

The characterization of  $\gamma$ H2AX as a biomarker of genotoxicity in *Oncorhynchus mykiss*

by

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## **AUTHOR'S DECLARATION**

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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## ABSTRACT

Environmental contamination due to by-products and waste from industrial activities is a growing universal concern, especially regarding the impact on water and the aquaculture industry. Conventional tests to diagnose genotoxicity in fish include the comet assay and the quantification of micronuclei; however, these techniques are uninformative as to the nature of DNA damage occurring and the resulting gene response of the organism. This study involves characterizing the sensitivity of H2AX phosphorylation ( $\gamma$ H2AX), a marker of double-stranded breaks, in rainbow trout using western blotting. I present RTbrain-W1 as an excellent fish cell line for characterizing  $\gamma$ H2AX in response to the model DNA damaging agents, bleomycin, MMS, and 4-NQO. Bleomycin, which was not cytotoxic to RTbrain-W1, induced robust H2AX phosphorylation at 12.5  $\mu$ g/mL that decreased with increasing concentrations up to 100  $\mu$ g/mL after a 24h exposure. At 100  $\mu$ g/mL,  $\gamma$ H2AX levels were rapidly and strongly increased by 4h. Cells exposed to MMS and 4-NQO exhibited a contrasting dose response to bleomycin; increasing MMS and 4-NQO doses were cytotoxic and increased H2AX phosphorylation dose-dependently. In MMS-treated cells,  $\gamma$ H2AX was first detectable at 0.01%, and an increase was observed in 0.015%-treated cells that remained stable through to a 0.025% treatment. Detectable induction with MMS at a concentration of 0.015% was much slower and weaker than bleomycin, and was first observed at 12h, increasing through 24h. Confocal microscopy was used to examine  $\gamma$ H2AX localization throughout these concentrations of bleomycin and MMS, and dose responses following treatment after 24h. MMS-treated cells showed a dose dependent increase in  $\gamma$ H2AX that was observed in the cytosol, likely due to cell death. Bleomycin-treated cells showed a strong dose-dependent increase of nuclear  $\gamma$ H2AX localization that stabilized, and slightly

decreased from 50  $\mu\text{g/mL}$  to 400  $\mu\text{g/mL}$ . Cells treated with 4-NQO showed a very subtle dose-dependent increase in  $\gamma\text{H2AX}$  levels at 1000 nM, which was confirmed by densitometry to be a 2x increase. These results show the sensitivity of H2AX phosphorylation in response to different genotoxicants, and suggest that  $\gamma\text{H2AX}$  may be a valuable biomarker for genotoxicity assays using rainbow trout cell lines.

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## **DEDICATION**

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# TABLE OF CONTENTS

<b>AUTHOR'S DECLARATION</b> .....	<b>ii</b>
<b>ABSTRACT</b> .....	<b>iii</b>
<b>ACKNOWLEDGEMENTS</b> .....	<b>v</b>
<b>DEDICATION</b> .....	<b>viii</b>
<b>TABLE OF CONTENTS</b> .....	<b>ix</b>
<b>LIST OF FIGURES</b> .....	<b>xi</b>
<b>LIST OF TABLES</b> .....	<b>xii</b>
<b>LIST OF ABBREVIATIONS</b> .....	<b>xiii</b>
<b>LIST OF GENE AND PROTEIN NAMES</b> .....	<b>xiv</b>
<b>CHAPTER ONE: GENERAL INTRODUCTION</b> .....	<b>1</b>
<b>1.1 Aquatic Toxicology</b> .....	<b>1</b>
1.1.1 Assaying for Waterborne Cytotoxicity.....	4
1.1.2 Assaying for Waterborne Genotoxicity .....	6
<b>1.2 Biomarkers</b> .....	<b>8</b>
1.2.1 Use in Aquatic Toxicology.....	9
<b>1.3 The DNA Damage Response</b> .....	<b>12</b>
1.3.1 The ATR-Chk1 Pathway .....	14
1.3.2 The ATM-Chk2 Pathway .....	15
<b>1.4 <math>\gamma</math>H2AX</b> .....	<b>19</b>
1.4.1 $\gamma$ H2AX in Aquatic Toxicology .....	21
<b>1.5 p53</b> .....	<b>22</b>
1.5.1 Regulation of p53 .....	24
1.5.2 Regulatory Functions of p53.....	25
1.5.3 p53 in Aquatic Toxicology.....	27
<b>1.6 Research Objectives</b> .....	<b>30</b>
<b>CHAPTER TWO: MATERIALS AND METHODS</b> .....	<b>31</b>
<b>2.1 Genotoxicants and Chemicals</b> .....	<b>31</b>
<b>2.2 Cell Lines and Culturing</b> .....	<b>31</b>
<b>2.3 Cytotoxicity Assays</b> .....	<b>32</b>
<b>2.4 Protein Extract Preparation</b> .....	<b>34</b>
<b>2.5 Electrophoresis and Western Blotting</b> .....	<b>35</b>
<b>2.6 Densitometry</b> .....	<b>36</b>
<b>2.7 Statistical Analysis</b> .....	<b>36</b>
<b>2.8 Immunocytochemistry</b> .....	<b>37</b>
<b>CHAPTER THREE: RESULTS</b> .....	<b>39</b>
<b>3.1 Comparison of H2AX Phosphorylation Across Fish Cell Lines</b> .....	<b>39</b>
3.1.1 H2AX Phosphorylation in Rainbow Trout Cell Lines .....	39
3.1.2 H2AX Phosphorylation in Other Fish Cell Lines .....	40

<b>3.2 Characterization of the <math>\gamma</math>H2AX Response in RTbrain-W1 .....</b>	<b>44</b>
3.2.1 Cytotoxicity of Bleomycin, MMS, and 4-NQO .....	44
3.2.2 H2AX Phosphorylation in Response to Bleomycin, MMS, and 4-NQO Exposure as Examined by Western Blotting.....	46
3.2.3 Effects of Bleomycin and MMS Exposure on $\gamma$ H2AX Foci as Examined by Confocal Microscopy .....	49
3.2.4 Time-course of H2AX Phosphorylation in Response to Bleomycin and MMS .....	52
3.2.5 Application of $\gamma$ H2AX as a Biomarker of Exposure to Environmental Contaminants .....	53
<b>CHAPTER FOUR: DISCUSSION.....</b>	<b>54</b>
<b>4.1 H2AX Phosphorylation in Fish Cell Lines.....</b>	<b>54</b>
4.1.1 Differences in $\gamma$ H2AX Response Between Different Fish Species .....	54
4.1.2 Differences in $\gamma$ H2AX Response Between Cell Lines of the Same Species.....	61
<b>4.2 The Relationship of H2AX Phosphorylation with Bleomycin Treatment.....</b>	<b>65</b>
<b>4.3 MMS Weakly Induces H2AX Phosphorylation .....</b>	<b>67</b>
<b>4.4 4-NQO Causes a Subtle Increase in H2AX Phosphorylation.....</b>	<b>68</b>
<b>4.5 Differences in <math>\gamma</math>H2AX Localization and Dose Response .....</b>	<b>70</b>
<b>4.6 Temporal Differences Between MMS- and Bleomycin-induced <math>\gamma</math>H2AX.....</b>	<b>73</b>
<b>CHAPTER FIVE: CONCLUSIONS .....</b>	<b>74</b>
<b>5.1 Future Considerations.....</b>	<b>75</b>
<b>BIBLIOGRAPHY .....</b>	<b>78</b>
<b>APPENDIX A: Multiple Sequence Alignment of the H2AX Peptide .....</b>	<b>96</b>
<b>APPENDIX B: p53 Expression in Response to MMS and Bleomycin .....</b>	<b>97</b>



## LIST OF FIGURES

FIGURE 1.1: THE CONDENSED DNA DAMAGE RESPONSE IN MAMMALS .....	13
FIGURE 1.2: BASIC p53 REGULATION AND FUNCTION .....	23
FIGURE 3.1: COMPARISON OF $\gamma$ H2AX LEVELS IN BLEOMYCIN- AND HU-TREATED RAINBOW TROUT CELL LINES .....	39
FIGURE 3.2: COMPARISON OF $\gamma$ H2AX LEVELS IN BLEOMYCIN- AND HU-TREATED FISH CELL LINES .....	41
FIGURE 3.3: COMPARISON OF $\gamma$ H2AX LEVELS IN MMS-TREATED FISH CELL LINES .....	43
FIGURE 3.4: EFFECT OF BLEOMYCIN, MMS, AND 4-NQO ON THE VIABILITY AND MORPHOLOGY OF RTbrain-W1 CELLS .....	45
FIGURE 3.5: EFFECT OF INCREASING CONCENTRATIONS OF BLEOMYCIN, MMS, AND 4-NQO ON $\gamma$ H2AX LEVELS IN RTbrain-W1 CELLS.....	47
FIGURE 3.6: EFFECT OF INCREASING BLEOMYCIN CONCENTRATIONS ON $\gamma$ H2AX LOCALIZATION IN RTbrain-W1 CELLS.....	50
FIGURE 3.7: EFFECT OF INCREASING MMS CONCENTRATIONS ON $\gamma$ H2AX LOCALIZATION IN RTbrain-W1 CELLS .....	51
FIGURE 3.8: EFFECT OF INCREASING EXPOSURE TIMES ON $\gamma$ H2AX LEVELS IN BLEOMYCIN- AND MMS-TREATED RTbrain-W1 CELLS .....	52
FIGURE A1: COBALT ALIGNMENT OF H2AX IN HUMANS AND FISH.....	96
FIGURE B1: COMPARISON OF BASELINE p53 LEVELS IN RAINBOW TROUT CELL LINES .....	97
FIGURE B2: COMPARISON OF p53 LEVELS IN Asimf20 AND RTbrain-W1 TREATED WITH BLEOMYCIN AND HU .....	98
FIGURE B3: p53 EXPRESSION IN RTbrain-W1 TREATED WITH BLEOMYCIN AND MMS ....	100

## LIST OF TABLES

**TABLE 1.1: GENOTOXICITY STUDIES USING  $\gamma$ H2AX AS A BIOMARKER IN FISH.....22**

**TABLE 1.2: GENOTOXICITY STUDIES USING p53 AS A BIOMARKER IN FISH.....28**

## LIST OF ABBREVIATIONS

4-NQO – 4-nitroquinoline-*N*-oxide  
AhR – aryl-hydrocarbon receptor  
AB – Alamar Blue  
BER – base excision repair  
BLEO - bleomycin  
CF – 5-carboxyfluorescein  
CFDA-AM - 5-carboxyfluorescein diacetate, acetoxyethyl ester  
ChIP – chromatin immunoprecipitation  
CHO – chinese hamster ovary cell line  
DDR – DNA damage response  
DMNA - N,N-dimethylnitrosamine  
DR – direct reversal  
DSB – double-stranded DNA break  
EAS – environmentally affected sample  
FBS – fetal bovine serum  
HR – homologous recombination  
HU - hydroxyurea  
MFO – multi-function oxidases  
mRNA – messenger RNA  
miRNA – micro RNA  
MMR – mismatch repair  
MMS – methyl methanesulfonate  
MN – micronucleus  
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide  
NA – naphthenic acids  
NBP – nuclear basic protein  
NEDD8 - neural precursor cell expressed, developmentally down-regulated 8  
NER – nucleotide excision repair  
NHEJ – non-homologous end joining  
NR – Neutral Red  
OSPW – oil sands processed water  
P/S – penicillin/streptomycin  
PAH – polyaromatic hydrocarbon  
PIKK – phosphoinositide-3-kinase related kinase  
RFU – relative fluorescent units  
ssDNA – single stranded DNA  
SUMO – small ubiquitin-like modifier  
TLS – translesion DNA synthesis

## LIST OF GENE AND PROTEIN NAMES

9-1-1 – Rad9-Hus1-Rad1  
14-3-3 – family of proteins involved in presenting ligands for degradation in cytosol  
53BP1 – p53-binding protein 1  
AP – alkaline phosphatase  
ATM – ataxia telangiectasia mutated  
ATR – ATM and Rad3-related  
ATRIP – ATR interacting protein  
BARD1 – BRCA1-associated RING domain 1  
Bax – Bcl-2-associated X  
Bcl-2 – B-cell lymphoma 2  
BRCA1/2 – breast cancer susceptibility type 1/2  
CAT - catalase  
Cdc – cell division cycle  
CDK– cyclin-dependent kinase  
Chk1/2 – checkpoint kinase 1/2  
CYP1A – cytochrome P4501A  
DNA-PK – DNA-dependent protein kinase  
DNMT – DNA methyltransferase  
ETAA1 – Ewing tumour-associated antigen 1  
Fbxo11 – F-box protein 11  
FoxM1 – forkhead box M1 protein  
Gadd45 - growth arrest and DNA damage inducible protein 45  
GPX – glutathione peroxidase  
GR – glutathione reductase  
GST – glutathione-S-transferase  
H2AX – histone 2A variant X, synonymous with yeast H2A  
HRP – horseradish peroxidase  
HSP – heat shock protein  $\gamma$ H2AX – histone 2A variant X phosphorylated at serine 139, synonymous with yeast  $\gamma$ H2A  
HAUSP – Herpesvirus-associated ubiquitin-specific protease  
Hus1 – checkpoint clamp component  
Mdc1 – mediator of DNA damage checkpoint protein 1  
Mdm2 – mouse double-minute 2  
MdmX – mouse double-minute X  
Mms2 – methyl methanesulfonate sensitive protein 2; non-canonical ubiquitin enzyme variant  
Mre11 –meiotic recombination 11  
MRN – Mre11-Rad50-NBS1 sensor complex for DSB in higher eukaryotes; MRX in yeast  
MRX – Mre11-Rad50-Xrs2 sensor complex for DSB in yeast; MRN in higher eukaryotes  
Nbs1 – Nijmegen breakage syndrome 1  
Noxa - phorbol-12-myristate-13-acetate-induced protein 1

Nub1 – NEDD8-interacting protein  
O<sup>6</sup>-MGMT – O<sup>6</sup> methylguanine DNA methyltransferase  
p21 – cyclin-interacting protein 1; CDK inhibitor  
p53 – tumour-suppressor protein  
p300/CBP – p300/CREBB-binding protein complex  
PCNA – proliferating cell nuclear antigen  
PIAS – protein inhibitors of activated STAT  
Pig3 – p53-inducible gene 3  
Pirh2 – synonymous with ZNF363  
PLK1 – Polo-like kinase 1  
PP1 – protein phosphatase 1  
Puma – p53-upregulated modulator of apoptosis  
RAP80 – receptor-associated protein 80  
Rif1 – Rap1-interacting factor 1  
RFC – replication factor C  
RING – really interesting new gene  
RNF8/168 – RING finger protein; E2 ubiquitin ligases  
RPA – replication protein A  
SOD – superoxide dismutase  
SBLP – stem-loop binding protein  
STAT – signal transducer and activator of transcription  
TopBP1 - DNA topoisomerase 2-binding protein 1  
TP53 – transformation protein 53; gene encoding p53  
XRCC1 – X-ray repair cross complementing 1  
YY1 – yin and yang 1  
ZNF – zinc finger C2H2-type protein

To preserving nature.

*It's the great, big, broad land 'way up yonder,  
It's the forests where silence has lease;  
It's the beauty that thrills me with wonder,  
It's the stillness that fills me with peace.*

Robert W. Service, 1953

# CHAPTER ONE: GENERAL INTRODUCTION

## 1.1 Aquatic Toxicology

Our environmental footprint has grown significantly over the last century with the increase in global industrialization. The impact of pollution has had adverse effects on the health of many organisms, including humans, and the ecosystems that they inhabit. This impact on nature has also reverberated in aquacultural industries. Since water is an important medium in which manmade pollutants spread, not to mention an essential resource to human health and industry, there has been considerable research into remediation and preservation efforts regarding waterborne pollution. Naturally, fish have emerged as excellent model organisms as indicators of aquatic toxicity, while also being used to monitor remediation efforts and improvements.

Two teleost species, the zebrafish (*Danio rerio*), and medaka (*Oryzias latipes*) to a lesser extent, have become popular over the past thirty years as models in toxicology and cancer studies. They form the majority of the scientific literature regarding fish owing to their quick development, large brood size, and ease of use in the laboratory (Krumnschnabel & Podrabsky, 2009; Scharl, 2014). However, conclusions drawn from these Asian fish species are not necessarily reflective of what occurs with fish native to North America. Molecular research on other teleost species is increasing, including walleye (*Sander vitreus*), Lake sturgeon (*Acipenser fulvescens*), American eel (*Anguilla rostrata*), and the rainbow trout (*Onchorhynchus mykiss*), to name a few. In particular, rainbow trout has risen in popularity due to its abundance in North American freshwater basins. This fish species is native to the west of North America, originally endemic to colder freshwater systems between Alaska and

Mexico (Scott & Crossman, 1973). It is believed to be native to the Okanagan lake system, though artificial stocking has introduced it for sport fishing to a majority of lakes across Canada (Coker et al., 2001; Evans et al., 2002). In colder freshwater environments, rainbow trout has also become invaluable in toxicology due to its sensitivity towards environmental toxicity and a hepatic response similar to humans in function, leading to its increased use in toxicological analyses at population, organismal, and cellular scales (Schartl, 2014). As such, the National Research Council of the Canadian government currently uses rainbow trout juveniles for acute lethality tests, and an extensive framework is in place to use this model in monitoring the toxicities of specific compounds, as well as whole effluents (Environment Canada, 2007; NRC, 1987).

Conventional frameworks of toxicity testing includes the use of the EC<sub>50</sub> (effective concentration) metric, pertaining to the concentration of a substance that is lethal to 50% of the population of treated test organisms over a specific time period (Hodgson, 2004; Trevan, 1927). Since it is one of the earliest toxicological metrics to be developed, it has been widely implemented in toxicity testing frameworks using fish, including regulatory guidelines set by the Organization for Economic Co-operation and Development (OECD), a thirty-five country international partnership including North American constituents.

