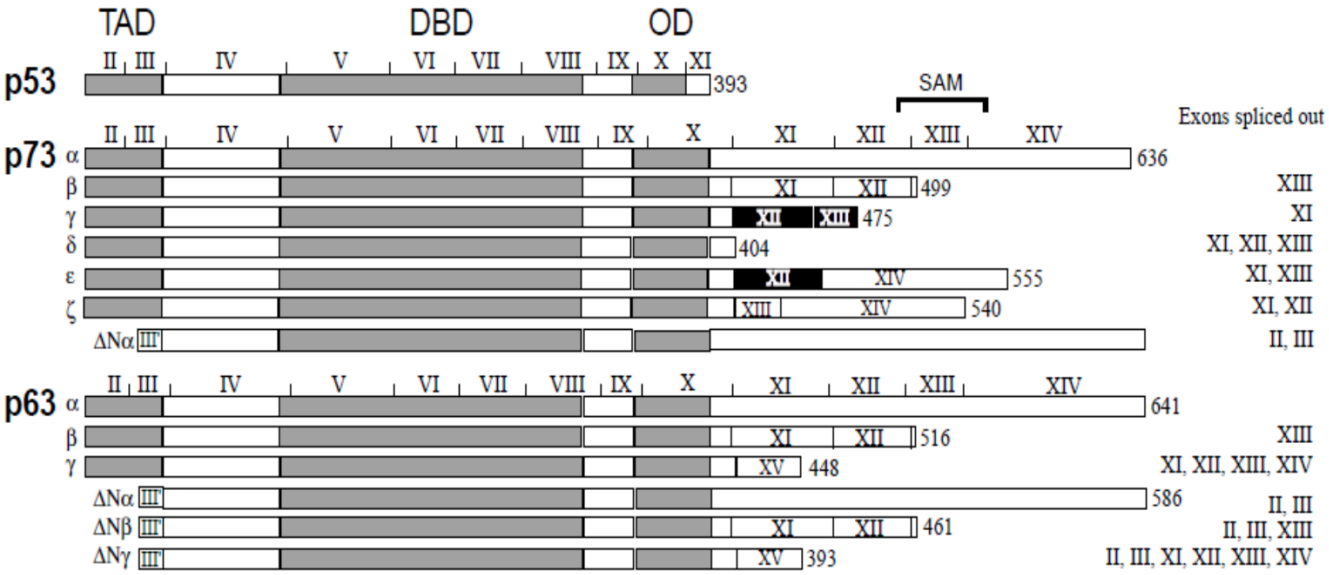

TAD
DBD
OD
p53
II III IV V VI VII VIII IX X XI
393
SAM
Exons spliced out
p73
α
β
γ
δ
ε
ζ
ΔNα
II III IV V VI VII VIII IX X XI XII XIII XIV
636
499
475
404
555
540
XIII
XI
XI, XII, XIII
XI, XIII
XI, XII
II, III
p63
α
β
γ
ΔNα
ΔNβ
ΔNγ
641
516
448
586
461
393
XIII
XI, XII, XIII, XIV
II, III
II, III, XIII
II, III, XI, XII, XIII, XIV

Figure 1.1: Schematic representation of the exon structures of human p53, p63 and p73. The transactivation domain (TAD), DNA binding domain (DBD) and the oligomerization domain (OD) are indicated in grey, the sterile alpha motif (SAM) domain is also indicated. The numbers of the exons are indicated in Roman numerals above each full length protein. Exons read out of frame with the α variant are in black. Exons spliced out in each isoform are indicated on the right. The ΔNP63 and ΔNP73 isoforms lacking the TAD, which are translated from an alternative ATG, are indicated in dotted grey. Adapted from Levrero *et al*. (2000).

1.4.1 The P53 protein

First discovered in 1979 (Lane and Crawford, 1979; Linzer and Levine, 1979), the 53 KDa tumor suppressor protein, P53, is one of the most studied proteins in the biological and medical field (Vogelstein *et al.*, 2000). The protein contains several domains: a transactivation domain (TAD), a DNA binding domain (DBD), and an oligomerization domain (OD). The *Trp53* gene has more than one transcription start site, yielding up to nine different mRNA variants that can lack the C-terminus OD (β and γ) or the N-terminus TAD (the ΔN isoforms) (Murray-Zmijewski*, et al.*, 2006). P53 is the central figure of a signaling pathway that controls vital cellular processes in response to a variety of intrinsic and extrinsic factors (Levrero*, et al.*, 2000).

Indeed, P53 and its signaling pathway have been studied extensively in relation to its role in tumor formation and cancer therapy; they have been conclusively shown to suppress and prevent tumor growth in animal studies (Donehower*, et al.*, 1992). In fact, about 50% of all human tumors are found to have a mutated and inactive P53 protein or defective P53 signaling pathway (Hainaut and Hollstein, 2000; Vogelstein*, et al.*, 2000). Due to its central role in protecting cells against tumorigenesis, it has been coined "the guardian of the genome" (Lane, 1992). However, there have been many studies that indicate an important role for P53 in other physiological processes, including mammalian embryonic development (Armstrong*, et al.*, 1995; Barkic *et al.*, 2009; Choi and Donehower, 1999; Hosako *et al.*, 2007; Sah *et al.*, 1995; Vousden and Lane, 2007; Zhang *et al.*, 2017).

1.4.1.1 P53 regulation

As P53 is a major determinant of the cell's response to stress factors, it is very tightly regulated. While current research reveals increasingly complex modes of P53 regulation and action, the classical model of P53 regulation and signaling is as follows: under normal conditions, P53 is maintained at low basal levels in the cytoplasm. It is bound by the E3-ubiquitin ligase, MDM2 (or its homolog, MDM4), which acts as a negative regulator of P53 by targeting it for proteasomal degradation via ubiquitination (Harris and Levine, 2005; Kruse and Gu, 2009; Shi and Gu, 2012). However, under stress conditions P53 undergoes extensive post-translational modifications; it can be phosphorylated on several serine and threonine residues by a variety of upstream kinases (e.g. ATM, ATR, CHK1/2, P38, JNK, ERK) and/or acetylated on several lysine residues by acetyl transferases (e.g. TIP60, CBP/P300). These modifications lead

to its dissociation from MDM2 (or MDM4) and its subsequent transactivation. Following this, P53 translocates to the nucleus where it binds to specific promotor sites on the DNA and acts as a transcription factor by up- or down-regulating the transcription of a variety of downstream gene targets. Through the expression of specific genes, P53 can arrest cell cycle progression (e.g. *Cdkn1a*, *Rb*), induce DNA damage repair (e.g. *R2*), autophagy (e.g. *Dram*), or apoptosis (e.g. *Puma*, *Noxa*), as well as affect other cellular processes (Vousden and Prives, 2009).

However, several recent findings challenge the established classical model, revealing an even more complex regulatory mechanism. As more tools were developed to study transcriptional activity and DNA binding *in vivo*, it was found that P53 has the ability to bind DNA in the absence of stress (Kruse and Gu, 2009). ChIP assays revealed that the sites at which P53 is bound under normal conditions do not necessarily contain the P53 consensus sequence binding site, suggesting that P53 controls the expression of a multitude of genes for normal homeostasis that are not directly involved in cell cycle arrest or apoptosis (Liu *et al.*, 2004). Moreover, it was found that under normal conditions P53 binds the promoter sites of its downstream effector targets, such as *Cdkn1a*, yet is unable to promote their transcription, possibly due to repression by MDM2, or its homologs, MDM4 and MDMX (Kaeser and Iggo, 2002). Indeed, MDM2, MDM4 and MDMX were found to complex with P53 at specific promoter sites, suggesting that the specificity of P53 in regulating the transcription of its downstream effectors depends largely on other co-factors, not just its post-translational modifications (Barboza *et al.*, 2008; Tang *et al.*, 2008). Yet, while it appears that P53 binding specificity to the promoter sites of its downstream targets does not rely on its post-translational modifications alone, its ability to activate their transcription, as well as the functional outcome, does. In response to DNA damage, the MAPK pathway initiates, with the ATM and ATR kinases phosphorylating their downstream targets, CHK1 and/or CHK2, which then activate the effector kinases, P38 and JNK (Appella and Anderson, 2001; Shieh *et al.*, 2000; Shieh *et al.*, 1997). P38 and JNK are able to preferentially phosphorylate specific serine residues on P53; P38 phosphorylates Ser15 (Ser18 in murine P53), while JNK phosphorylates Ser20 (Ser23 in murine P53). Knock-in mutation studies conducted in mice showed that while P53 activation is not dependent on the phosphorylation of just one residue, the phosphorylation of both residues is required for the dissociation of the MDM2-P53 complex and the transactivation of P53 (Chao *et al.*, 2003; Chao *et al.*, 2006; MacPherson *et al.*, 2004). These modifications usually lead to a

transcriptional activity mediated outcome, whereupon depending on the severity of the stress, P53 modulates the expression of specific genes that prime the cell for cell cycle arrest, senescence or apoptosis.

Other types of post-translational modifications exist as well; P53 acetylation by the histone acetyltransferase, TIP60, is essential for P53-mediated apoptosis but not cell cycle arrest (Sykes *et al.*, 2006; Tang *et al.*, 2006). In addition, other types of P53 modifications, such as methylation, sumoylation and neddylation, can control its binding affinity and transcriptional activity. The methylation of Lys372 of P53 by the methyltransferase SET9 induces the P53-mediated expression of *Cdkn1a* (Chuikov *et al.*, 2004), while the dimethylation of Lys370 and Lys382 allows P53 to bind the P53BP1 promoter site, a DNA damage repair factor (Huang *et al.*, 2007). Methylation by SMYD2 and SET8 can repress P53 transcriptional activity, and while not yet determined *in vivo,* both sumoylation and neddylation are believed to act to retain P53 in the cytoplasm and prevent its activity (Abida *et al.*, 2007; Carter *et al.*, 2007; Huang *et al.*, 2006; Shi *et al.*, 2007).

These findings reveal a complex regulatory mechanism for the stabilization and activation of P53 that occurs both at the cytoplasmic and at the DNA level. The involvement of a multitude of co-factors and post-translational events ensures a tightly-controlled, yet rapid and specific, cellular response to stress.

1.4.1.2 P53 functions under stress and normal conditions

Generally, P53 is known for its roles as a tumor suppressor and as a survival factor against cytotoxic stress, by causing the cell to undergo cell cycle arrest or programmed-cell death via apoptosis. Various external stress factors lead to the stabilization and activation of P53. These include RNOS-mediated oxidative stress, heat shock, and DNA-damaging agents (e.g. UV rays, γ-irradiation, cyclophosphamide, and hydroxyurea) (Sablina *et al.*, 2005; Seillier M., 2012).

While the type of stress can influence the method of P53 activation differentially, most cause P53 to elicit either cell cycle arrest or apoptosis. Initially, these effects were thought to rely solely on P53's transcriptional activity (Fig 1.1), where P53 upregulates genes involved in cell cycle inhibition (e.g. *Cdkn1a*) or apoptosis (e.g. *Puma, Noxa*) (Kruse and Gu, 2009). However, in addition to its transcription-based functions, P53 has non-transcriptional activities. Once

stabilized in the cytoplasm, P53 can migrate to the mitochondrion, where it interacts with the pro-apoptotic proteins BAX and BAK and the anti-apoptotic protein BCL to induce apoptosis (Chipuk *et al.*, 2004; Leu *et al.*, 2004; Mihara *et al.*, 2003). It is well-established that the mitochondrion is essential for the initiation and propagation of the intrinsic apoptotic cascade; P53 binds BAX and BAK, forming pores in the outer mitochondrial membrane that allow for the release of cytochrome C, which then signals APAF-1 and pro-caspase 9 to form the apoptosome, that subsequently activates pro-caspase 3 into its cleaved effector form. At the same time, P53 binds the anti-apoptotic BCL-X and BCL2 proteins, preventing them from abrogating the effects of BAX and BAK (Elmore, 2007). Thus, P53 can induce apoptosis in response to stress in a transcription independent manner (Fig 1.2).

It is important to note that P53 seems to exert a balancing act when it comes to the path the cell takes in response to stress; low-level stress signals promote P53-mediated cell-cycle arrest, while sustained or high-level stress pushes P53 towards the pro-apoptotic route (Eliaš *et al.*, 2014; Vousden and Prives, 2009). The proposed theory behind this phenomenon stipulates that since the main aim of P53 is to protect the cell from external insults, such as genotoxic compounds, it preferentially promotes cell cycle arrest to give time for the damage to be repaired by the cell's repair machinery (e.g. the MRN complex for DNA strand breaks) after which the cell can resume its normal cycle (Gatz and Wiesmuller, 2006; Robison *et al.*, 2005). However, if the damage is irreparable then P53 opts for "sacrificing" the cell via apoptosis, to prevent the passing on of damaged DNA to daughter cells and thus preserve tissue integrity (Geske *et al.*, 2001; Vousden and Prives, 2009).

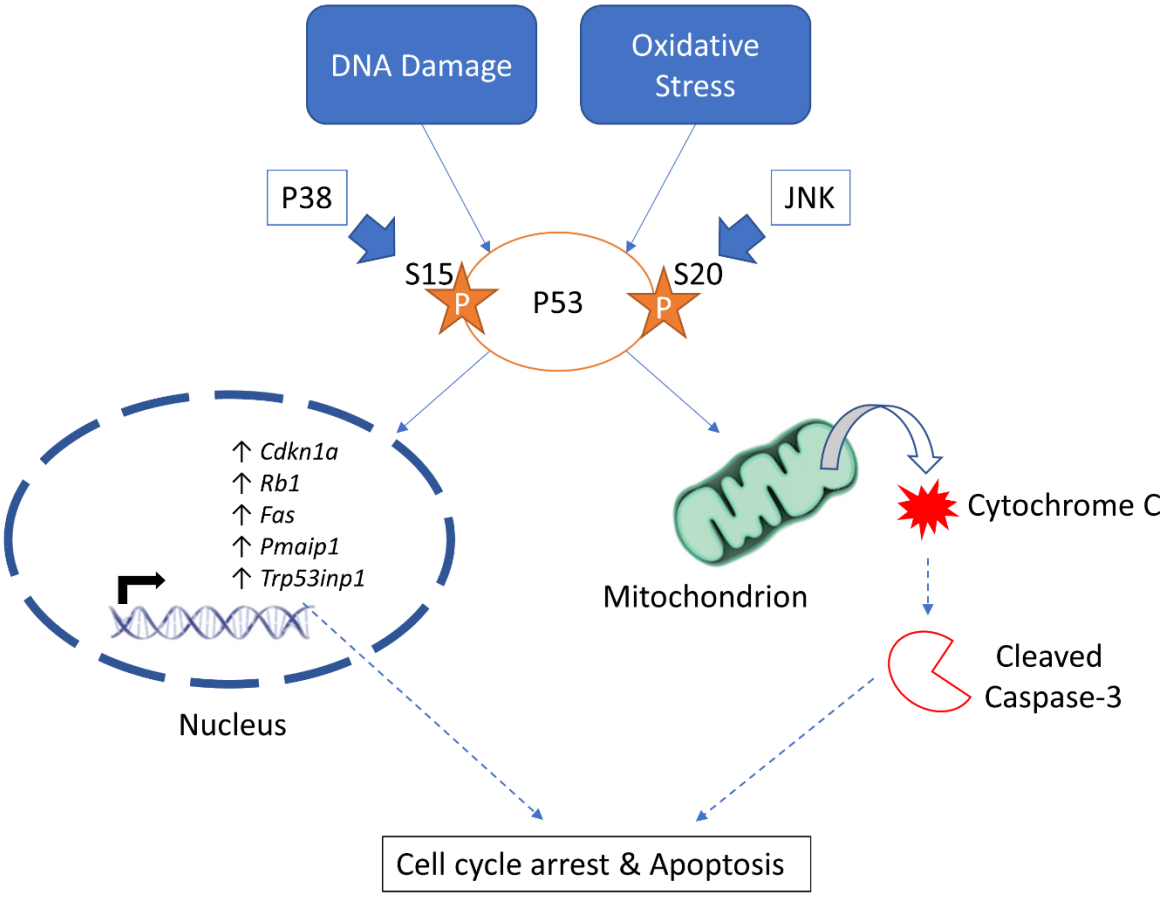

DNA Damage
Oxidative Stress
P38
JNK
S15
P
P53
S20
P
↑ Cdkn1a
↑ Rb1
↑ Fas
↑ Pmaip1
↑ Trp53inp1
Nucleus
Mitochondrion
Cytochrome C
Cleaved Caspase-3
Cell cycle arrest & Apoptosis

Figure 1.2: P53 signaling in response to stress.

Upon stimulus from DNA damage or oxidative stress, several upstream kinases (e.g. P38 and JNK) phosphorylate P53 on specific residues (i.e. S15 and S20 respectively) leading to its stabilization and transactivation. Subsequently, P53 can translocate to the nucleus, where it acts as a transcription factor and upregulates genes involved in cell cycle arrest (e.g. *Cdkn1a, Rb1*) and apoptosis (e.g. *Fas, Pmaip1, Trp53inp1*). P53 can also induce apoptosis by migrating to the mitochondrion and promoting the release of cytochrome C, eventually leading to the cleavage and activation of the effector apoptotic protein, Caspase-3.

Other than its function in response to stress, P53 also has basal level activity, regulating several cellular processes to maintain proper homeostasis. P53 has a role in the regulation of glycolysis; under normal conditions P53 inhibits the expression of the glucose transporters GLUT1 and GLUT4, decreases fructose-2,6-bisphosphate levels by increasing *Tigar* expression (which increases fructose bisphosphatase levels), while also modulating NF-κB levels, ultimately leading to the downregulation of glycolysis (Bensaad *et al.*, 2006; Kawauchi *et al.*, 2008; Schwartzenberg-Bar-Yoseph *et al.*, 2004). Conversely, P53 is a positive regulator of oxidative phosphorylation; P53 upregulates the cytochrome c oxidase subunit I, and cytochrome c oxidase synthase 2 (SCO2), both of which are components of the mitochondrial oxidative phosphorylation cascade that is the main source for ATP production (Matoba *et al.*, 2006; Okamura *et al.*, 1999). These effects help maintain a positive metabolic outcome for the cell's energy requirements.

Concomitant with its role in mitochondrial oxidative phosphorylation, P53 maintains the mitochondrion's integrity under both normal and stress conditions. P53 upregulates P53R2, a subunit of the ribonucleotide reductase enzyme that maintains mitochondrial DNA (Bourdon *et al.*, 2007), and preserves mitochondrial DNA copy number by regulating the mitochondrion's damage checkpoint pathway (Kulawiec *et al.*, 2009).

In addition, P53 plays an important role in maintaining redox homeostasis and promoting the cell's anti-oxidant defenses. Even though P53 tends to promote RNOS production and release under stress conditions to instigate apoptosis, under normal conditions P53 reduces intracellular RNOS levels to prevent DNA oxidation, fatty acid peroxidation and the formation of protein adducts (Sablina*, et al.*, 2005). P53 performs its anti-oxidant functions by regulating a number of downstream targets involved in preserving redox status, such as aldehyde dehydrogenase (*Aldh4a1*), glutathione peroxidase (*Gpx1*), Mn-superoxide dismutase (*Sod2*), catalase (*Cat*), and Sestrins 1 & 2 (*Sesn1, Sesn2*) (Hussain *et al.*, 2004; O'Connor *et al.*, 2008; Yoon *et al.*, 2004). These transcripts code for enzymes that are proven anti-oxidants, which break down or neutralize potent RNOS, thus protecting the cell's organelles and DNA against damaging oxidation. Interestingly, the P53 protein itself is redox-sensitive; several cysteine residues of P53 are susceptible to the formation of di-sulfide bridges with glutathione (GSH) in oxidative stress conditions (Velu *et al.*, 2007). These cysteine residues lie within P53's DNA binding domain and are important for its binding to promoter sequences (Sun *et al.*, 2003). In addition, P53 activity

can be enhanced by several redox proteins, such as thioredoxin (TRX) and redox factor 1 (REF-1) (Hanson *et al.*, 2005; Seemann and Hainaut, 2005). Thus, P53 is capable of both influencing and being influenced by cellular redox status under basal and stress conditions.

Although P53 primarily promotes cell death via apoptosis under stress conditions, it is also involved in regulating other cell death mechanisms, such as autophagy. Autophagy is the process by which the cell removes damaged organelles and parts of the cytoplasm by sequestration and digestion, to maintain the cell and the genome, and to recycle cellular components to be used in biosynthetic pathways (Levine and Kroemer, 2008; Mathew *et al.*, 2007). The main component of autophagy is the autophagosome, a double membrane vesicle that absorbs cellular debris and organelles from the cytoplasm, and fuses with lysosomes containing digestive enzymes for their eventual degradation (Xie and Klionsky, 2007). Under basal conditions, cells experience autophagy to maintain homeostasis and promote cell survival, but upon stress, the rate of autophagy increases, leading to a form of programmed cell death (Berry and Baehrecke, 2007; Yu *et al.*, 2004). P53 both inhibits and promotes autophagy, depending on the context. Although the exact mechanism is still unclear, cytoplasmic (but not nuclear) P53 inhibits autophagy under basal conditions by downregulating AKT, a positive regulator of autophagy, and upregulating mTOR, a negative regulator of autophagy (Tasdemir *et al.*, 2008). Under conditions of extreme or sustained stress, P53 upregulates the expression of DRAM, SESN1 and SESN2, which induce autophagy (Budanov and Karin, 2008; Crighton *et al.*, 2006). Thus, P53 is capable of regulating several forms of programmed cell death under basal and stress conditions, adding to the complexity of P53's role in determining cell fate.

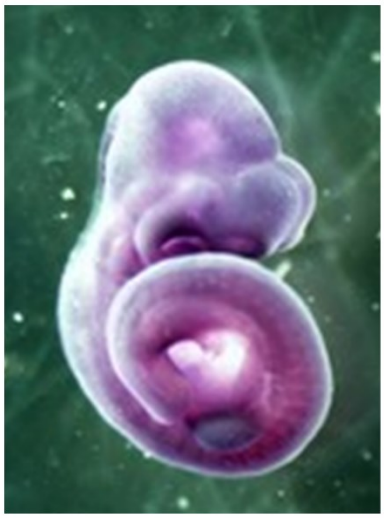
Another function of P53 under basal conditions is its involvement in stem cell renewal. P53 mRNA and protein have been detected in mouse and human embryonic stem cells (ESCs) (Chandrasekaran *et al.*, 1981; Qin *et al.*, 2007); however, during late stages of embryogenesis – particularly when cells have undergone terminal differentiation – the levels of P53 decrease sharply (Lin *et al.*, 2005; Schmid *et al.*, 1991). In addition, ESCs lacking P53 exhibit increased proliferation and a significantly decreased ability to spontaneously differentiate (Qin*, et al.*, 2007). Moreover, P53 inhibits the expression of both NANOG and OCT4, two factors that are essential for maintaining stem cell pluripotency (Loh *et al.*, 2006). P53 was also found to inhibit stem cell renewal in adult stem cells, including hematopoietic progenitor cells and neural stem cells (Liu *et al.*, 2009; Meletis *et al.*, 2006). As expected, P53 also functions as a stress response

factor in embryonic stem cells; hydroxyurea promotes P53-mediated apoptosis in mouse embryonic stem cells by activating P38 and increasing P53 acetylation (Heo *et al.*, 2014). Thus, P53 plays a role as a promoter of cellular differentiation and quiescence in embryonic and adult stem cells, under basal conditions.

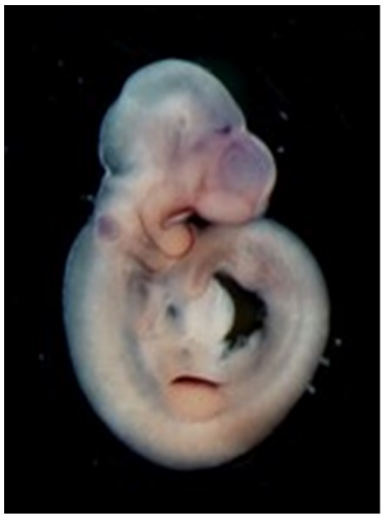
Its ability to exert functions in the cytoplasm and the nucleus under both basal and stress conditions, along with its ability to directly or indirectly control the expression of thousands of genes, makes P53 a central node in the regulation of many crucial cellular processes (Riley *et al.*, 2008). These processes include cell division, senescence, programmed cell death, redox homeostasis, and cell differentiation, all of which are crucial elements to ensure proper embryonic development. Thus, P53 may have consequential roles in how the embryo develops under both basal and stress conditions.

1.4.1.3 The role of P53 in embryonic development

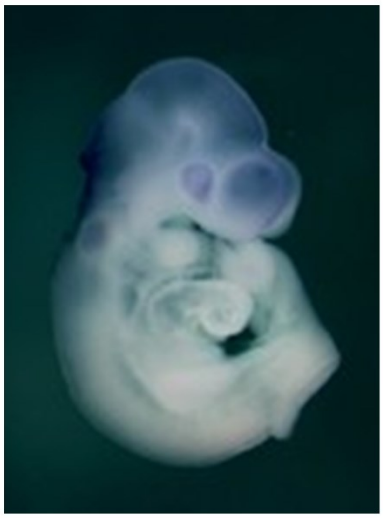
Under normal conditions during embryonic development, *Trp53* expression can be detected as early as GD 8.5 in the mouse embryo, when organogenesis commences (Schmid*, et al.*, 1991). The expression of *Trp53* at this stage is rather homogenous and can be found in proliferating but not terminally differentiated cells (Chen *et al.*, 2015). The reason for this may be that P53 promotes cellular differentiation during this phase of development, as tissues and organs become more specialized in function and morphology; indeed, P53 has been shown to induce cellular differentiation and inhibit stem cell renewal both *in vivo* and *in vitro* (Gil-Perotin *et al.*, 2006; Mazzaro *et al.*, 1999; Meletis*, et al.*, 2006; Porrello *et al.*, 2000). At later stages of development (i.e. > GD 12.5) *Trp53* expression becomes more heterogeneous and sharply declines in cells that are terminally differentiated. In addition, P53 activity seems to be restricted to the developing nervous system, even in later stages of embryogenesis (Gottlieb *et al.*, 1997; Komarova *et al.*, 1997). Overall, basal expression remains low throughout development except during the organogenesis phase, thus rendering the murine embryo especially sensitive to P53 activation between GD 8 to GD 12 (Fig. 1.3).



Trp53



Trp63



Trp73

Figure 1.3: Expression patterns of *Trp53*, *Trp63* and *Trp73* in GD 9 mouse embryos.
Lateral view of GD 9 mouse embryos showing the expression of *Trp53* (EMAGE #25302), *Trp63* (EMAGE #26006) and *Trp73* (EMAGE #26081) using whole mount *in situ* hybridization. *Trp53* is present in the forelimb bud and neural tube. *Trp63* is present in the AER of the forelimb bud. *Trp73* levels are barely detectable at GD 9 but start to be observed from GD 10 onwards. All images were obtained from the EMAGE database (Richardson *et al.*, 2014) on October 2017.

While initial knockout animal studies showed that P53 is not essential for normal mammalian development, subsequent studies showed that *Trp53*$^{-/-}$ mouse embryos exhibited some congenital malformations. These included exencephaly, anencephaly, ocular abnormalities and increased lethality, particularly in female embryos (Armstrong*, et al.*, 1995; Sah*, et al.*, 1995). Strangely, female embryos were overwhelmingly affected, even when different strains of *Trp53*$^{-/-}$ mice were used (i.e. 129sv/C57BL6, BALBc, SWR and 129/Ola) (Armstrong*, et al.*, 1995).