There are several disadvantages with animal experimentation. First, there is the loss of life associated with toxicological animal testing that is required for appropriate analysis of short- and long-term exposure to toxicants (Scholz et al., 2013). Second, the effects observed with concentrations of compounds potent enough to cause acute lethality are often less important to understand than those obtained with lower, more environmentally relevant sublethal concentrations that an organism is likely to face, and subsequently survive, outside



of the laboratory. Third, the EC<sub>50</sub> metric does not address the molecular mechanisms of how chemicals exert their toxicity. Attempts to address these shortcomings have been made through the use of cell-culture bioassays and *in vitro* testing of chemical toxicity, showing potential for alternatives to animal use (Scholz et al., 2013). *In vitro* systems also allow for easy measurement of minute toxicant concentrations, and assaying for molecular responses to toxicant exposure, in response to both purified chemicals and environmental water samples (Behrens et al., 2001; Dayeh et al., 2005; Schirmer et al., 2004). With advances in the research of cellular stress, *in vitro* protocols have been established in order to characterize the cytotoxicity and genotoxicity (ability of a compound to cause cytological or genetic stress, respectively) of environmentally relevant compounds. Cell-culture is ideal for such tests because it is rapid, it can be high throughput, and it can replace the need for live animals.

Cell lines derived from fish tissues have been shown to be valuable in aquatic toxicology for several reasons. Most importantly, fish cells are genetically representative of the species they are derived from. Unlike mortal mammalian cell lines, most piscine cell lines appear to spontaneously immortalize without treatment, making them easy to culture. In addition, they can grow in the absence of carbon dioxide, and most cell lines proliferate at ambient temperatures in conventional media with modest serum requirements (Bols et al., 2005). Finally, protocols using fish cells have been developed that measure receptor-mediated toxicity, including estrogen and aryl-hydrocarbon (AhR) receptor activation, whose ligands are two of the more widely studied toxicants in the environment concerning fish (Bols et al., 2005). The exploitation of the latter in rainbow trout cell lines has allowed research of the basic toxicity mechanisms of polycyclic aromatic hydrocarbons (PAHs), persistent environmental pollutants created from various industrial processes which activate

AhR and induce genes for PAH metabolism (Behrens et al., 2001; Schirmer et al., 1998, 2004). Several other cell-based assays have emerged using trout, including embryonic toxicity tests, acute microplate toxicity tests using cultured cells, and high throughput “omics” analyses (OECD, 2012).

### *1.1.1 Assaying for Waterborne Cytotoxicity*

Cytotoxicity assays used in environmental analysis commonly employ indicator dyes that reflect the metabolic activity, structural integrity, and overall viability of cells in a culture. These dyes fluoresce and can be analyzed by spectrometry, and the data generated from treated groups is normalized against solvent or mock-treated controls. Two well-established examples of these dyes are Alamar Blue (AB) and 5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester (CFDA-AM).

Alamar Blue is a resazurin-based dye that measures the reducing power of a cell. Resazurin easily crosses cellular membranes, is non-toxic, and monitors the reducing environment of living cells. When reduced by metabolically active cells, resazurin becomes the fluorescent resorufin product. Reduction has been attributed to cellular oxidoreductases in the cytoplasm and mitochondria and to the oxidation-reduction activity of the mitochondrial electron transport chain which can be quantified using spectrophotometry (Nakayama et al., 1997; O’Brien et al., 2000; Petrenko et al., 2001; Rampersad, 2012). In addition, AB has been adapted for high-throughput use in multiplates to assess cytotoxicity of xenobiotics to fish cell lines (Dayeh et al., 2005; Schirmer et al., 1997).

CFDA-AM is an esterase substrate that is converted by the esterases of viable cells into a fluorescent, non-polar dye. The unmetabolized CFDA-AM rapidly enters cells whereas

the fluorescent product, 5-carboxyfluorescein (CF), slowly diffuses out. Measuring CF production most directly measures esterase activity. When being used to monitor esterase activity in cell cultures that began with the cells attached to the surface of a culture well and subsequently had the medium removed prior to the addition of CFDA-AM, CF formation provides a measure of plasma membrane integrity. The integrity of the plasma membrane supports esterase activity in three ways. Functioning plasma membranes help keep cells on the plastic surface, retain esterases inside the cells, and maintain the cytoplasmic environment in order to support esterase activity. Different experimental treatments might impact differently these three ways that plasma membranes contribute to esterase activity, which may account for those rare situations where the CFDA-AM assay appears at odds with other viability endpoints (Schirmer et al., 2004a). Nonetheless, a decline in CFDA-AM metabolism is indicative of diminishing plasma membrane activity (Dayeh et al., 2005; Ganassin & Bols, 2000).

Other commonly employed dyes include Neutral Red, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), calcein, and trypan blue. Using such dyes that exploit the status of processes within a cell allows researchers to observe the different broad cytological changes that occur in acute or chronic response to a toxicant. These can be applied in studying xenobiotic cytotoxicity and in monitoring the efficacy of remedial work done to remove xenobiotics or other harmful agents from the environment (Dayeh et al., 2002, 2013). Fortunately, well-established protocols exist using rainbow trout cell lines for acute and chronic toxicity assays using microplates. The  $EC_{50}$  metric can easily be applied to *in vitro* cytotoxicity assays such as these to elucidate toxicity profiles of tested compounds. In addition, data is easy to process using microplates and spectrophotometric readers, and internal replicates of each plate lend statistical power to the experiment.

### *1.1.2 Assaying for Waterborne Genotoxicity*

Although cytotoxicity assays give a measure of the viability of a cell culture, they do not give specific insight on the mechanisms of a substance's toxicity. The symptoms that indicator dyes exploit are often downstream effects of a genetic response. To augment our ability in monitoring the environmental effects on an organism, much research has been focusing on the damage that substances inflict on an organism's DNA and the gene induction that follows. Assays have been developed to observe the genetic effects of both chronic and acute sublethal chemical exposures. The most commonly used techniques are the sister chromatid test, the chromosome aberration test, the comet test, and the micronucleus (MN) test (Luzhna et al., 2013; Osman, 2014). All four were initially developed for analysis of mammalian cells; however, they have recently been adapted for use in aquatic species due to emerging issues in environmental pollution (Osman, 2014). The comet and MN assays are the most popular and convenient of conventional methods, and will be the only ones elaborated on further.

The comet assay, or single cell gel electrophoresis assay, is a common laboratory procedure that was developed by *Cook, et al.* in 1976. By lysing cells post-treatment, isolating nuclei, and running the nucleic acids on an agarose gel, the integrity of the genome can be observed by the intensity of the comet-like tail the degraded genome leaves behind after electrophoretic migration (Collins, 2004; Cook et al., 1976). This also allows the researcher to indirectly observe DNA repair activity in cells by taking intermittent samples and comparing the extent of degradation over time. As sensitive as this technique is, it is cumbersome and lacks resolution in that only nuclear DNA integrity is studied, and not the induction of genes involved in checkpoint responses and/or repair mechanisms. In addition,

high-throughput analysis is difficult, although new advances have been made to address this and make the comet assay possible *in vivo* (Brunborg et al., 2014). A major limitation of this technique is that there is not a standardized protocol and experimental parameters differ between cell types being studied. The gel is run in alkaline conditions, which can affect basal levels of smearing observed based on alkali-labile sites throughout the genome which have been shown to exist in fish cells (Moretti et al., 1998; Osman, 2014). In addition, scoring parameters of genome degradation can vary between each experiment by use of different reference compounds, and even technically by different research groups, which can lead to biases or inconsistencies in measuring DNA damage (Osman, 2014; Wirzinger et al., 2007)

Like the comet assay, the MN test is a sensitive indicator of genotoxicity. Upon exposure to a DNA-damaging agent, the damaged chromatin fragments lag during anaphase of mitosis and are left in a small nuclear enclosure, forming the MN and resulting in subsequent chromosomal loss from the much larger nucleus (Evans et al., 1959; Fenech, 2011; Fenech & Morley, 1985; Luzhna et al., 2013). This can be due to chromosome missegregation caused by improper repair of DNA damage by homologous recombination (HR) or non-homologous end joining (NHEJ) prior to mitosis, hypomethylation of satellite repeats involved in spindle fibre formation and chromosome separation, or losses of chromosomes altogether (Fenech, 2011). As such, a linear relationship has been observed between MN formation and increasing concentrations of model genotoxicants, rendering this phenomenon the basis of an assay (Luzhna et al., 2013). In addition, protocols for this assay has moved from simple slide-smears and manual counting to flow cytometry based quantitation using molecular markers of MN formation, making it more sensitive, high-throughput, and easier to accurately count.

These genotoxicity assays demonstrate the current state of technology in the field of environmental toxicology. As a result, these techniques, and the comet assay in particular, have been adapted for various aquatic cell lines. Interestingly, the comet and MN assays have even been optimized for some freshwater bivalves in monitoring freshwater genotoxicity (Buschini et al., 2003; Klobučar et al., 2003; Lacaze et al., 2011) in addition to studying the effect of temperature on DNA integrity (Buschini et al., 2003). However, there are limitations in comparing and interpreting data across studies using the comet and MN assays (Frenzilli et al., 2009). There are instances in the literature where data from the two methods contradict one another, for example, in a 2007 study by *Wirzinger et al.*, where the comet and MN tests showed positive and negative correlations to genotoxicity, respectively, in field-collected sticklebacks. This reaffirms the importance of using multiple assays to assess genotoxicity in conjunction to obtain a more accurate evaluation and in identifying false negatives or positives. In response to these issues, a significant effort has recently gone into research of gene expression and protein expression/modification as markers of genotoxicity and cytotoxicity.

## 1.2 Biomarkers

A biological marker, or biomarker, is an umbrella term for an assayable indicator within a biological system that can be used to monitor an event or the condition of that system, either directly or by proxy. The definition has also grown to include vague uses of the term “signal”, including not only molecular signalling but results from toxicity assays as signals themselves, such as a positive and consistent result using the comet assay or MN test. Biomarkers can fall into three broad categories: exposure, effect, and susceptibility, as defined by the National Research Council of Canada in 1987. In terms of exposure, this may

include the existence and/or interactions of an exogenous substance within a system. Biomarkers of effect include a measure of the functional capacity or altered state of a system that can be associated with disease or toxicological response. Finally, those of susceptibility include indicators that suggest a system is sensitive or predisposed to an exogenous substance (NRC, 1987). The particular interest of biomarkers in the context of this thesis is to use the expression and induction of stress and DNA-damage response (DDR) related proteins as biomarkers of exposure and effect. Not only are these biomarkers indicative of the specific types of stress fish endure, but the capability of assaying for DDR gene induction, protein expression, and post-translational modifications involved in signalling allows us to observe the molecular response a cell or organism may exhibit during toxicological challenge. The consequences of acute and chronic exposure of cells to xenobiotics and other exogenous compounds may act as an early warning system of potentially harmful genotoxicity, and may prove to be as sensitive as current genotoxicity assays depending on the biomarkers being monitored.

### *1.2.1 Use in Aquatic Toxicology*

An extensive review by J.P. Sherry (2003) categorizes the vast majority of biomarkers studied in aquatic toxicology into effect and exposure as proposed by the NRC (NRC, 1987). For markers of exposure, research groups have studied the transcription, translation, and catalytic activity of mixed function oxidases (MFOs), heat shock proteins (HSPs), metallothioneins, apoptotic markers, metabolic enzymes, and multixenobiotic resistance enzymes. Chemical markers have been studied including bile metabolites, those involved in physiological stress responses such as elevated cortisol levels, and metabolic/physiological parameters such as blood composition (Sherry, 2003). Molecular

markers of effect include cholinesterases such as acetylcholinesterase, an enzyme involved in neurotransmitter production; components of antioxidant systems such as glutathione peroxidase and superoxide dismutase, reproductive markers which can include hormones and their downstream inducible protein products; factors involved in immunological function, stimulation, and suppression; and those associated with DNA adducts and breaks (Regoli et al., 2011; Valavanidis et al., 2006). Visual markers of effect include histo/morphological features, and MN formation (Sherry, 2003). Finally, markers of susceptibility include factors associated with tumourigenesis as well as those involved in adaptations to environmental stress (Sherry, 2003). Tumour formation in response to a toxicant can be used, however, genetic predisposition and unknown viral activity can complicate analysis and give false results (Baumann, 1992; Sherry, 2003). In terms of adaptation, organisms under chronic stress can form persistent changes in response to cope with toxicity (i.e. lower expression of defense systems by adapted resident animals in environments that would otherwise elicit a response by previously unexposed organisms), and these changes can even be inherited by offspring. However, this can also give false results as to the actual toxicity of an environmentally affected sample (Hahn, 1998; Sherry, 2003).

Of the aforementioned markers, the application of mixed function oxidases (MFOs), metallothioneins, and antioxidant systems have been extensively studied in fish due to their ability to indicate the oxidative potential of contaminated waters. MFOs are located predominantly in the liver, where detoxification of contaminants or xenobiotics occurs. These oxidases include cytochrome P4501A (CYP1A) which is responsible for oxidizing planar aromatic hydrocarbons such as dioxins in order to render them susceptible to further degradation (Sherry, 2003). When induced, these are indicative of many planar, aromatic



xenobiotics including the presence of polyaromatic hydrocarbons (PAHs), a common contaminant in water and sediment that fish are exposed to (Schirmer et al., 1998). In contrast, metallothioneins are enzymes rich in cysteine that bind to and detoxify transition and heavy metals (Lyons-Alcantara et al., 1998; Morcillo et al., 2016; Risso-de Faverney et al., 2001; Sherry, 2003). Antioxidants are responsible for the catalytic removal of free radicals, removal of metal ions that promote the formation of free radicals, protection of macromolecules, and reduction of free radicals within a cell (Limón-Pacheco & Gonsebatt, 2009). Though great markers, they can both be constitutively expressed in certain cell types and species (Sherry, 2003), and yield information focused on cytotoxicity rather than genotoxicity. They are also not indicative of cellular health or genetic integrity, but rather effectors of detoxification within cells.

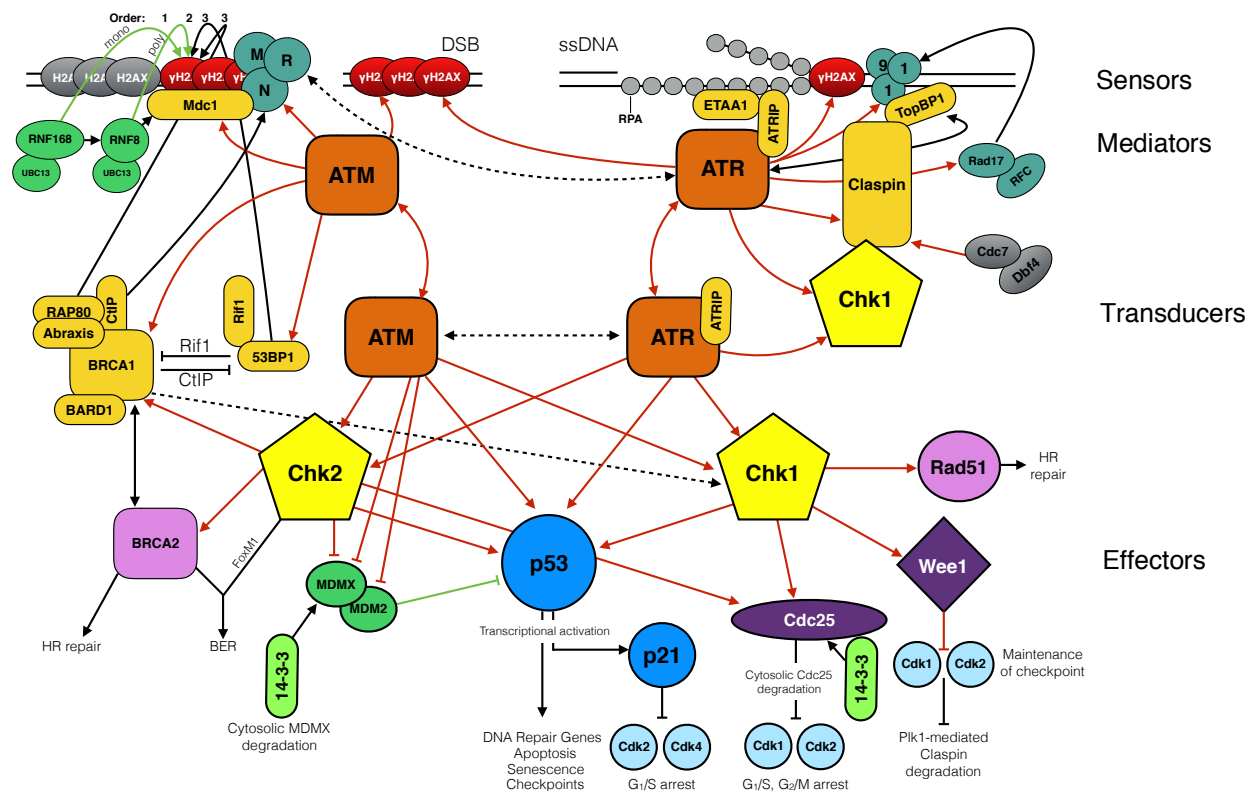
The concept of using expressional changes of DDR genes and proteins (including post-translational modifications) as biomarkers of genotoxic stress in the environment has been previously suggested (Anderson et al., 1994; Depledge, 1996, 1998), and there has been a broad effort to apply these biomarkers to characterize the genotoxicity of environmental samples and purified compounds that may be environmentally relevant. Technological advances have increased the accuracy and throughput of current methods; however, a major limiting factor in using the expression of DDR genes and proteins as toxicological biomarkers is the breadth and depth of basic research in the DDR of aquatic species. DDR mechanisms in non-model organisms such as the rainbow trout are far less studied than zebrafish and medaka, the collective research on which is dwarfed by that for mammalian models, meaning that the extent of conservation of the complex regulatory and interactive/signalling networks governing the cell cycle in fish is largely unknown. This is

also problematic in terms of a lack of reagents necessary for monitoring biomarkers, such as primers for gene transcripts and antibodies for proteins (especially phosphorylation-specific antibodies for signalling events), which are well characterized and widely available for mammalian research. Nonetheless, the use of DDR and cell cycle proteins as biomarkers in fish is growing in popularity, and these genes are continually being identified and their expression studied in new species of fish.

### 1.3 The DNA Damage Response

Life has evolved an intricate and well-coordinated cascade of events that becomes active in response to genotoxic stress, whether exogenous or endogenous in origin. Mechanisms have evolved for coping with such stress to allow the replication and repair of genetic material with high fidelity from one generation to the next. Ultimately, these mechanisms monitor genetic integrity and can halt cell cycle progression at “checkpoints” - committal steps in the eukaryotic cell cycle where progression may introduce or propagate heritable genetic errors. These checkpoints can be generally characterized as G<sub>1</sub>/S, intra-S-phase, and G<sub>2</sub>/M. Proteins involved in the DDR are loosely categorized by function as sensors, mediators, transducers, and effectors, with respect to their role in the signal cascade (Niida & Nakanishi, 2005). After the formation of a genetic lesion, sensor proteins interact with and recognize the type of damage in order to recruit the necessary mediator proteins that, through signal transducers, mobilize the response of the cell through effector proteins. Ultimately, these effector proteins execute signals to proteins that arrest the cell cycle, facilitate repair, induce apoptosis, or initiate senescence. If repairable, the type of damage and sensor proteins that detect it will direct the cell towards one of seven repair mechanisms: direct reversal (DR), base excision repair (BER), nucleotide excision repair (NER), mismatch

repair (MMR), homologous recombination (HR), non-homologous end joining (NHEJ), or translesion DNA synthesis (TLS) (Broustas & Lieberman, 2014). The complex interaction network that these DDR proteins participate in has been extensively studied using mammalian cell and yeast models due to the functional similarity that exists between the two. The collective knowledge of DDR pathways in aquatic species is substantially less, with most research using *Xenopus laevis* or *Danio rerio* models. In addition, many of these



**Figure 1.1: The Condensed DNA Damage Response in Mammals**

A schematic representation of the core DDR in mammalian models. On the left, the basic transduction pathway is represented for double-stranded break detection and recruitment of repair factors. On the right, the basic signal transduction pathway for recognition and recruitment of factors for single-stranded DNA and stalled replication fork damage. Proteins that reside closer to the middle of the two pathways are more central to the DNA damage response, and implicated in cross-talk. **Colours:** teal shapes represent sensor complexes, and loading complexes that are required for their function. Green shapes are involved in ubiquitylation and proteasomal degradation. Dark orange shapes are signal transducers. Tan shapes mediate the response. Yellow shapes are the main effectors of the DDR. Dark blue shapes include canonical p53 targets, while light blue represents cyclin-dependent kinases. Light purple proteins are involved in HR repair and dark purple proteins interact directly with Cdks. Grey proteins are arbitrarily coloured. **Lines:** Flathead lines indicate an inhibition, while arrowhead lines indicate a positive interaction. Double-headed lines indicate a reciprocated interaction. Dotted lines indicate cross-talk between pathways. In terms of colouring, black represents a physical interaction, red represents a phosphorylation event, and green represents a ubiquitylation event, where “mono” and “poly” refer to the type of ubiquitylation.

proteins have multiple roles in the cell cycle, sometimes redundant with one another, and new non-canonical functions of checkpoint proteins are continually being discovered. For simplicity and a more unified explanation, only the core network of the mammalian DDR pathway will be further summarized. A graphical representation of core interactions from the DDR is presented in **Figure 1.1**.

### *1.3.1 The ATR-Chk1 Pathway*

Upon formation of stalled replication forks and/or single-stranded DNA (ssDNA) damage, the ssDNA is coated by replication protein A (RPA) to initiate the DDR and protect exposed bases from reannealing (Byun et al., 2005). This is necessary to recruit ataxia telangiectasia and Rad3-related (ATR) to damaged sites, a phosphoinositide-3-kinase related kinase (PIKK) signal transducer, which occurs via the ATR interacting protein (ATRIP) regulatory subunit, a mediator protein (review by Harper & Elledge, 2007; review by Niida & Nakanishi, 2005; Zou & Elledge, 2003)

ATR responds to a broad range of genetic damage, and is a transducer that has a role in cross talk between repair pathways, in activating downstream transducer and effector proteins, and in activating itself via autophosphorylation in order to amplify the signal. Importantly, ATR helps facilitate loading of the Rad9-Hus1-Rad1 (9-1-1) complex, a reparative clamp, to damaged loci (Harper & Elledge, 2007; Parrilla-Castellar et al., 2004; Zou & Elledge, 2003) by phosphorylating 9-1-1 directly as well as Rad17, part of the replication factor C (RFC) complex that facilitates 9-1-1 loading onto DNA (Bermudez et al., 2003; Ellison & Stillman, 2003; Majka & Burgers, 2003; Parrilla-Castellar et al., 2004). The presence of this clamp allows tethering of downstream repair machinery to damaged DNA

(Bermudez et al., 2003; Ellison & Stillman, 2003; Harper & Elledge, 2007; Majka & Burgers, 2003). The loaded 9-1-1 is recognized and bound by topoisomerase 2-binding protein 1 (TopBP1), another mediator protein, which binds to ATR/ATRIP to stimulate ATR kinase activity and recruitment in a positive feedback loop (Harper & Elledge, 2007; Kumagai et al., 2006; Mordes et al., 2008). A third mediator protein, Claspin, is recruited by TopBP1 and phosphorylated by ATR at foci in order to bind checkpoint kinase 1 (Chk1), a crucial effector kinase (Liu et al., 2006).

Chk1 and ATR phosphorylate p53 (refer to Section 1.5), protecting it from degradation by preventing its nuclear export (Zhang & Xiong, 2001). p53 is then able to transcriptionally activate p21<sup>Waf1/Cip1</sup>, which prevents S-phase entry by binding cyclin-dependent kinases 2 and 4 (CDK2 & CDK4) (Harper et al., 1993). In addition, activated p53 is able to direct severely damaged cells towards apoptosis (Fridman & Lowe, 2003). It is important to note that there is extensive crosstalk between this pathway and the ATM-Chk2 pathway, adding to the complex redundancy and many fail-safes of the DDR. This will be addressed in the section below.

### *1.3.2 The ATM-Chk2 Pathway*

The consequences of signalling within the ATM-Chk2 pathway are similar to that of ATR-Chk1 signalling, with most differences involving damage type, damage sensing, or mediation of the signal. The ATM-Chk2 pathway is the predominant cellular response to the formation of double-stranded DNA breaks (DSBs), and begins with the damage-sensing Mre11-Rad50-Nbs1 (MRN) complex, a heterotrimeric nuclease and end-processor that detects DSBs and ssDNA overhangs (van den Bosch et al., 2003). The presence of these

lesions, as well as nucleic acid byproducts from MRN end-processing, enhances ATM kinase activity and rapid localization of ATM, in addition to increased affinity of ATM for ligands and DNA (Jazayeri et al., 2008; Lee & Paull, 2004). In addition, Nijmegen breakage syndrome 1 (Nbs1) is phosphorylated by ATM upon DNA damage, which may stimulate ATM activity by recruiting downstream substrates (Lee & Paull, 2004). Then, the rapid and robust phosphorylation of histone 2A variant X (H2AX; to form  $\gamma$ H2AX; refer to Section 1.4) occurs around lesions in chromatin (Rogakou et al., 1998). This is facilitated redundantly by the damage-sensing and signal-transducing PIKKs such as the aforementioned ATR, ATM, as well as DNA-dependent protein kinase (DNA-PK), a primary coordinator of NHEJ (Andegeko et al., 2001; Burma et al., 2001; Stiff et al., 2004; Wang et al., 2005). The formation of  $\gamma$ H2AX acts as a beacon for DDR machinery to localize to damaged DNA, quickly forming repair foci.

The mediator protein, mediator of DNA damage checkpoint protein 1 (Mdc1) is also phosphorylated by ATM and binds  $\gamma$ H2AX, which is essential for the association of several downstream factors with repair foci and forming high densities of  $\gamma$ H2AX at DSBs (Kolas et al., 2007; Paull et al., 2000; Stucki et al., 2005). In addition, these two phosphorylations aid in further anchoring/recruiting MRN to foci via Nbs1 and further activating ATM, creating a positive-feedback loop and preventing deactivation of the checkpoint (Chapman & Jackson, 2008; Stucki et al., 2005). Interestingly, DDR-related K63 polyubiquitylation has emerged in recent years to be critical to foci formation of proteins downstream of Mdc1, acting in concert with ATM phosphorylation (Al-Hakim et al., 2010). It is possible that while H2AX phosphorylation initiates the DDR, its ubiquitylation by RNF8 and RNF168 (RING finger proteins) is what promotes the downstream response and pushes signalling irreversibly

towards checkpoint activation (Al-Hakim et al., 2010). Compared to canonical ubiquitylation, K63 polyubiquitylation is implicated in signalling events unrelated to proteasomal degradation.

Two key mediators, p53-binding protein 1 (53BP1) and breast cancer susceptibility type 1 (BRCA1) are recruited to ubiquitylated H2A/ $\gamma$ H2AX in order to recruit factors for NHEJ and repair, respectively, as well as stimulate ATM autophosphorylation and Chk2 activation (Panier & Boulton, 2014; Wilson & Stern, 2008). It is thought that 53BP1 contributes to NHEJ initiation by binding DSB ends, preventing resection and subsequent HR; the Rap1-interacting factor 1 (Rif1) and PP1 phosphatase have recently emerged as key effectors of this pathway (Panier & Boulton, 2014). Importantly, the ATM-dependent phosphorylation of 53BP1 allows recruitment of Rif1 to damaged foci, which is then able to prevent the initiation of end resection and loading of HR machinery, thus promoting NHEJ (Chapman et al., 2013; Feng et al., 2013; Zimmermann et al., 2013). In contrast, BRCA1 is localized to ubiquitylated repair foci via its interacting partner, receptor-associated protein 80 (RAP80), which is brought into contact with BRCA1 via an adaptor protein, Abraxis (Kim et al., 2007; Sobhian et al., 2007; Wang et al., 2007). Interestingly, it has been shown via super-high resolution microscopy that abundance of both BRCA1 and 53BP1 at repair foci on chromatin fluctuates, and that BRCA1 physically excludes 53BP1 from foci outside of G<sub>1</sub> phase, showing the temporal pattern of both DNA repair pathways during the cell cycle (Chapman et al., 2012). This switch-like mechanism also facilitates cross-talk between the ATM and ATR directed pathways, as the resection of DSBs during the initiation of HR repair creates ssDNA overhangs that have been shown to stimulate the ATR-Chk1 signalling pathway, reinforcing the maintenance of intra-S and G<sub>2</sub>/M checkpoints during repair

(Kousholt et al., 2012). Chk1 is then able to phosphorylate an essential HR repair protein, Rad51, which can localize to DSBs (Sørensen et al., 2005). In addition, it has been shown that the phosphorylation of BRCA1 (when in complex with one of its binding partners, BARD1; BRCA1-associated RING domain 1) is essential for p53 activation by both ATM and ATR, suggesting that the influence of BRCA1 is that of an adaptor and scaffold for binding partners that communicate between both pathways, and its activation influences ATM/ATR activity on substrates (Fabbro et al., 2004).

Downstream of the extensive mediator cross talk between 53BP1, BRCA1, and their involvement with ATM and ATR resides checkpoint kinase 2 (Chk2), an effector kinase with similar functions to Chk1. To date, it is known to interact with 24 proteins involved in the cell cycle, p53-mediated apoptosis, and its own inactivation (Zannini et al., 2014). Activated ATM phosphorylates Chk2 upon DNA damage, leading to its dimerization and subsequent autophosphorylation to become fully active in a monomeric state. It is then able to direct the cell towards cell cycle arrest, DNA repair via BER and HR, p53-related functions, and apoptosis (Zannini et al., 2014). Like Chk1, Chk2 is able to execute the G<sub>1</sub>/S and G<sub>2</sub>/M checkpoints by targeting cell division cycle 25 (Cdc25) and inducing p53-dependent activation of p21<sup>Waf1/Cip1</sup> (Chehab et al., 2000; Hirao et al., 2000; Mailand et al., 2000). Unlike Chk1, Chk2 has the capability of also directly inhibiting the main negative feedback pathway of p53 ubiquitin-based degradation. Interestingly, Chk2 has also been shown to be activated by ATR cross-talk to signal to p53 and initiate apoptosis. Chk1 is degraded rapidly in this scenario, possibly to focus the signal towards apoptosis and prevent survival when damage is too severe (Pabla et al., 2008). The ubiquitylation of Chk2 has also been shown to be important for checkpoint regulation, often involving p53. Protein turnover is facilitated by



removal of ATM-phosphorylated residues and ubiquitylation courtesy of the p53-inducible E3 ligase, Pirh2 (synonymous to ZNF363), possibly as a negative feedback loop (Bohgaki et al., 2013; Kass et al., 2007). In addition, the autophosphorylation of Chk2 is necessary for a separate ubiquitylation event by a Cullen1-containing E3 ubiquitin ligase complex, required for p53-mediated apoptosis (Lovly et al., 2008). Finally, Chk2 promotes repair by directly activating BRCA1 and BRCA2, which leads to focus formation with Rad51 of HR repair machinery at DSBs (Bahassi et al., 2008; Lee et al., 2000). With BRCA2 activation, Chk2 also promotes BER by stabilizing the forkhead box M1 (FoxM1) transcription factor and leading to increased expression of DNA repair genes, including X-ray repair cross complementing 1 (XRCC1) (Tan et al., 2007). Chk2 is also capable of promoting senescence in both p53-dependent and independent mechanisms, with help from ATM (Zannini et al., 2014).

#### 1.4 $\gamma$ H2AX

The average eukaryotic genome is many folds larger than those of most prokaryotes and must be compressed into the confines of the nucleus. In human cells for example, DNA equalling almost two metres in length must be compacted into an area ten microns in diameter, and be organized in such a way that it can be remodelled easily for access to and activation of genes necessary for all aspects of life (Cooper, 1997). To address this challenge, eukaryotes express small nuclear basic proteins (NBPs) rich in arginine and lysine that are able to closely attract the negatively charged phosphodiester backbone of DNA and modulate the spatial conformation of chromatin. These NBPs include histone proteins (H1 through H5) and are amongst the highest conserved eukaryotic proteins (Mardian & Isenberg, 1978).

In the nucleus, DNA is wrapped every ~147bp around the nucleosome, an octamer consisting of pairs of H2A, H2B, H3, and H4, to form a structure resembling that of a “beaded string” (Luger et al., 1997). Pairs of H3 and H4 associate to form the core tetramer where DNA interacts, while pairs of H2A and H2B associate near the entry and exit points of DNA to complete the nucleosome (Sullivan et al., 2002; Weber & Henikoff, 2014). Histones 1 and 5 are structurally dissimilar and are not part of the nucleosome, although they aid in organizing chromatin by binding to the nucleosomes and the chromatin in between, respectively (Luger et al., 1997). Each of the core histones can also be replaced at different points of the cell cycle with variants that differ in exposed amino acid sequence, most often the exposed C'-terminal “tails” that extrude from the nucleosome complex. They can be modified by kinases, acetylases, and ubiquitinases in order to initiate chromatin reorganization and other signal cascades (Thatcher & Gorovsky, 1994; Weber & Henikoff, 2014). These signals can involve processes of chromatin remodelling, transcription, and DNA repair. Of the core histones, H2A has the most conserved and well-studied variants, including variant X (H2AX), which is of considerable interest for this thesis.

Mammalian H2AX comprises between 2-25% of the H2A pool within chromatin depending on the cell or tissue type (Furuta et al., 2003; Redon et al., 2002; Rogakou et al., 1998). Of importance to H2AX in the DDR is an SQE motif at position 139 in humans, an exposed serine just short of the C'-terminus that is not part of mammalian H2A (Rogakou et al., 1998) (refer to **Appendix A - Figure A1**). This residue is phosphorylated (referred to as  $\gamma$ H2AX) by several sensor kinases responsible for the initiation of several different pathways involving chromatin remodelling, most notably by ATM, ATR, and DNA-PK as mentioned above to initiate the DDR to address DSBs caused either by mitotic failure, genotoxicants or

radiation. The presence of the SQE motif is conserved between mammals, fish, and yeast with slight variations in surrounding sequence. Aside from DDR proteins, it has been shown that  $\gamma$ H2AX interacts directly with the INO80 chromatin remodelling complex (Downs et al., 2004). Interestingly,  $\gamma$ H2AX has also been implicated in chromosome silencing in yeast. It has been found that  $\gamma$ H2AX is central to silenced loci in a Sir-dependent manner (silent information regulator proteins), and that Sir2 and Sir3 proteins also associate with  $\gamma$ H2AX at DSBs (Kirkland et al., 2015; Kirkland & Kamakaka, 2013). The ubiquitylation of  $\gamma$ H2AX has also gained attention, as mentioned earlier, for the loading for various DDR mediators onto chromatin (Kolas et al., 2007).