P53 may be involved in osteogenesis and skeletal development; *Trp53*$^{-/-}$ adult mice exhibit increased bone mass and bone formation. P53 has been shown to inhibit osteoblastogenesis by inhibiting the expression of the osteoblast differentiation factor, *Osterix* (Wang *et al.*, 2006). In addition, P53 binds to the osteoblast-promoting transcription factor, RUNX2, in response to DNA damage (Ozaki *et al.*, 2013), and decreases its expression in osteoblast progenitor cells (Lengner *et al.*, 2006).

Another organ that relies on P53 for its proper development is the limb bud. Pruning of the AER and interdigital regions occurs by apoptosis and is essential for proper digit formation (Scott, 1979). This process must be tightly controlled by P53 to prevent abnormal limb development; abnormal limb development was observed in cultured limbs exposed to P53-activating chemicals (e.g. cyclophosphamide) (Moallem and Hales, 1995), while limbs lacking P53 showed spontaneous malformations that increased significantly when exposed to cyclophosphamide (Moallem and Hales, 1998). In addition, low P53 levels are necessary for the initiation and regeneration of limb buds; salamanders exhibit decreased P53 levels during blastemal formation which then revert to normal levels when the limb starts to differentiate (Yun *et al.*, 2013).

While a causal link between P53 and human congenital malformations is difficult to establish, studies on mutant *Trp53* knock-in mice, in which P53 is activated, showed embryos exhibiting a phenotype similar to that of the CHARGE syndrome (Van Nostrand *et al.*, 2014). This congenital defect presents with a myriad of structural and growth abnormalities, such as: coloboma of the eye, atresia of the nasal choanae, retardation of growth, genital/urinary abnormalities, and inner ear defects (Zentner *et al.*, 2010). Interestingly, in over 70% of CHARGE syndrome sufferers, the *CHD7* gene, which regulates chromatin remodeling, is mutated. CHD7 was found to bind to the *Trp53* promotor and reduce P53 expression, thus

increased P53 activity may be related to the occurrence of CHARGE-like defects in *CHD7* mutant individuals (Van Nostrand*, et al.*, 2014).

A recent study also showed that under conditions of low and sustained stress, the P53 pathway is responsible for maintaining tissue fitness during embryogenesis. In genetically mosaic murine embryos containing $Mdm2^{+/-}$ $Mdm4^{+/-}$ and wild type cells, the double knockout cells were outcompeted during embryogenesis via a P53 based mechanism (Zhang*, et al.*, 2017). This phenomenon occurred in a non-cell autonomous fashion, where cells exhibiting a mildly higher activity of P53 than their surrounding wild type counterparts had reduced proliferation, without necessarily undergoing cell death.

Taken together, these findings suggest that P53 regulates a variety of important physiological processes during organogenesis, which can be negatively affected if P53 regulation and activity are disrupted, resulting in improper mammalian development and the possible manifestations of congenital malformations.

1.4.1.4 The role of P53 in the embryonic response to developmental toxicants

As observed in adult tissue, P53 expression in the developing embryo increases significantly in response to stress-including that caused by known teratogens (Torchinsky and Toder, 2010). In several teratology studies, P53 has been found to be activated in the organogenesis stage embryo by compounds that elicit oxidative stress (e.g. phenytoin, benzo[a]pyrene), DNA damage (e.g. UV light), and apoptosis (e.g. 2-chloro-2-deoxyadenosine, cyclophosphamide). However, whether P53 acts as a teratological suppressor – by protecting the embryo against teratogenic insults – or as a teratological inducer – by promoting the teratogenicity of developmental toxicants – is not clear. The divergence in the incidence of certain malformations caused by different teratogens in *Trp53* wild type and *Trp53*-deficient embryos suggests that the role of P53 as a suppressor or inducer of teratogenesis depends on the timing of exposure and the mechanism of action of the teratogen in question (Torchinsky and Toder, 2010).

In studies examining the effects of oxidative stressinducing chemicals in *Trp53*-deficient embryos, benzo[a]pyrene was found to increase the incidence of malformations in the litters of $Trp53^{+/-}$ dams more than in those of $Trp53^{+/+}$ dams (Nicol *et al.*, 1995). In addition, in the litters of benzo[a]pyrene-treated $Trp53^{+/-}$ dams, more intrauterine deaths (i.e. resorptions) were

observed in *Trp53*$^{-/-}$ embryos compared to their *Trp53*$^{+/+}$ littermates. Intriguingly, although all dams were mated with *Trp53*$^{+/-}$ males, the percentage of *Trp53*$^{-/-}$ embryos produced from untreated *Trp53*$^{+/-}$ dams was less than the expected Mendelian ratio (17% vs. 25%), suggesting that many of these embryos died before implantation. Thus, a role for P53 as a teratological suppressor was established, but whether the instigation of apoptosis is the mechanism by which P53 exerts this protective role is unclear, at least with oxidative-stress inducing agents.

γ-Radiation leads to the activation of P53 in virtually all tissues of the GD 8.5 mouse embryo – except the heart – and much of the caudal and rostral regions of the GD 10 embryo (Gottlieb*, et al.*, 1997). Consequentially, γ-irradiation at 2 Gy of GD 9.5-10.5 *Trp53*$^{-/-}$ embryos at the low rate of 1.2 mGy/min resulted in a 12% increase in malformations, versus no malformations in the wild type (Kato *et al.*, 2001). Similarly, 2 Gy of X-irradiation of *Trp53*$^{+/+}$ and *Trp53*$^{-/-}$ embryos on GD 9.5 led to an increase in the incidence of malformations and a decrease in fetal deaths in the *Trp53*$^{-/-}$ fetuses, compared to *Trp53* wild types; 70% of the *Trp53*$^{-/-}$ embryos exhibited anomalies, with only 7% dying, while the wild type embryos had an incidence of 20% anomalies and 60% death (Norimura *et al.*, 1996). Interestingly, the number of apoptotic cells in *Trp53*$^{-/-}$ embryos exposed to ionizing radiation was much less than in their wild type counterparts, suggesting that in cases of radiation-induced DNA damage, P53 mediates its teratogenic response by activating apoptosis in an effort to rid the embryo of damaged - and potentially maldeveloped - tissues.

This suggestion is supported by studies conducted with agents that are known to induce apoptosis; cyclophosphamide is a DNA alkylating agent that forms DNA crosslinks, resulting in apoptosis (Hall and Tilby, 1992). When treated with its pre-activated analog, 4-hydroperoxycyclophosphamide (4OOH-CPA), *Trp53*$^{-/-}$ cultured murine limbs showed more malformations and less DNA fragmentation – a marker of apoptosis – than their wild type counterparts (Moallem and Hales, 1998). Torchinsky et al. showed that wild type embryos treated with cyclophosphamide showed increased malformations in the limbs and little accumulation of P53 - compared to the head and liver - suggesting that the lack of sufficient P53 accumulation is the reason why the limbs were more sensitive to teratogenic insult to cyclophosphamide (Torchinsky *et al.*, 1999). These studies suggest that P53 acts as a teratological suppressor by reducing the incidence of malformed tissue via the induction of apoptosis in damaged cells.

In contrast, studies conducted with the DNA nucleotide analog, 2-chloro-2-deoxyadenosine (2-CdA), suggested a different role for P53. Wild type mouse embryos treated on GD 8 exhibited an increase, rather than a decrease, in the incidence of a specific eye defect, micropthalmia, compared to *Trp53*$^{-/-}$ embryos (Wubah *et al.*, 1996). Interestingly, this effect was also correlated with an increase of apoptosis in the malformed, wild type embryos, suggesting that 2-CdA causes a P53-mediated induction of apoptosis that contributes to the malformation. Thus, at least for this type of defect and teratogen, P53 acts as a teratological inducer rather than a suppressor.

A common finding of these studies is the involvement of P53 in the embryo's response to teratogen exposure, particularly compounds that cause oxidative stress and DNA damage. The explanation for why P53 acts as a teratological suppressor in some cases, but a teratological inducer in others, is still unclear. However, possible factors that influence this discrepancy are the type of tissue affected by each teratogen, the level of P53 activity and expression in different embryonic tissues, the time and duration of teratogenic exposure (early vs. late, acute vs. chronic), and the mechanism of action of the teratogen in question. Whether the other members of the P53 family of proteins play a role in determining P53's response to teratogenic stress is unknown.

1.4.2 The P63 and P73 proteins

When it was first discovered in the late 70s, P53 was not known to have any homologs. However, that changed with the discovery of P63 and P73 almost two decades later (Kaghad *et al.*, 1997; Yang *et al.*, 1998). Both proteins were found to be very similar in structure to P53, containing an acidic N-terminal transactivation (TA) domain, a core DNA-binding domain (DBD), and a C-terminal oligomerization domain (OD). In addition, they can bind to the majority of P53 DNA-binding sites and act as transcription factors, upregulating many P53 downstream gene targets (e.g. *Cdkn1a, Fas, Rb1,* etc). Moreover, they are able to respond to cellular stress stimuli (e.g. oxidative stress, DNA damage, UV light) and induce caspase-mediated apoptosis (Celardo *et al.*, 2013; Jost *et al.*, 1997; Ramadan *et al.*, 2005; Satija and Das, 2016). Lastly, similar to P53 (Murray-Zmijewski*, et al.*, 2006), the gene structure of *Trp63* and *Trp73* contains two transcription start sites which allow for the formation of two classes of P63 and P73, a TA-domain containing isoform (TAP63 & TAP73), and a N-terminus truncated

isoform (ΔNP63 & ΔNP73) which lacks the TA domain. In addition, each isoform can form several C-terminus-modified variants, resulting in a total of three variants for each of the TA and ΔN isoforms of P63 (α, β, γ), and seven variants for P73 (α-ζ) (Yang and McKeon, 2000).

Yet, there are some fundamental differences between P53 and its more recently discovered homologs (Table 1.1). First, while they do share many domains, both P63 and P73, but not P53, contain a sterile alpha motif (SAM) at the C-terminus. Secondly, while a clear role for P53 in tumor suppression has been well-established, that is not the case with P63 and P73; although there is some evidence P63 and P73 mutations and deletions are associated with specific types of cancers, and heterozygous mice deficient for P63 and/or P73 develop certain types of tumors with a higher burden than those generated from P53-knockout mice, homozygous knockout animals do not develop tumors and not many human cancers are associated with mutated P63 and P73 compared to P53. (Dohn *et al.*, 2001; Flores *et al.*, 2005; Kaghad*, et al.*, 1997; Parsa *et al.*, 1999). However, both P63 and P73 were shown to be essential for the proper development of key organs and systems during embryogenesis (Moll and Slade, 2004).

1.4.2.1 The P63 protein

As mentioned earlier, P63 is a homolog of the tumor suppressor protein P53. While it does exhibit properties similar to P53, it has unique functions and expression patterns in response to stress and during development.

P63 is found in the cytoplasm under normal conditions, where it exists in a variety of isoforms. The full-length isoform, TAP63α, contains all the P53 domains with the addition of a sterile alpha motif (SAM), while the β and γ isoforms closely resemble P53 structure (Moll and Slade, 2004). In addition, P63 is targeted by E3-ubiquitin ligases, such as Itch, which are believed to negatively regulate P63 protein levels under normal conditions, targeting it for proteasomal degradation (Rossi *et al.*, 2006a).

Upon activation, P63 oligomerizes and translocates to the nucleus where it binds to promoter binding sequences to control the transcription of a variety of downstream targets. Genotoxic stress can induce P63 phosphorylation by c-ABL on Tyr149, Tyr171, Tyr289, prompting P63 to stabilize and translocate to the nucleus (Gonfloni *et al.*, 2009). Likewise, other kinases, such as CDK2 and ATM, have been shown to phosphorylate and stabilize P63 in

response to stress (Huang *et al.*, 2008). Nuclear translocation and DNA binding of P63 induce the transcription of genes involved in cell cycle arrest and DNA damage repair and can trigger an apoptosis cascade that leads to cell death via the upregulation of pro-apoptotic proteins (e.g. NOXA, PUMA, BAX) that inhibit the pro-survival BCL2 factors (Dohn*, et al.*, 2001; Jacobs *et al.*, 2005; Kerr *et al.*, 2012).

While phosphorylation of TAP63 isoforms leads to their stabilization and activation, the opposite is true for the ΔN isoforms. These isoforms act as negative regulators, counteracting the effects of their TA counterparts. Therefore, under stress conditions, when the effects of the TA isoforms are required by the cell, the ΔN isoforms are targeted for degradation by phosphorylation in order to allow the TA isoform to exert its effect to the fullest (Kartasheva *et al.*, 2002; Petitjean*, et al.*, 2008; Westfall *et al.*, 2005). Intriguingly, TAP63 isoforms can bind to the promoter sites of ΔNP63 and ΔNP73 and induce their transcription, thus maintaining a fine balance of negative feedback to control and regulate the cell's response to genotoxic stress and prevent rampant cell cycle disruption or cell death (Lanza *et al.*, 2006; Rocco *et al.*, 2006).

1.4.2.1.1 The role of P63 in embryonic development

The *Trp63* gene is highly expressed in adult normal tissue, specifically in the nuclei of basal cells of the skin, cervix, tongue, esophagus, mammary glands, prostate and urothelium (Yang*, et al.*, 1998). It is most abundant in squamous epithelia but, in a shared characteristic with *Trp53*, the levels of *Trp63* decrease in terminally differentiated cells. Thus, P63 is found to be highly expressed in keratinocytes with high proliferative potential, such as stem cells, but is low in cells undergoing terminal differentiation (Parsa*, et al.*, 1999; Pellegrini *et al.*, 2001). Indeed, *Trp63*-null epithelial progenitor cells fail to undergo the characteristic asymmetric cell division that is required for the maintenance and regeneration of stem cells (Lu *et al.*, 1998). Moreover, upon stimulus, these *Trp63*-null cells tend to undergo differentiation rather than maintain basal levels of progenitor cells, suggesting that P63 is required to preserve and regenerate stem cell populations, at least within the epithelium (Barrandon and Green, 1987; Watt, 1998; Yang and McKeon, 2000). Indeed, ΔNP63 has been found to maintain the survival of neural stem cells, mainly by counteracting the effects of P53 (Cancino *et al.*, 2013; Dugani *et al.*, 2009; Fatt*, et al.*, 2014).

During murine embryonic development, *Trp63* can be detected as early as GD 9 in the embryonic ectoderm (Fig 1.3), including the forelimb and – to a more restricted degree – the hindlimb buds (Chung *et al.*, 2011; Tadeu and Horsley, 2013). While homozygote *Trp63* knockout mice do not develop tumors, the fetuses die at birth and display severe congenital malformations: a complete absence of all squamous epithelial tissue, including skin, mammary glands, whiskers and teeth, as well as stunted or absent limbs (Mills *et al.*, 1999). The affected limbs show absence of the radii, carpals and digits of the forelimb, as well as the femur, tibia, fibula, talus and digits of the hindlimb. Developmental studies suggest that the absence of epithelial tissue is due to the disruption of stem cell maintenance, for which P63 is responsible, while the limb defects are a result of the failure of the formation of the AER, a structure that is essential for limb growth and progression; the AER exhibits high P63 expression during organogenesis (Yang*, et al.*, 1999). Thus, P63 plays an essential role during embryonic development, particularly in the formation of limbs and bones.

Indeed, mutations in the *Trp63* gene have been associated with a number of human congenital syndromes that include limb malformations (Brunner *et al.*, 2002). Mutations of *Trp63* have been found in patients with ectrodactyly, ectodermal dysplasia, clefting (EEC) syndrome (Celli *et al.*, 1999); nonsyndromic split hand/foot malformation (SHFM) (Ianakiev *et al.*, 2000); ankyloblepharon, ectodermal dysplasia, clefting (AEC) syndrome (McGrath *et al.*, 2001); acro-dermato-ungual- lacrimal-tooth (ADULT) syndrome (Amiel *et al.*, 2001); and limb-mammary syndrome (LMS) (van Bokhoven *et al.*, 1999). Almost all mutations detected in the families that exhibit one of these syndromes lie within the DNA binding domain of P63, with some found within the SAM region. While the exact consequence of these mutations on P63 function is undetermined, it appears likely that missense or frameshift mutations within the DNA binding domain result in the disruption of P63's structural integrity and/or its ability to bind the DNA, thus leading to a loss-of-function effect. However, a report by Chitayat et al. shows that deletion of the 3q27 region of the genome, in which the *Trp63* lies, did not result in the occurrence of limb deformities (Chitayat *et al.*, 1996). In addition, the fact that *Trp63* can yield a number of isoforms, each with transactivating and repressive functions, adds another layer of complexity, whereby mutations in these isoforms can result in partial-loss-of-function, dominant-gain-of-function, or a change of function (Celli*, et al.*, 1999). Thus, it is likely that disrupting the

activity and regulation of the various isoforms of P63 contributes to the manifestation of congenital malformations, including limb defects.

1.4.2.2 The P73 protein

The other member of the P53 family of proteins is P73. Like P63, it shares many domains and sequence structures with P53: a N-terminal transactivation (TA) domain, a DNA binding domain, and a C-terminal oligomerization domain. It also has two promoter sites in its gene structure, yielding TA and ΔN classes of isoforms, with its α isoform containing a SAM at the C-terminus (Levrero *et al.*, 1999).

Like P53 and P63, P73 is able to regulate the cell cycle and promote cell death via apoptosis in response to DNA damage and oxidative stress. While the exact mechanism of its regulation is not as well established as that of P53, it appears to share many of the basic components of P53 regulation. Under normal conditions P73 can be bound and ubiquitinated by E3-ubiquitin ligases which target it for proteasomal degradation. These ubiquitin ligases include TRIM28, a DNA damage response protein that has been shown to interact with and assist the P53-negative regulator MDM2 (Noon *et al.*, 2010; Wang *et al.*, 2005). Yet, under specific genotoxic stress conditions (e.g. etoposide), P73 dissociates from TRIM28 when it is phosphorylated on Tyr99 by the kinase c-ABL, leading to its stabilization and transactivation (Satija and Das, 2016). Other kinases that are known to interact with and regulate P73 are the checkpoint kinases, CHK1 and CHK2, as well as the stress-response related kinase, JNK (Jones *et al.*, 2007; Urist *et al.*, 2004). Recall that these kinases are also upstream regulators of P53. Activated P73 can translocate to the nucleus and bind DNA, including the promoter binding sites to which P53 and P63 bind, and can upregulate cell cycle arrest (e.g. *Cdkn1a*, *Gadd45*), DNA damage repair (*Brca2*, *Rad51*), and cell death-related transcripts (e.g. *Puma*, *Bax*) (Lin *et al.*, 2009; Satija and Das, 2016). However, P73 has also been shown to upregulate genes that are not commonly regulated by P53. These genes include DNA repair genes (e.g. *Mre11*) and genes involved in the Notch pathway signaling (e.g. *Jag2*), among others (Fontemaggi *et al.*, 2002; Lin*, et al.*, 2009; Sasaki *et al.*, 2002).

An interesting characteristic of P73, which it shares with P63, is the interaction between the TA and ΔN isoforms. While the TA variants function as transcription factors that maintain DNA integrity and promote cell cycle arrest and cell death in response to stress, the ΔN variants

have the opposite effects by promoting the cell's survival and replication potential. Indeed, while ΔNP73 forms are unable to promote gene expression, they can affect the function of TAP73, as well as P53, in a dominant-negative fashion to inhibit their transcriptional activity (Melino*, et al.*, 2002). ΔNP73 can interact with the oligomerized TAP73 (Ishimoto *et al.*, 2002) and can compete with P53 at its DNA binding sites (Stiewe *et al.*, 2002), ultimately leading to an inhibition of apoptosis. Interestingly, the second promoter site of the *Trp73* gene, which is the starting site for the transcription of ΔNP73, can be bound by both P53 and TAP73, suggesting that ΔNP73 contributes to a negative feedback loop that maintains a balance between the pro-apoptotic P53 and TAP73 and the pro-survival ΔNP73 (Nakagawa *et al.*, 2002). Furthermore, the levels of ΔNP73 have been found to be increased in cells of various types of cancers, while those of TAP73 are relatively repressed, showing that the TA and ΔN isoforms of P73 act in a tug-of-war like scenario to maintain cell senescence, proliferation and survival (Di *et al.*, 2013; Douc-Rasy *et al.*, 2002; Guan and Chen, 2005).

1.4.2.2.1 The role of P73 in embryonic development

Trp73 expression is detectable around GD 10 in the mouse embryo, with peak levels at GD 15 (Fig. 1.3). It is mostly restricted to the brain, specifically the Cajal-Retzius neurons of the hippocampus, the fimbria of the hippocampus (a.k.a the cortical hem area) and the choroid plexus (Cabrera-Socorro *et al.*, 2007; Diez-Roux *et al.*, 2011; Meyer *et al.*, 2004). Consequentially, P73 was found to play an essential role in embryonic development, especially in the development of the central nervous system (CNS). While *Trp73*-null mice (which lack all P73 isoforms) are viable at birth, many of them die prematurely due to neurological, inflammatory and pheromonal defects. The absence of P73 increases the incidence of hydrocephaly and cortical hypoplasia, leading to a severe disorganization of the CA1, CA3 and the dentate gyrus regions of the hippocampus, as well as a decrease in Cajal-Retzius neurons (Yang*, et al.*, 2000). These effects are probably the cause of the neuro-behavioral deficits observed in older animals; adult mice deficient in P73 exhibit increased anxiety, loss of memory and learning, impaired reflex and neuromuscular function, as well as disrupted sensorimotor coordination (Niklison-Chirou *et al.*, 2013; Talos *et al.*, 2010).

Interestingly, the ΔNP73 isoform seems to play a major role in neurodevelopment. In the mouse it is expressed as early as GD 12 in the choroid plexus, vomeronasal area, stria terminalis,

preoptic area and the hippocampal Cajal-Retzius neurons (Pozniak *et al.*, 2000; Tissir *et al.*, 2009). Mice that were specifically deleted for the ΔNP73 isoform showed a slightly distinct phenotype from their TAP73-null (which contained only the ΔNP73 isoform) or fully ablated P73 (which lacked all P73 isoforms) counterparts. Even though they did display cortical hypoplasia and loss of Cajal-Retzius neurons – like mice lacking the full *Trp73* gene (i.e. both isoforms) – the ΔNP73-null mice showed milder neurodegeneration in general and their behavior was not affected as much (Niklison-Chirou *et al.*, 2016; Wilhelm*, et al.*, 2010). In addition, the TAP73-null mice display a phenotype that is intermediate between that exhibited by full P73 ablation and ΔNP73-deficient mice. The fact that ΔNP73 has anti-apoptotic effects in neurons suggests that both isoforms of P73 contribute to neurodevelopment and may have opposing and complementary functions in maintaining neuronal survival and promoting neurogenesis (Pozniak*, et al.*, 2000; Tissir*, et al.*, 2009; Wilhelm*, et al.*, 2010).

Like P53 and P63, P73 appears to play a role in stemness and differentiation, particularly of neuronal stem cells. Both TAP73 and ΔNP73 are expressed in neuronal stem cells (NSCs) and their levels increase as the cells differentiate (Agostini *et al.*, 2010). In addition, P73 and TAP73 knockout mice have significantly lower numbers of stem cells in the sub-granular zone of the hippocampus and the sub-ventricular zone, which both normally contain progenitor cells for neurogenesis (Ehninger and Kempermann, 2008; Noctor *et al.*, 2004). However, P73 expression increases significantly in myeloid leukemic cells and neuroblastomas undergoing differentiation (De Laurenzi *et al.*, 2000; Tschan *et al.*, 2000). Thus, P73, like P53, promotes cell differentiation and, like P63, also plays a role in maintaining stem cell renewal. Interestingly, P73 has been shown to interact with P63 to counteract the effects of P53 and thus promote cell proliferation and stem cell regeneration (Dugani*, et al.*, 2009). The diverging roles of P73 in stem cell maintenance and differentiation may depend upon the effects of its various isoforms and how they interact with the other P53 family proteins, the stem cell lineage, as well as the stage of embryonic development (Dugani*, et al.*, 2009; Fatt*, et al.*, 2014; Fujitani *et al.*, 2010; Pozniak *et al.*, 2002; Talos*, et al.*, 2010).

1.4.3 The role of P63 and P73 in response to genotoxic and oxidative stress-inducing agents

Both P63 and P73 can and do respond to several genotoxicants that induce DNA double or single strand breaks. In addition, there seems to be a complex interplay between all three

members of the P53-family in response to DNA damage, with the aim of managing the cell's response to enact DNA repair, cell cycle arrest or cell death. This response varies depending on the toxicant, the dose, the cell type affected and the stage of development.

Genotoxic agents that lead to the activation of P63 include doxorubicin, a chemical that inhibits DNA replication by intercalating the DNA helix and stabilizing topoisomerase II (Tacar *et al.*, 2013). Once exposed, cells exhibit a rapid increase in the TAP63 isoform, which becomes phosphorylated, most likely by c-ABL kinase (Petitjean*, et al.*, 2008). Interestingly, doxorubicin treatment also induces *ΔNP73* transcript levels; as mentioned earlier, the ΔN isoforms of P73 and P63 can negatively regulate the activities of their TA isoform counterparts, as well as that of P53. Indeed, ΔNP73 has been found to inhibit the transcriptional activity of TAP63γ, TAP73 and P53 (Grob*, et al.*, 2001; Kartasheva*, et al.*, 2002), while ΔNP63α inhibits the activity of TAP73-mediated apoptosis (Leong *et al.*, 2007; Rocco*, et al.*, 2006). Moreover, *ΔNP73* is a transcriptional target of P53, TAP63α/γ and TAP73 (Kartasheva*, et al.*, 2002; Lanza*, et al.*, 2006). The fact that P63 controls the expression of DNA repair enzymes under normal and stress conditions supports a role for P63 in the DNA damage response pathway (Lin*, et al.*, 2009; McDade *et al.*, 2014).

A similar function for P73 exists in the context of genotoxic stress; a wide range of DNA damaging agents (e.g. doxorubicin, cisplatin, camptothecin, etoposide) induce the expression, phosphorylation and acetylation of TAP73, leading to cell cycle arrest or apoptosis (Costanzo *et al.*, 2002; Irwin *et al.*, 2003; Jost*, et al.*, 1997; Satija and Das, 2016; Urist*, et al.*, 2004). Interestingly, the ability of TAP73 to promote apoptosis depends on the intensity of DNA damage as well as the availability of functional P53. Mutant P53 has been shown to inhibit the proapoptotic activity of TAP73 (Di Como *et al.*, 1999), while TAP73 is able to repress P53-mediated apoptosis under conditions of low DNA damage (Chen *et al.*, 2014). There seems to exist an interdependent relationship between P53 and P73 that maintains a "check and balance" mechanism in response to certain types of stress; P53 can upregulate TAP73 transcript levels in response to DNA damage, maintaining an auto-inhibitory loop that fine-tunes the cell's fate between survival and senescence as it tries to repair the damage (Wang *et al.*, 2007).