#### *1.4.1 $\gamma$ H2AX in Aquatic Toxicology*

The appearance of  $\gamma$ H2AX foci is a well-studied phenomenon that has been applied as a biomarker of DSB formation, especially in cancerous mammalian cells and in studying the effects of chemotherapeutic compounds (Banáth & Olive, 2003). It is a canonical marker of severe DNA damage that has been used in damage and repair studies in many terrestrial model organisms, and to a lesser extent zebrafish, and the quantification of damage-induced foci has become a well-established index for severity of DNA damage in cells (Pilch et al., 2003; Watters et al., 2009). Naturally, it has been proposed by several different sources to be a good potential biomarker of genotoxicity in the environment (Gerić et al., 2014; Smart et al., 2011); however, its application in fish cell lines has been predominantly with those derived from zebrafish, in far fewer studies than have been conducted with mammalian cells (Ismail & Hendzel, 2008; Smart et al., 2011). With H2A being a core histone, the H2AX variant is also very well conserved across eukaryotes (refer to **Appendix A**) (Thatcher & Gorovsky, 1994) and is likely to have a similar role in fish DDR to mammalian DDR, acting

**Table 1.1:** Genotoxicity Studies Using  $\gamma$ H2AX as a Biomarker in Fish

Organism	Cell Line / Tissue Type	Genotoxin	Antibody	Application	Author
<i>D. rerio</i> Zebrafish	ZF4: embryonic fibroblast	Uranium (1-250 $\mu$ M)	Upstate Biotechnology- Euromedex	Confocal microscopy	Pereira et al., 2012
<i>D. rerio</i> Zebrafish	ZF4: embryonic fibroblast	$\gamma$ -radiation (0.1-2.0 Gy/d)	Upstate Biotechnology- Euromedex	Confocal microscopy	Pereira et al., 2011
<i>D. rerio</i> Zebrafish	Liver	Silver nanoparticles (30-120 mg/L Ag)	Cell Signalling Technology	Western blot	Choi et al., 2010
<i>D. rerio</i> Zebrafish	ZF4: embryonic fibroblast	Cadmium (0-100 $\mu$ M) Aluminum (0-100 $\mu$ M)	Upstate Biotechnology- Euromedex	Confocal microscopy	Pereira et al., 2013
<i>O. mykiss</i> Rainbow trout	Liver, Heart, Spleen, Brain, Kidney	Tritium (7000-30000 Bq/L)	Millipore: #16-202A	FACS, Confocal microscopy	Festarini et al., 2016
<i>O. mykiss</i> Rainbow trout	RTgill-W1: gill epithelial RTH-149: liver epithelial hepatoma	Cadmium (10-500 $\mu$ M)	New England Biolabs: #9718	FACS, Confocal microscopy	Krumschnabel et al., 2010
<i>O. mykiss</i> Rainbow trout	RTbrain-W1: brain glial RTgill-W1: gill epithelial	Bleocin (20-200 $\mu$ g/mL)	Santa Cruz Biotechnology	Western blot	Liu et al., 2011

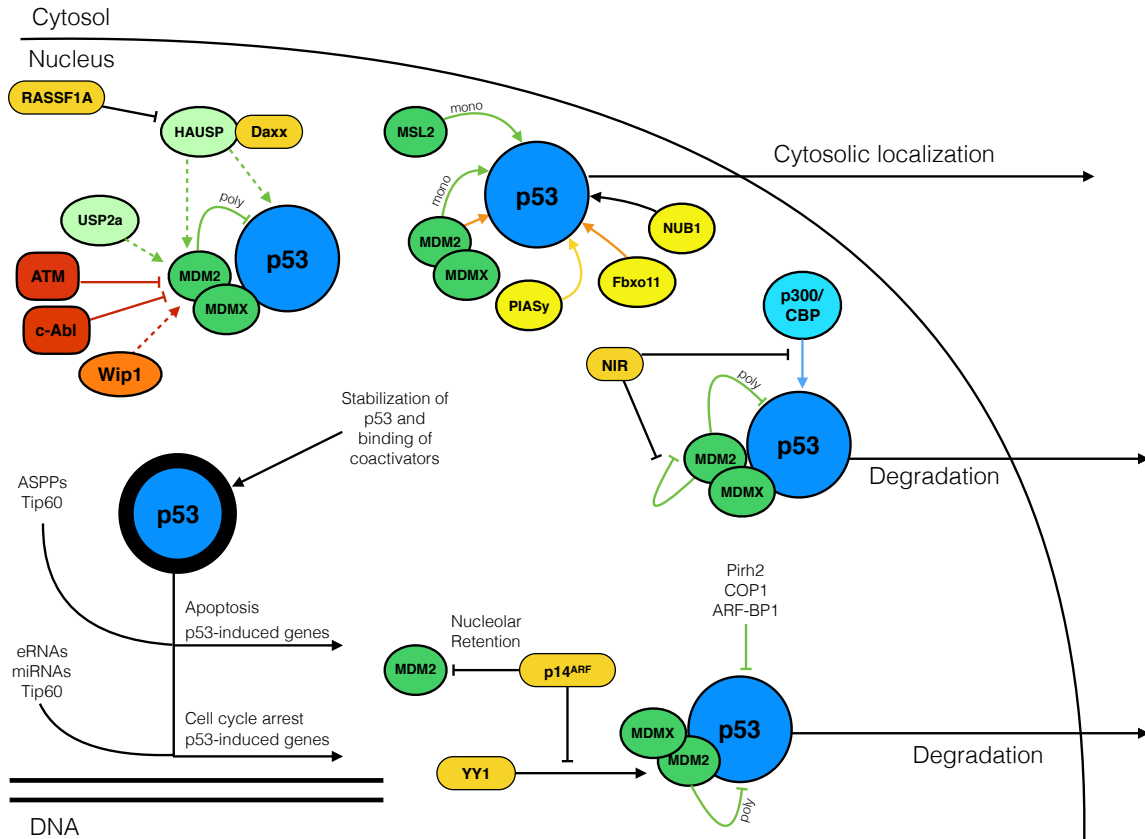
as a scaffold for downstream repair and transducing proteins that amplify the repair response. However, neither histone ubiquitylation or protein-protein interactions have been studied in fish, so it is quite possible that the DDR pathways are functionally different, especially since model chemotherapeutics have been shown to elicit varying responses between fish and mammals (Embry et al., 2006). Nevertheless, the quantification of  $\gamma$ H2AX induction has had limited use in teleost fish species for assaying genotoxicity via flow cytometry, foci counting (by confocal microscopy), and western blot (refer to **Table 1.1**), especially with studying responses to nanoparticle-based genotoxicity, with the most popular method involving the counting of  $\gamma$ H2AX foci by either fluorescence-activated cell sorting (FACS) or confocal microscopy.

## 1.5 p53

Though not the main focus of this thesis, this background information is included to help orient the reader with the results shown in **Appendix B** regarding p53 response to model

genotoxicants. A resonating quote in an extensive review by Vousden and Prives (2009) truly summarizes the complexity of p53:

“If genius is the ability to reduce the complicated to the simple, then the study of p53



**Figure 1.2: Basic p53 Regulation and Function**

A schematic representation of the regulation and cellular function of p53 in normal mammalian cells. **Colours:** dark green shapes represent ubiquitylating enzymes, while light green shapes are deubiquitylating enzymes. Red shapes are kinases, while orange shapes are phosphatases. Tan shapes interact physically without modifying substrates post-translationally. Yellow shapes represent ubiquitin-like modifiers, such as SUMOylators and NEDDylators. The p53 with the thick border represents an activated state, and the curved arrows show the transcriptional activities of p53. **Lines:** flathead lines indicate an inhibition, while arrowhead lines represent a positive interaction. Dotted lines represent the removal of the moiety that the colour represents (ie. dotted red is dephosphorylation, dotted green is deubiquitylation). Red lines indicate phosphorylation, green lines indicate ubiquitylation, orange lines represent NEDDylation, and yellow lines represent SUMOylation. Black lines represent interactions in lieu of causing a post-translational modification.

makes fools of us all.”

Since its discovery, it has become one of the largest puzzles that genetics has to offer researchers. Often termed the “master regulator” of the cell, *TP53* is one of the most widely studied genes in the human genome due to its wide involvement in regulating cellular processes, but primarily for the role of *TP53* mutations in cancer. Identified in 1979, it was the first tumour-suppressor gene (accidentally) discovered in the human genome (Kress et al., 1979). Mutations in “hotspots” of p53 have been found in more than half of all cancers studied, demonstrating its importance in cellular transformation (Muller & Vousden, 2013; Vogelstein et al., 2000). It is a regulator of the cell cycle that responds to broad types of cellular stress and can directly guide the fate of the cell towards DNA repair, cell cycle arrest, senescence, apoptosis, and autophagy. It carries out its activity in three phases: stabilization (derepression), binding to DNA as a transcription factor of target genes, and subsequent activation; however, this canonical model is constantly evolving to include new dynamics of regulation (Kruse & Gu, 2009). In addition, p53 can be methylated, ADP-ribosylated, glycosylated, SUMOylated (small ubiquitin-like modifiers), NEDDylated (neural precursor cell expressed, developmentally down-regulated 8), acetylated, and phosphorylated at multiple sites, adding tremendous complexity to its regulation. The main known regulators and functions of mammalian p53 in the DDR will be elaborated below, and the core activities of p53 and Mdm2 are illustrated in **Figure 1.2**.

### *1.5.1 Regulation of p53*

The canonical negative regulation of p53 occurs courtesy of mouse double-minute 2 (Mdm2) during unperturbed conditions (Muller & Vousden, 2013; Vousden & Prives, 2009).

Mdm2 homodimers possess E3 ubiquitin ligase activity, responsible for the ubiquitin-mediated proteasomal degradation of p53 and preventing formation of its active state as a tetramer (Wade et al., 2013). Mdm2 itself is subject to several modes of regulation, all sharing a common theme of altering the Mdm2-p53 interaction, including post-translational modifications of Mdm2 (Kruse & Gu, 2009; Li et al., 2001; Song et al., 2008).

The activity of p53 is also modulated by conjugation of ubiquitin-like modifiers including SUMO and NEDD8. Both Mdm2 (Xirodimas et al., 2004) and F-box protein 11 (Fbxo11) (Abida et al., 2007) are able to NEDDylate p53 at mutually exclusive sites, inhibiting the transcriptional ability of p53. Interestingly a NEDD8-interacting protein, Nub1 (negative regulator of ubiquitin-like proteins 1), is able to bind NEDDylated p53 and, dependent on Mdm2-mediated ubiquitylation of p53, helps promote its monoubiquitylation or cytoplasmic localization (G. Liu & Xirodimas, 2010). As for SUMOylation, the PIAS (Protein Inhibitors of Activated STAT) family of E3 SUMO ligases, specifically PIASy, has been shown to interact with and SUMOylate p53 (Stehmeier & Muller, 2009) in a Mdm2-mediated monoubiquitylation-dependent manner, promoting monoubiquitylation and nuclear export of p53 (Carter et al., 2007). Conversely, PIASy has also been shown to activate p53 via SUMOylation in order to promote senescence (Bischof et al., 2006).

### *1.5.2 Regulatory Functions of p53*

When activated, p53 becomes a transcription factor for a multitude of downstream genes that contain the p53 response element (Bode & Dong, 2004; Riley et al., 2008). A battery of different coactivating factors can interact with activated p53 to tailor the response towards transcribing certain genes. To add to the overwhelming complexity of p53 as a

transcription factor, it has been proven by chromatin immunoprecipitation (ChIP) that p53 is bound to promoters of *MDM2* and *p21<sup>Waf1/Cip1</sup>*, a downstream target of p53 and potent CDK inhibitor, without causing activation (Kaesler & Iggo, 2002; Szak et al., 2001), further supporting a role for additional factors in initiating transcription of p53 target genes. Furthermore, p53 can also directly trigger the transcription of non-coding enhancer RNAs (eRNA) to enhance its own transcriptional ability (Melo et al., 2013). For example, these eRNAs have been shown contribute to the activation of *p21<sup>Waf1/Cip1</sup>* during the DDR when coupled with p53 (Melo et al., 2016). Thus, the specific action of p53 is a function of regulation of itself, the proteins that regulate it, and association of transcription factors with p53 at p53-inducible promoters.

As mentioned previously, one of the critical roles p53 can play in the DDR is initiating apoptosis. A protein family named the apoptosis stimulating proteins of p53 (ASPP) play a major role in influencing p53 to transactivate proapoptotic genes over genes leading to cell cycle arrest. ASPP1 and 2 bind to activated p53 and the transcription factor complex initiates transcription of proapoptotic factors such as *BAX* (Bcl2-associated X), *PUMA* (p53-upregulated modulator of apoptosis), *NOXA* (phorbol-12-myristate-13-acetate-induced protein 1), and *PIG3* (p53-induced gene 3).

In addition to apoptotic programs, p53 also directly initiates cell cycle arrest when activated as a transcription factor. Similar to the previously mentioned *p21<sup>Waf1/Cip1</sup>*-dependent inhibition of CDK2 and CDK4, p53 can also activate Gadd45 (growth arrest and DNA damage inducible protein 45), which specifically inhibits CDK1 activity by preventing Cdc2 from interacting with cyclin B1 at the G<sub>2</sub>/M checkpoint (Zhan et al., 1999). p53 also maintains the G<sub>2</sub>/M checkpoint by inducing expression of 14-3-3, a family of proteins



responsible for anchoring Cdc2 in the cytoplasm for degradation (Taylor & Stark, 2001). Interestingly, screens have identified p53-inducible microRNAs (miRNA) that induce the G<sub>1</sub>/S checkpoint, as well as apoptosis. This occurs via precursors miR-34a and miR-34a, respectively, by cooperating with transcription factors regulating downstream target genes (Tarasov et al., 2007) revealing another layer of sophistication in the seemingly ubiquitous influence p53 has on cellular processes. This has brought focus to other miRNAs, such as miR-192 and miR-215, two p53-inducible miRNAs which aid in G<sub>1</sub>/S and G<sub>2</sub>/M arrest through similar regulatory targets (Georges et al., 2008). It is important to note that although the interactions described throughout this section may seem extensive, our knowledge of the regulatory and physical interaction network of p53 is constantly expanding, and new involvements of p53 are being documented in areas including metabolism, hypoxia, DNA synthesis and repair, and of course apoptosis and the cell cycle. It is likely that the vast majority of genes involved in these processes are either directly or indirectly regulated by p53, and vice versa, which will be the aim of future research regarding p53 for years to come.

### *1.5.3 p53 in Aquatic Toxicology*

The notion of using p53 and DDR transcripts or proteins as biomarkers of environmental genotoxic stress in fish has been suggested before (Bhaskaran et al., 1999; Kienzler, Bony, & Devaux, 2013); however, the basic research of DDR mechanics in and across fish species is relatively unknown compared to mammalian models. Nevertheless, p53 has been investigated in several fish species as a potential biomarker, predominantly by quantifying mRNA expression in response to genotoxic stimuli. A listing of key genotoxicity studies in fish regarding p53 can be found in **Table 1.2**. Interestingly, bleomycin (a radiomimetic drug) induced p53 expression in flounder (Geng et al., 2012), congruent with mammalian literature

(Patel et al., 2000); however, cell lines from rainbow trout (Liu et al., 2011; Embry et al., 2006) and tophead minnow (Embry et al., 2006) appear non-inducible in this regard.

**Table 1.2:** Genotoxicity Studies Using p53 as a Biomarker in Fish

Organism	Cell Line / Tissue Type	Toxicant	Antibody	Application	Study
<i>A. nebulosus</i> Brown bullhead	Liver, gill, spleen, kidney	Benzo[a]pyrene (0.087 M) Sediment of Trenton Channel, Canada	Santa Cruz Biotechnology: #SC-126	Western blot, qPCR	Williams & Hubberstey, 2014
<i>D. rerio</i> Zebrafish	Liver	Silver nanoparticles (30-120 mg/L Ag)	Cell Signalling Technology	Western blot, qPCR	Choi et al., 2010
<i>G. rarus</i> Chinese rare minnow	Liver	Benzo[a]pyrene (0-10 µg/L)	N/A	qPCR	Yuan et al., 2017
<i>K. marmoratus</i> Mangrove killifish	Brain, eye, gonad, intestine, liver, muscle, skin	Bisphenol A (300 µg/L) 4-tert-Octylphenol (150 µg/L) 4-Nonylphenol (150 µg/L)	N/A	qPCR	Lee et al., 2007
<i>K. marmoratus</i> Mangrove killifish	Liver	Diethylnitrosamine (9, 160 mg/L)	Oncogene Science: #Ab-1	Immunohistochemistry	Goodwin & Grizzle, 1994a, 1994b
<i>O. latipes</i> Japanese medaka	Liver	Silver nanoparticles and silver nitrate (1, 25 µg/L)	N/A	qPCR	Chae et al., 2009
<i>O. latipes</i> Japanese medaka	Liver, gill, intestine	Diclofenac (1 µg/L, 8 mg/L)	N/A	qPCR	Hong et al., 2007
<i>O. latipes</i> Japanese medaka	Liver	Propiconazole (2.5-250 µg/L)	N/A	qPCR	Tu et al., 2016
<i>O. mykiss</i> Rainbow trout	RTbrain-W1: brain glial RTgill-W1: gill epithelial RTgutGC: gut epithelial *RTG-2: gonad fibroblastic RTLiver-W1: hepatocyte RTS11: spleen haematopoietic Brain, gill, liver, spleen, heart	Bleocin (20-200 µg/mL) Hydroxyurea (20-200 mM)	Made in-house	Western blot	Liu et al., 2011
<i>O. mykiss</i> Rainbow trout	RTgill-W1: gill epithelial	2-phenylethylsulfonamide (0-5.25 µg/mL)	Made in-house	Western blot	Zeng et al., 2014
<i>O. mykiss</i> Rainbow trout	**RTLiver-W1: hepatocyte **Primary hepatocyte culture	Bleomycin (0-100 µM) Camptothecin (0-20 µM) Cisplatin (0-400 µM)	Santa Cruz Biotechnology: #4246	Western blot	Embry et al., 2006
<i>O. mykiss</i> Rainbow trout	RTgill-W1: gill epithelial	Pifithrin-a (5.25 µg/mL)	Made in-house	Western blot	Zeng et al., 2016
<i>O. mykiss</i> Rainbow trout	Head kidney	Benzo[a]pyrene and cyclopenta[c]phenanthrene (10 mg/kg injection)	N/A	qPCR	Brzuzan et al., 2011
<i>M. edulis</i> Blue mussel	*Haemocytes	Raw water; Pictou Harbour, Canada	p53: Calbiochem p53-73: In-house, Univ. New Hampshire	Western blot	St-Jean et al., 2005
<i>O. niloticus</i> Tilapia	Blood cells	Simulated acid rain (pH ~5.3)	Made in-house	Western blot, qPCR	Mai et al., 2010
<i>O. niloticus</i> Tilapia	Gill, heart, spleen, liver, muscle, blood cells	Etoposide and cadmium chloride (10, 100 µg/kg) Camptothecin (0-20 µM) Cisplatin (0-100 µM) Etoposide (0-200 µM) 5-fluorouracil and Mitomycin C (0-25 µM)	Made in-house	Western blot, qPCR	Mai et al., 2012
<i>P. lucida</i> Desert topminnow	**PLHC-1: hepatoma		Santa Cruz Biotechnology: #4246	Western blot	Embry et al., 2006

\*No basal expression of p53 was observed in this cell or tissue

\*\*No induction of p53 was observed following exposure

Evidently, this shows that p53 activity differs between fish species, though it is possible that this phenomenon is specific to the cell lines under study. Species-specific differences in p53 regulation have been documented. For example, when Mdm2 was overexpressed in *Xenopus laevis* (African clawed frog), tumour formation occurred (Wallingford et al., 1997), whereas apoptosis was robustly triggered in zebrafish (Neel et al., 2000), suggesting fundamentally different functions of Mdm2-mediated negative regulation between these species. Further studies have shown that zebrafish p53 induces p21<sup>Waf1/Cip1</sup> similarly to mammals (Langheinrich et al., 2002).

High-throughput techniques have also made it possible to identify potential p53-responsive genes within zebrafish, and recently seven new genes were discovered to be regulated by p53 (Mandriani et al., 2016). With regards to rainbow trout, p53 has been cloned and characterized (Liu et al., 2011), but such high-throughput studies have yet to be performed. It should be noted that at this moment, the development of antibodies targeting post-translational modifications of fish p53 are more-or-less non-existent, and have to be developed in-house. The only exception is zebrafish, which is a popular model organism; however, these antibodies sometimes are not cross-reactive with other fish species such as rainbow trout. As such, assays of the protein are confined to global expression of p53 itself, which is only indicative of stability and expression. As mentioned in the previous sections, a much higher resolution explanation of p53 activity is given by detecting post-translational modifications, rather than just protein levels. There have also been studies that assay the mutagenic properties of compounds on the *TP53* gene itself in aquatic species including flounder (Sueiro et al., 2000) and medaka (Krause et al., 1997), revealing insight as to what

functions of p53 might be differentially altered in aquatic species based on the roles of affected motifs.

Taken together, the analysis of p53 expression, mutation, modification, and regulation (including regulation of p53 itself) will be important in the future for identifying functional similarity of the DDR between fish and mammalian species, and will help to generate novel biomarkers of genotoxicity in fish. These markers will not only allow us to draw parallels between p53 activity in mammals and fish, but will also address any differences in genotoxic response across different aquatic species. Of course, this will allow for the generation of more specific diagnostic tools (i.e. probing for type-specific signalling events in response to DNA damage), as well as resolve any erroneous assumptions made regarding p53-involved DDR mechanics by extrapolating from mammalian literature.

## 1.6 Research Objectives

The objectives of this thesis were two-fold:

- 1) To identify a fish cell line with robust biomarker activation for experimentation with genotoxicants and;
- 2) To characterize the induction of  $\gamma$ H2AX by the candidate in response to model DNA damaging agents.

Taken together, the overall goal of this thesis was to characterize checkpoint biomarkers in fish that could perhaps be used in an *in vitro*, biomarker-based assay for environmental genotoxicity testing.

## CHAPTER TWO: MATERIALS AND METHODS

### 2.1 Genotoxicants and Chemicals

Biomarker testing was performed using methyl methanesulfonate (MMS; CAS 66-27-3, #129925), 4-Nitroquinoline *N*-oxide (4-NQO; CAS 56-57-5, #N8141), glyphosate (GLY; CAS 1071-83-6, #45521), 2-mercaptobenzothiazole (2-MBTH; CAS 149-30-4, #M3302), benzo[*a*]pyrene (B[*a*]P; CAS 50-32-8, #B1760), hydroxyurea (HU; CAS 127-07-1, #H8627) and bleocin (proprietary name for bleomycin, referred to as BLEO; CAS 55658-47-4, #203408-M) which were all purchased from Sigma-Aldrich. 4-NQO, GLY, and B[*a*]P were dissolved in dimethyl sulfoxide (DMSO; CAS 67-68-5, #472301) from Sigma-Aldrich for dosing. The final concentration of DMSO was 0.5% v/v and was not cytotoxic on its own to cells (Schnell et al., 2009). BLEO, MMS, and 2-MBTH were dissolved in ultrapure deionized water for dosing, where the solvent never exceeded 1% of total exposure volume. These solvents were used as mock-treated negative controls for exposure of the respective chemicals they were used to dissolve. Nutlin-3 (N3; CAS 548472-68-0, #N6287), an Mdm2-inhibitor, was used in **Appendix B** (Sigma-Aldrich).

### 2.2 Cell Lines and Culturing

Adherent fish cell lines that were tested for biomarker induction include intestinal myofibroblasts from Atlantic salmon, *Salmo salar* (Asimf20: Kawano et al., 2010); neuroglial brain cells from American eel, *Anguilla anguilla* (EelB: Bloch et al., 2015; Wagg & Lee, 2005); astroglial-like brain cells from walleye, *Sander vitreus* (WEB5: unpublished), blastula-stage embryonic cells from zebrafish, *Danio rerio* (ZEB2J: Xing et al., 2008); and glial brain cells from lake sturgeon, *Acipenser fulvescens* (SB3: unpublished). Those from

rainbow trout (*Oncorhynchus mykiss*) include intestinal endothelial cells (RTgutGC: A. Kawano et al., 2011), gill epithelial cells (RTgill-W1: Bols et al., 1994), liver epithelial cells (RTLiver-W1: Lee et al., 1993), and glial brain cells (RTbrain-W1: Steinmoeller et al., 2009). RTbrain-W1 has seen limited use in the literature (Fischer et al., 2011; Liu et al., 2011; Lončar et al., 2010; Steinmoeller et al., 2009; Vo et al., 2015). Cells were routinely grown in 75 cm<sup>2</sup> (T75) polystyrene tissue culture flasks (BioLite, Thermo Fisher Scientific) at room temperature (RT; 20 ± 2°C) in L-15 basal medium (HyClone, GE Healthcare) supplemented with fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific) and a 1% penicillin-streptomycin cocktail (P/S; HyClone, GE Healthcare). During routine culturing and seeding of multiplates, RTbrain-W1 cells were supplemented with 15% FBS while all other cell lines were supplemented with 10% FBS. During exposure, all cell lines were exposed in L-15 with 10% FBS and 1% P/S. Routine passaging and seeding of multiwell plates was performed using 0.25% v/v trypsin (HyClone, GE Healthcare) diluted in Dulbecco's phosphate-buffered saline (DPBS; from HyClone, GE Healthcare), and RTB cells were used between their 5<sup>th</sup> and 25<sup>th</sup> passages.

### 2.3 Cytotoxicity Assays

Cytotoxicity was evaluated as described by (Dayeh et al., 2005) using two fluorescent indicator dyes: Alamar Blue (AB; Invitrogen) and 5-carboxyfluorescein diacetate (CFDA-AM; Sigma-Aldrich, CAS 124412-00-6). AB was used to monitor metabolism of cells in 96 well plates using a protocol that has been described previously in a step-by-step fashion (Dayeh et al., 2005, 2013). The AB stock solution comes in water, and was diluted in DPBS to form a working solution of 5% v/v. The CFDA-AM was ordered in crystalline form from Life Technologies (Thermo Fisher Scientific) and solubilized in DMSO to form a 4 mM stock.

Once evenly mixed, the concentrated CFDA-AM was added to the working solution containing AB to a working concentration of 4  $\mu\text{M}$ . Both AB and CFDA-AM can be applied individually as outlined previously (Ganassin & Bols, 2000). However, in all experiments AB was combined with CFDA-AM so that the two different viability endpoints were simultaneously measurable on the same culture wells, as performed previously (Schirmer et al., 1997).

To prepare experiments, 200  $\mu\text{L}$  of cells in growth medium were seeded on a 96-well multiplate (BioLite, Fisher Scientific) at a density of  $4 \times 10^4$  cells per well. All wells were inoculated except the top row, which was to be kept void of cells as a zero during spectrophotometry for media containing genotoxicants. Plates were sealed in Parafilm and incubated in the dark at RT for 24h. Once a dilution series of the genotoxicant to be tested was made and ready to be administered, the growth medium was aspirated and cells were treated with 200  $\mu\text{L}$  of the genotoxicant dilution in described exposure medium, resealed, and incubated for 24h in the dark at RT. The bottom row of the plate was not dosed, but rather left in original growth medium as an internal control to ensure that the mechanical effect of dosing had no significant impact on viability. Thus, 6 wells from each column were subjected to genotoxic challenge, with two columns set aside for a mock-treatment solvent control and a positive control for loss of viability, which was 0.05% v/v MMS. The remaining 10 columns were challenged with various concentrations of the genotoxicant. Following 24h, the entire plate was aspirated, gently washed twice with DPBS and incubated with 100  $\mu\text{L}$  of the working solution for 1h at RT in the dark. Relative fluorescent units (RFUs) of each well were then measured with a fluorescence multiplate reader, the CytoFluor 4000 (PerSeptive Biosystems). RFUs from the bottom row (untreated viability control) were used for the experimenter's reference only, and were not included in calculations. The top row was

averaged and subtracted from the mean of each treatment column in order to correct for background absorbance. Mean values of the 10 experimentally treated columns were corrected and normalized to the mean corrected mock-treated control values.

## 2.4 Protein Extract Preparation

To prepare experiments, 1 mL of cells in growth medium was seeded at a density of  $9 \times 10^4$  cells/mL in each well of a 6-well multiplate (BioLite, Thermo Fisher Scientific). The plate was sealed with Parafilm and cells were allowed to adhere for 24h at RT in the dark. After adherence, a dilution series of the genotoxicant to be tested was prepared in exposure medium, including a mock-treated solvent control. The growth media was aspirated, the wells were dosed, and the plate was resealed and incubated for 24h at RT in the dark. Following exposure, cells were washed once with DPBS and scraped off the surface of the 6-well multiplate. The cells were transferred to 1.5 mL microcentrifuge tubes and centrifuged briefly at 4°C to remove the supernatant, additionally the pellet was lysed in ice-cold modified radioimmunoprecipitate assay (RIPA) buffer (50mM HEPES-KOH, pH 7.5, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate) supplemented with Halt! (Roche) protease inhibitor. Cell debris was separated via centrifugation (21 x g) at 4°C and protein concentration of each lysate supernatant was assayed by a bicinchoninic acid (BCA) kit (Pierce) using a VICTOR X3 1420 multilabel plate reader (PerkinElmer Inc.). A standard curve using bovine serum albumin was prepared in parallel for each experiment and used as a reference for quantitation.



## 2.5 Electrophoresis and Western Blotting

Protein extracts were prepared for electrophoresis by addition of Laemmli buffer (2% SDS, 10% glycerol, 60 mM Tris-HCl pH 6.8, 0.01% bromophenol blue) with  $\beta$ -mercaptoethanol (0.1%) and boiled for 10 min in a 95°C hot plate. Extracts were immediately immersed in ice or frozen at -80°C for storage. If proceeding, 50  $\mu$ g aliquots of each sample were loaded into and resolved by 12% polyacrylamide gels via SDS-PAGE using a Mini-Protean™ electrophoretic apparatus (Bio-Rad) using standard running buffer (192 mM glycine, 0.1% SDS, 24.8 mM Tris base, pH 8.3). Immediately after, extracts were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad) for 2h or overnight at 4°C with stirring in an Owl™ wet transfer apparatus (Thermo Fisher Scientific) using Towbin buffer (192 mM glycine, 8.25 mM Tris base, 20% MeOH, pH 8.6) until proteins of high molecular weight had fully transferred. Resultant blots were stained for extract quality and equal loading by Ponceau stain from Sigma-Aldrich (0.1% w/v Ponceau S, 5% glacial acetic acid v/v). After 3 rinses in dH<sub>2</sub>O to remove excess stain, blots were imaged using an Epson Perfection V600 scanner. Blots were destained in tris-buffered saline with Tween-20 (TBS-T; 136mM NaCl, 2.68mM KCl, 24.8mM Tris base, 0.1% Tween-20) and blocked with TBS-T containing 5% bovine serum albumin (BSA; Sigma-Aldrich) for 1h at RT with shaking or overnight at 4°C without shaking. Blots were then sequentially incubated with rabbit anti- $\beta$ -actin polyclonal antibody (A2066, Sigma-Aldrich) at a 1:1000 dilution in TBS-T with 5% BSA for 1h at RT with shaking. After washing with TBS-T, blots were incubated with rabbit anti- $\gamma$ H2AX monoclonal antibody (Cell Signaling Technology, #9718) at a 1:1000 dilution in TBS-T with 5% BSA for 1h at RT with shaking. The antibody specifically recognizes phosphorylated Ser139 of the H2AX C' terminus, and not the unphosphorylated

residue. After washing with TBS-T, blots were incubated with rabbit anti-p53 polyclonal antibody as described and generated previously (Liu et al., 2011) at a 1:1000 dilution in TBS-T with 5% BSA overnight at 4°C for experiments shown in **Appendix B**. After washing with TBS-T, blots were incubated with a goat anti-rabbit secondary IgG antibody conjugated to horseradish peroxidase (HRP) (Bio-Rad) at a 1:5000 dilution in TBS-T with 5% skim milk for 1h at RT with shaking. Blots were washed in TBS-T and developed for 5 min in the dark with Clarity™ ECL substrate (Bio-Rad) and imaged using a ChemiDoc™ MP imaging system (Bio-Rad).

## 2.6 Densitometry

Densitometry and post-image processing was performed using ImageLab™ software (Bio-Rad). This software allows the densitometric analysis of raw data directly from the ChemiDoc™ MP and does not allow the user to manipulate images prior to analysis. However, one is able to superficially alter the contrast, brightness, and other various settings of images after densitometry without affecting values. Nevertheless, the individual intensities of p53 expression and  $\gamma$ H2AX induction were normalized to  $\beta$ -actin expression in each lane. The ratios of each treatment concentration were then normalized to mock-treated solvent control values and expressed as a fold-induction.

## 2.7 Statistical Analysis

For cytotoxicity assays, each treatment was performed in two independent trials (n=2). The mean cell viability at each dosage was averaged across both trials, and plotted. Error bars represent standard deviation from the mean. For densitometry, normalized  $\gamma$ H2AX

levels were averaged at each dosage and the Student's paired t-test (n=2) was used to calculate significance.

## 2.8 Immunocytochemistry

To examine the occurrence of  $\gamma$ H2AX foci in response to DNA damage, confocal microscopy was employed. Cells were suspended in growth medium at a density of  $1 \times 10^6$  cells/mL and 200  $\mu$ L of the suspension was seeded into each well of an 8-well Millicell™ EZ glass slide (EMD Millipore, #PEZGS0816) and sealed with Parafilm. The cells were allowed to adhere in the dark at RT. After 24 h, one well (mock-treated solvent control) was dosed only with solvent (either DMSO or H<sub>2</sub>O); a second well (positive control) received 1 mM H<sub>2</sub>O<sub>2</sub>, (Sigma-Aldrich) for the generation of the expected foci; and a third well (2<sup>nd</sup> antibody control) was set aside for no subsequent primary antibody treatment to account for any background binding of the 2<sup>nd</sup> antibody. The remaining wells (test wells) were dosed with the potential genotoxicant over a range of concentrations. The slide was resealed with Parafilm and incubated at RT in the dark for 24h after which the medium in the wells was removed by gentle aspiration. The cells were gently rinsed twice with cold DPBS and fixed with ice-cold MeOH at -20°C for 20 min. The fixative was aspirated and the cells permeabilized with 0.1% v/v Triton X-100 diluted in DPBS for 10 min at RT. Each well then received blocking buffer (3% v/v BSA, 10% v/v goat serum, 0.1% v/v Triton X-100; in DPBS). The slide was incubated at RT with shaking and after 1h the blocking buffer was aspirated off. The rabbit anti- $\gamma$ H2AX monoclonal primary antibody at a concentration of 1:1000 in the described blocking buffer was added to all wells except for 2<sup>nd</sup> antibody control. Following 1h incubation at RT with shaking, the wells were washed twice with DPBS and the 2<sup>nd</sup> antibody, goat anti-rabbit AlexaFluor® 488 (Thermo Fisher Scientific) at a concentration of 1:1000 in

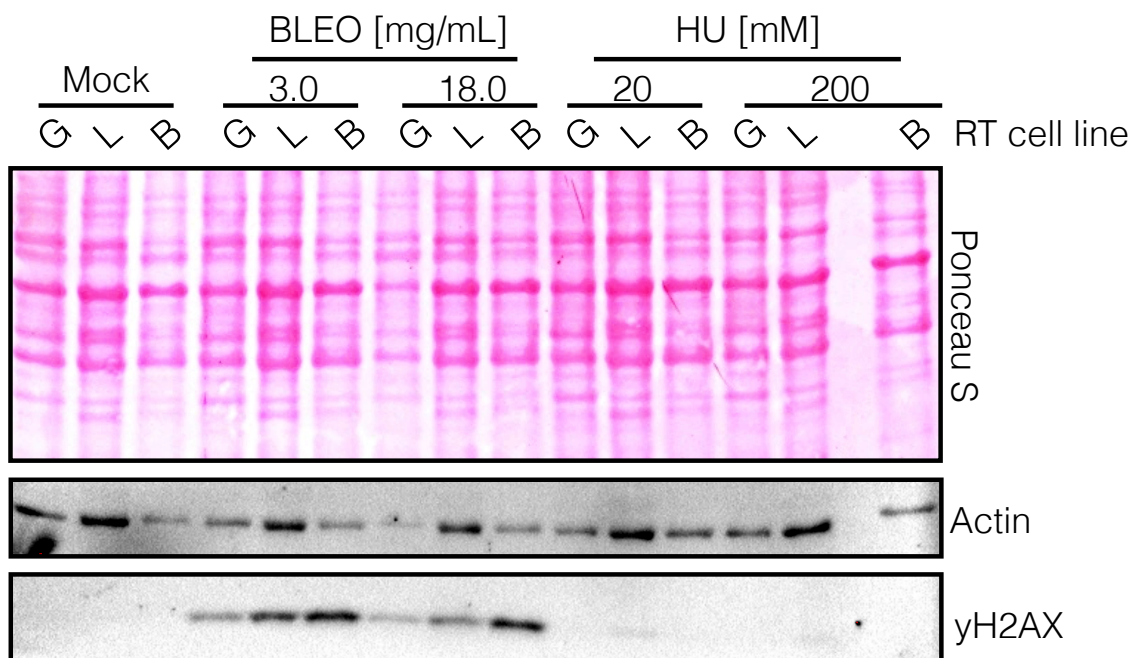
DPBS, was applied to all wells. The slide was incubated at RT with shaking in the dark for 1h prior to removal of unbound antibody by aspiration. The wells were rinsed twice with DPBS, followed by the addition of Fluoroshield™ mounting medium (Thermo Fisher Scientific), which contains the nuclear stain DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride). After the addition of a coverslip (Fisher Scientific), the slide was left at RT in the dark for 24h, and then examined with a Carl Zeiss LSM 700 confocal microscope. Post-image processing was performed using the supplied software, ZEN 2011.