Another form of stress that can induce P63 and P73 activity is oxidative stress. Both proteins are involved, either directly or indirectly, in redox homeostasis and the regulation of oxidative stress response factors. P63, specifically the TAP63γ and ΔNP63γ isoforms, can

directly bind to the promoter of *Gpx2* – which encodes a glutathione peroxidase – and upregulate it in response to oxidative stress. Ultimately this enzyme is able to rescue cells from oxidative stress-induced P53-mediated apoptosis (Yan and Chen, 2006). In addition, the expression of *Redd1* – a hypoxia-sensitive factor with antioxidant properties – is regulated by both P63 and P53 in embryonic and adult tissue. Interestingly, the expression pattern of *Redd1* in the developing mouse embryo parallels that of *Trp63*; in wild type embryos *Redd1* can be found in the AER of limb buds, the developing epidermis and the whisker pads; however, its levels are almost depleted in *Trp63*-null embryos (Ellisen *et al.*, 2002). Similarly, P73 has been shown to mitigate oxidative stress damage; TAP73 itself is upregulated in response to oxidative stress and mouse embryonic fibroblasts that lack P73 show increased accumulation of ROS and a greater susceptibility to cell death (Du *et al.*, 2013). Moreover, neurons treated with the antioxidant, N-acetyl cysteine, show a dramatic decrease in c-ABL mediated P73 activation, suggesting that P73 is responsive to RNOS (Klein *et al.*, 2011). Additionally, PCBP2 – an RNA binding protein that stabilizes *Trp73* and thus regulates P73 expression – is capable of upregulating P73 in response to oxidative stress. In PCBP2-deficient cells, P73 expression decreases while RNOS levels increase (Ren *et al.*, 2016). This effect is possibly mediated by GLS2 – a glutaminase that replenishes the cell's pools of reduced glutathione – which is a downstream target of P73 (Velletri *et al.*, 2013). Taken together, these findings show that both P63 and P73 are capable of regulating the expression of redox-sensitive factors and inducing antioxidant effects, suggesting that they play a role in the cell's oxidative stress response.

To our knowledge, no studies have been conducted to study the role of P63 and P73 to developmental toxicants. While studies have established a clear and essential role for P63 and P73 in proper embryonic development, none have examined whether they exhibit functions similar to that of P53 in terms of their ability to respond to teratogenic insult. The fact that both P63 and P73 are essential for development, coupled with their similarity to P53 in structure, function and signaling mechanisms, and the apparently complex interplay between them and their various isoforms, renders the question of their involvement in the embryonic stress response of immense importance. It is possible that they respond to genotoxic and oxidative stress during organogenesis in a similar fashion to what has been observed in immortalized and differentiating cell lines, and that P53's function as a teratogenic suppressor or inducer relies partially on the activity and regulation of P63 and P73. In addition, it is not known whether P63 and P73 can act

in a redundant fashion to teratogenic insult during embryogenesis, and so would be capable of instigating an embryonic stress response in the absence of P53.

Our understanding of the role of the P53 family and its signaling pathways in the embryonic stress response will enhance our ability to predict, identify, monitor and prevent potential teratogenic agents, and further our understanding of developmental biology.

1.4 Hypothesis & objectives

We propose that the P53 signaling pathway is involved in the early embryonic stress response to hydroxyurea in the organogenesis-stage murine embryo. We hypothesize that exposure to hydroxyurea activates the P53 protein, allowing it to function as a transcription factor that regulates apoptosis and cell cycle arrest pathways, ultimately leading to the development of malformations and embryonic death. We have tested this hypothesis through achieving the following objectives:

Objective 1: Determine whether the P53 signaling pathway is activated in the early embryonic stress response to hydroxyurea.

Objective 2: Determine if the P53 family proteins, P63 and P73, respond to hydroxyurea in a similar fashion to P53 in the early embryonic stress response.

Objective 3: Determine if P53 acts as a suppressor or inducer of hydroxyurea embryotoxicity.

Our ultimate objective is to understand the molecular mechanisms of the embryonic stress response to teratogenic insult in order to identify potential embryotoxic pharmaceutical or environmental chemicals and prevent their dissemination.

CHAPTER II

Hydroxyurea Exposure Activates the P53 Signaling Pathway in Murine Organogenesis-Stage Embryos

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ABSTRACT

Hydroxyurea, an anticancer agent and potent teratogen, induces oxidative stress and activates a DNA damage response pathway in the gestation day (GD) 9 mouse embryo. To delineate the stress response pathways activated by this drug, we investigated the effect of hydroxyurea exposure on the transcriptome of GD 9 embryos. Timed pregnant CD-1 mice were treated with saline or hydroxyurea (400 mg/kg or 600 mg/kg) on GD 9; embryonic gene and protein expression were examined 3 h later. Microarray analysis revealed that the expression of 1346 probe sets changed significantly in embryos exposed to hydroxyurea compared to controls; the P53 signaling pathway was highly affected. In addition, P53 related family members, P63 and P73, were predicted to be activated and had common and unique downstream targets. Western blot analysis revealed that active phospho-P53 was significantly increased in drug-exposed embryos; confocal microscopy showed that the translocation of phospho-P53 to the nucleus was widespread in the embryo. Furthermore, qRT-PCR showed that the expression of P53-regulated genes (*Cdkn1A*, *Fas*, *Trp53inp1*) was significantly upregulated in hydroxyurea-exposed embryos; the concentration of the redox sensitive P53INP1 protein was also increased in a hydroxyurea dose-dependent fashion. Thus, hydroxyurea elicits a significant effect on the transcriptome of the organogenesis stage murine embryo, activating several key developmental signaling pathways related to DNA damage and oxidative stress. We propose that the P53 pathway plays a central role in the embryonic stress response and the developmental outcome after teratogen exposure.

INTRODUCTION

During gestation embryos may be exposed to a variety of harmful environmental and therapeutic agents with detrimental effects on development, resulting in birth defects, miscarriage, or even embryonic death. Organogenesis is a particularly vulnerable stage of embryo development (Carney *et al.*, 2004). During this time exposure to a wide variety of teratogens, including thalidomide, ethanol, anticonvulsants or anticancer drugs, may compromise normal embryo development (Kovacic and Somanathan, 2006). The ability of embryos to respond to the stress triggered by teratogenic exposures is likely to be an important determinant of their developmental outcome. Many such exposures activate specific stress response signaling pathways in the organogenesis-stage conceptus; these include the oxidative stress, DNA damage response, P53, hypoxia, NFκB, and mitogen-activated protein kinases (MAPKs) signaling pathways (Torchinsky and Toder, 2010; Vinson and Hales, 2003; Wells*, et al.*, 2009).

Stress responses may play an adaptive role in protecting the embryo from insult, for example by regulating cell proliferation or cycle checkpoints and providing the time to repair damaged DNA, thus ensuring embryo survival and normal development (Aylon and Oren, 2007). Alternatively, activation of these stress response pathways may "tip the balance" in favor of excess cell death and altered cell differentiation pathways, leading to abnormal development or embryolethality (Brill *et al.*, 1999; Faustman, 2012). Genetic or pharmacological manipulation of a number of these pathways has been shown to alter embryo fate after a teratogen exposure (Nicol*, et al.*, 1995; Yan and Hales, 2006). There is a clear need to identify the stress response factors that are at play in the embryo during organogenesis. It is also important to understand how they are coordinated. Does exposure to a teratogen activate distinct stress response signaling pathways or does it disrupt several interactive pathways? The goal of this study was to analyze the effects of exposure to a model teratogen, hydroxyurea, on the organogenesis stage murine embryo transcriptome.

Hydroxyurea, a drug used clinically in the treatment of sickle cell anemia and chronic myeloid leukemia, inhibits ribonucleotide reductase, depleting the endogenous pool of ribonucleotides and inhibiting DNA synthesis, leading to DNA replication fork stalling and strand breaks (Timson, 1975). Hydroxyurea is a potent teratogen in animal models; it causes severe hind-limb (syndactyly, polydactyly), tail (curled or absent) and craniofacial (cleft palate, exencephaly) defects in murine embryos exposed during organogenesis (DeSesso, 1979;

DeSesso*, et al.*, 2000; Yan and Hales, 2005). We have shown that exposure to teratogenic doses of hydroxyurea induces oxidative stress (Schlisser *et al.*, 2010; Yan and Hales, 2005), activates the P38 and c-Jun N-terminal kinase (JNK) signaling pathways (Yan and Hales, 2008) and triggers a DNA damage response (Banh and Hales, 2013) in organogenesis-stage murine embryos. Glutathione depletion (Yan and Hales, 2006) or the overexpression of superoxide dismutase (Larouche and Hales, 2009), altering the oxidative stress response, or inhibition of the activation of the P38 or JNK MAPK signaling pathways (Yan and Hales, 2008) will modify the teratogenicity of hydroxyurea.

Our goal is to use a genome-wide approach to delineate the major cellular pathways that are activated in the murine embryo in response to a teratogenic dose of hydroxyurea during organogenesis. Our data show that hydroxyurea exposure has a significant impact on the gene expression profile in these embryos. Genome-wide pathway analysis of the genes with changes in expression has revealed that many are downstream of the tumor suppressor protein P53.

MATERIALS AND METHODS

Experimental Animals

Timed-pregnant CD1 mice were purchased from Charles River Canada Ltd (St. Constant, QC, Canada) and housed in the McIntyre Animal Resource Centre (McGill University, 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. Mice were mated by the supplier between 8:00 and 10:00 AM on gestation day (GD) 0. Between 8:00 and 10:00 AM on GD 9 pregnant dams were treated with either saline (control) or hydroxyurea (Aldrich Chemical Co., Madison, WI) at 400 or 600 mg/kg (designated as HU400 and HU600, respectively) by intraperitoneal injection. Previous studies showed a decrease in fetal weights and an increase in the incidence of caudal malformations in the 400 mg/kg treatment group; these effects were more severe and were accompanied by embryolethality in the 600 mg/kg treatment group (Schlisser and Hales, 2013). The dams were euthanized by CO_2 asphyxiation and cervical dislocation after 3 h; this time point was chosen based on previous reports that hydroxyurea activation of stress response mechanisms in GD9 embryos, such as P38 MAPK, peaked at 3 h (Yan and Hales, 2008). The uteri were removed and embryos were explanted in Hanks' balanced salt solution (Invitrogen Canada, Inc., ON, Canada). At the time of collection, whole embryos from each litter were separated for future sample processing: 2-3 embryos were stored in 4% paraformaldehyde for immunofluorescence experiments; 6-10 embryos were flash frozen in liquid nitrogen for Western blot analysis; 2 embryos were stored in RNAlater Stabilization Reagent (Qiagen, Mississauga, ON, Canada) for qRT-PCR.

RNA Extraction, Microarray Probe Preparation and Hybridization

Agilent SurePrint G3 Mouse GE 8x60k Microarrays (Agilent Technologies, Mississauga, ON, Canada) were used to probe embryonic gene expression in control and hydroxyurea-treated embryos (HU400) at 3 h post treatment. Total RNA was extracted using RNeasy® Plus Mini Kits (Qiagen, Mississauga, ON, Canada). The RNA concentration and purity of each sample were assessed by spectrophotometry using a NanoDrop1000 spectrophotometer (Fisher Scientific, Wilmington, DE, USA) and Agilent 2100 BioAnalyzer (Agilent Technologies, Mississauga, ON, Canada). For each sample, 600 ng of Cy3 labeled cDNA was used for the single-color microarray. After 17 h of hybridization at 65°C, the microarray slides were washed as per the

recommendations for the Agilent hybridization kit. Arrays were scanned with Agilent DNA Microarray Scanner. Whole litters were pooled for each sample and each experiment was replicated 6 times.

Microarray Analysis

Analysis of the scanned microarrays was done using Agilent's Feature Extraction software version 11.5.1.1. Statistical analysis, clustering and principal component analysis (PCA) were done using GeneSpring Gx version 13.1 (Agilent Technologies). Detected flags were filtered based on expression (20^{th}-100^{th} percentiles) and 1.5-fold change. Pathway statistical analysis and prediction of activated upstream regulators were done using the Ingenuity Pathway Analysis™ (IPA) software (Qiagen). Pathway Studio™ (Elsevier) was used to generate the schematic representation of the P53 pathway with highlighted downstream targets. Venny v2.0.2, a free online tool, was used to create the Venn diagrams and lists of P53/P63/P73 overlapping and unique targets (Oliveros, 2007-2015). Several arrays were determined to be of low quality by PCA and quality analysis (Supplementary Fig. S2.1 and S2.2) and were not included, reducing the number of replicate experiments for each treatment to four.

Western Blotting

Whole tissue lysates were prepared for detection and quantification of P53, phospho-P53 (S15), P53INP1 and Actin protein concentrations. Each sample, consisting of 6-10 embryos, was lysed and processed as previously described (Banh and Hales, 2013). Total protein content from each sample was quantified using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Ltd., Mississauga, ON, Canada). Proteins (20 µg) were loaded and separated on 10% SDS-PAGE and transferred at 14 volts overnight at 4ºC to PVDF membranes (Bio-Rad Laboratories, Ltd.). Afterwards, membranes were blocked with either 5% BSA or milk in 1X TBS containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature. Membranes were incubated with primary antibodies against P53 (1:1000, cs2524, Cell Signaling Technology, Inc., Danvers, MA), phospho-P53 (S15) (1:1000, cs12571, Cell Signaling Technology), P53INP1 (1:1000, ab202026, Abcam Toronto, ON, Canada) or Actin (1:1000, sc-1616, Santa Cruz Biotechnology, TX, USA) overnight at 4º C. Membranes were washed five times for five minute intervals with 1X TBST-T and then incubated for 1 h at room temperature with horseradish peroxidase conjugated anti-

mouse (1:5000, cs-7076, Cell Signaling Technology), anti-rabbit (1:5000, NA934V, GE Healthcare, Buckinghamshire, UK) 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 quantified using ImageJ software (National Institutes of Health, MD, USA) where the area under the curve represents band intensity. All intensities were normalized to that of the Actin loading control.

Real-Time qRT-PCR

RNA extracted from whole embryos was diluted to a working solution of 2 ng RNA/µl and transcripts were quantified using the Power SYBR Green RNA-to-C_T 1-Step Kit (Applied Biosystems, Foster City, CA) 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, 55° C for 30 s, 72º C for 30 s and melting curve at 95° C for 15 s, 60° C for 15 s and 95º C for 15 s. Primer sets were purchased from Qiagen: TNF receptor superfamily member 6 (*Fas,* QT00095333); transformation related protein 53 (*Trp53,* QT00101906); tumor protein p53 inducible nuclear protein 1 (*Trp53inp1,* QT00112910); cyclin-dependent kinase inhibitor 1A (*Cdkn1a,* QT00137053); hypoxanthine phosphoribosyltransferase 1 (*Hprt1*, QT00166768). Serial dilutions of whole embryo RNA pooled from all treatment groups were used as an internal reference and to create a standard curve for optimizing primer efficiency and concentration. Each reaction was conducted in triplicate, 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 by the StepOnePlus™ Software (version 2.3).

Preparation of Slides for Immunofluorescence

Whole embryo sagittal sections were prepared as described previously (Banh and Hales, 2013). Phospho-P53 immunoreactivity was detected as follows: sections were blocked with 10%

goat serum (0.5% BSA, 0.1% Triton X-100, 10% goat serum in PBS) for 1 h at room temperature in a dark humidified chamber followed by incubation with the phospho-P53 primary antibody (1:100, cs12571, Cell Signaling Technology) overnight at 4º C. Next, slides were washed three times for five minute intervals in PBS, followed by a 1 h incubation with AlexaFluor®-594 goat anti-rabbit polyclonal secondary antibody (1:200, A-11037, Life Technologies Inc., Burlington, ON) at room temperature. Slides were then washed three times for five minute intervals in PBS, mounted with Vectashield mounting medium supplemented with 4',6-diamidino-2-phenylindole (DAPI) as a counterstain (H-1200, Vector Laboratories Inc., Burlington, ON) and covered with glass cover slips. Each slide was mounted with 2-3 embryos from each treatment group. Negative control experiments were done in parallel by blocking the phospho-P53 primary antibody for 1 h at room temperature with phospho-P53 blocking peptide (1:50, SAB 51094, Signalway Antibody LLC, Baltimore, MD) then proceeding with the above protocol.

Confocal Microscopy

Visualization of phospho-P53 immunoreactivity and cellular localization was done using a Leica TCS 8MP multiphoton confocal microscope. For whole embryo imaging, tile images were scanned at 400 Hz with an optical slice of 5 μm, zoom factor equal to 1, and a pinhole setting of 600 μm using a HC PL APO CS2 20x/0.75 water immersion objective lens. The tiled images were then stitched together using the proprietary Leica Image Analysis software (version 3.2). For cellular localization of phospho-P53, a HC PL APO CS2 63x/1.40 oil immersion lens was used to acquire z-stack images of two randomly selected regions within three different tissues, the heart (H), the caudal neuroepithelium (CNE), and the rostral neuroepithelium (RNE) of 1-2 embryos per treatment group, from 4-5 different litters. All images were captured at a 1024 x 1024 resolution and optical and laser settings were optimized and maintained within each imaging experiment; the 522 nm laser was used for phospho-P53 fluorescence excitation, while the multiphoton laser line was set at 730 nm for DAPI excitation.

Quantitative Image Analysis

Phospho-P53 intensity and nuclear localization were analyzed using Imaris™ image analysis software (version 8.1.2, Bitplane AG, Zurich, Switzerland). For whole embryo analysis,

stitched tile scan images acquired from the Leica software were cropped and imported into Imaris™. The mean intensity of the red-channel, representing phospho-P53, was isolated and measured by creating a red-channel specific surface mask, utilizing a region-of-interest approach to neutralize any background signal and to determine the optimal threshold intensity, subsequently applying the optimal parameters to the whole image. Parameters for the surface mask were selected by using the images from the HU600 treatment group. The average mean intensities of phospho-P53 from 4-5 litters per treatment group were compared.

Nuclear localization of phospho-P53 was quantified as follows: z-stack images taken at 63x magnification were quantified by applying a whole image surface mask specific to the blue-channel, representing the DAPI counterstain. The mean intensity of the red-channel within the mask was quantified, reflecting the content of phospho-P53 immunoreactivity within the nuclear staining, as shown in the movie file (Supplementary data movie file, movie1.mpg). The nuclear localization of phospho-P53 was compared between three different tissues within each embryo and across the treatment groups. These regions were selected based on earlier studies that showed significant levels of phospho-P38 MAPK intensity as a result of hydroxyurea exposure (Banh and Hales, 2013). The average mean nuclear intensity was determined from 1-2 embryos per litter from each treatment group.

Statistical Analyses

All data were statistically analyzed using the GraphPad Prism Software (version 5, Graph Pad Software Inc., La Jolla, CA) except for the microarray and pathway analysis results. The microarray data were analyzed in GeneSpring using an unpaired t-test with a Benjamini-Hochberg multiple comparison correction to compare differences between the control and hydroxyurea treatment group. Pathway analysis was conducted using the Fisher's Exact test in the IPA™ software.

Data from all other experiments were log transformed then tested using 1-way or 2-way ANOVA, followed by a Bonferroni *post-hoc* multiple comparison correction, to detect statistical differences between the control and hydroxyurea treated groups. The 2-way ANOVA test was used to analyze statistical differences of phospho-P53 nuclear translocation between the control and hydroxyurea treated groups as well as differences between the three selected tissues, and any

statistically significant interaction between the treatment and tissue variables. The level of significance for all statistical tests was set to $p < 0.05$.

RESULTS

Hydroxyurea exposure activates the P53 pathway

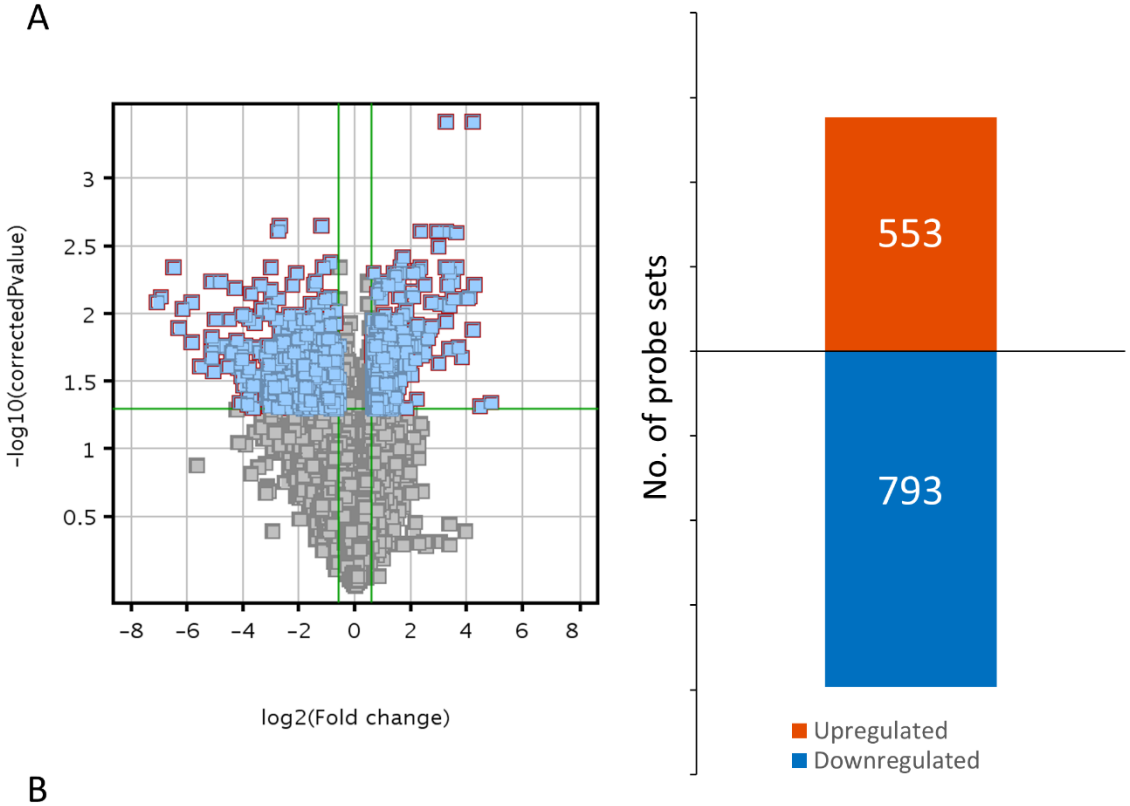
Microarray experiments were conducted using GD 9 embryos exposed to saline or hydroxyurea (400 mg/kg) for 3 h. All raw data are deposited in the GEO repository, under study number GSE54579 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54579). A total of 28,080 probe sets were detected under both conditions; principal component analysis revealed a clear separation between the control and the hydroxyurea treated group (Fig. S2.2). Data filtration and analysis of statistical differences revealed that the expression of 1346 transcripts was significantly changed by at least 1.5 fold in hydroxyurea-treated embryos compared to control; 553 were upregulated while 793 were downregulated (Fig.2.1A). Upregulated transcripts with the greatest fold change included cell cycle checkpoint regulators (*Ptprv*, *Cdkn1a, Ccng1),* programmed cell death factors (*Pmaip1, Fas, Tp53inp1, Phlda3, Anxa8*) and DNA damage response factors (*Zfp365, Ddit4l*) (Table 2.1). Thus, hydroxyurea exposure alters the expression of genes that regulate cell cycle arrest and cell death by apoptosis, in addition to inducing a DNA damage response.

To delineate the cellular pathways and upstream regulators that are activated during organogenesis in response to a teratogenic dose of hydroxyurea, we ran a pathway analysis on the microarray data. Of the 1346 transcripts detected in the microarray, only 743 transcripts were assigned a functional pathway by the IPA software. The most significantly activated pathway in GD 9 embryos exposed to hydroxyurea was the P53 pathway (p= 1.6e-5) (Fig. 2.1B); two MAPK pathways, the P38 and JNK pathways, were also predicted to be activated (data not shown). As anticipated based on the individual genes that were highly upregulated, pathway analysis revealed that hydroxyurea exposure activated cell death/pro-apoptotic pathways and cell cycle checkpoint pathways; in addition, it affected mitochondrial function and aryl hydrocarbon receptor signaling (Fig. 2.1B, Tables 2.2 and 2.3).

The p53 pathway was the highest predicted activated upstream regulator after hydroxyurea treatment; indeed, many of the most affected genes were downstream targets of P53 transcriptional regulation (Fig. 2.2A, Table 2.4). Other predicted upstream regulators related to the P53 pathway and protein family included BRCA1, P63 and P73 (Table 2.5). A comparative analysis of genes predicted to be regulated by p53 family members showed that 90 genes were downstream targets of P53; there were four and five unique genes that were targets of P63 and

P73, respectively. Interestingly, there were a number of genes that were shared or common targets of all three proteins, P53, P63 and P73, as depicted in the overlapping regions of the Venn diagram (Fig. 2.2B). Together, these data show that the tumor-suppressor protein P53 and its related family members, P63 and P73 play a major role in the response of embryos to exposure to a teratogenic dose of hydroxyurea during organogenesis.

A


-log10(correctedPvalue)
3
2.5
2
1.5
1
0.5
-8
-6
-4
-2
0
2
4
6
8
log2(Fold change)
No. of probe sets
553
793
Upregulated
Downregulated

B

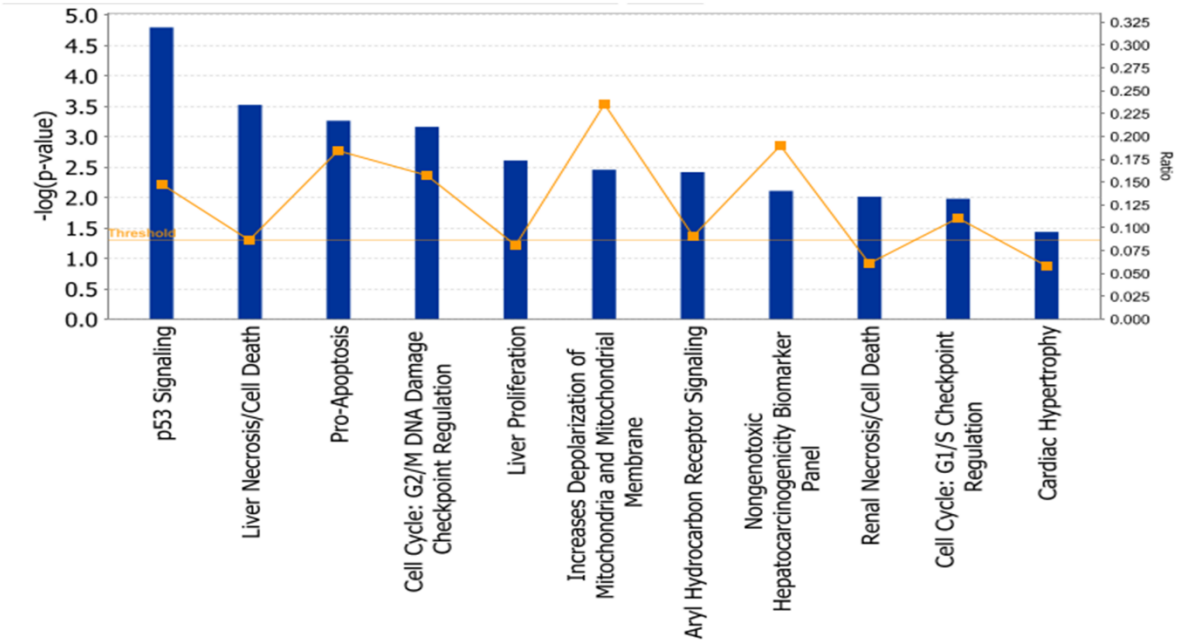

-log(p-value)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Threshold
Ratio
0.325
0.300
0.275
0.250
0.225
0.200
0.175
0.150
0.125
0.100
0.075
0.050
0.025
0.000
p53 Signaling
Liver Necrosis/Cell Death
Pro-Apoptosis
Cell Cycle: G2/M DNA Damage Checkpoint Regulation
Liver Proliferation
Increases Depolarization of Mitochondria and Mitochondrial Membrane
Aryl Hydrocarbon Receptor Signaling
Nongenotoxic Hepatocarcinogenicity Biomarker Panel
Renal Necrosis/Cell Death
Cell Cycle: G1/S Checkpoint Regulation
Cardiac Hypertrophy

Figure 2.1: Hydroxyurea significantly impacts gene expression profiles in GD 9 embryos and activates several cell cycle and cell death related pathways.