## CHAPTER THREE: RESULTS

### 3.1 Comparison of H2AX Phosphorylation Across Fish Cell Lines

#### 3.1.1 H2AX Phosphorylation in Rainbow Trout Cell Lines

The first step for characterizing H2AX phosphorylation as a biomarker of genotoxicity was to identify a candidate cell line with robust  $\gamma$ H2AX formation in response to DNA damaging agents, and to examine differences in these responses across different piscine cell lines. Preliminary screens were performed in rainbow trout cell lines using two



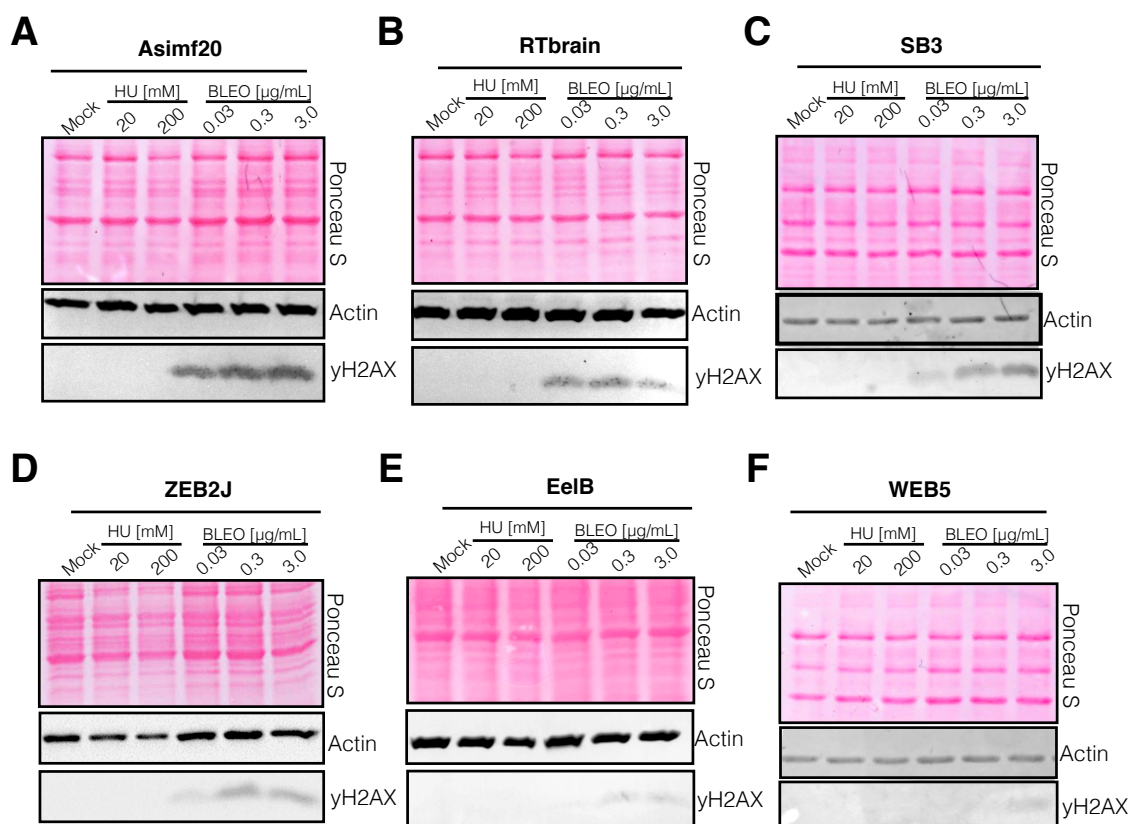
**Figure 3.1: Comparison of  $\gamma$ H2AX levels in Bleomycin- and HU-treated Rainbow Trout Cell Lines**

Cell lines were exposed to different concentrations of bleomycin and HU and analyzed for  $\gamma$ H2AX induction after a 24h exposure. Protein extracts were subjected to SDS-PAGE electrophoresis and western blotting. The letters **G**, **L**, and **B** correspond to RTgill-W1, RTliver-W1, and RTbrain-W1 respectively. Ponceau S staining of protein extracts is shown at the top of the figures followed by detection of actin and  $\gamma$ H2AX. The primary antibody for actin was a rabbit anti- $\beta$ -actin polyclonal antibody (A2066, Sigma) and for  $\gamma$ H2AX, the primary antibody was a rabbit anti- $\gamma$ H2AX monoclonal antibody (9718, Cell Signal). The secondary antibody was an HRP-conjugated goat anti-rabbit IgG.

model genotoxicants: bleomycin, a radiomimetic compound that intercalates DNA and generates DSBs in an iron- and oxygen-dependent manner (Giloni et al., 1981; Lown & Sim, 1977), and hydroxyurea (HU), a ribonucleotide reductase inhibitor that results in the depletion of cellular dNTP pools and subsequent replication fork collapse (Koç et al., 2004). Three commonly studied rainbow trout cell lines in the Bols laboratory were chosen including RTbrain-W1, RTgill-W1, and RTliver-W1, which were treated with bleomycin (3, 18 mg/mL) and HU (20, 200 mM) for 24h and analyzed for H2AX phosphorylation by western blot (**Figure 3.1**). The concentrations of bleomycin (Steinmoeller et al., 2009) and HU (Liu et al., 2011) were used previously in RTbrain-W1 and RTgill-W1 cells, and did not appear to alter cell morphology under the phase contrast microscope (data not shown). RTbrain-W1 appeared to have substantially higher  $\gamma$ H2AX levels after bleomycin treatment compared to RTliver-W1 and RTgill-W1, the latter showing the lowest  $\gamma$ H2AX levels in response. Interestingly, only RTliver-W1 showed an increase in H2AX phosphorylation following HU treatment, though this was subtle and could have been due to greater loading.

### *3.1.2 H2AX Phosphorylation in Other Fish Cell Lines*

Next, we compared RTbrain-W1 biomarker induction to several different fish cell lines in the Bols laboratory. Levels of  $\gamma$ H2AX were assayed via western blotting in response to 24h exposures of bleomycin (0.03, 0.3, 3.0 mg/mL), HU (20 mM, 200 mM), and MMS (0.015%, 0.03%), the latter being a mutagen that acts by forming monoalkyl methyl adducts with DNA, which must be removed by BER and NER (Lindahl & Wood, 1999). If left unrepaired, these single-stranded lesions will degrade into DSBs that must be repaired by HR. It is also a reported clastogen that causes detectable micronuclei and genotoxicity via the comet assay (Tao et al., 1993).



**Figure 3.2: Comparison of  $\gamma$ H2AX levels in Bleomycin- and HU-treated Fish Cell Lines**

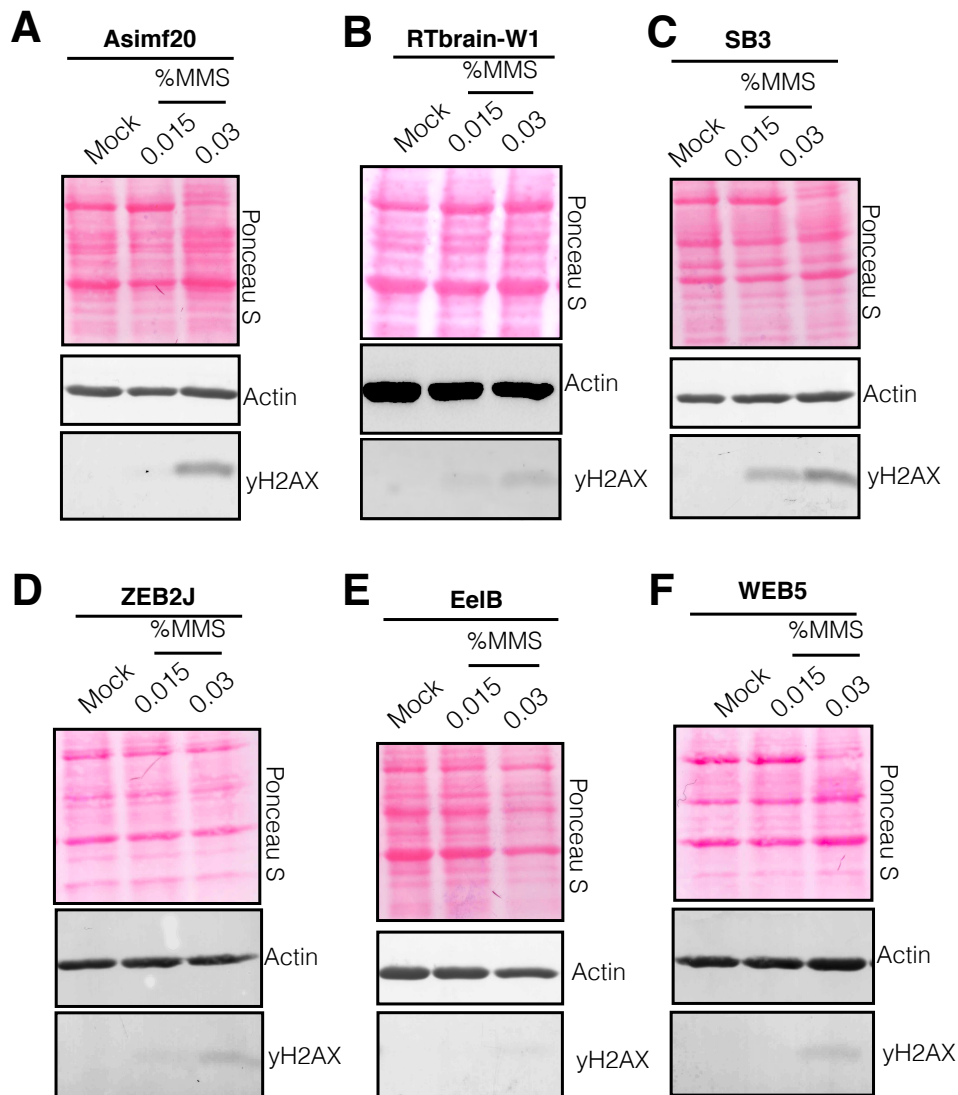
Cell lines were exposed to different concentrations of bleomycin and HU and analyzed for  $\gamma$ H2AX induction after a 24h exposure. Protein extracts were subjected to SDS-PAGE electrophoresis and western blotting. Ponceau S staining of protein extracts is shown at the top of the figures followed by detection of actin and  $\gamma$ H2AX. The primary antibody for actin was a rabbit anti- $\beta$ -actin polyclonal antibody (A2066, Sigma) and for  $\gamma$ H2AX, the primary antibody was a rabbit anti- $\gamma$ H2AX monoclonal antibody (9718, Cell Signal). The secondary antibody was an AP-conjugated goat anti-rabbit IgG. Panels A & B, D & E, and C & F are pairs from the same blot.

Results of bleomycin and HU exposure are presented in **Figure 3.2**, and results of MMS exposure are presented in **Figure 3.3**. No cell lines showed any response to HU treatment. After exposure to bleomycin and MMS, however, all cell lines showed varying levels of induction. Three cell lines emerged as particularly sensitive amongst the group: Asimf20, RTbrain-W1, and SB3. SB3 and Asimf20 appeared to show a positive dose-response to bleomycin, with Asimf20 being the most robust in response compared to all cell

lines tested (**Figure 3.2A**). SB3 showed modest induction at 0.03 mg/mL, with a typical-looking increase in dose-response through 0.3 and 3.0 mg/mL concentrations (**Figure 3.2C**). The response of RTbrain-W1 to bleomycin (**Figure 3.2B**) was less than Asimf20 but comparable to SB3, and a decrease was observed at the highest concentration, similar to observations from **Figure 3.2A**, though it should be noted that the concentrations are much higher. ZEB2J (**Figure 3.2D**), EelB (**Figure 3.2E**), and WEB5 (**Figure 3.2F**) showed comparatively less induction of  $\gamma$ H2AX at the concentrations of bleomycin tested.

When looking at MMS-mediated  $\gamma$ H2AX formation, SB3 was noticeably more sensitive at the concentrations tested than the other cell lines (**Figure 3.3C**). Asimf20 showed a comparable response at the highest concentration, 0.03% (**Figure 3.3A**). RTbrain-W1 showed slight induction in a positive dose-response to these concentrations (**Figure 3.3B**), though not as strong as Asimf20 and SB3. However, unlike Asimf20, it showed sensitivity at a medium concentration (0.015%), like SB3. All cell lines displayed moderate cell death at this medium concentration, and were almost completely dead at 0.03% as shown by phase contrast microscopy (data not shown). Similar to **Figure 3.2**, ZEB2J (**Figure 3.3D**), EelB (**Figure 3.3E**), and WEB5 (**Figure 3.3F**) showed induction to a lesser extent, although ZEB2J did show a weak induction of  $\gamma$ H2AX at 0.015%, like RTbrain-W1. Interestingly, RTgutGC was also treated with the same concentrations of bleomycin, HU, and MMS, and no  $\gamma$ H2AX was observed (data not shown).





**Figure 3.3: Comparison of  $\gamma$ H2AX levels in MMS-treated Fish Cell Lines**

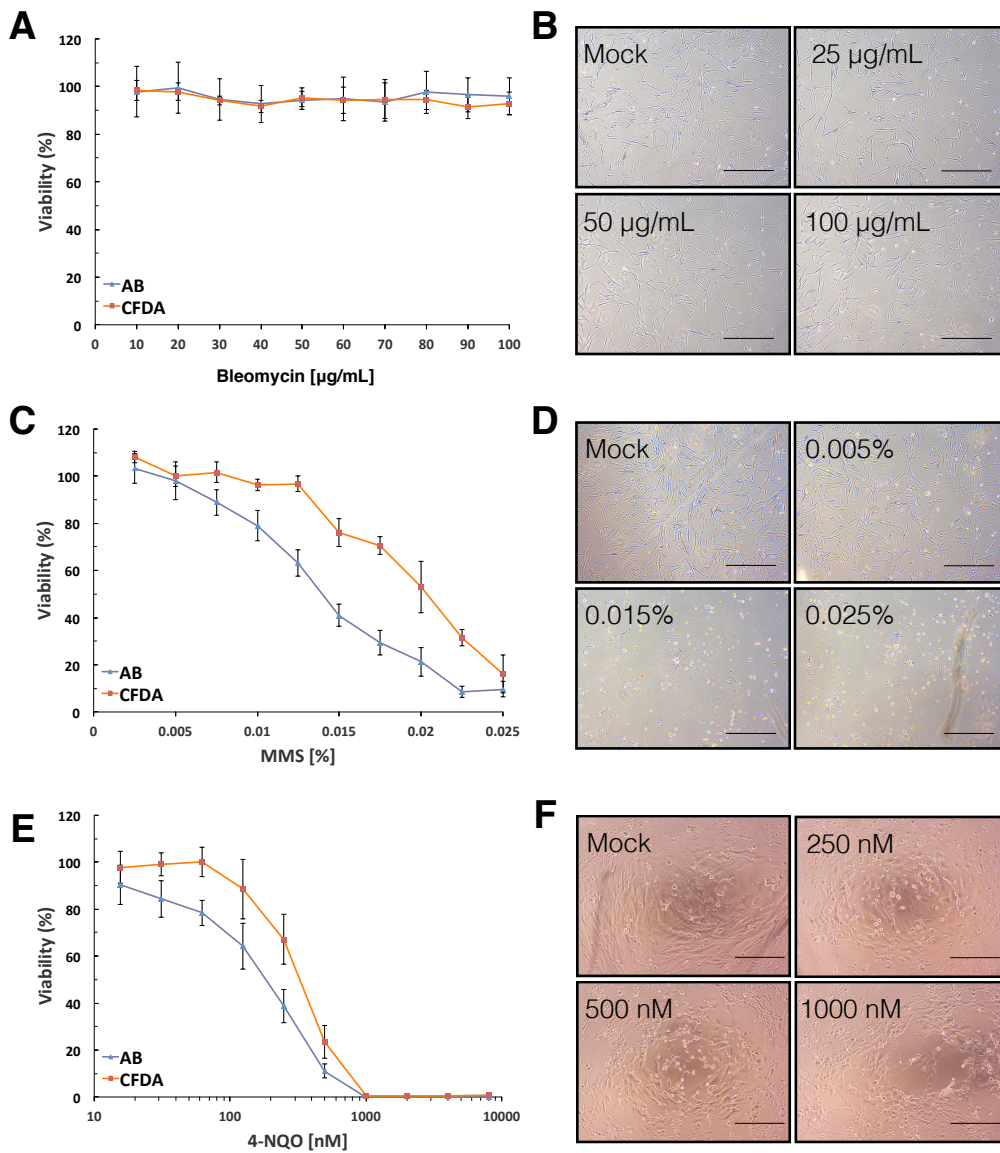
Cell lines were exposed to different concentrations of MMS and analyzed for  $\gamma$ H2AX induction after a 24h exposure. Protein extracts were subjected to SDS-PAGE electrophoresis and western blotting. Ponceau S staining of protein extracts is shown at the top of the figures followed by detection of actin and  $\gamma$ H2AX. The primary antibody for actin was a rabbit anti- $\beta$ -actin polyclonal antibody (A2066, Sigma) and for  $\gamma$ H2AX, the primary antibody was a rabbit anti- $\gamma$ H2AX monoclonal antibody (9718, Cell Signal). The secondary antibody was an AP-conjugated goat anti-rabbit IgG. Panels A, C, and E, and B, D, and F are from the same blots

Though it was not the most sensitive in either trial (Asimf20 and SB3 were more sensitive to bleomycin and MMS), it was comparably sensitive amongst the two, and more sensitive than ZEB2J, EelB, and WEB5. A very large reason for using RTbrain-W1 was due to the production of an anti-p53 antibody specific to rainbow trout, and the ability to examine both p53 and  $\gamma$ H2AX could not be done with other non-rainbow trout cell lines (refer to **Appendix B**) In addition, RTbrain-W1 is easiest to culture compared to the other cell lines tested, and grows to a high density. On the basis of these results, RTbrain-W1 was chosen as the candidate cell line to further study the dynamics of  $\gamma$ H2AX formation.