A) *Left*: Volcano plot of all detected entities in the microarray. Blue dots indicate transcripts that were significantly changed (-Log p-value = 1.3; $p < 0.05$) by at least 1.5 fold. *Right*: Bar graph of detected probes that were significantly upregulated and downregulated by at least 1.5 fold in response to hydroxyurea treatment in GD 9 embryos.

B) Data collected from GeneSpring were analyzed using the Ingenuity Pathway Analysis™ (IPA) software to predict and examine the molecular pathways that were most affected by hydroxyurea exposure. Vertical bars indicate the level of significance of each pathway indicated by –Log p-value; boxes (■) indicate the ratio between detected genes in the microarray and total number of known genes in the database for that pathway. Statistical analysis was conducted with the IPA software. N=4 for each treatment group.

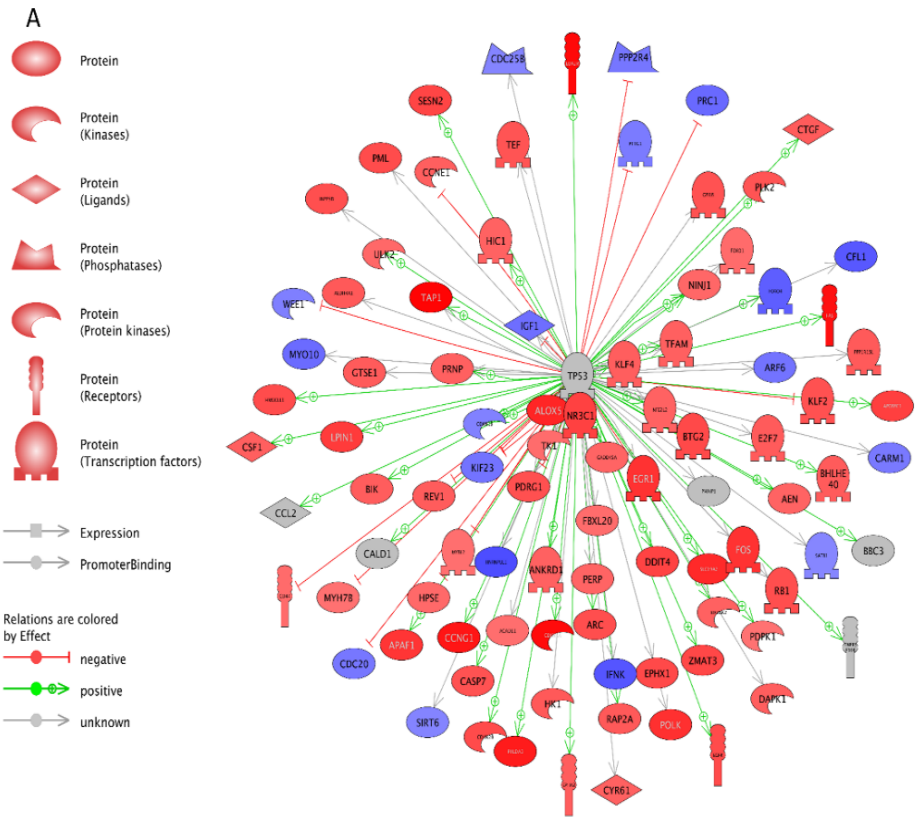

A
Protein
Protein (Kinases)
Protein (Ligands)
Protein (Phosphatases)
Protein (Protein kinases)
Protein (Receptors)
Protein (Transcription factors)
Expression
PromoterBinding
Relations are colored by Effect
negative
positive
unknown
TP53
CDC25B
PPP2R4
SESN2
PRC1
CTGF
TEF
PML
CCNE1
PLK2
HIC1
ULK2
CFL1
NINJ1
WEE1
TAP1
IGF1
TFAM
MYO10
GTSE1
PRNP
KLF4
ARF6
ALOX5
NR3C1
KLF2
LRN1
CSF1
BTG2
E2F7
CARM1
KIF23
TK1
BHLHE40
BIK
REV1
PDRG1
EGR1
AEN
CCL2
FBXL20
CALD1
FOS
BBC3
ANKRD1
DDIT4
PERP
MYH7B
HPSE
RB1
ARC
APAF1
CCNG1
PDPK1
CDC20
IFNK
EPHX1
ZMAT3
CASP7
DAPK1
HK1
SIRT6
RAP2A
POLK
CYR61
B
P53
P63
P73
90
10
10
15
4
1
5

Figure 2.2: Pathway analysis predicts P53 as the most activated upstream regulator in response to hydroxyurea exposure in GD 9 embryos

A) Schematic representation of interactions between P53 and downstream targets detected in the microarray. Colors indicate the level of transcript expression as determined by the Log fold change extracted from the microarray data by GeneSpring; red denotes upregulation, blue denotes downregulation, while grey denotes no effect.

B) Venn diagram of common and unique genes associated with the transcription factors P53, P63 and P73. Data were extracted from analysis of predicted activated upstream regulators as determined by the IPA™ software.

Table 2.1: Genes upregulated in embryos exposed to 400 mg/kg hydroxyurea compared to control, filtered by fold change > 9.0 and p < 0.05, sorted by descending order

Gene Symbol	*Description*	*Fold Change*	*p -value*
Agtr1b	angiotensin II receptor, type 1b	18.87	0.0002
Ptprv	protein tyrosine phosphatase, receptor type, V	18.49	0.0007
1700007K13Rik	RIKEN cDNA 1700007K13 gene	17.71	0.0000
Tap1	transporter 1, ATP-binding cassette, sub-family B	16.46	0.0003
Svop	SV2 related protein	16.06	0.0003
Eda2r	ectodysplasin A2 receptor, transcript variant 3	14.11	0.0011
Cdkn1a	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	12.70	0.0009
Pmaip1	phorbol-12-myristate-13-acetate-induced protein 1	12.17	0.0002
Zfp365	zinc finger protein 365	12.08	0.0001
Prrg4	proline rich Gla (G-carboxyglutamic acid) 4 (transmembrane)	11.32	0.0003
Fas	Fas (TNF receptor superfamily member 6)	10.99	0.0002
Ddit4l	DNA-damage-inducible transcript 4-like	10.26	0.0002
Zfp750	zinc finger protein 750	10.00	0.0004
P2ry6	pyrimidinergic receptor P2Y, G-protein coupled, 6	9.70	0.0002
Ltb4r1	leukotriene B4 receptor 1	9.54	0.0002
Eva1c	eva-1 homolog C (C. elegans)	9.43	0.0002
D630023F18Rik	RIKEN cDNA D630023F18 gene	9.33	0.0006
Trp53inp1	transformation related protein 53 inducible nuclear protein 1	9.32	0.0001
Ccng1	cyclin G1	9.17	0.0000

Table 2.2: G1/S cell cycle checkpoint regulation pathway genes that were upregulated by >1.5 fold in embryos exposed to 400 mg/kg hydroxyurea

Gene Symbol	*Gene Name*	*Fold Change*	*p-Value*
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	12.70	0.0004
CDKN2B	cyclin-dependent kinase inhibitor 2B	2.55	0.0026
RB1	retinoblastoma 1	2.40	0.0008
CCNE1	cyclin E1	1.87	0.0017
FOXO1	forkhead box O1	1.62	0.0027
ATM	ATM serine/threonine kinase	1.57	0.0010

Table 2.3: Pro-apoptotic pathway genes that were upregulated by >1.5 fold in embryos exposed to 400 mg/kg hydroxyurea

Gene Symbol	*Gene Name*	*Fold Change*	*p-Value*
FAS	Fas cell surface death receptor	10.99	0.0002
TNFRSF10A	tumor necrosis factor receptor superfamily member 10a	4.99	0.0002
APAF1	apoptotic peptidase activating factor 1	3.90	0.0003
BBC3	BCL2 binding component 3	2.73	0.0007
CASP7	caspase 7, apoptosis-related cysteine peptidase	2.26	0.0008
BIK	BCL2-interacting killer (apoptosis-inducing)	1.92	0.0011
DAPK1	death-associated protein kinase 1	1.89	0.0037

Table 2.4: P53 signaling pathway genes that were upregulated by >1.5 fold in embryos exposed to 400 mg/kg hydroxyurea

Gene Symbol	*Gene Name*	*Fold Change*	*p-Value*
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	12.70	0.0009
FAS	Fas cell surface death receptor	10.99	0.0002
TP53INP1	tumor protein p53 inducible nuclear protein 1	9.32	0.0001
CCNG1	cyclin G1	9.17	0.0000
TNFRSF10A	tumor necrosis factor receptor superfamily, member 10a	4.99	0.0002
APAF1	apoptotic peptidase activating factor 1	3.90	0.0003
PIDD1	p53-induced death domain protein 1	3.35	0.0010
BBC3	BCL2 binding component 3	2.73	0.0007
PML	promyelocytic leukemia	2.50	0.0011
RB1	retinoblastoma 1	2.40	0.0008
PERP	PERP, TP53 apoptosis effector	1.77	0.0008
GADD45A	growth arrest and DNA-damage-inducible, alpha	1.76	0.0017
ATM	ATM serine/threonine kinase	1.57	0.0010
KAT2B	K(lysine) acetyltransferase 2B	1.56	0.0034

Table 2.5: Top predicted activated upstream regulators in GD 9 embryos exposed to 400 mg/kg hydroxyurea by descending p-value order

Upstream Regulator	*Molecule Type*	*Predicted Activation State*	*Activation Z-Score*	*p-Value Of Overlap*
TP53	transcription regulator	Activated	5.74	3.12E-23
BRCA1	transcription regulator	Activated	3.17	5.49E-10
CCND1	transcription regulator	-	0.63	5.09E-09
CDKN1A	kinase	-	0.11	2.81E-08
TP63	transcription regulator	Activated	3.49	4.78E-08
TOPBP1	other	-	-1.89	1.54E-07
TP73	transcription regulator	Activated	3.01	1.69E-07
IGF1	growth factor	-	1.68	2.77E-07
PTH	other	-	0.69	3.75E-07

P53 and phospho-P53 protein concentrations are significantly increased after hydroxyurea exposure

Since P53 activity is largely regulated post-transcriptionally the next question was whether hydroxyurea exposure affected the protein concentrations of P53 and phospho-P53 in treated embryos. Using Western blots P53 and phospho-P53 protein (Ser15) levels were found to be significantly increased in both hydroxyurea treatment groups compared to the control group (Fig. 2.3A & B). Quantitative analysis showed that in the HU400 and HU600 treated embryos P53 concentrations were increased by 3 and 4 fold respectively, while phospho-P53 levels were increased 3 and 5.5 fold. These results indicate that phosphorylated P53 accumulates in murine embryos in response to a teratogenic dose of hydroxyurea.

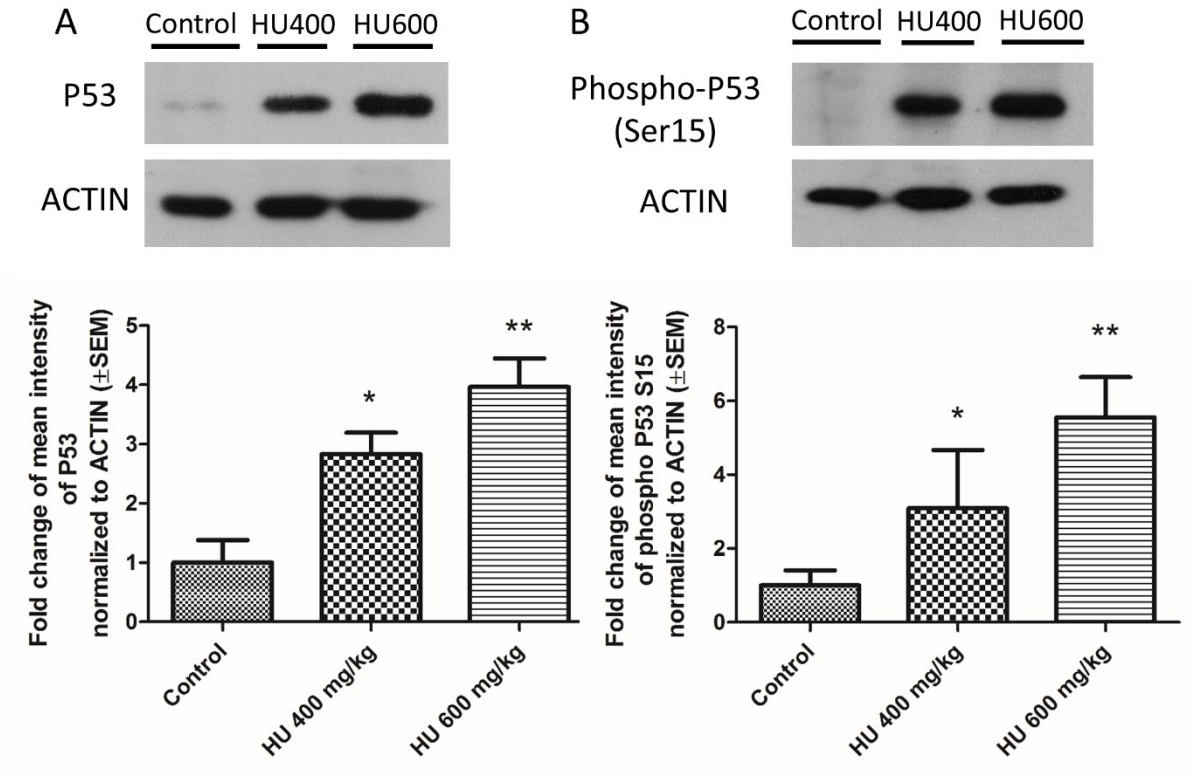

A
Control
HU400
HU600
P53
ACTIN
B
Control
HU400
HU600
Phospho-P53
(Ser15)
ACTIN
Fold change of mean intensity of P53 normalized to ACTIN (±SEM)
0
1
2
3
4
5
*
**
Control
HU 400 mg/kg
HU 600 mg/kg
Fold change of mean intensity of phospho P53 S15 normalized to ACTIN (±SEM)
0
2
4
6
8
*
**
Control
HU 400 mg/kg
HU 600 mg/kg

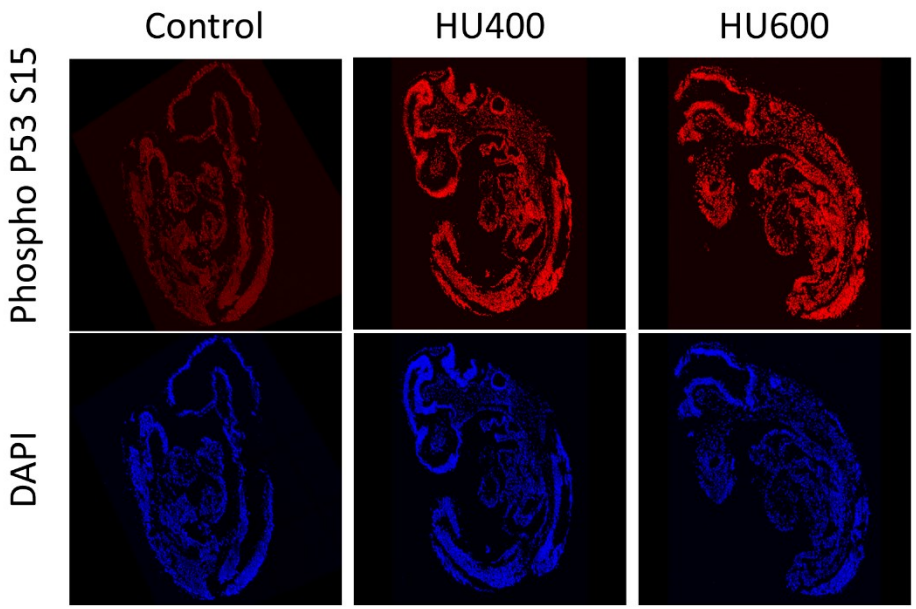
Figure 2.3: P53 and Phospho-P53 protein levels increased in a dose-dependent fashion after hydroxyurea exposure

A) *Top*: Representative blots of P53 protein expression. *Bottom*: Quantification of P53 levels in embryos from the 400 mg/kg hydroxyurea treatment group (HU400) (Fold change [FC]= 2.83, SEM= ±0.36) and the 600 mg/kg group (HU600) (FC= 3.96, SEM= ±0.48). B) *Top*: Representative blots of phospho-P53 protein expression. *Bottom*: Quantification of phospho-P53 levels in HU400 (FC= 3.1, SEM= ±1.5) and HU600 treated embryos (FC= 5.55, SEM= ±1.2). P53 and phospho-P53 levels were significantly upregulated in both hydroxyurea treated groups compared to control. All protein levels were normalized to Actin protein expression. N=4-5 for each treatment group. * $p<0.05$, ** $p<0.01$, 1-way ANOVA with Bonferroni *post-hoc* test.

Hydroxyurea treatment induces phospho-P53 immunoreactivity and nuclear localization in various embryonic tissues

To determine whether hydroxyurea activates P53 in a tissue-specific manner phospho-P53 immunoreactivity was visualized in paraffin-embedded whole embryo sections using confocal microscopy. Phospho-P53 immunoreactivity significantly increased in a dose-dependent fashion throughout the embryo (Fig. 2.4A), with a significant 20-fold increase in the highest hydroxyurea dose group, HU600 (Fig. 2.4B). The extent to which phospho-P53 was translocated to the nucleus was determined by quantifying the co-localization of phospho-P53 and DAPI. The nuclear concentrations of phospho-P53 in three regions, the heart, the caudal neuroepithelium (CNE) and the rostral neuroepithelium (RNE), were compared (Fig. 2.5A; these embryo regions are depicted in Supplementary data Fig. S2.3). Nuclear concentrations of phospho-P53 were significantly elevated in all three regions of the embryos exposed to hydroxyurea treatment, compared to controls (Fig. 2.5B). There were no significant differences of phospho-P53 nuclear translocation between the three different tissues within each treatment group.

A


Control
HU400
HU600
Phospho P53 S15
DAPI

B

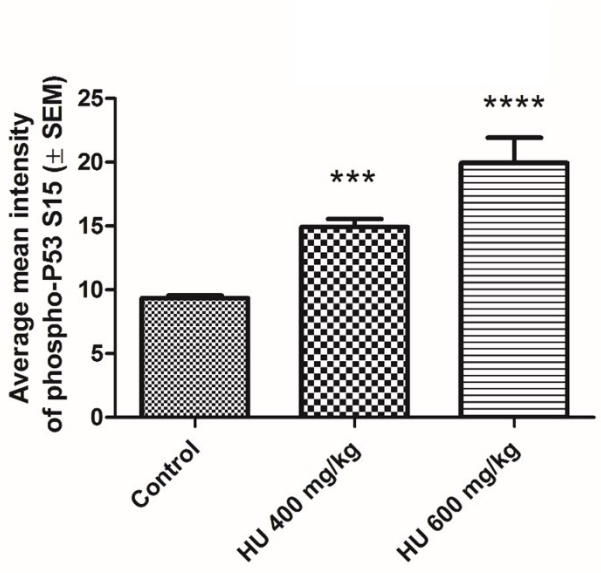
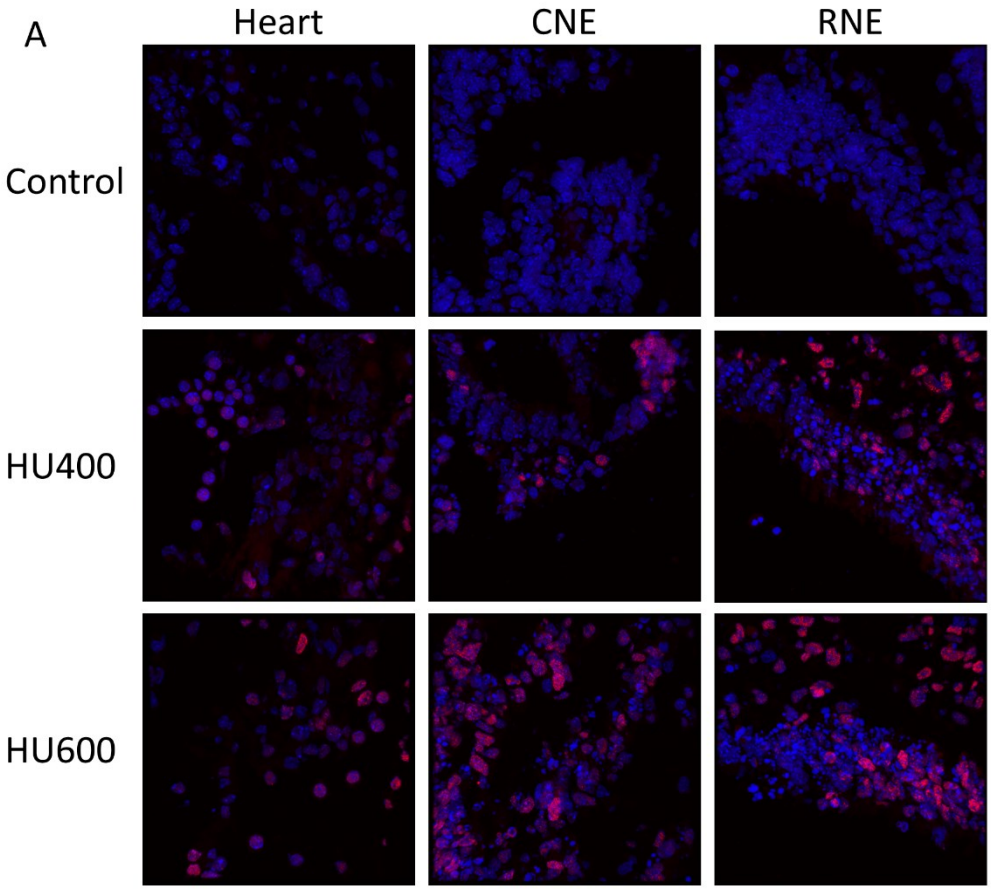

Average mean intensity of phospho-P53 S15 (± SEM)
25
20
15
10
5
0
***
****
Control
HU 400 mg/kg
HU 600 mg/kg

Figure 2.4: Hydroxyurea exposure induced a widespread increase in phospho-P53 immunoreactivity

A) Representative multiphoton and confocal microscopy images taken at 20x of whole embryo sections showing increasing phospho-P53 immunoreactivity (red, top panel) and the DAPI nuclear counterstain (blue, bottom panel). B) Average mean intensity of phospho-P53 immunoreactivity in whole embryo sections. A significant increase in phospho-P53 intensity was detected in the HU400 and HU600 groups. N=4-5 for each treatment group. *** $p<0.001$, ****$p<0.0001$, 1-way ANOVA with Bonferroni *post-hoc* test.


A
Heart
CNE
RNE
Control
HU400
HU600

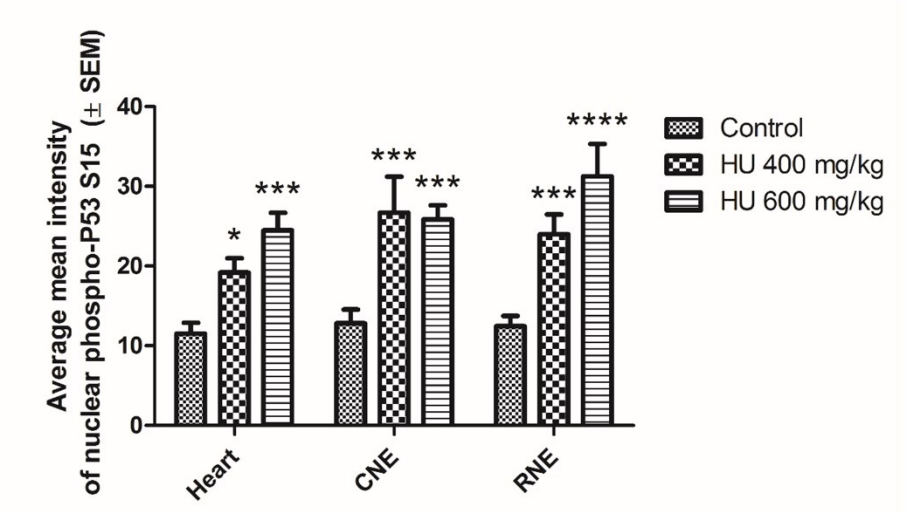

B
Average mean intensity of nuclear phospho-P53 S15 (± SEM)
40
30
20
10
0
*
***
***
***
***
****
Heart
CNE
RNE
Control
HU 400 mg/kg
HU 600 mg/kg

Figure 2.5: Nuclear translocation of phospho-P53 increased significantly with hydroxyurea treatment

A) Representative multiphoton and confocal images of phospho-P53 immunoreactivity taken at 63x magnification. 3 tissues were analyzed per embryo across all treatment groups (Heart, CNE: caudal neuroepithelium, RNE: rostral neuroepithelium). B) Quantification of phospho-P53 colocalization with DAPI. N=4-5 for each tissue and treatment group. * $p<0.05$, *** $p<0.001$, **** $p<0.0001$ compared to control, 2-way ANOVA with Bonferroni *post-hoc* test. There were no statistically significant differences in phospho-P53 nuclear translocation between tissues in the embryo, and no statistically significant interaction between the treatment and tissue variables.

P53 downstream transcription targets are significantly upregulated by hydroxyurea

The induction of specific P53 regulated genes was quantified using qRT-PCR to assess P53 pathway activation and to validate the microarray data. Although *Trp53* transcript levels did not change with hydroxyurea exposure compared to control (Fig. 2.6A), *Cdkn1a* (Cyclin-Dependent Kinase Inhibitor 1A [also known as P21, CIP1]), *Fas* (Fas Cell Surface Death Receptor) and *Trp53inp1* (Tumor protein p53-inducible nuclear protein 1) were significantly upregulated in both the HU400 and HU600 treatment groups (Fig. 2.6B-D). We examined the protein expression level of P53INP1 in response to hydroxyurea exposure as a marker for P53 downstream pro-apoptotic effects. While P53INP1 was almost undetected in control embryos, expression increased significantly in response to hydroxyurea exposure in both the HU400 and HU600 treatment groups (Fig. 2.7).

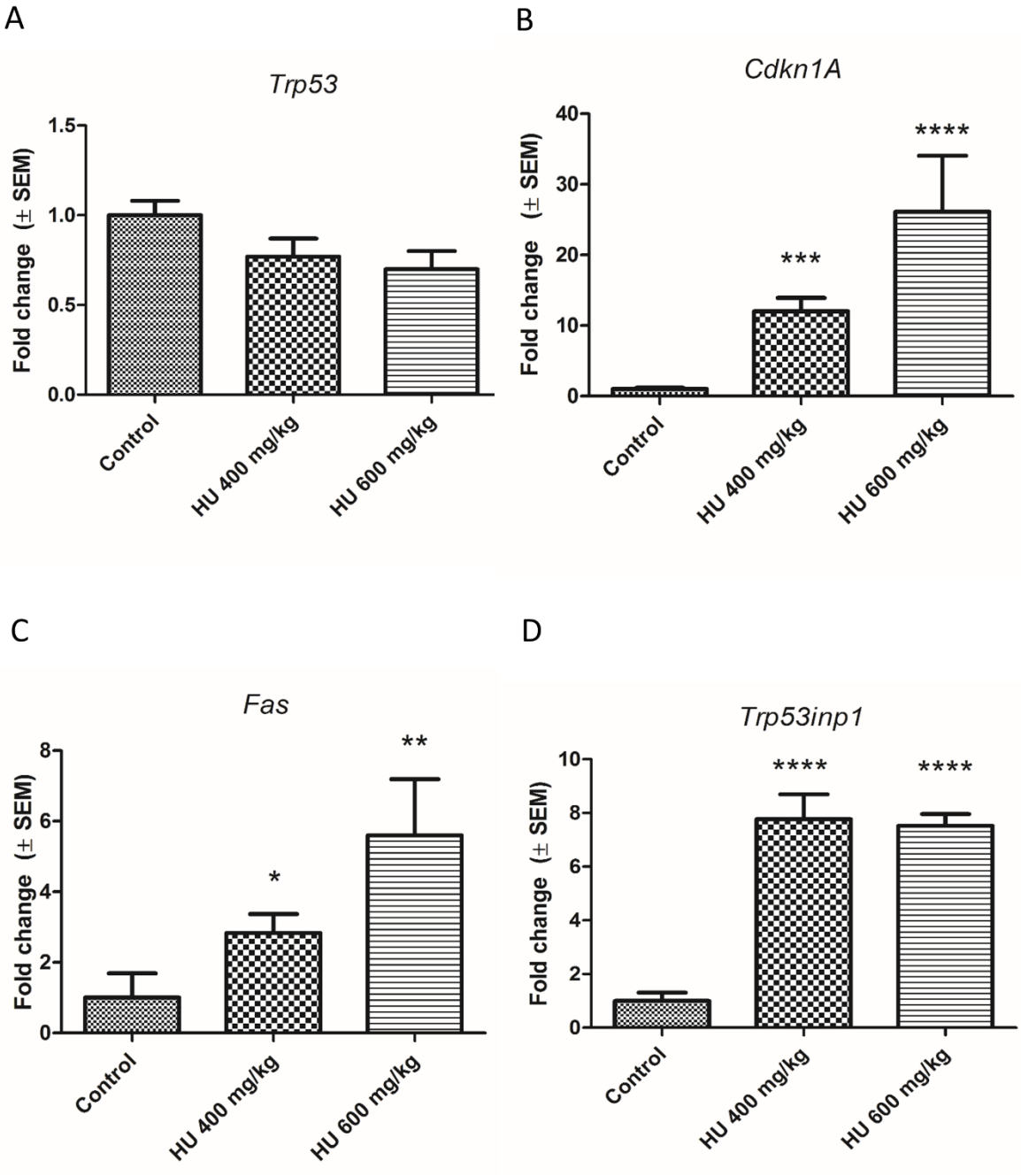
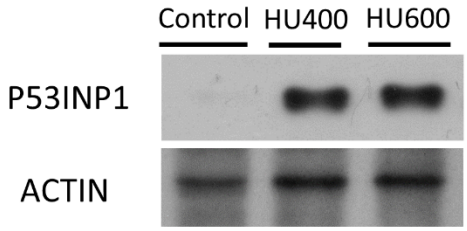

A
Trp53
Fold change (± SEM)
1.5
1.0
0.5
0.0
Control
HU 400 mg/kg
HU 600 mg/kg
B
Cdkn1A
Fold change (± SEM)
40
30
20
10
0
***
****
Control
HU 400 mg/kg
HU 600 mg/kg
C
Fas
Fold change (± SEM)
8
6
4
2
0
*
**
Control
HU 400 mg/kg
HU 600 mg/kg
D
Trp53inp1
Fold change (± SEM)
10
8
6
4
2
0
****
****
Control
HU 400 mg/kg
HU 600 mg/kg

Figure 2.6: Transcription levels of P53 downstream targets in response to hydroxyurea exposure

A) *Trp53* transcript levels after hydroxyurea treatment were not significantly different compared to control, N=3. B-D) Hydroxyurea exposure significantly induced transcript levels of *Cdkn1a* (HU400 FC=12.02, SEM= ±1.90; HU600 FC= 26.11, SEM= ±7.95, N=5), *Fas* (HU400 FC=2.83, SEM= ±0.54; HU600 FC= 5.6, SEM= ±1.59, N=4-5) and *Trp53inp1* (HU400 FC= 7.77, SEM= ±0.93; HU600 FC= 7.52, SEM= ±0.44, N=5). Each bar represents the fold change of the mean quantity of the transcript relative to *Hprt1*. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ****$p<0.0001$, 1-way ANOVA followed by a Bonferroni *post-hoc* test

A


Control
HU400
HU600
P53INP1
ACTIN

B

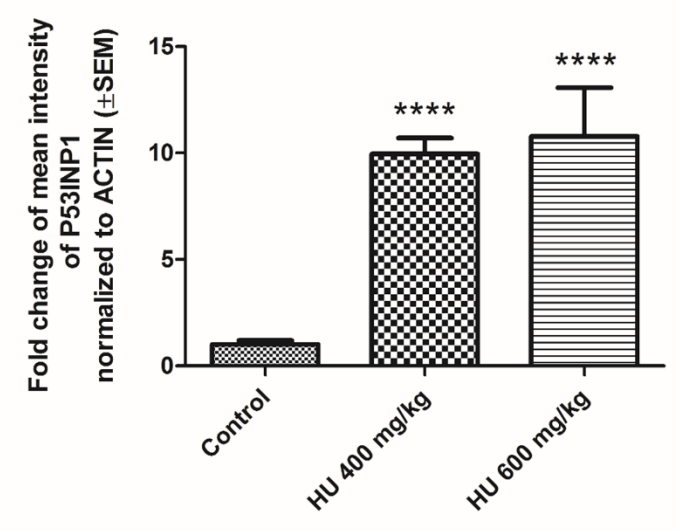

Fold change of mean intensity of P53INP1 normalized to ACTIN (±SEM)
15
10
5
0
****
****
Control
HU 400 mg/kg
HU 600 mg/kg

Figure 2.7: Upregulation of P53INP1 in response to hydroxyurea treatment

A) Representative blot of P53INP1 protein expression levels in embryos exposed to hydroxyurea compared to controls. B) Quantification of immunoblots showed that hydroxyurea treatment significantly upregulated P53INP1 in the HU400 (FC= 9.95, SEM= ±0.75) and HU600 (FC= 10.78, SEM= 2.29) groups. N=4-5 for each treatment group. **** $p<0.0001$, 1-way ANOVA followed by a Bonferroni *post-hoc* test.

DISCUSSION

Hydroxyurea exposure has a significant impact on the gene expression profile of organogenesis-stage mouse embryos. Genome-wide pathway analysis reveals that many significantly changed genes are downstream targets of the tumor suppressor protein P53. Indeed, P53 is the hub in the regulation of a wide gene network in these embryos. We show here that hydroxyurea exposure significantly increases overall P53 protein levels in the embryo and specifically increases the concentrations of phosphorylated P53. Phospho-P53 immunoreactivity was widespread throughout hydroxyurea exposed embryos.

P53, the "guardian of the genome", is a transcription factor that regulates the expression of numerous annotated genes in response to stress (Chang *et al.*, 2014); P53 activates the expression of genes that are important in cell cycle regulation, the DNA damage response, apoptosis, senescence, cellular metabolism, mitochondrial function, and the oxidative stress response (Ashcroft *et al.*, 1999; Aylon and Oren, 2007). P53 has important roles in murine embryonic development in addition to its role as a tumor suppressor gene; P53 has been shown to regulate the differentiation and modeling of embryonic progenitor nephrons in the kidney, of osteoblasts during bone formation, and of the interdigital tissue of developing limbs (Aboudehen *et al.*, 2012; Armstrong*, et al.*, 1995; Lorda-Diez *et al.*, 2015; Schmid*, et al.*, 1991; Wang*, et al.*, 2006). *In situ* hybridization studies showed that P53 transcripts are widely expressed in organogenesis-stage murine embryos up to GD 10 (Schmid*, et al.*, 1991). Using mice in which the p53-dependent promoter of *Mdm2* was tagged with lacZ, Gottlieb and co-workers (Gottlieb*, et al.*, 1997) demonstrated that the pattern of P53 activation in response to ionizing radiation-induced DNA damage became progressively more restricted with embryo age. P53 expression was strongly activated throughout the embryo on GD 8.5 but by GD 10.5 positive staining was observed in the brain regions, the branchial arches, the maxillary area and the limb buds; no staining was observed in the heart. Here we report that immunoreactive phospho-P53 is widespread throughout the embryo after hydroxyurea exposure, however the translocation of phospho-P53 to the nucleus appears to be treatment and dose-dependent but not embryo region-specific. A previous study from our lab showed that phospho-P38α expression was increased in the neuroepithelium and neural tube, but not in the somites or the heart, after hydroxyurea exposure (Banh and Hales, 2013); P53 phosphorylation (serine 18 in the mouse is equivalent to

serine 15 in human P53) is catalyzed by the P38 MAPK kinase in response to DNA strand breaks (Sluss *et al.*, 2004).

Pathway analysis revealed that cell cycle checkpoint pathways and pro-apoptotic factors are activated in embryos in response to hydroxyurea exposure; these include *Cdkn1A* (P21), a cyclin-dependent kinase inhibitor that binds and deactivates CDK1, a major driver of the cell cycle (Harper *et al.*, 1993), *Fas,* which is involved in the extrinsic apoptotic pathway, and the autophagy-related factor *Trp53inp1* (Adachi *et al.*, 1997; Seillier *et al.*, 2012). Whether these downstream factors elicit their effects in a pattern that mimics the embryo-wide expression and activation of P53 is yet to be determined. The expression of other genes is also highly upregulated in response to hydroxyurea. These include *Ptprv*, which transcribes a G1/S cell cycle checkpoint regulator that acts downstream of P53, *Pmaip1*, which transcribes the protein NOXA that induces mitochondrial mediated apoptosis, *Phdla3*, which represses the Akt1 pathway and thereby induces apoptosis, and *Zfp365*, which transcribes a zinc-finger protein that is involved in the DNA damage repair of stalled replication forks (Doumont *et al.*, 2005; Kurata *et al.*, 2008; Zhang *et al.*, 2013). Thus, hydroxyurea induces DNA damage repair factors as well as apoptotic and cell-cycle regulators.

Other P53 family members, namely P63 and P73, might possibly play a role in the response of the embryo to teratogenic doses of hydroxyurea as they also have been shown to respond to DNA damage from other anti-cancer agents (Lin*, et al.*, 2009; Wilhelm*, et al.*, 2010). These transcription factors have overlapping as well as distinct roles from P53 and are expressed in a region-specific manner during organogenesis (Levrero*, et al.*, 2000); when P63 is mutated or knocked out in mice, the offspring suffer from developmental defects that are similar to the malformations that are observed after hydroxyurea exposure (e.g. truncated or absent fore- and hind-limbs) (Duijf *et al.*, 2003; Mills*, et al.*, 1999). Our microarray data analysis revealed that several transcripts are common targets of two or all of these tumor suppressor family members but each of these upstream regulators is also associated with its unique set of downstream targets. The possible roles of these upstream regulators in the embryonic response to teratogenic stress and the functions of their unique downstream targets are not clear.

In utero exposure to hydroxyurea activates MAPK signaling, inducing the phosphorylation and activation of P38 and JNK (Yan and Hales, 2008), which are known to respond to both DNA damage and oxidative stress. Our pathway analysis and qRT-PCR data

revealed a DNA damage response but did not detect an increase in the oxidative stress response at the transcript level. Previous studies have shown an increase in 4-hydroxynonenal (4-HNE) protein adducts after hydroxyurea exposure, suggesting that oxidative stress is induced by this drug (Schlisser*, et al.*, 2010). However, we did observe a significant upregulation of P53INP1 at both the transcript and protein levels in this study. P53INP1, a P53 downstream effector that is known to respond to oxidative stress, interacts with P53 via a specific binding domain to regulate its pro-apoptotic function (Peuget *et al.*, 2014; Seillier, 2012). P53 itself is also redox sensitive (Forsberg and Di Giovanni, 2014). Thus, any oxidative stress response in the embryo after 3 h of exposure to hydroxyurea may be best detected at the post-translational level.

P53 is upregulated in embryos exposed to several known teratogens (e.g. ionizing radiation, cyclophosphamide, valproic acid) (Hosako*, et al.*, 2007; MacCallum *et al.*, 1996; Paradis and Hales, 2015). A number of labs have investigated the role of P53 in mediating the response of embryos to teratogen exposures. *Trp53* deficient embryos exhibit more malformations than their wild type counterparts when exposed to teratogenic doses of benzo[a]pyrene or ionizing radiation, suggesting that p53 plays a protective role (Nicol*, et al.*, 1995; Norimura*, et al.*, 1996). Other labs have reported that *Trp53* null embryos are more resistant than their wild type counterparts to teratogen exposures that included 2-chloro-2'-deoxyadenosine and cyclophosphamide (Pekar *et al.*, 2007; Wubah*, et al.*, 1996). While each of these teratogens may damage DNA, it is important to note that they activate different pathways in the embryo; while benzo[a]pyrene induces oxidative stress, ionizing radiation, 2-chloro-2'-deoxyadenosine and cyclophosphamide activate a strong apoptotic response (Torchinsky and Toder, 2010). In addition, the timing and duration of exposure to a teratogen during development may be an important factor in determining developmental outcome. Thus, it is possible that P53 plays a role as both a teratological inducer and suppressor depending on the type and timing of the insult.

Our data demonstrate that organogenesis-stage embryos respond to exposure to a potent teratogen and genotoxic agent by activating the P53 signaling pathway. P53 may also play a central role in cross-talk with other stress response pathways, such as the oxidative stress and DNA damage response pathways in the developing embryo after a teratogenic insult.

ACKNOWLEDGMENTS

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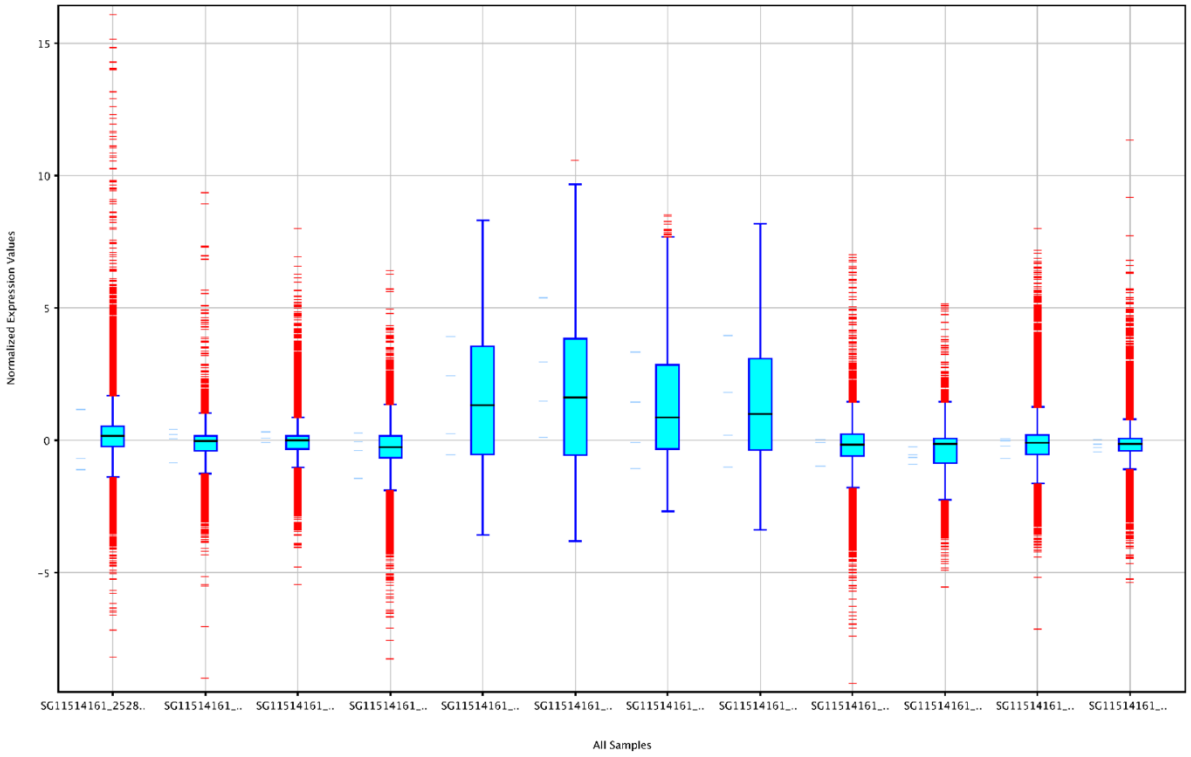
SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfrodjounrals.org.

SUPPLEMENTARY FIGURES: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5
0
-5
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SG11514161_..
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SG11514161_..
SG11514161_..
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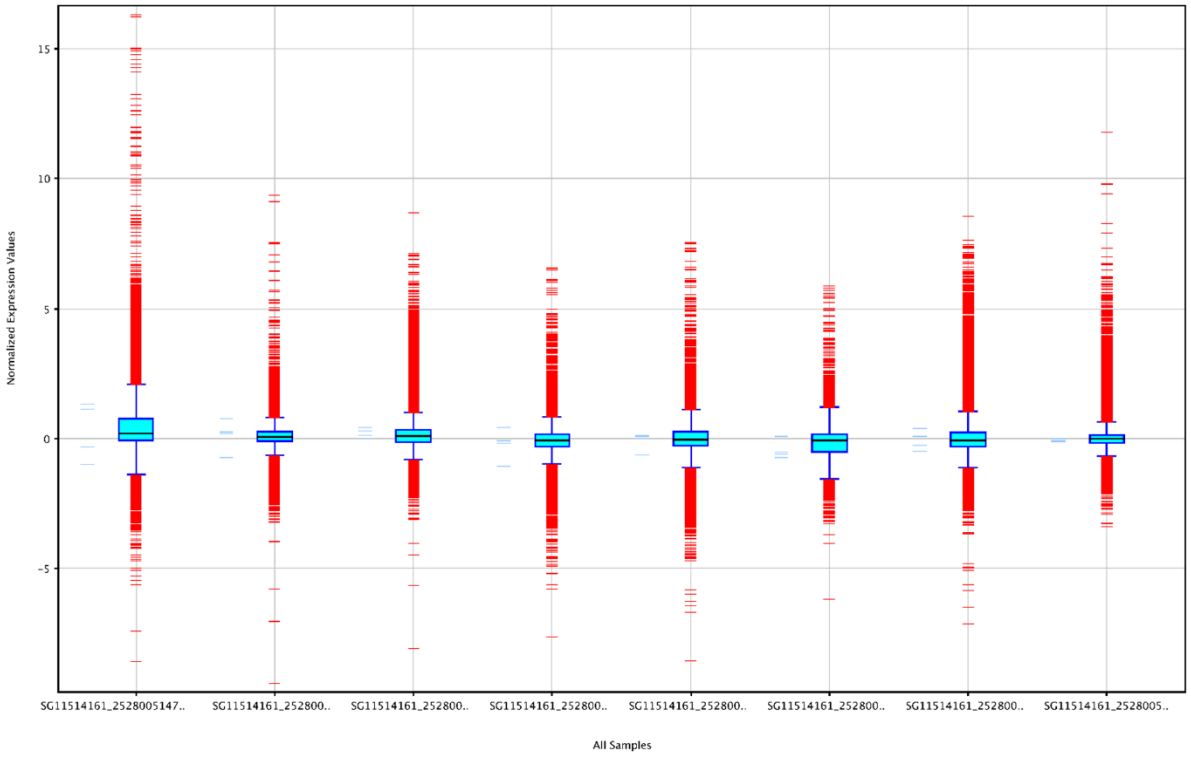
SG11514161_..
SG11514161_..
SG11514161_..
All Samples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SG11514161_252800..
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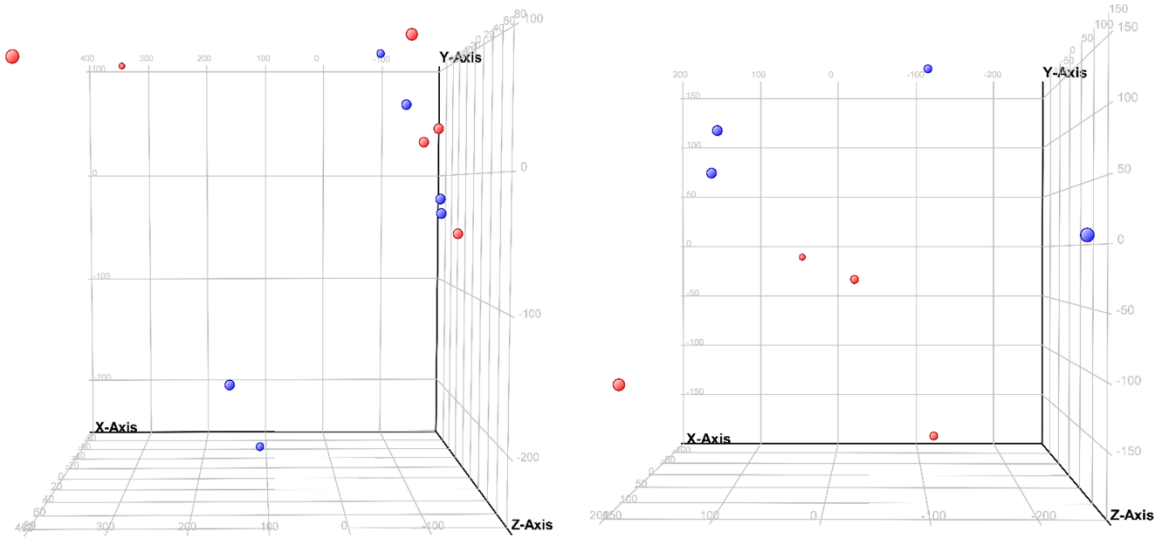
SG11514161_252800..
SG11514161_252800..
SG11514161_2528005..
All Samples

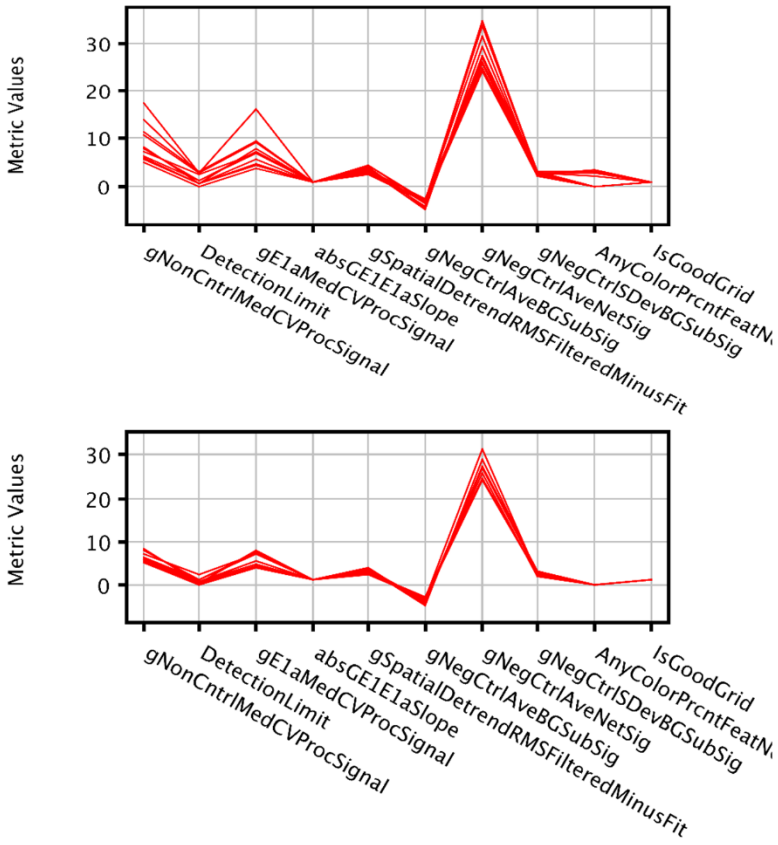
Figure S2.1: Box plots of arrays from the microarray chips.

Top: Box plots of the arrays from three microarray chips each containing two control and two treated samples (N=6 per treatment group)**.** *Bottom*: Box plots of the arrays after removing the chip with the greatest variation, leaving two microarray chips each containing two control and two treated samples (N= 4 per treatment group) y-axis: normalized expression values, x-axis: sample identification number.

A


Y-Axis
X-Axis
Z-Axis
Y-Axis
X-Axis
Z-Axis

B


Metric Values
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gSpatialDetrendRMSFilteredMinusFit
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IsGoodGrid
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gSpatialDetrendRMSFilteredMinusFit
gNegCtrlAveBGSubSig
gNegCtrlAveNetSig
gNegCtrlSDevBGSubSig
AnyColorPrcntFeatN
IsGoodGrid

Figure S2.2: Principal component and quality analysis of microarray chips

A) Three-dimensional graphs depicting principal component analysis (PCA) before (left) and after (right) removal of low quality chip. Red dots indicate expression profiles from HU-treated samples, blue dots indicate expression profiles from control samples

B) Graphs showing quality control parameters before (top) and after (bottom) removal of low quality chip. Descriptions of error parameters can be found on the GeneSpring™ website.

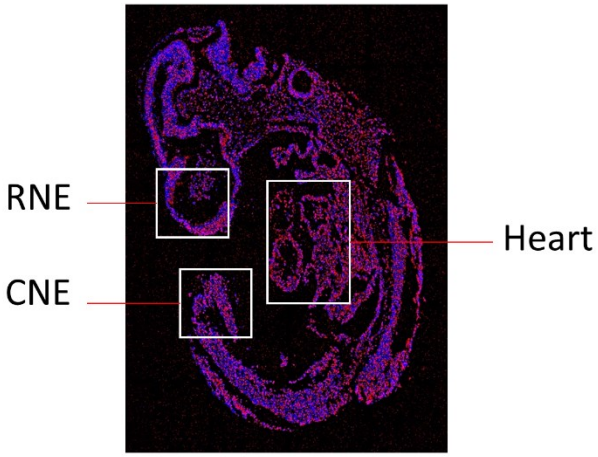
RNE
CNE
Heart

Figure S2.3: Embryonic regions examined for nuclear localization of phospho-P53

Image of paraffin-embedded section of GD9 embryo showing three regions selected for quantification of phospho-P53 nuclear localization: RNE=rostral neuroepithelium, CNE= caudal neuroepithelium. Blue color indicates DAPI nuclear staining, red color indicates phospho-P53 staining.

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CONNECTING TEXT

In the previous chapter we established that the P53 signaling pathway is involved in the embryonic stress response to hydroxyurea exposure. The P53 protein is highly expressed and phosphorylated in embryos exposed to hydroxyurea, ultimately translocating to the nucleus and upregulating downstream transcripts involved in cell cycle arrest and cell death. We also found that other upstream regulators are potentially activated in response to hydroxyurea exposure, including the P53 homologs, P63 and P73. In the following chapter, we examine the roles of P63 and P73 in the organogenesis stage embryo under normal and stressed conditions, and whether they act similarly to P53 in response to hydroxyurea exposure.

CHAPTER III

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

Nazem El Husseini and Barbara F. Hales

Toxicological Sciences (2017) 162, 439-449

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 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 (MST-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.

INTRODUCTION

Hydroxyurea, 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 hydroxyurea 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 hydroxyurea 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 is present at low concentrations during organogenesis and is largely inactive (Choi and Donehower, 1999). In contrast, 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; Yang*, et al.*, 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 two transcription start sites which produce protein isoforms with transactivation domains (TA), 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).