## 3.2 Characterization of the $\gamma$ H2AX Response in RTbrain-W1

### 3.2.1 Cytotoxicity of Bleomycin, MMS, and 4-NQO

Alamar Blue and CFDA-AM were used to measure viability in response to a broad range of concentrations of each genotoxicant after a 24h exposure. There was no significant loss in cell viability over all tested concentrations of bleomycin as shown in **Figure 3.4A**, and cell morphology appeared unchanged as depicted by phase contrast microscopy in **Figure 3.4B**. In contrast, MMS exposure caused a loss of metabolic activity in cells in a dose-dependent fashion as indicated by Alamar Blue starting at the second-lowest dose of 0.005%, while CFDA-AM indicated first signs of membrane degradation at 0.0125% MMS (**Figure 3.4C**). Indeed, phase contrast microscopy of cells treated with MMS (**Figure 3.4D**) reveals symptoms of cellular stress including membrane shrinking, detachment of cells from substrate and one another, and membrane lysis. The cytotoxic effects of the final genotoxicant we tested, 4-nitroquinoline-*N*-oxide (4-NQO), are shown in **Figure 3.4E**. 4-NQO was included in the following studies as an additional way of forming DSBs. Like MMS, it is a mutagen that acts instead by forming quinoline adducts with DNA, which are



**Figure 3.4: Effect of Bleomycin, MMS, and 4-NQO on the Viability and Morphology of RTbrain-W1 Cells**

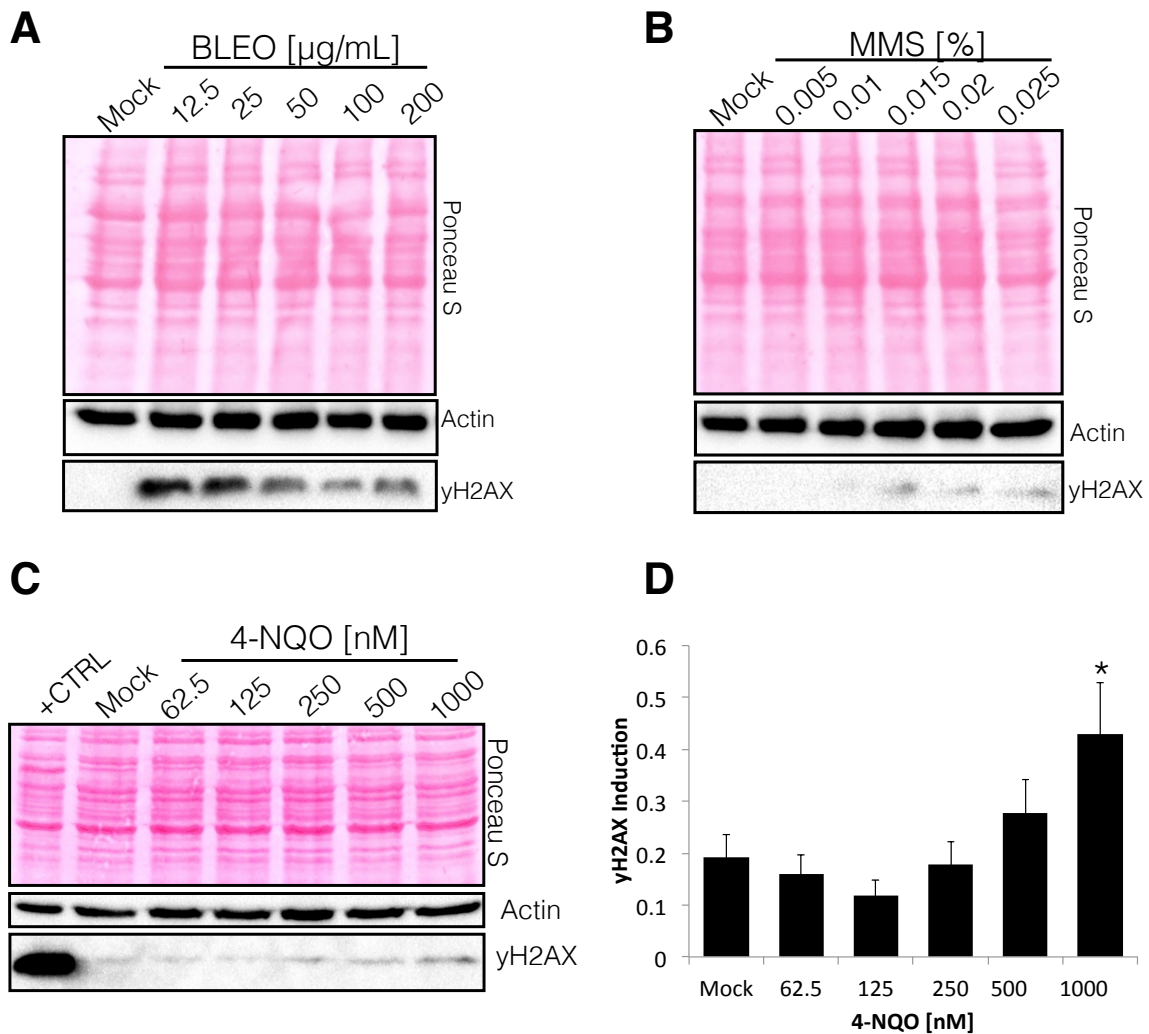
Cell viability was evaluated with Alamar Blue (AB) and 5-carboxyfluorescein diacetate (CFDA-AM) 24h after cultures had been exposed to increasing concentrations of bleomycin (A), MMS (C), and 4-NQO (E) and expressed relative to control cultures (solvent only). Each data point represents the mean with standard deviation (n=2). Phase contrast micrographs illustrate the appearance of cells 24h after exposure to bleomycin (B), MMS (D), and 4-NQO (F). Images taken at 160X magnification. Scale bar represents 500 µm.

also removed by BER, NER (Lindahl & Wood, 1999), and if left unrepaired, HR. Despite a complete loss in cellular viability at 1000 nM, cells appeared considerably healthier under the phase contrast microscope (**Figure 3.4F**). In addition, similar to MMS cytotoxicity depicted in **Figure 3.4C**, a loss in metabolic activity was observed before membrane esterase activity as shown by Alamar Blue, suggesting that MMS and NQO may exhibit a similar mechanism of cytotoxicity.

The results of these cytotoxicity assays identified both lethal and sublethal concentrations of bleomycin, MMS, and 4-NQO that we used in order to examine the dynamics of H2AX phosphorylation in RTbrain-W1.

### *3.2.2 H2AX Phosphorylation in Response to Bleomycin, MMS, and 4-NQO Exposure as Examined by Western Blotting*

All three genotoxicants induced  $\gamma$ H2AX formation after a 24h exposure with varying intensities compared to controls (Figure 3.5). Concentrations within the ranges of the cytotoxicity assays were tested for bleomycin (12.5, 25, 50, 100, 200  $\mu$ g/mL), MMS (0.005, 0.01, 0.015, 0.02, 0.025%), and 4-NQO (62.5, 125, 250, 500, 1000 nM). Bleomycin caused the most robust H2AX phosphorylation of the three drugs at the lowest tested concentration, 12.5  $\mu$ g/mL, and decreasing  $\gamma$ H2AX levels were observed with increasing bleomycin concentrations up to 100  $\mu$ g/mL as shown in Figure 3.5A. MMS exposure caused a small increase in  $\gamma$ H2AX formation that was first detectable at 0.01%, increasing at 0.015%, and remaining about the same through to 0.025% (Figure 3.5B). Finally, 4-NQO showed a subtle but detectable increase in H2AX phosphorylation at 1000 nM, the highest concentration (**Figure 3.5C**). Densitometry was used to normalize the relative signal of  $\gamma$ H2AX to actin, showing a 2.0x increase in  $\gamma$ H2AX (**Figure 3.5D**).  $\beta$ -actin was used as a housekeeping



**Figure 3.5: Effect of Increasing Concentrations of Bleomycin, MMS, and 4-NQO on  $\gamma\text{H2AX}$  Levels in RTbrain-W1 Cells**

RTbrain-W1 cultures were exposed to bleomycin (A), MMS (B), or 4-NQO (C) for 24h and subjected to SDS-PAGE electrophoresis and western blotting. Ponceau S staining of protein extracts is shown at the top of the figures followed by detection of actin and  $\gamma\text{H2AX}$ . The primary antibody for actin was a rabbit anti- $\beta$ -actin polyclonal antibody (A2066, Sigma) and for  $\gamma\text{H2AX}$ , the primary antibody was a rabbit anti- $\gamma\text{H2AX}$  monoclonal antibody (9718, Cell Signal). The secondary antibody was an HRP-conjugated goat anti-rabbit IgG. Positive control in (C) corresponds to RTbrain-W1 cells treated with 100  $\mu\text{g/mL}$  bleomycin for 24h. (D) Densitometric analysis of  $\gamma\text{H2AX}$  levels were normalized to actin expression using ImageLab. Bars represent mean normalized  $\gamma\text{H2AX}$  induction across both trials. Error bars represent standard deviation across both trials. (\*) indicates significance using the paired Student's t-test ( $p < 0.05$ )

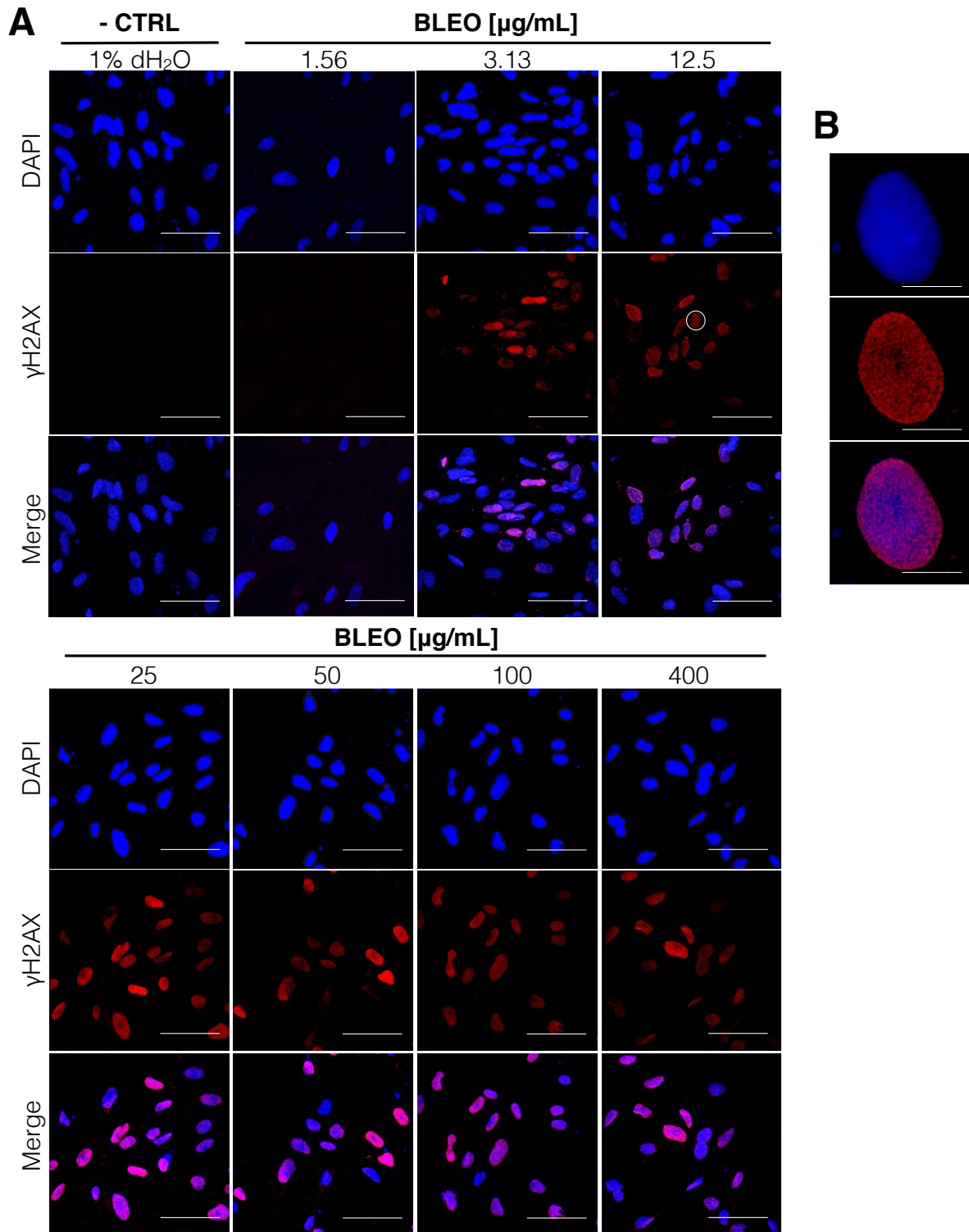
protein to normalize  $\gamma$ H2AX induction because there are no instances in the literature where bleomycin, MMS, and 4-NQO affect  $\beta$ -actin expression. As shown by the representative microscopic images in **Figure 3.4B, D, & F**, bleomycin, MMS, and 4-NQO had contrasting morphological effects on cells after treatment. Bleomycin-treated cells looked healthy at all concentrations and MMS-treated cells showed a loss of viability around 0.015%. Bleomycin treatment showed a dose-dependent decrease in  $\gamma$ H2AX formation at sublethal concentrations, which was opposite to the increases in  $\gamma$ H2AX observed in response to MMS and 4-NQO. It should be noted that after 24h exposure, cells at the two highest MMS concentrations were almost entirely detached from the plate prior to protein collection. This could possibly explain why  $\gamma$ H2AX induction ceased, presumably since most cells had died and were no longer actively phosphorylating H2AX.

$\gamma$ H2AX was inducible by bleomycin at all sublethal concentrations, however, densitometry confirmed a lack of detectable induction in cells treated with 0.005% MMS (data not shown). At this concentration, cells had shown complete viability according to the cytotoxicity assay shown in **Figure 3.4C**, suggesting that with exposure to a monoalkylating agent such as MMS, sublethal  $\gamma$ H2AX induction does not occur in RTbrain-W1, at least after an acute 24h exposure. However, at the semi-lethal concentration of 0.01%, MMS induced a detectable increase in H2AX phosphorylation. Similarly with 4-NQO exposure shown in **Figure 3.5C**, there was an increase in  $\gamma$ H2AX induction at semi-lethal and lethal concentrations, evident with 500 nM and 1000 nM concentrations, respectively.

### *3.2.3 Effects of Bleomycin and MMS Exposure on $\gamma$ H2AX Foci as Examined by Confocal Microscopy*

Next, we wanted to visualize the formation of  $\gamma$ H2AX foci in response to genotoxicity using confocal microscopy. This allows one to directly visualize the formation of foci within individual cells of a population, as well as observe the spatial distribution of response across a population of cells. As such, we tested bleomycin and MMS concentrations within the ranges previously tested in **Figures 3.4** and **3.5**. Differences in  $\gamma$ H2AX localization appeared in RTbrain-W1 as shown in **Figures 3.6** and **3.7**.  $\gamma$ H2AX signal confined to the nucleus was observed in bleomycin-treated cells as low as 3.125  $\mu$ g/mL and persisted throughout exposure to the maximum tested concentration, 400  $\mu$ g/mL (**Figure 3.6A**). In response to MMS,  $\gamma$ H2AX signal was observed in the cytosol of cells. A very low level of  $\gamma$ H2AX formation was seen at 0.005% compared to the control and an increase was seen in 0.015%, appearing roughly the same in 0.025% treated cells (**Figure 3.7**).