Since 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 three 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 (e.g. *Cdkn1a, Rb1*) and apoptosis (e.g. *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 M., 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 three transcription factors are capable of inducing pro-apoptotic factors (e.g. *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 pro-Caspase-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). MST-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 (e.g. 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 hydroxyurea 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 hydroxyurea 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, ME, USA) 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 10:00 AM the following day (designated as gestation day 0, or GD 0). Between 8:00 and 10:00 AM on GD 9 pregnant dams were treated with either saline (control) or 400 mg/kg hydroxyurea (HU) (Aldrich Chemical Co., Madison, WI) by intraperitoneal injection.

All dams were euthanized by CO_2 asphyxiation and cervical dislocation 3 h after hydroxyurea 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 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, Mississauga, ON, Canada). 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, DE). Proteins were precipitated from the remaining embryonic lysate using ice-cold 100% acetone and dissolved in protein lysis buffer (8M urea, 2M thiourea, 0.5% CHAPS). 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 above 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, MO, USA). Samples were then centrifuged at 20,000g 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 precipitate the 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, CA) 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 15s, 60° C for 30s, and 72° C for 30s. Subsequently, the melt-curve stage was as follows: 95° C for 15s, 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 (~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: CAGCCTCTGTTCCACATACACT; common reverse: TGGATGGTGGTATACTCAGAGC) 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, VA). 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 4º C (Bio-Rad Laboratories, Ltd.). Afterwards, membranes were blocked with 5% milk in 1X TBS containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature. Membranes were incubated overnight, at 4º C, with primary antibodies against P53 (1:1000, cs2524, Cell Signaling Technology, Inc., Danvers, MA), P63 (1:1000, PA121739, Thermo Fisher Scientific, Waltham, MA), 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, TX). Membranes were washed three 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, MD, USA) 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 inter-blot 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, Foster City, CA) 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 72º 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); 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

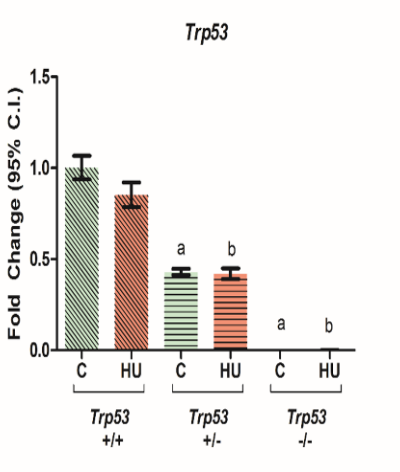
All data were analyzed using GraphPad Prism Software (version 5, Graph Pad Software Inc., La Jolla, CA). Data were tested by 2-way ANOVA, followed by a Bonferroni *post-hoc* multiple comparison correction, to detect statistical differences between the control and hydroxyurea-treated groups and the different genotypes. The litter was used to define the statistical unit (n). The level of significance for all statistical tests was set to $p < 0.05$.

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 (Fig. 3.1A). As expected, the levels of *Trp53* decreased by 50% in the heterozygote embryos (*Trp53*$^{+/-}$) and *Trp53* was not detected in the homozygote knockout (*Trp53*$^{-/-}$) embryos in either the control or hydroxyurea-treated groups. Transcript levels of *Trp63* were significantly reduced in all drug-treated embryos (Fig. 3.1B). This effect was independent of the *Trp53* gene as the levels of *Trp63* were not statistically different between hydroxyurea-treated embryos that expressed or lacked *Trp53*. The transcript levels of *Trp73* were significantly increased by hydroxyurea exposure in *Trp53*$^{+/+}$ embryos (Fig. 3.1C). Interestingly, hydroxyurea-increased *Trp73* expression was dependent on the gene dosage of *Trp53*; the induction of *Trp73* expression by hydroxyurea 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.

a.


Trp53
Fold Change (95% C.I.)
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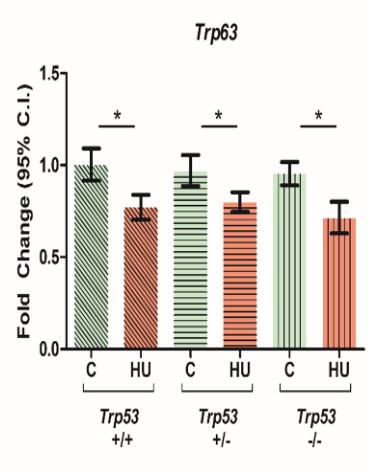
1.0
0.5
0.0
a
b
a
b
C
HU
C
HU
C
HU
Trp53 +/+
Trp53 +/-
Trp53 -/-

b.


Trp63
Fold Change (95% C.I.)
1.5
1.0
0.5
0.0
*
*
*
C
HU
C
HU
C
HU
Trp53 +/+
Trp53 +/-
Trp53 -/-

c.

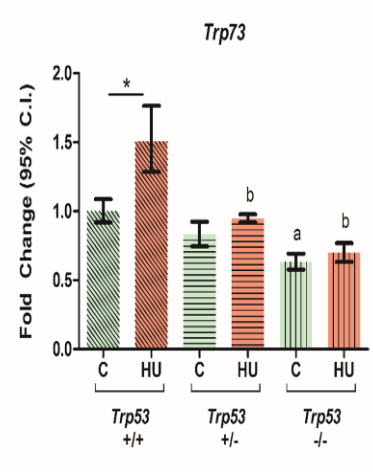

Trp73
Fold Change (95% C.I.)
2.0
1.5
1.0
0.5
0.0
*
b
a
b
C
HU
C
HU
C
HU
Trp53 +/+
Trp53 +/-
Trp53 -/-

Figure 3.1: Effects of hydroxyurea 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. $p<0.05$, 2-way ANOVA followed by a Bonferroni post-hoc test, n=3. (*) denotes significant change compared to control group with the same genotype, (a) denotes significant change compared to the *Trp53*$^{+/+}$ control group, (b) denotes significant change compared to the *Trp53*$^{+/+}$ hydroxyurea-treated group.

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 (Fig. 3.2A). Hydroxyurea treatment of *Trp53*$^{+/+}$ embryos induced a significant increase in P53 protein levels (Fig. 3.2A-B). The amounts of P53 detected in hydroxyurea-exposed embryos reflected the *Trp53* gene dosage; P53 decreased by 50% in *Trp53*$^{+/-}$ embryos and was not detected in *Trp53*$^{-/-}$ embryos. Since an essential component of P53 stabilization is its post-translational 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, 2009). In hydroxyurea-treated *Trp53*$^{+/+}$ and *Trp53*$^{+/-}$ embryos a higher molecular weight band (~55 KDa), representing phosphorylated P53, appeared above the P53 band (~53 KDa) (Fig. 3.2C). As expected, no detectable levels of phosphorylated P53 were found in *Trp53*$^{-/-}$ embryos.

TAP63α, the P63 full length isoform, is shown in Fig. 3.2D. In contrast to P53, P63 was readily detected in control embryos from all genotypes. Furthermore, hydroxyurea treatment did not significantly alter the expression of TAP63α (Fig. 3.2E). Moreover, no higher molecular weight TAP63α band was observed in Phos-tag™ gels, suggesting that hydroxyurea treatment did not induce P63 phosphorylation (Fig. 3.2F).

P73 protein immunoreactivity was detected in control embryos (Fig. 3.2G); hydroxyurea treatment did not alter P73 protein concentrations in embryos with or without *Trp53* (Fig. 3.2H). In addition, no higher molecular weight form of P73 was observed in Phos-tag™ gels (Fig. 3.2I). Thus, both P63 and P73 proteins are present during organogenesis; however, they are not responsive to hydroxyurea treatment and their expression is not affected by the absence of P53.

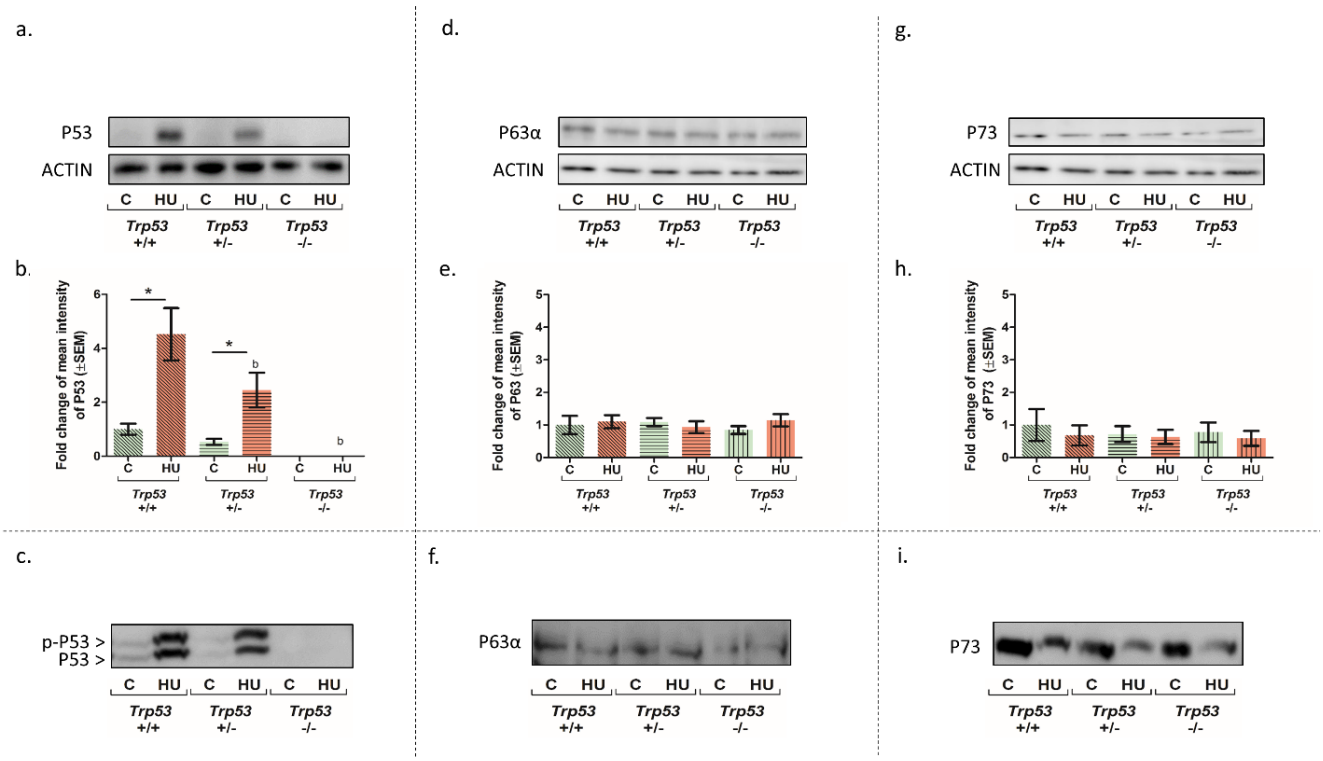

a.
P53
ACTIN
C HU C HU C HU
Trp53 +/+
Trp53 +/-
Trp53 -/-
b.
Fold change of mean intensity of P53 (±SEM)
*
*
b
b
c.
p-P53 >
P53 >
d.
P63α
e.
Fold change of mean intensity of P63 (±SEM)
f.
P63α
g.
P73
h.
Fold change of mean intensity of P73 (±SEM)
i.
P73

Figure 3.2: Effects of hydroxyurea treatment on P53, P63 and P73 protein expression and phosphorylation status in embryos

Top: Representative Western blots and quantification of P53 (***A-B***), P63 (***D-E***) and P73 (***G-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. $p<0.05$, 2-way ANOVA followed by a Bonferroni post-hoc test, n=3. (*) denotes significant change compared to control group with the same genotype, (b) denotes significant change compared to the $Trp53^{+/+}$ hydroxyurea-treated group.

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 hydroxyurea-induced stress in the organogenesis-stage embryo

In our previous study, we identified a number of transcripts with altered expression in hydroxyurea-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 3.1).

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*) (Fig. 3.3A), under control and hydroxyurea-treated conditions, in the presence and absence of *Trp53. Trp53inp1* transcript levels were significantly upregulated by hydroxyurea in $Trp53^{+/+}$ embryos; this effect was abolished in *Trp53* null embryos (Fig. 3.3B). In contrast, the transcript levels of *Pax9* and *Tbx5* decreased in response to hydroxyurea treatment and were not affected by the presence or absence of *Trp53* (Fig. 3.3C-D). Lastly, hydroxyurea treatment induced *Jag2* transcript levels in a *Trp53*-dependent manner (Fig. 3.3E).

We then assessed the level of transcripts that are commonly regulated by all three P53 family members (*Cdkn1a, Fas*), by P53 and P63 (*Pmaip1*) or by P53 and P73 (*Rb1*) (Fig.3.4A). For each of these downstream target genes, transcript levels were significantly increased in embryos exposed to hydroxyurea; this induction was ablated by the deletion of *Trp53* (Fig. 3.4B-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 hydroxyurea 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.

Table 3.1: Prediction analysis of unique and common markers of P53, P63 and P73 transcriptional activity.

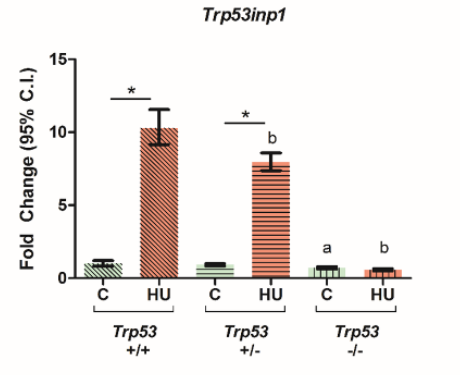
Protein	Gene	Prediction	References
P53	*Trp53inp1*	↑	(Kenzelmann Broz *et al.*, 2013; Okamura *et al.*, 2001)
	Cdkn1a	↑	(Kenzelmann Broz, *et al.*, 2013; Nayak and Das, 2002)
	Fas	↑	(Lima *et al.*, 2011; Munsch *et al.*, 2000; Thiery *et al.*, 2005)
	Pmaip1	↑	(Hamard *et al.*, 2013; Zhu *et al.*, 2010)
	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 hydroxyurea if each of the P53 proteins is activated; upregulation (green), downregulation (red). Prediction analysis is based on Ingenuity Pathway Analysis (IPA™) software using microarray data from (El Husseini, *et al.*, 2016).

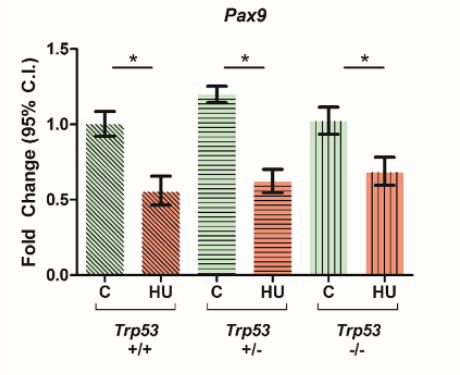
a.

Unique P53	Unique P63	Unique P73	Common to P63/P73
Trp53inp1	*Pax9*	*Tbx5*	*Jag2*

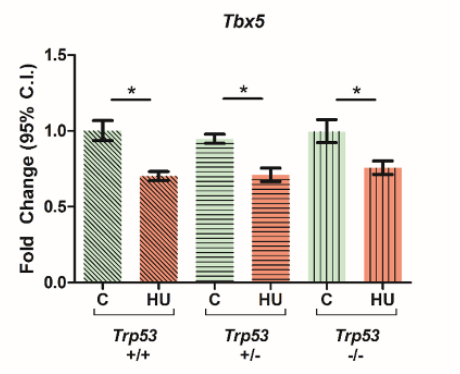
b.



c.



d.



e.

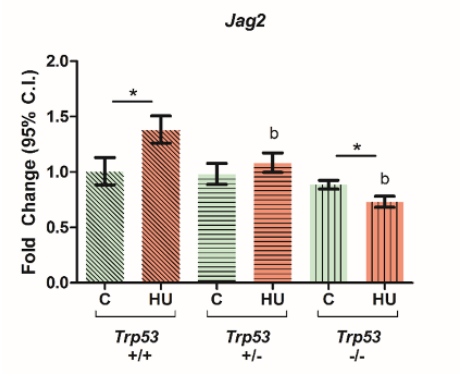


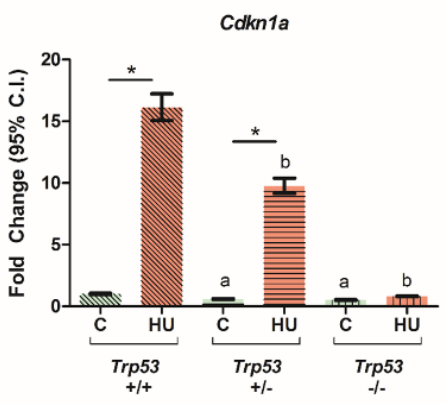
Figure 3.3: Effects of hydroxyurea exposure in embryos on the relative expression of genes that are downstream targets of unique P53 family proteins.

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. $p<0.05$, 2-way ANOVA followed by a Bonferroni post-hoc test, n=3. (*) denotes significant change compared to control group with the same genotype, (b) denotes significant change compared to the $Trp53^{+/+}$ hydroxyurea-treated group.

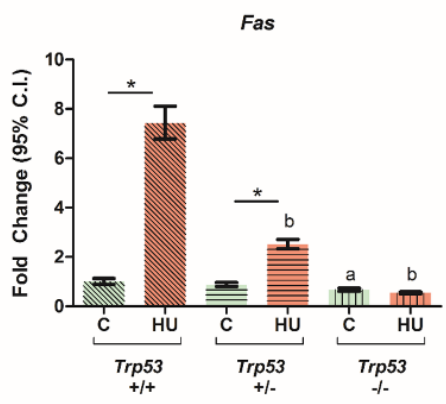
a.

Common to P53 & P63 & P73	Common to P53 & P63	Common to P53 & P73
Cdkn1a, Fas	*Pmaip1*	*Rb1*

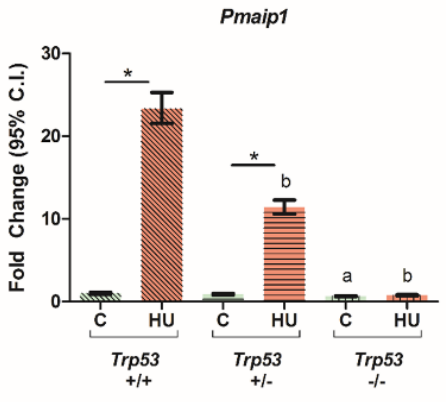
b.



c.



d.



e.

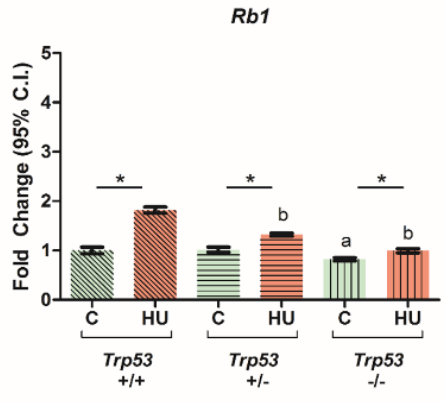


Figure 3.4: Effects of hydroxyurea exposure in embryos on the relative expression of transcripts that control cell cycle progression and apoptosis and are regulated by P53 family proteins.

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. $p<0.05$, 2-way ANOVA followed by a Bonferroni post-hoc test, n=3. (*) denotes significant change compared to control group with the same genotype, (a) denotes significant change compared to the $Trp53^{+/+}$ control group, (b) denotes significant change compared to the $Trp53^{+/+}$ hydroxyurea-treated group.

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 hydroxyurea-treated embryos as a marker of apoptosis. Cleaved Caspase-3 was not detected in control embryos from any genotype (Fig. 3.5A). A significant increase in cleaved Caspase-3 was observed in hydroxyurea-treated *Trp53*$^{+/+}$ embryos and *Trp53*$^{+/-}$ embryos (Fig. 3.5B). However, no appreciable cleaved Caspase-3 was observed in hydroxyurea-treated *Trp53*$^{-/-}$ embryos (Fig. 3.5A), indicating that hydroxyurea-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 hydroxyurea-treated *Trp53*$^{+/+}$ embryos; the levels of cleaved MST-1 decreased in a dose-dependent manner relative to *Trp53* concentration (Fig. 3.5C-D), reflecting the trend observed with cleaved Caspase-3. In contrast, all hydroxyurea-treated embryos, including the *Trp53*$^{+/-}$ and *Trp53*$^{-/-}$ embryos, exhibited strong upregulation of γH2AX levels compared to controls (Fig. 3.5E-F). However, after hydroxyurea treatment, γH2AX levels were significantly lower in *Trp53*$^{-/-}$ embryos compared to their *Trp53*$^{+/+}$ littermates (Fig. 3.5F).

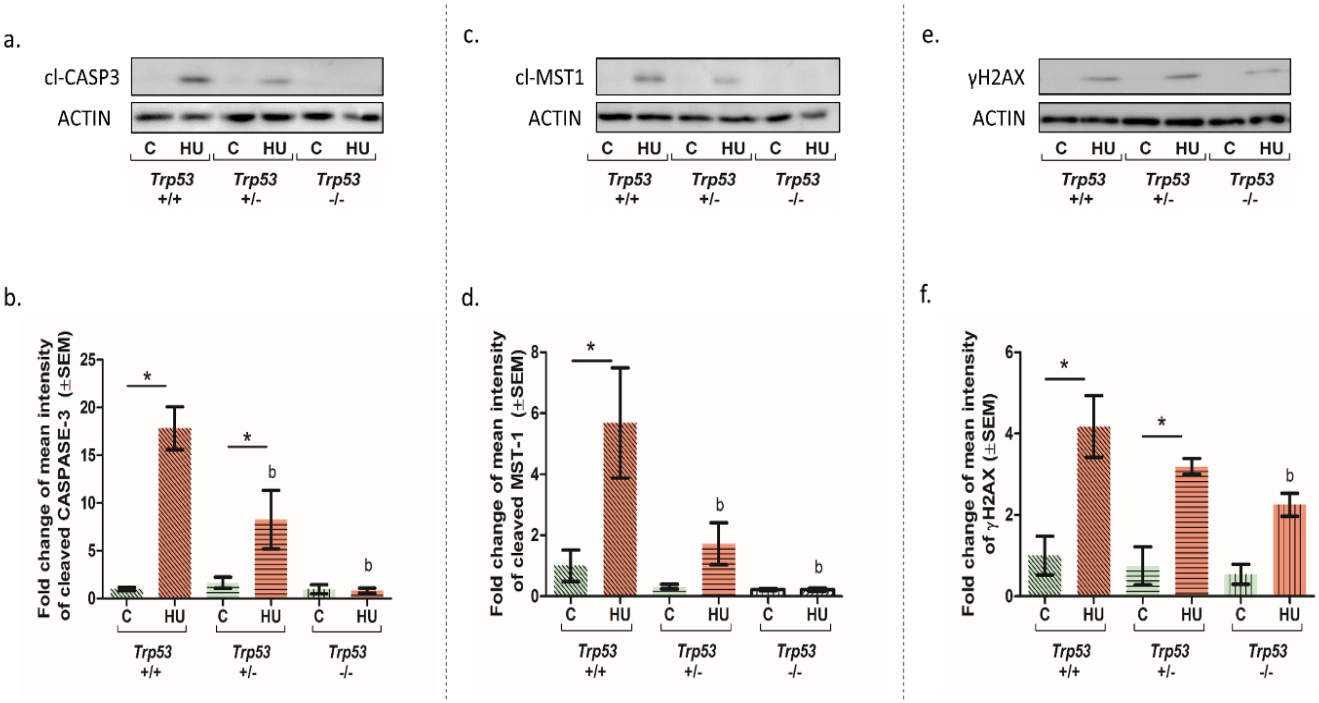

a.
cl-CASP3
ACTIN
C HU C HU C HU
Trp53 +/+
Trp53 +/-
Trp53 -/-
b.
Fold change of mean intensity of cleaved CASPASE-3 (±SEM)
c.
cl-MST1
ACTIN
d.
Fold change of mean intensity of cleaved MST-1 (±SEM)
e.
γH2AX
ACTIN
f.
Fold change of mean intensity of γH2AX (±SEM)

Figure 3.5: Effects of hydroxyurea exposure on the cleavage of Caspase-3 and MST-1 and H2AX phosphorylation in embryos.

Representative Western blots and quantification of cleaved Caspase-3 (***A-B***), cleaved MST-1 (***C-D***) and γH2AX (***E-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. $p<0.05$, 2-way ANOVA followed by a Bonferroni post-hoc test, n=3. (*) denotes significant change compared to control group with the same genotype, (b) denotes significant change compared to the *Trp53*$^{+/+}$ hydroxyurea-treated group.

DISCUSSION

The exposure of organogenesis-stage embryos to a DNA damaging agent, hydroxyurea, 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. While *Trp63* levels decrease with hydroxyurea 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 *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.*, 2006b; 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 two proteins during organogenesis differs from that of P53 since P63 and P73 are essential for the maintenance of normal embryo development (Yang*, et al.*, 1999; Yang*, et al.*, 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; Levrero*, et al.*, 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 hydroxyurea 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 *Jag2* suggests that they may be partially involved in mediating the embryotoxicity of hydroxyurea; *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 hydroxyurea-exposed embryos and the concomitant decrease in *Pax9* and *Tbx5* transcript levels suggest that other upstream regulators (i.e. not P63 or P73) may be involved in the transcriptional suppression of these genes. Indeed, *Pax9* transcription may be regulated by the sonic hedgehog (SHH) 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 hydroxyurea. 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 three P53 family members. This induction was ablated in the absence of *Trp53,* indicating that P53 is the main transcriptional activator in their response to hydroxyurea 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 hydroxyurea in the organogenesis-stage embryo. Indeed, P53 responds to hydroxyurea-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 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 hydroxyurea 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 pre-activated 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 hydroxyurea-exposed embryos; however, γH2AX accumulation was significantly lower in *Trp53*$^{-/-}$ embryos treated with hydroxyurea 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, ATM and ATR, 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).

While P63 and P73 are essential for crucial developmental processes under normal conditions (Yang*, et al.*, 1999; Yang*, et al.*, 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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CONNECTING TEXT

In chapter III, we observed that P53 is the primary member of the P53 family of transcription factors that is activated in the embryonic stress response to hydroxyurea exposure. While P63 and P73 are detected under normal and stress conditions in the embryo, their levels of expression and activation are unaffected by hydroxyurea; only P53 is capable of inducing the common downstream targets that are involved in cell cycle arrest and cell death in hydroxyurea-exposed embryos. In chapter IV, we use *Trp53* null fetuses to inquire whether the activation of P53 acts to suppress or induce the embryotoxic effects of hydroxyurea.

CHAPTER IV

P53 Suppresses Hydroxyurea Embryotoxicity

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ABSTRACT

Hydroxyurea is a potent teratogen in mice, causing severe limb and skeletal defects. Treatment of timed pregnant mice on gestational day (GD) 9 with hydroxyurea elicits an early embryonic stress response that involves activation of the P53 transcription factor. Whether P53 activation is a mechanism of suppression or enhancement of hydroxyurea embryotoxicity is unknown. To address this question we treated *Trp53*$^{+/-}$ timed pregnant mice with saline or hydroxyurea (200 or 400 mg/kg) on GD 9; fetuses were examined for viability and external malformations on GD 18. Treatment with saline resulted in a low incidence of resorptions and malformations in *Trp53*$^{+/-}$ and *Trp53*$^{-/-}$ fetuses. After treatment with 200 mg/kg hydroxyurea, the incidence of resorptions was elevated for *Trp53*$^{+/-}$ fetuses; malformations were observed among both the *Trp53*$^{+/-}$ and *Trp53*$^{-/-}$ fetuses. Treatment with 400 mg/kg hydroxyurea elicited an increased incidence of resorptions among fetuses from all three genotypes; none of the *Trp53*-deficient fetuses survived. Moreover, at this dose of hydroxyurea exencephaly and limb and tail defects were observed in a high proportion of the surviving *Trp53*$^{+/+}$ and *Trp53*$^{+/-}$ fetuses. These data demonstrate that fetal P53 genotype is an important determinant of the effects of hydroxyurea during organogenesis; P53 acts to suppress the embryotoxicity of hydroxyurea.

INTRODUCTION

P53 is a tumor suppressor protein that acts as a transcription factor in response to various stress stimuli, including genotoxic and oxidative stress (Kruse and Gu, 2009). Under normal conditions P53 concentrations are tightly controlled; in response to stress P53 is post-translationally modified and translocated to the nucleus where it upregulates the transcription of several downstream targets involved in apoptosis and cell cycle arrest (El Husseini, *et al.*, 2016; Granetto *et al.*, 1996). P53 deletions (e.g. in *Trp53*-deficient mice) and gene mutations (in mice and humans) are associated with an increase in the incidence of tumors (Donehower, *et al.*, 1992; Hollstein *et al.*, 1991).

In the murine embryo, *Trp53* expression is maintained at low concentrations throughout development, but increasing levels are detected during the critical stage of organogenesis, as early as gestational day (GD) 8 and up to GD 12 (Chen, *et al.*, 2015; Schmid, *et al.*, 1991). P53 is important for proper embryonic development; a subset of *Trp53*-null embryos display birth defects, such as exencephaly and limb malformations (Armstrong, *et al.*, 1995; Donehower, *et al.*, 1992; Sah, *et al.*, 1995). In response to genotoxic stress, P53 activity increases dramatically in several regions of the organogenesis-stage embryo (Gottlieb, *et al.*, 1997; Komarova, *et al.*, 1997). Exposure to teratogens that induce genotoxic stress leads to significant upregulation of P53 protein levels and of the transcript levels of many of the P53 downstream targets that are involved in apoptosis (Gottlieb, *et al.*, 1997; Mikheeva, *et al.*, 2004; Moallem and Hales, 1998; Norimura, *et al.*, 1996). Embryos lacking P53 show higher rates of malformations when exposed to various genotoxic teratogens (Moallem and Hales, 1998; Nicol, *et al.*, 1995; Norimura, *et al.*, 1996). However, other teratogens, such as 2-chloro-2-deoxyadenosine, show a reduced incidence of specific malformations in the absence of P53 (Wubah, *et al.*, 1996). Thus, the role of P53 as a suppressor or inducer of embryotoxicity is uncertain.

Hydroxyurea is an anti-neoplastic agent that is used to treat chronic myeloid leukemia and sickle cell anemia (Bristol-Myers-Squib, 2016; Davies and Gilmore, 2003). It is a genotoxicant that inhibits ribonucleotide reductase, resulting in the depletion of deoxyribonucleotides and the stalling of the DNA replication fork (Fishbein and Carbone, 1963). It induces cell cycle arrest at the G1/S phase and leads to cell death by apoptosis in proliferating cells (Heo, *et al.*, 2014; Kovacic, 2011; Philips, *et al.*, 1967). Since organogenesis is dependent on rapidly proliferating cells, embryos at this stage of development are highly susceptible to the

effects of hydroxyurea (Aliverti*, et al.*, 1980; DeSesso*, et al.*, 2000; DeSesso and Jordan, 1977). The treatment of timed pregnant CD-1 mice with hydroxyurea on GD 9 produces dose-dependent increases in the incidence of resorptions and fetuses with severe limb (e.g. syndactyly, oligodactyly), tail (e.g. hypoplastic or curly tails) and skeletal defects (e.g. amelia, short ribs) (Schlisser and Hales, 2013; Yan and Hales, 2005). Exposure to hydroxyurea also elicits an early stress response in the embryo that involves the upregulation of P53 and its downstream signaling pathways (El Husseini*, et al.*, 2016). The activation of P53 promotes caspase-mediated apoptosis via the cleavage and activation of Caspase-3 (Chapter III). Whether the activity of P53 in the early embryonic stress response influences the outcome of hydroxyurea embryotoxicity remains to be determined. Here we have tested the hypothesis that P53 acts as a suppressor of hydroxyurea embryotoxicity.

MATERIALS AND METHODS

Experimental Animals

Trp53 transgenic mice (B6.129S2-Trp53tm1Tyj/J) were purchased from The Jackson Laboratory (Sacramento, CA, USA) and housed in the McIntyre Animal Resource Centre (Montreal, QC, Canada) to maintain and expand colonies. *Trp53* heterozygote females were mated with *Trp53* heterozygote males so that only the fetal genotypes differ within each litter. One breeding pair per cage was housed overnight and the females were separated from the males at 10:00 AM the following day (designated as GD 0). Animal treatments were conducted in accordance with the guidelines outlined in the Canadian Guide to the Care and Use of Experimental Animals. Between 8:00 and 10:00 AM on GD 9 pregnant dams were treated with either saline (SAL, n= 5), 200 mg/kg (HU200, n= 4) or 400 mg/kg (HU400, n=4) hydroxyurea (Aldrich Chemical Co., Madison, WI) by intraperitoneal injection.

Examination of Pregnancy Outcome and External Malformations

On GD 18, all dams were euthanized by CO_2 asphyxiation and cervical dislocation. The uteri were removed and examined for implantation sites and resorptions. Fetuses that did not respond to prodding stimuli were considered dead. All fetuses were numbered according to their site of implantation in ascending order, from left to right. Live fetuses were weighed after explantation and then euthanized by hypothermia. Crown-rump lengths (CRL) were determined using a digital caliper. External malformations were then recorded, including the type, incidence and severity. Internal tissues, as well as tissues from resorption sites, were collected from each transgenic fetus for genotyping.

DNA Extraction

The DNA of *Trp53* adult and fetal transgenic mice was extracted from tail tips or internal tissues 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, MO, USA). Samples were then centrifuged at 20,000g for 10 min to remove fur and debris, followed by two additions of 100% ethanol to the supernatant. Lastly, the sample was centrifuged to precipitate the DNA, the ethanol was discarded and the pellet left to air dry. DNA

was then dissolved in distilled water and the concentration was determined by a NanoDrop1000 spectrophotometer (Fisher Scientific, Wilmington, DE, USA).

Genotyping by High Resolution Melt-Curve Analysis

To determine the zygosity of adult and fetal samples, the Power SYBR Green RNA-to-C_T 1-Step Kit (Applied Biosystems, Foster City, CA) and the StepOnePlus Real-Time PCR System (Applied Biosystems) were used to carry out high resolution melt-curve analysis (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 mM 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 as 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 (~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: CAGCCTCTGTTCCACATACACT; common reverse: TGGATGGTGGTATACTCAGAGC) and were synthesized by Alpha DNA (Montreal, QC, Canada).

Statistical Analyses

All data were statistically analyzed using the GraphPad Prism Software (version 5, Graph Pad Software Inc., La Jolla, CA.). Implantation sites, resorptions and malformations were tabulated. Total percentages were calculated based on the total number of implantation sites or fetuses examined, of the same genotype, within each treatment group, regardless of the litter source. Data were tested by the Fisher's Exact test or 2-way ANOVA followed by a Bonferroni *post-hoc* multiple comparison correction, as appropriate. The level of significance for all statistical tests was set to $p < 0.05$.

RESULTS

Trp53$^{-/-}$ fetuses are more susceptible to hydroxyurea-induced embryolethality and malformations than their *Trp53*$^{+/+}$ littermates

The numbers of implantation sites, resorptions and malformed fetuses that were observed when GD 9 heterozygote (*Trp53*$^{+/-}$) timed pregnancy dams were treated with saline or hydroxyurea are shown in Table 4.1. The overall incidence of resorptions among the saline-treated litters was similar to that found in wild type (C57BL/6J) saline-treated animals (data not shown) and can be attributed to the background resorption rate that is inherent to this inbred mouse strain. A trend towards an elevated resorption rate after treatment with 400 mg/kg of hydroxyurea was observed, with the highest rates of resorptions in the *Trp53*$^{+/-}$ and *Trp53*$^{-/-}$ fetuses (Table 4.1).

Several malformations were observed in the saline-treated litters among the *Trp53*$^{+/-}$ and *Trp53*$^{-/-}$ fetuses. These included exencephaly, digit defects and tail defects (Table 4.2). After treatment with 200 mg/kg of hydroxyurea the number of malformed fetuses increased and their genotype was found to be either *Trp53*$^{+/-}$ or *Trp53*$^{-/-}$ (Table 4.1). After treatment with 400 mg/kg hydroxyurea all of the *Trp53*$^{-/-}$ fetuses were resorbed and a trend towards an elevated incidence of malformations was observed among the *Trp53*$^{+/+}$ and *Trp53*$^{+/-}$ fetuses (Table 4.1).

Interestingly, the deletion of *Trp53* did not affect fetal growth, as assessed by fetal weights and crown-rump lengths (CRLs) (Fig. 4.1). In addition, hydroxyurea treatment had no significant effect on fetal weights or CRLs.

Table 4.1: The fetal *Trp53* zygosity of implantation sites, resorptions and malformed fetuses in transgenic litters treated with saline or hydroxyurea.

Treatment	SAL			HU200			HU400		
Dams (n)	5			4			4		
Conceptal Genotype	*Trp53*$^{+/+}$	*Trp53*$^{+/-}$	*Trp53*$^{-/-}$	*Trp53*$^{+/+}$	*Trp53*$^{+/-}$	*Trp53*$^{-/-}$	*Trp53*$^{+/+}$	*Trp53*$^{+/-}$	*Trp53*$^{-/-}$
Implantation Sites (n)	8	27	12	4	17	8	6	16	4
Resorptions (n)	0	2	1	0	3	0	2	6	4
% Resorptions	0	7	8	0	18	0	33	38	100
Viable Fetuses (n)	8	25	11	4	14	8	4	10	0
Malformed Fetuses (n)	0	1	3	0	2	6	4	6	NA
% Malformed Fetuses	0	4	27	0	14	75	100	60	NA

Trp53-deficient fetuses tended to exhibit a greater percentage of resorptions (in HU400) and malformations (in SAL and HU200 groups) than their *Trp53*$^{+/+}$ littermates.

Percentages are represented as the resorbed or malformed fetuses out of the total implantation sites and fetuses examined, respectively, per genotype within each treatment group.

SAL = saline, HU = hydroxyurea, NA = not applicable.

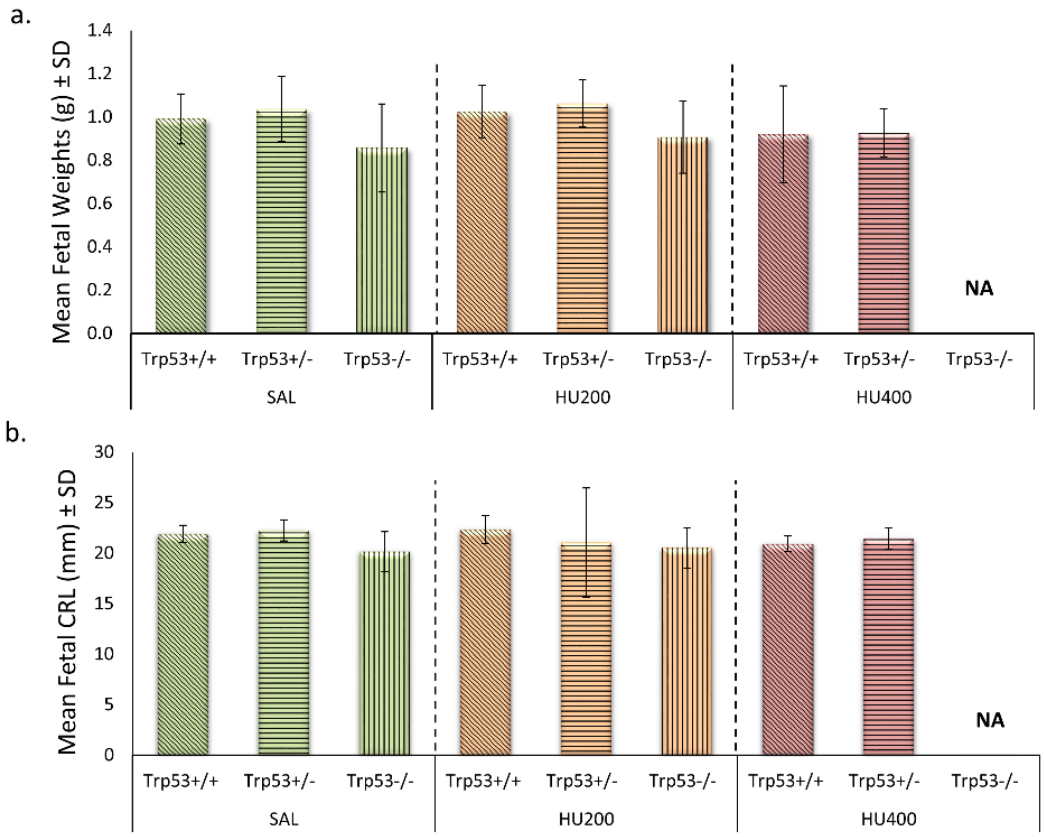
a.
Mean Fetal Weights (g) ± SD
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NA
Trp53+/+
Trp53+/-
Trp53-/-
SAL
HU200
HU400
b.
Mean Fetal CRL (mm) ± SD
30
25
20
15
10
5
0
NA

Figure 4.1: Hydroxyurea did not affect fetal weights or CRLs in transgenic litters.
A: Fetal weights in transgenic fetuses did not differ between the three possible *Trp53* genotypes in any treatment group. Each bar represents the mean weight in grams of the fetuses examined in each treatment group. ***B***: Fetal CRLs in transgenic fetuses did not differ between the three possible *Trp53* genotypes in any treatment group. Each bar represents the mean weight in grams of the fetuses examined in each treatment group. SAL= saline, HU= hydroxyurea, NA= not applicable.

The majority of malformations induced by hydroxyurea are in the caudal region of the fetus

Several *Trp53*$^{-/-}$ fetuses among the litters that were exposed to saline (i.e. control) exhibited exencephaly and tail defects at levels that tended to be greater than what was observed in their *Trp53*$^{+/+}$ littermates (Table 4.2). The range of malformations observed among fetuses exposed to hydroxyurea included craniofacial anomalies (e.g. exencephaly), abdominal defects (e.g. gastroschisis), and a multitude of limb and tail abnormalities (Table 4.2). The *Trp53*$^{-/-}$ fetuses exposed to 200 mg/kg hydroxyurea showed a trend towards a higher incidence of syndactyly and hypoplastic tails compared to their *Trp53*$^{+/+}$ littermates (Table 4.2).

Table 4.2: Types and incidence of malformations in transgenic litters by *Trp53* zygosity, in saline or hydroxyurea treatment groups.

		SAL			HU200			HU400		
		Trp53$^{+/+}$	*Trp53*$^{+/-}$	*Trp53*$^{-/-}$	*Trp53*$^{+/+}$	*Trp53*$^{+/-}$	*Trp53*$^{-/-}$	*Trp53*$^{+/+}$	*Trp53*$^{+/-}$	*Trp53*$^{-/-}$
Craniofacial	Exencephaly	-	-	2 (18.2)	-	-	-	-	-	NA
	Cleft Lip	-	-	-	-	-	1 (12.5)	-	-	NA
Abdominal	Gastroschisis	-	-	-	-	-	2 (25.0)	-	-	NA
	Edema	-	-	-	-	-	-	1 (25.0)	3 (30.0)	NA
Forelimb	Syndactyly	-	-	1 (8.3)	-	-	-	-	1 (10.0)	NA
	Oligodactyly =1	-	-	-	-	-	-	-	1 (10.0)	NA
Hindlimb	Syndactyly	-	1 (4.0)	-	-	2 (14.3)	2 (25.0)	2 (50.0)	1 (10.0)	NA
	Oligodactyly =1	-	-	-	-	-	-	3 (75.0)	-	NA
Tail	Curly/ Hypoplastic	-	1 (4.0)	3 (27.3)	-	-	3 (37.5)	1 (25.0)	4 (40.0)	NA

Percentages (in parentheses) are represented as the malformed fetuses out of the fetuses examined per genotype within each treatment group; a single fetus may be represented more than once in listing individual defects. SAL = saline, HU = hydroxyurea, NA = not applicable. Oligodactyly =1 indicates missing one digit on any limb; oligodactyly >1 indicates missing more than one digit on any limb.

DISCUSSION

The overall impact of hydroxyurea exposure during organogenesis was enhanced in P53 heterozygote and null fetuses. Specifically, a trend towards higher rates of resorptions and malformations were observed in *Trp53*$^{-/-}$ fetuses compared to their *Trp53*$^{+/+}$ littermates. This suggests that P53 plays a role in limiting the embryotoxicity of hydroxyurea. The P53 protein has been implicated in the ability of embryos to cope with a teratogenic exposure in several studies (Torchinsky and Toder, 2010). Ionizing radiation, cyclophosphamide, benzo[a]pyrene and phenytoin all increased the incidence of resorptions and malformations in fetuses lacking *Trp53* compared to their *Trp53*$^{+/+}$ counterparts (Kato, *et al.*, 2001; Moallem and Hales, 1998; Nicol, *et al.*, 1995; Norimura, *et al.*, 1996; Wells, *et al.*, 2009). All these agents are well-characterized teratogens that share a common mechanistic outcome: the induction of apoptosis as a result of oxidative stress and DNA damage (Banh and Hales, 2013; Chaube and Murphy, 1966; DeSesso, *et al.*, 2000; Schlisser and Hales, 2013; Torchinsky and Toder, 2010).

We observed a trend towards a higher incidence of malformations in saline-treated *Trp53*$^{-/-}$ fetuses than their *Trp53*$^{+/+}$ counterparts. These malformations included tail defects and exencephaly, which are reminiscent of observations from earlier studies of *Trp53*$^{-/-}$ embryos (Sah, *et al.*, 1995). During organogenesis, P53 activity is tightly controlled and is mainly restricted to the limb buds, where it helps "sculpt" the digits of the hind- and fore-limbs, and the neural tube, where it regulates the local cell turnover that is essential for proper neural tube closure (Choi and Donehower, 1999; Mori, *et al.*, 1995). These processes are driven by P53-regulated cell cycle progression and apoptosis (Donehower, *et al.*, 1992; Mendrysa *et al.*, 2011). Thus, an imbalance in P53-mediated regulation of cell cycle progression and apoptosis would disrupt limb development, leading to merged or absent digits, and prevent neural tubes from closing properly, resulting in the defects that are observed.

P53-mediated apoptosis increases in several tissues after exposure to a DNA damaging agent, resulting in the selective disposal of irreparable damaged cells to enable preservation of the integrity of the surrounding tissue and allow proper organ development (Appella and Anderson, 2001; Jacobson *et al.*, 1997; Zhang, *et al.*, 2017). Interestingly, hydroxyurea treatment of GD 9 *Trp53*$^{-/-}$ embryos failed to activate Caspase-3 and to induce the expression of pro-apoptotic factors (*Fas, Trp53inp1, Pmaip1*) (Chapter III). Previous studies reported that *Trp53*$^{+/+}$ fetuses exposed to ionizing radiation displayed lower rates of malformation than

Trp53$^{-/-}$ fetuses, yet showed an increase in apoptotic cells that was not observed in *Trp53*$^{-/-}$ fetuses (Kato*, et al.*, 2001; Norimura*, et al.*, 1996). Other studies have shown that a decrease in apoptosis may lead to unregulated forms of cell death, such as necrosis (Kerr *et al.*, 1972; Kuida *et al.*, 1996). Indeed, *Trp53*-deficient murine limb buds exposed to teratogenic doses of 4-hydroperoxycyclophosphamide displayed higher rates of malformation, compared to wild type, and underwent necrosis, as shown by the rupture of cell membranes and lack of DNA fragmentation (Moallem and Hales, 1998). As such, it is possible that the absence of P53 renders the embryo more susceptible to hydroxyurea embryotoxicity due to its inability to dispose of damaged cells via apoptosis, causing widespread irrevocable tissue damage, resulting in an elevated incidence of malformations and resorptions.

The ability of P53 to limit hydroxyurea embryotoxicity may be dose-dependent. In transgenic litters; 200 mg/kg hydroxyurea resulted in a higher percentage of malformed *Trp53*$^{-/-}$ fetuses than *Trp53*$^{+/+}$, however at 400 mg/kg the rate of malformations in *Trp53*$^{+/+}$ fetuses increased and was almost equal to that of *Trp53*$^{+/-}$ fetuses. Earlier reports showed that when *Trp53*$^{+/+}$ fetuses were exposed to a high rate of ionizing radiation (1.06 Gy/min) a 70% increase in the incidence of malformations occurred, while the incidence of malformations was not higher than the control at 1.2 mGy/min (Kato*, et al.*, 2001). The ability of the embryo to counter damaging effects appears to rely on a native characteristic of P53 function during embryonic development; Zhang et al. demonstrated that mildly-stressed embryonic cells are marked for elimination by P53 and are "out-competed" by healthier surrounding cells as an adaptive response that aims to protect organs from maldevelopment (Zhang*, et al.*, 2017). On the other hand, higher genotoxic stress levels may result in P53 activity reaching a "tipping point", going from an adaptive response to an adverse response, where P53-mediated apoptosis surpasses the adaptive threshold and results in irrevocable tissue death, failure to replace this tissue leads to malformed organs (Currier *et al.*, 2016; Vousden and Lane, 2007). Thus, P53 can suppress hydroxyurea-induced birth defects up to a certain "threshold".

While most studies indicate that P53 acts as a suppressor of the embryotoxicity of various teratogens, a few studies indicate the opposite. Wubah et al. demonstrated that *Trp53*$^{-/-}$ fetuses treated with the nucleoside analog 2-chloro-2-deoxyadenosine on GD 8 had lower incidences of eye defects (i.e. microphthalmia) than their wild type counterparts (Wubah*, et al.*, 1996). This effect appears to rely on the ability of P53 to induce apoptosis; after treatment with 2-chloro-2-

deoxyadenosine *Trp53*$^{+/+}$ fetuses displayed more rampant apoptosis in the eye compared to *Trp53*$^{-/-}$ fetuses (Wubah *et al.*, 2001). In addition, a correlation between increasing 2-chloro-2-deoxyadenosine dose and P53 induction was observed in wild type CD-1 (i.e. outbred) mice, showing that P53 responds to 2-chloro-2-deoxyadenosine -induced damage (Wubah*, et al.*, 2001). A possible explanation for the discrepancy between studies that indicate P53 is acting as a suppressor of embryotoxicity and others that indicate that it induces embryotoxicity, is the degree of stress induced by the different teratogens that were examined. It is likely that the DNA lesions caused by 2-chloro-2-deoxyadenosine initiate a more acute and restricted response in the embryo than do hydroxyurea, benzo[a]pyrene, cyclophosphamide or phenytoin, all of which may cause widespread oxidative and genotoxic stress, (Torchinsky and Toder, 2010; Wells*, et al.*, 2009). Furthermore, the ocular lens seems to be highly sensitive to P53-dependent apoptosis, increasing the chance of malformations due to irrevocable tissue death compared to other organs after exposure to P53-activating teratogens (Nakamura *et al.*, 1995; Pan and Griep, 1995). Thus, depending on the sensitivity of the organ, and the mechanism of action and/or the level of stress that the teratogens induce, P53 activity may produce different outcomes (Kato*, et al.*, 2001; Wubah*, et al.*, 1996).

While more litters and sample numbers are required to reach a definitive conclusion, we herein demonstrated a trend towards increasing susceptibility to malformations at 200 mg/kg hydroxyurea, and to resorptions at 400 mg/kg, in *Trp53*-deficient fetuses compared to *Trp53*$^{+/+}$ littermates. At the same time, we showed that increasing genotoxic stress increases the rate of malformations in *Trp53*$^{+/+}$ embryos. Thus, our findings suggest a paradigm in which P53 is capable of acting as a suppressor of embryotoxicity up to a certain threshold.

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CHAPTER V

Discussion

5.1 Summary

The purpose of the research in this thesis is to better understand the embryonic response to stress during the sensitive developmental stage of organogenesis. In response to hydroxyurea, several signaling pathways are activated in the murine embryo on GD 9 that are involved in cell cycle regulation and apoptosis, including the P53 signaling pathway. We found that the P53 protein is robustly responsive to teratogenic doses of hydroxyurea, leading to the upregulation of transcripts involved in cell cycle arrest, DNA damage repair and apoptosis. The other P53 homologs, P63 and P73, do not respond in a similar fashion and fail to regulate factors involved in cell cycle progression and cell death in the absence of P53. Lastly, we show that P53 acts to suppress hydroxyurea's embryotoxicity since embryos lacking the *Trp53* gene are noticeably more susceptible to resorptions and malformations compared to their $Trp53^{+/+}$ littermates. These findings indicate a crucial role for P53 in the embryonic stress response to teratogenic insult, and the manifestation of congenital malformations associated with hydroxyurea exposure in murine embryos.

5.2 The early embryonic response to oxidative and genotoxic stress

The organogenesis stage embryo is susceptible to various forms of extrinsic stress that can have lasting damaging effects that manifest as congenital malformations (Wells*, et al.*, 2010). The sensitivity of the embryo to various forms of insult at this stage stems from its immature and limited capacity to adapt to and repair the damage caused by such stresses, including oxidative and genotoxic stress.

We found that hydroxyurea induces the expression of several transcripts involved in DNA damage repair, cell cycle arrest and apoptosis in embryos within 3 hours of treatment. Interestingly, transcripts coding for antioxidant elements, such as glutathione regulating enzymes, were not affected at the transcriptional level, although in earlier studies the GSH/GSSG ratio was disrupted by hydroxyurea exposure, leading to a significant decrease in GSH levels compared to GSSG (Schlisser and Hales, 2013). In addition, hydroxyurea exposure increased the levels of 4-HNE protein adducts in a dose-dependent fashion (Schlisser*, et al.*, 2010). A possible explanation for the lack of a strong antioxidant response is the immaturity of the embryonic antioxidant system in early organogenesis (Choe*, et al.*, 2001; Hansen, 2006). During this period, the embryo experiences an increased oxidative physiological environment as

a result of switching from anaerobic to aerobic respiration, which makes its redox balance very delicate and susceptible to extrinsic oxidizing agents. Thus, it may be that once exposed to hydroxyurea the embryo is incapable of efficiently adapting to the disruption in its redox balance, such as by increasing the transcription of glutathione regulating factors that can restore the balance in the long term. Instead, the affected cells have a more immediate, short-term response at the protein level which includes activating P53, increasing P53INP1 concentrations and promoting the transcription of cell cycle arrest and pro-apoptotic factors as a means to remove irreparable cells and thus limit the damage to the conceptus.

Hydroxyurea exposure also increased expression of transcripts involved in DNA damage repair, such as *Zfp365* and *Atm*, however many other DNA repair factors were not significantly affected (i.e. *Xrcc1, Lig1, Apex, Ung*). We observed increased γH2AX concentrations in *Trp53*$^{+/+}$ embryos (Chapter III), and it was previously shown that hydroxyurea exposure increased γH2AX foci mainly in the caudal region of the embryo, where most malformations were manifested (Banh and Hales, 2013). Hydroxyurea is known to be a potent genotoxicant, leading to DNA replication fork stalling and causing DNA strand breaks (Kovacic, 2011). Several teratogens are potent genotoxicants, such as UV light, benzo[a]pyrene and cyclophosphamide (Moallem and Hales, 1998; Nicol*, et al.*, 1995; Norimura*, et al.*, 1996). The capability of the embryo to repair DNA damage due to strand breaks and oxidative damage is quite limited during early organogenesis; many – but not all – of the DNA repair factors are in low abundance and fail to overcome the damage induced by extrinsic genotoxic agents (Vinson and Hales, 2002a).

Thus, the weak antioxidant defenses and immature DNA repair mechanisms of the embryo render it susceptible to insult from hydroxyurea, leading to DNA strand breaks, the disruption of protein function and the activation of stress response pathways (Wells*, et al.*, 2009). With teratogenic doses of hydroxyurea, the resulting stress response "tips the balance" from an adaptive to a more adverse effect leading to the manifestation of malformations and embryolethality (Brill*, et al.*, 1999; Hood, 2016).

5.3 Expression of skeletal and limb developmental genes in the embryonic response to genotoxic stress

Interestingly, CD-1 embryos exposed to hydroxyurea showed changes in the expression of several genes involved in skeletal and limb development (Chapter II). The analysis of microarray data using Ingenuity Pathway Analysis™ (IPA) revealed that the expression of several transcripts related to skeletal and limb development was significantly affected by at least 1.5 fold compared to control. One of these transcripts is *Jag2*, which is involved in the Notch signaling pathway and is important for the formation of the limb apical ectodermal ridge (AER). It also ensures proper digit formation by controlling the apoptosis of the interdigital space; knocking out *Jag2* leads to a disrupted AER and the fusion of digits in mouse embryos (Jiang*, et al.*, 1998; Pan *et al.*, 2005; Xu*, et al.*, 2010). Interestingly, our studies showed that *Jag2* expression in response to hydroxyurea is influenced by P53 (Chapter III). Previous reports have linked other P53 family proteins, particularly P63 and P73, to *Jag2* regulation (Fontemaggi*, et al.*, 2002; Sasaki*, et al.*, 2002; Wu*, et al.*, 2003). While P53 is not generally considered a direct regulator of *Jag2* transcription, it is possible that the effects we observe are mediated via the effects of P53 on P73 regulation; using qRT-PCR we showed that *Trp73* expression is influenced by P53 (Chapter III), which is capable of binding to and activating the promoter of *Trp73* (Wang*, et al.*, 2007).

Pax9 and *Tbx5,* both important transcription factors necessary for proper embryonic development*,* are two other transcripts that are downregulated by hydroxyurea (Chapter III). *Pax9* is an important factor in bone and tooth formation and responds to sonic hedgehog (SHH) signaling (McGlinn*, et al.*, 2005). It is expressed during organogenesis in the distal anterior mesenchyme compartment of limb buds, as well as the maxillary and mandibular arches that give rise to the palate and teeth (LeClair*, et al.*, 1999; Peters*, et al.*, 1998). *Tbx5* is an early marker for limb bud fields, promoting the initiation of the fore-limb bud in particular, which fails to form in *Tbx5* knockout mouse embryos (Agarwal*, et al.*, 2003). Disrupting the levels of such important developmental regulators could partially contribute to the teratogenic effects of hydroxyurea, which mainly manifest as skeletal and limb deformities.

5.4 The P53 signaling pathway in the early embryonic stress response

The mechanisms by which the embryo responds to oxidative and genotoxic stress are complex and involve multiple factors and signaling pathways. Even at the time of early organogenesis, several stress-sensitive signaling pathways are present and responsive. Here, we showed that exposure to hydroxyurea induces several significant changes at the transcriptional level in the organogenesis stage embryo merely 3 hours after treatment (Chapter II). Most of these transcripts cluster within signaling pathways that are involved in cell cycle regulation, apoptosis and DNA damage repair. The most prominent canonical pathway predicted by IPA™ to be activated is the P53 signaling pathway. Indeed, exposure to hydroxyurea increased P53 protein and phosphorylation levels in several tissues in the embryo, including the heart and the rostral and caudal neuroepithelium. This induction coincided with an increase in P53 transcriptional activity, as measured by elevated levels of *Cdkn1a*, *Trp53inp1* and *Fas*, which are all direct P53 downstream targets that are involved in cell cycle regulation and apoptosis.

The P53 protein is itself a downstream effector of several stress-response pathways, including the MAPK pathway, the DNA damage response pathway, and the oxidative stress response pathway. P53 is highly dependent on phosphorylation by several upstream kinases to promote its stability and dissociation from its negative regulator MDM2 (Kruse and Gu, 2009). These upstream regulators include DNA damage response kinases, such as Chk1/2 and ATR, which are activated in response to hydroxyurea (Ward and Chen, 2001). In addition, previous studies have shown that organogenesis-stage embryos exposed to hydroxyurea have an activated MAPK pathway, which involves the upregulation and activation of the P53-phosphorylating enzymes, P38 and JNK (Banh and Hales, 2013; Yan and Hales, 2008). Similarly, hydroxyurea activates the AP-1 transcription factor, which is a known redox-sensitive factor that induces antioxidant mechanisms and regulates P53 activity (Shaulian and Karin, 2001; Yan and Hales, 2005). In addition, P53 itself contains several redox-sensitive cysteine residues that alter its conformation and promote its transactivation and DNA binding capability once the redox status of the cell is disrupted (Hafsi and Hainaut, 2011). Thus, P53 is at the convergence of several upstream pathways that are activated in response to hydroxyurea, making it a signaling hub that directs the fate of cells – and consequently of embryos – in response to damaging stress. This makes P53 and its signaling pathway of great importance for the survival of the embryo after exposure to potentially damaging xenobiotics and hence a necessary subject of study.

Indeed, data gleaned from our studies and those of others can be integrated in predictive toxicological frameworks, known as adverse outcome pathways (AOPs). AOPs allow for a more comprehensive understanding of the molecular events that are initiated by environmental or pharmaceutical agents that may have toxic and teratogenic events (Vinken, 2013). They can serve as a supplemental tool that helps us to predict the potential toxicity of novel synthetic compounds, or untested chemicals, on the cellular, organismal and population levels. Efforts are currently underway to use data extracted from the ToxCast high-throughput screening and testing platforms to model the potential effects of environmental and pharmaceutical compounds on embryonic viability and development (Sipes *et al.*, 2011). This form of predictive systems toxicology relies on understanding the P53 signaling pathway during embryogenesis; a recent study examining the toxic effects of 774 chemicals, using *in silico* modeling coupled with ToxCast data, showed the P53 signaling pathway was affected in a subset of chemicals involved in male reproductive toxicity (Leung *et al.*, 2016). A similar study utilized P53 assays in high-throughput screening to predict the dysregulation of mitochondrial processes by chemicals that disrupt angiogenesis in an embryonic context, showing that P53 signaling was activated by 343 chemicals out of the initial 1032 used in the screen (Leung, 2016). Indeed, about 10% of the 10,000 chemicals included in the Tox21 library of testing batteries are capable of disrupting mitochondrial membrane potential, and thus may affect P53 signaling in both embryonic and adult cells (Attene-Ramos *et al.*, 2015; Green *et al.*, 2013; Green *et al.*, 2011). This shows that our increasing understanding of the involvement of P53 signaling in embryonic development and stress response will prove critical to enriching our knowledge of AOPs and bolstering the goals of predictive toxicology and the Tox21 principles.

5.5 The P53 family of proteins in the early embryonic stress response

Our studies show that the P53 protein responds robustly to genotoxic stress induced by teratogenic doses of hydroxyurea in the organogenesis-stage embryo. P53 concentrations increase significantly in hydroxyurea-treated embryos, where it acts as a transcription factor that regulates cell cycle arrest and apoptosis. This is supported by the nuclear translocation of phosphorylated P53 and the upregulation of cell cycle arrest transcripts (e.g. *Cdkn1a, Rb1*) and pro-apoptotic transcripts (e.g. *Fas, Pmaip1, Trp53inp1*), as well as the cleavage of Caspase-3. Caspase-3 cleavage is a marker for the occurrence of apoptosis and is highly dependent on P53

activity; *Trp53*$^{-/-}$ embryos exposed to hydroxyurea showed no detectable levels of Caspase-3 (Chapter III).

The P53 protein is well established as an inducer of cell cycle arrest and apoptosis under stress conditions, but is known to regulate basal functions during development under normal conditions as well (Kruse and Gu, 2009). In the mouse embryo, P53 expression is limited to certain periods of development and in specific tissues under normal conditions; by GD 10 it is mainly expressed in the central nervous system and the developing limb buds (Choi and Donehower, 1999; Donehower, *et al.*, 1992). The developmental functions of P53 include the formation and closure of the neural tube; we observed a number of saline-treated *Trp53*$^{-/-}$ fetuses exhibiting exencephaly (Chapter IV), which results from a failure to close the neural tube. This observation is noted in the literature; a subset of *Trp53* knockout embryos (~16%) was reported to display exencephaly (Sah, *et al.*, 1995). Neural tube closure relies on the proper formation of neural folds that depend on local changes in cell turnover. This programmed turnover is largely a consequence of P53 regulation of cell cycle progression and apoptosis (Choi and Donehower, 1999). As such, an excessive activation of P53 is also associated with the formation of congenital defects; embryos that have over-activated P53 display exencephaly as well as a number of other defects related to the CHARGE syndrome (Van Nostrand, *et al.*, 2014). Thus, an imbalance in P53-mediated regulation of cell cycle progression and apoptosis would prevent neural tubes from closing properly. This is also true for the development of other organs; embryonic limbs normally express P53 to "sculpt" the digits from the surrounding inter-digital space, which are removed by apoptosis (Mori, *et al.*, 1995). However, lack of P53, or over-activation by genotoxicants, results in limb malformations (Armstrong, *et al.*, 1995; Moallem and Hales, 1998; Wang *et al.*, 2000). In our studies, we observed a higher incidence of limb malformations, including syndactyly, oligodactyly and amelia, in *Trp53*$^{-/-}$ embryos exposed to hydroxyurea compared to their *Trp53*$^{+/+}$ littermates (Chapter IV). Together, these results show that a fine balance of P53 activity needs to be maintained during gestation to ensure a properly developed fetus, as the P53 protein is important for embryonic development and is susceptible to external influences from xenobiotic agents and stress factors.

Interestingly, our studies showed that other P53 family members, P63 and P73, are not influenced in the same manner as P53 by genotoxic stress during organogenesis. Although both proteins are readily detected under normal conditions, their levels are not altered by hydroxyurea

treatment (Chapter III). In addition, they are not phosphorylated by upstream kinases in the embryo, in contrast to what is observed in immortal cell lines and other *in vitro* models (Rossi*, et al.*, 2006a; Satija and Das, 2016). Moreover, while both proteins are known to act as transcription factors that regulate cell cycle arrest and apoptosis in response to stress, we observed that in the absence of P53, neither protein was capable of inducing the expression of *Cdkn1a, Rb1, Fas,* or *Pmaip1*, transcripts that are known to be downstream targets of these transcription factors (Chapter III). Thus, neither P63 nor P73 responds the same way as P53 does to hydroxyurea exposure. A possible explanation for this differential role in stress response during development may be the functions that P63 and P73 are responsible for during embryonic development that preclude them from acting as stress-response factors, like P53.

Both P63 and P73 are essential for embryonic development; lack of *Trp63* produces fetuses with severe limb deformities and skin defects that leads to their death soon after birth, while lack of *Trp73* produces fetuses with neurological, pheromonal and inflammatory defects (Yang*, et al.*, 1999; Yang*, et al.*, 2000). Interestingly, animals lacking either *Trp63* or *Trp73* do not develop tumors and inactivating mutations are not present in human cancers, unlike the case with *Trp53* (Moll and Slade, 2004). On the other hand, the majority of *Trp53* deficient embryos do not display congenital malformations while they are highly susceptible to cancers as they age (Donehower*, et al.*, 1992). This shows that while all three family members share similar structural homology, their functions during embryonic development differ significantly, with P63 and P73 playing a more essential part in maintaining proper gastrulation than P53.

It is possible that P63 and P73 play different roles at different stages of development (i.e. embryo vs. adult). Both P63 and P73 have been shown to be important for maintaining sufficient pools of embryonic stem cells (ESCs) during development, as well as driving cell differentiation after ESCs exit their pluripotent state (Wang *et al.*, 2017; Watt, 1998; Yang and McKeon, 2000). Using triple knockout (TKO) chimeric mouse embryos generated by CRISPER/Cas9 genome editing, Wang et al. showed that P53, P63 and P73 are essential for the differentiation of the embryonic germ layers; they regulate the expression of Wnt3 and co-operate with Smad2/3 signaling to promote genes involved in mesendodermal differentiation (Wang*, et al.*, 2017). Interestingly, most TKO embryos failed to gastrulate beyond GD 8, showing that these proteins are essential for embryonic development and tissue differentiation. However, whether the P53 family proteins play redundant roles in development and differentiation is still unclear. Van

Nostrand et al. used traditional breeding of *Trp53*, *Trp63* and *Trp73* knockout mice to generate double and triple knockout embryos, showing that the three P53 family members acted non-redundantly in embryonic development. While most of the embryos that lacked one or more of the P53 proteins displayed one or more malformations, these effects were not exacerbated in double or triple knockout embryos (Van Nostrand *et al.*, 2017). Indeed, one embryo that lacked all three P53 family proteins had relatively normal development at GD 10 and no sign of failed gastrulation, although it did display malformations typical of *Trp63*-deficiency (Van Nostrand*, et al.*, 2017). This shows that P63 and P73 are essential for proper embryonic development, while P53 is dispensable for gastrulation under normal conditions. Yet, as we have shown, in the context of genotoxic or oxidative damage during development, P53 may be the only member of this family that is capable of reacting to stress as a transcription factor that drives cell cycle arrest or apoptosis, while P63 and P73 remain relatively inactive (Chapter III). Although *in vitro* studies using immortal cell lines and adult tissue models showed P63 and P73 are capable of regulating cell cycle progression and apoptosis in response to DNA damage (Levrero*, et al.*, 2000; Moll and Slade, 2004), the functions of P63 and P73 in response to DNA damage in an embryonic context have not been explored previously. Since P63 and P73 are essential for preserving gastrulation, it may be that they remain committed to this task in genotoxicant-exposed embryos, while relegating the greater responsibility of the embryonic stress response to P53.

Indeed, this may be a trait related to the evolutionary origins of P53, P63 and P73. While the gene structure of the three family members is remarkably similar - where they all contain the three major domains (TAD, DBD and OD) and can produce both TA and ΔN isoforms from different transcription start sites - there are distinct differences (Melino *et al.*, 2003). First, the full-length forms of P63 and P73 are much longer than that of P53, with 3 additional exons and a C-terminal SAM domain (Melino*, et al.*, 2003). Secondly, gene homology across species indicates that lower organisms, including protostomes such as squid and *Drosophila*, possess only one P53-like gene that resembles the structure of TA-P63 (with or without a SAM domain), while P53 and P73 only appear in more highly evolved organisms such as *Xenopus*, mouse and human (Yang*, et al.*, 2002). Thirdly, the sequence identity between P63 and P73 is far greater than between both of them and P53 (TAD: 60% vs. 25%, DBD: 85% vs. 65%, OD: 60% vs. 35%, SAM: 50% vs. 0%) (Yang*, et al.*, 2002). Lastly, while most mutations in P53 are

associated with tumor development, most mutations in P63 are associated with human congenital malformations, many of which are in the SAM domain that is absent from P53 (Brunner*, et al.*, 2002; Yang*, et al.*, 2002). This suggests that P53 originates from a prototypical gene that greatly resembles P63 and P73, and that the original function of this ancestral gene was to preserve embryonic development, mainly through stem cell maintenance. With the advent of P53, however, tumor suppression (in the form of regulating of cell cycle progression and apoptosis in response to extrinsic stressors) became a secondary, more specialized, function.

Another possible explanation for the difference in the response of the P53 family members to DNA damaging agents during organogenesis lies in the functions of their various isoforms and their efficiency in DNA-binding and induction of apoptosis compared to P53. Some reports show that the capacity of TA-P63α and TA-P73α to bind DNA and upregulate the transcription of downstream targets, such as *Cdkn1a* and *Bax*, is lower than that of P53 (Bamberger and Schmale, 2001; Ozaki*, et al.*, 1999; Yang*, et al.*, 1998). This appears to be due to the structures of TA-P63α and TA-P73α themselves; their SAM domains can auto-inhibit their activities, hindering their ability to transactivate and bind to the DNA (Ozaki*, et al.*, 1999; Serber *et al.*, 2002). Interestingly, this domain is absent from P53 which may be why P53 is more robust to transactivation and the induction of cell cycle arrest and apoptotic mechanisms compared to P63 and P73. Using Western blots and specific antibodies, we were able to detect several isoforms of P63 in organogenesis-stage embryos, but only the α form of P73 (Chapter III, data not shown). While other isoforms of P63, such as β and γ, do not have the SAM domain, they are still less efficient in binding to the promoters of downstream targets (e.g. *Cdkn1a* and *Bax*) compared to P53 (Bamberger and Schmale, 2001; Yang*, et al.*, 1998). Hence, it appears that the structure of P53 allows it to be more sensitive and responsive to extrinsic stressors, permitting it to take a leading role in the embryonic stress response.

5.6 The role of P53-mediated apoptosis in the suppression of embryotoxicity

The ability to respond quickly to a DNA damaging event, such as hydroxyurea exposure, shows that the embryo is capable of adapting to extrinsic detrimental effects. The activation of pathways that inhibit cell cycle progression and promote DNA damage repair is an attempt by the embryo to counter the damaging effects of hydroxyurea. However, embryos at the organogenesis stage have a weak arsenal of DNA damage repair factors (Vinson and Hales,

2002a) and thus may be particularly sensitive at this stage to damage from genotoxicants, like hydroxyurea.

A cell-process that is activated early in the embryo in response to hydroxyurea is apoptosis. The apoptotic machinery is present in the embryo from very early in development (Brill*, et al.*, 1999). Apoptosis, a form of programmed cell death, may be triggered when a cell is deemed to be damaged beyond repair (Elmore, 2007), or as part of normal development, such as when cells in the interdigital space undergo apoptosis to define and separate the digits (Mori*, et al.*, 1995). Apoptosis allows the dying cell to be consumed by neighboring cells, via phagocytosis, to prevent its toxic metabolites from damaging surrounding tissue (Elmore, 2007). Apoptosis can be triggered in the embryo by many stress factors, including hydroxyurea, which leads to the upregulation of pro-apoptotic factors (e.g. *Fas, Trp53inp1, Pmaip1*) and the expression of cleaved Caspase-3, the effector molecule involved in the caspase-cascade (Porter and Janicke, 1999).

The rate of apoptosis during development is tightly controlled by several signaling pathways, including that of P53 (Chipuk*, et al.*, 2004). At the same time, P53 is considered the most prominent factor in the response to DNA damaging agents (Brill*, et al.*, 1999). While apoptosis occurs normally during development, excessive levels (induced by high concentration of activated P53, for example) could disrupt normal development and cause malformations and/or embryonic death (Scott, 1977). Similarly, if a cell with damaged DNA is incapable of inducing apoptosis, it runs the risk of propagating the damaging effects to surrounding tissue and/or "daughter" cells, ultimately leading to the manifestation of congenital malformations (Wells*, et al.*, 2009). Cells that are destined to die but cannot induce apoptosis may also undergo necrosis, a form of unregulated cell death that can cause damaging effects to surrounding tissue (Elmore, 2007; Kerr*, et al.*, 1972). Moallem and Hales (1998) showed that *Trp53*$^{-/-}$ murine limbs exposed to a 4-hydroperoxycyclophosphamide displayed several malformations without any apoptotic hallmarks (e.g. DNA fragmentation and nuclear condensation). Indeed, closer examination of the cellular morphology of *Trp53*$^{-/-}$ limbs revealed cells displaying ruptured membranes and disintegrated organelles, a clear indication of necrosis.

These findings shed some light on the ability of P53 to suppress the embryotoxicity of known teratogens via its ability to regulate apoptosis. We observed that B6.129S2-Trp53tm1Tyj/J embryos lacking *Trp53* had a higher incidence of malformations when exposed

to hydroxyurea compared to their *Trp53*$^{+/+}$ littermates (Chapter IV). Using Western blots, we showed that *Trp53*$^{-/-}$ embryos do not express cleaved Caspase-3 when exposed to hydroxyurea, whereas *Trp53*$^{+/+}$ do (Chapter III). These results suggest that when embryos are exposed to hydroxyurea, P53 suppresses the formation of defects by mediating apoptosis. Similar conclusions were drawn by other groups studying the effects of P53 in countering the embryotoxicity of different teratogens.

Nicol et al. was the first to demonstrate that *Trp53*-deficient embryos were more susceptible to the teratogenic effects of benzo[a]pyrene; these embryos displayed a higher incidence of an array of malformations, including short snout, club foot, curly tails and low set ears, as well as a higher rate of resorptions and postpartum lethality compared to wild type (Nicol*, et al.*, 1995). Similarly, several studies examined the effects of ionizing radiation on inducing apoptosis in *Trp53*-deficient embryos; in wild type embryos, the embryotoxicity of ionizing radiation was linked to P53-mediated apoptosis (Kato*, et al.*, 2001; Norimura*, et al.*, 1996). Interestingly, 2 Gy of ionizing radiation did not increase apoptotic cells in *Trp53*$^{-/-}$ embryos exposed on GD 9.5, yet around 70% displayed malformations - a rate higher than that detected in *Trp53*$^{+/+}$ embryos - reflecting our findings with hydroxyurea (Norimura*, et al.*, 1996). Moreover, Kato at al. showed that P53-mediated apoptosis was responsible for the removal of unrepaired cells that had sustained damage; GD 9.5 *Trp53*$^{+/+}$ embryos exposed to 2 Gy of ionizing radiation at a rate of 1.2 mGy/min displayed no increase in the rate of malformations, whereas *Trp53*$^{-/-}$ embryos displayed malformations at levels that were 12% above control (Kato*, et al.*, 2001). Interestingly, this was coupled with a 20% increase in apoptotic cells in the neural tube of *Trp53*$^{+/+}$ embryos, which were not detected in *Trp53*$^{-/-}$ embryos. Taken together, these results suggest that P53 protects the embryo from developing malformations by inducing apoptosis in tissues that are beyond repair and are destined to mal-develop, with the goal of replacing them with healthier tissue. This appears to be a native characteristic of P53 during embryonic development, where mildly-stressed cells are marked for elimination by P53 and thus are "out-competed" by healthier surrounding cells (Zhang*, et al.*, 2017).

However, an excessive dose of a teratogen may overwhelm any P53 dependent adaptive responses, forcing it beyond its protective threshold and tipping it towards rampant apoptosis that produces malformed or resorbed fetuses (Currier*, et al.*, 2016; Torchinsky and Toder, 2010). It is likely that the ability of P53 to suppress the embryotoxicity of various DNA damaging

teratogens is limited by the dose or level of exposure. In our studies, the highest doses of hydroxyurea administered caused much higher incidences of malformations and resorptions in wild type embryos from both the CD1 strain (600 mg/kg vs. 400 mg/kg) (Chapter II) and the C57BL/6 strain (400 mg/kg vs. 200 mg/kg) (data not shown). In addition, *Trp53*$^{+/+}$ fetuses from transgenic litters exposed to 400 mg/kg hydroxyurea had a similar rate of malformation to that of *Trp53*$^{+/-}$ fetuses, and a higher rate than *Trp53*$^{+/+}$ fetuses exposed to 200 mg/kg (Chapter IV). Similarly, Kato et al. observed a 70% increase in the rate of malformations in wild type embryos exposed to a higher rate of ionizing radiation (1.06 Gy/min vs. 1.2 mGy/min) (Kato*, et al.*, 2001). This dose-dependent effect is in line with Wilson's sixth principle of teratology which states "manifestations of deviant development increase in frequency and degree as dosage increases, from no-effect to the totally lethal level" (Wilson, 1959). In other words, the higher the intensity or dose of the teratogen, the greater the incidence and severity of malformations displayed, with the ultimate detrimental effect being embryolethality (i.e. resorption). Our work suggests that in the context of a DNA damaging teratogen, such as hydroxyurea, this effect is governed by P53-mediated apoptosis, which under low levels of genotoxic stress is capable of suppressing the rate of malformation, yet with increasing dose results in higher rates of resorption and malformation due to excessive apoptosis (as a result of increasing activated P53 concentrations). This describes a paradigm in which P53 is capable of acting as a suppressor of embryotoxicity up to a certain threshold.

5.7 Conclusions

1) The results of our present studies show that the organogenesis-stage embryo is sensitive and responsive to teratogenic doses of hydroxyurea. The embryo is capable of instigating an early stress response at both the transcriptional and protein levels, with the significant involvement of the P53 signaling pathway, as well as cell death, cell cycle arrest and DNA damage repair mechanisms. P53 responds rapidly to hydroxyurea exposure and translocates to the nucleus in almost all major regions of the embryo (e.g. heart, CNE and RNE) to mediate cell cycle arrest and apoptosis via its transcriptional activity.

2) While the P53 homologs, P63 and P73, are present during organogenesis, their protein levels are not affected by hydroxyurea exposure, unlike P53. Also, P63 and P73 phosphorylation

does not occur. Moreover, neither P63 nor P73 can compensate for the absence of P53 in the transcriptional regulation of cell cycle arrest and apoptotic factors (e.g. *Cdkn1a, Rb1, Fas, Pmaip1*), and the activation of the caspase-cascade in response to hydroxyurea. Lastly, hydroxyurea dysregulates the transcription of several limb and skeletal development genes. Thus, P53 is the main member of the P53 family that mediates cell cycle arrest and apoptosis in response to hydroxyurea in the organogenesis-stage embryo.

3) The ultimate function of P53 is to protect the embryo against teratogenic damage caused by hydroxyurea. The absence of P53 renders the embryo more susceptible to hydroxyurea embryotoxicity, which is manifested by increased rates of malformations and resorptions in *Trp53*$^{-/-}$ embryos vs. *Trp53*$^{+/+}$ littermates. Thus, P53 acts as a suppressor of hydroxyurea embryotoxicity.

ORIGINAL CONTRIBUTIONS

1) Hydroxyurea elicits an early embryonic stress response during organogenesis that involves the P53 signaling pathway. P53 phosphorylation occurs in several embryonic tissues, including the heart, RNE and CNE. Hydroxyurea induces the upregulation of P53INP1, a redox-sensitive pro-apoptotic factor, at the transcript and protein levels.

2) Hydroxyurea exposure does not affect the protein or phosphorylation levels of the P53 family members, P63 and P73. Both proteins are expressed under normal conditions during organogenesis, yet neither can compensate for the absence of P53 to regulate cell cycle arrest and pro-apoptotic factors, such as *Cdkn1a, Rb1, Fas* and *Pmaip1*.

3) Hydroxyurea activates P53 during organogenesis, which then controls apoptosis, cell cycle arrest and DNA damage repair by regulating the expression levels of *Trp53inp1*, *Cdkn1a*, *Rb1*, *Fas*, *Pmaip1*, cleaved Caspase-3 and MST-1.

4) Hydroxyurea exposure disrupts regulation of the expression of *Pax9*, *Tbx5* and *Jag2* during organogenesis, all of which are involved in limb and skeletal development and may contribute to the teratogenic effects of hydroxyurea.

5) P53 acts to suppress the embryotoxicity of hydroxyurea by limiting the rate of malformations and resorptions.

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