In cultures treated with 3.13  $\mu$ g/mL bleomycin, most cells expressed above-background but relatively low  $\gamma$ H2AX signal. A small population of cells showed more intense  $\gamma$ H2AX signal than their neighbours and this population intensified and increased up to 25  $\mu$ g/mL. In 50  $\mu$ g/mL-treated cells and onwards, the majority of cells showed higher levels of  $\gamma$ H2AX induction in a more uniform manner from cell to cell, and those individuals with significantly more  $\gamma$ H2AX induction were less. The lowest tested bleomycin concentration in this experiment, 1.56  $\mu$ g/mL, did not show any induction. The dose-response shown by confocal microscopy somewhat coincides with the dose-dependent decrease as seen in **Figure 3.5A** with regards to  $\gamma$ H2AX signal, though the trend is not nearly as striking,



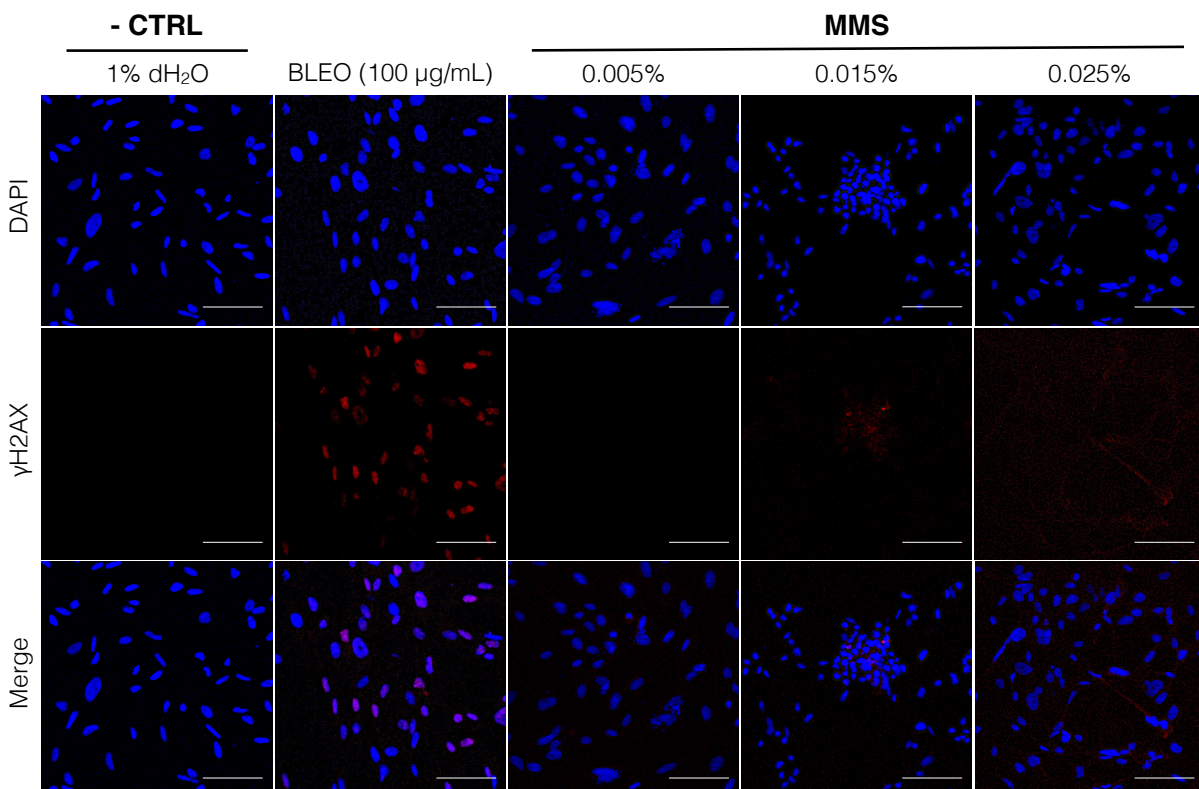
**Figure 3.6: Effect of Increasing Bleomycin Concentrations on  $\gamma$ H2AX Localization in RTbrain-W1 Cells**

(A) RTbrain-W1 cultures were exposed to various concentrations of bleomycin for 24h and visualized for  $\gamma$ H2AX localization in the nuclei of cells. The primary antibody for  $\gamma$ H2AX was a rabbit anti- $\gamma$ H2AX monoclonal antibody (9718, Cell Signal), and nuclei were counterstained with DAPI. Scale bars represent 30  $\mu$ m (B) A single cell shown in the white circle of the 12.5  $\mu$ g/mL treatment group was focussed on to show typical  $\gamma$ H2AX localization within nuclei. Scale bar represents 5  $\mu$ m.



and the highest induction may appear to occur in cells treated with 25  $\mu\text{g}/\text{mL}$  rather than 12.5  $\mu\text{g}/\text{mL}$ . A high magnification image of typical nuclear  $\gamma\text{H2AX}$  localization in response to bleomycin (12.5  $\mu\text{g}/\text{mL}$ ) is shown in **Figure 3.6B**.

The pattern of  $\gamma\text{H2AX}$  induction in response to MMS was different than the results of bleomycin treatment. As depicted by sample images shown in **Figure 3.7**, there was a subtle increase in overall  $\gamma\text{H2AX}$  induction across the population of cells within the field of view between 0.005% and 0.015%, and this intensity appeared roughly the same in the 0.025%-treated cells. This, similar to **Figure 3.5B**, could be due to the fact that many cells had died in both 0.015% and 0.025%-treated groups prior to fixation and processing for microscopy, and



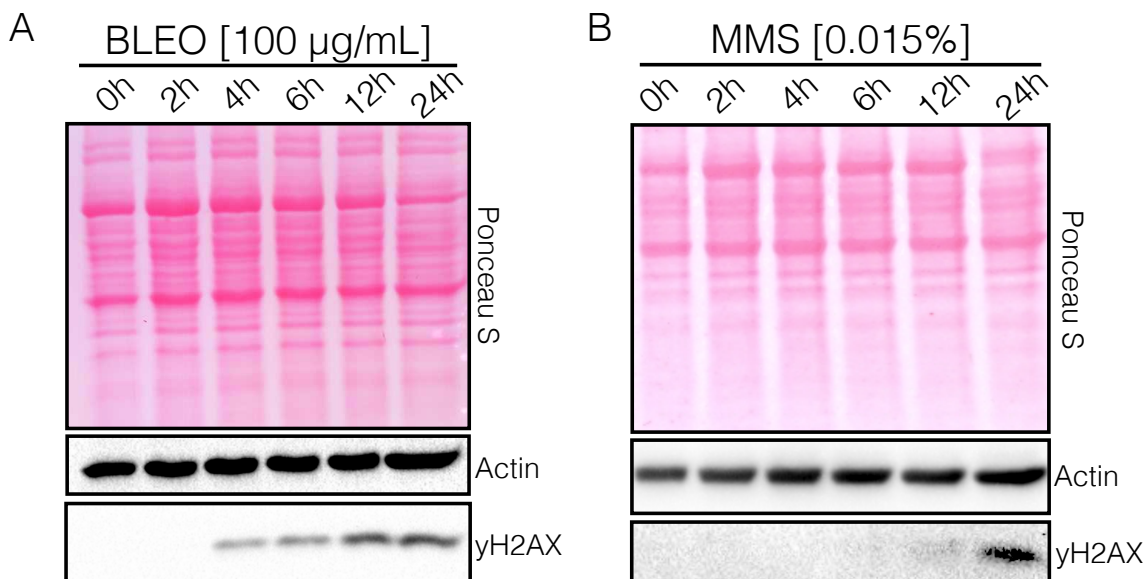
**Figure 3.7: Effect of Increasing MMS Concentrations on  $\gamma\text{H2AX}$  Localization in RTbrain-W1 Cells**  
 RTbrain-W1 cultures were exposed to various concentrations of MMS for 24h and visualized for  $\gamma\text{H2AX}$  localization in the nuclei of cells. A culture treated with 100  $\mu\text{g}/\text{mL}$  bleomycin was included to demonstrate a positive  $\gamma\text{H2AX}$  signal. The primary antibody for  $\gamma\text{H2AX}$  was a rabbit anti- $\gamma\text{H2AX}$  monoclonal antibody (9718, Cell Signal), and nuclei were counterstained with DAPI. Scale bars represent 50  $\mu\text{m}$ .

were not actively phosphorylating H2AX.

The next logical step in characterizing  $\gamma$ H2AX induction was to examine the time-course of  $\gamma$ H2AX formation in response to bleomycin and MMS via western blotting.

### 3.2.4 Time-course of H2AX Phosphorylation in Response to Bleomycin and MMS

Cells were treated with either 100  $\mu$ g/mL bleomycin (Figure 3.8A) or 0.015% MMS (Figure 3.8B) and protein samples were collected at various time points up until 24h. Despite a strong  $\gamma$ H2AX induction at 12.5  $\mu$ g/mL in Figure 3.5A, a concentration of 100  $\mu$ g/mL was chosen because it also showed a strong response in confocal images presented in Figures 3.6 and



**Figure 3.8: Effect of Increasing Exposure Times on  $\gamma$ H2AX levels in Bleomycin- and MMS-Treated RTbrain-W1 Cells**

RTbrain-W1 cultures were exposed to 100  $\mu$ g/ml bleocin (A) or 0.015% MMS (B) and analyzed for  $\gamma$ H2AX induction at various time points up to 24h. Protein extracts were subjected to SDS-PAGE electrophoresis and western blotting. Ponceau S staining of protein extracts is shown at the top of the figures followed by detection of actin and  $\gamma$ H2AX. The primary antibody for actin was a rabbit anti- $\beta$ -actin polyclonal antibody (A2066, Sigma) and for  $\gamma$ H2AX, the primary antibody was a rabbit anti- $\gamma$ H2AX monoclonal antibody (9718, Cell Signal). The secondary antibody was an HRP-conjugated goat anti-rabbit IgG.

**3.7.** The mid-range MMS concentration, 0.015%, was chosen because it elicited a consistent  $\gamma$ H2AX response in confocal images (**Figure 3.7**) and western blot (**Figure 3.5B**), and is semi-lethal. Bleomycin induced  $\gamma$ H2AX formation as early as 4h and continued to increase and reach a maximal induction around 12h.  $\gamma$ H2AX induction appeared to remain approximately the same from 12h to 24h, indicating a sustained DDR in response to sustained genotoxic injury. The course of  $\gamma$ H2AX induction in response to MMS; however, was delayed in comparison to what was observed for bleomycin. An initial and very weak response was observed at 12h and grew substantially by 24h. Cells treated with MMS did not appear stressed via phase contrast microscopy images (data not shown) after 12h, but by 24h roughly 50% of cells had detached from substrate, similar to observations from **Figure 3.4D**.

### *3.2.5 Application of $\gamma$ H2AX as a Biomarker of Exposure to Environmental Contaminants*

After examining  $\gamma$ H2AX induction in response to well-studied DNA damaging agents, the response of  $\gamma$ H2AX was examined via western blot during 24h exposures of RTbrain-W1 to several purified pollutants. Those tested include: glyphosate, the active reagent of the Roundup® pesticide; 2-mercaptobenzothiazole, an environmentally persistent de-icing agent and rust protector whose cytotoxicity has been extensively studied in rainbow trout in this laboratory (Zeng et al., 2016); and benzo[*a*]pyrene, a known carcinogen and PAH that is an EROD-inducer in rainbow trout (Schirmer et al., 2004). Though these compounds were cytotoxic to RTbrain-W1,  $\gamma$ H2AX induction was not observed at any tested concentration, and thus the datasets for these exposures are not included in this thesis.

## CHAPTER FOUR: DISCUSSION

### 4.1 H2AX Phosphorylation in Fish Cell Lines

In preliminary assays that were used to identify a candidate fish cell line, striking differences in sensitivity were observed across cell lines tested in parallel to the same genotoxicants. The cell lines were morphologically identical under the phase contrast microscope (data not shown), appearing healthy at all concentrations of bleomycin and HU (**Figures 3.1** and **3.2**), and showing dose-dependent increases in cell death after MMS treatment (**Figure 3.3**) at concentrations of 0.015% and 0.03%. However, each cell line tested differed in their extent of  $\gamma$ H2AX induction, which will be elaborated on further below.

#### *4.1.1 Differences in $\gamma$ H2AX Response Between Different Fish Species*

It has been documented in the literature that cell lines between different mammalian species differ in their ability to phosphorylate H2AX in response to DNA damaging agents, with the majority of research occurring in well-studied human, mouse, and hamster cell lines (Macphail et al., 2003). It is rational to assume then, that different fish species would not exhibit the same damage responses as one another even when challenged with the same genotoxicant. This could be justified by several reasons, including functional/expressional differences in the repair and defense machinery of different organisms, differences in cell cycle progression, differences in susceptibility of the genome to DNA damage, and differences in the H2AX sequence itself across species.

The basic molecular defenses against abiotic stressors have evolved across fish of different species, and for fish in different environments (Martínez-Lvarez et al., 2005). These defenses include antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), as well as glutathione-containing enzymes such as glutathione-S-transferase (GST), glutathione peroxidase (GPX), and glutathione reductase (GR), as well as more primitive non-enzymatic antioxidant molecules such as vitamins K, C, and E, uric acid, and flavonoids that are either dietary or endogenously produced. Enzymatic antioxidant defenses have been characterized across fish species, and their expression has been found to differ with species complexity as well as environmental conditions, including water temperature/salinity and the presence/absence of xenobiotic contamination (Martínez-Lvarez et al., 2005; Winston, 1991). Interestingly, fish have been found to differ by species both in which antioxidant enzymes are expressed during oxidative stress, as well as biases in which tissues are most active in producing antioxidants. For example, in a study exposing channel catfish (*Ictalurus punctatus*) and rainbow trout to kraft milling effluents, catfish was found to respond with only CAT expression, whereas trout expressed both CAT and SOD (Mather-Mihaich & Di Giulio, 1991). Since many compounds exert their genotoxicity via oxidation, such as bleomycin, it is possible that cell lines from species with higher basal expression of antioxidants, or a greater variety of inducible antioxidants, may be able to prevent genotoxicity from occurring at lower doses and dampen the resulting damage response (ie. H2AX phosphorylation).

In a similar line of thought, the differences observed in **Figures 3.2** and **3.3** could be due to how fish species differ from one another in their capacity to repair DNA damage. In an experiment testing the DNA repair activity of hepatic tissue of eight fish species in

response to DMNA (N,N-dimethylnitrosamine; a methylating agent), it was found that medaka was the most effective at repairing DNA, as judged by measuring O<sup>6</sup>-methylguanine DNA methyltransferase (O<sup>6</sup>-MGMT) activity, an enzyme that removes the methyl adducts from DNA. Operating at nearly half the activity was arctic lamprey, followed closely by goldfish, African lungfish, Masu salmon, rainbow trout, flamingo cichlid, and Japanese char (Nakatsuru et al., 1987), illustrating clear differences in repair activity between different species of fish. This type of damage, as previously mentioned regarding MMS and 4-NQO, is corrected by the two most commonly studied repair processes in fish - BER and NER, which have even been adapted for use in genotoxicity assays such as the comet assay (Kienzler et al., 2013). It has also been documented that fish species differ greatly in their susceptibility to carcinogenesis, with some notable examples including goldfish and yaritanago (*Acheilognatus lanceolatus*), which have been unable to form tumours in laboratory experiments (Ishikawa & Takayama, 1979), likely due to functional differences in genetic repair machineries and damage surveillance. Studied to a lesser extent are the mechanisms involved in repairing DSBs, which is limited to medaka and zebrafish. It has been shown that zebrafish possess more efficient HR (Fan et al., 2006) and NHEJ (Hagmann et al., 1998) than *Drosophila* and *Xenopus laevis* (Hagmann et al., 1996), at least in embryonic cells. However, no such studies regarding DSB repair have been performed in any of the species in this thesis whose cells have been studied. Nevertheless, differences in repair activities are evident in identical cell types across different fish species. Though interspecies comparisons in this thesis can only be made amongst equivalent cell types across fish species, it could possibly explain the reasons for differences in H2AX phosphorylation. One would expect that a cell type with higher repair activity would have less  $\gamma$ H2AX accumulation, which may be

occurring in **Figures 3.2** and **3.3**. It is also possible that sensor proteins and signal transducers such as ATM, ATR, and DNA-PK may be expressed differently across these species, which would also affect the rate that  $\gamma$ H2AX occurs.

The regulation of chromatin-bound histone levels is cell cycle dependent, and could lead to differences in H2AX availability between fish species, and thus  $\gamma$ H2AX induction. According to mammalian studies there exists a tight regulation of H2AX protein availability that is regulated both at transcriptional and post-transcriptional levels. In actively cycling somatic cells, it has been discovered that there are pools of replication-dependent and – independent histone mRNAs that dictate relative abundance and persistence of histone subtypes throughout the cell cycle (Marzluff et al., 2008; Wu & Bonner, 1981). Replication-dependent histone mRNAs contain a 3' tail devoid of a polyA termination sequence, but rather a stem-loop which is processed by histone-specific translation machinery (SLBP – stem-loop binding protein) that is also cell cycle-dependent (Bonner et al., 1993; Mannironi et al., 1989; Townley-Tilson et al., 2006). The translation of replication-dependent canonical histones (H1, H2A – including H2AX, H2B, H3, H4) is highest in cells entering S-phase (Connor et al., 1984). Importantly, histone translation is highest during S-phase only when DNA is replicating (Sariban et al., 1985). The half-life of histone mRNA during DNA-replication is approximately 40 minutes, but when replication is inhibited this half-life decreases to almost 8 minutes, revealing that translation and DNA replication regulate transcript stability (Heintz et al., 1983). This has been justified by the logic that as DNA replicates, histone proteins are produced and immediately incorporated into DNA, as shown in HeLa cells (Connor et al., 1984; Heintz et al., 1983). Alternatively, the replication-independent transcripts (polyadenylated) are generated at all stages of the cell cycle as a

small basal pool, comprising roughly 10% of the histone mRNA pool at maximal transcription during S-phase, and these isoforms are much more resistant in terms of stability to inhibition of DNA replication and translation (Wu & Bonner, 1981). Interestingly, the stem-loop isoform of H2AX appears to be differentially regulated, where the impact of inhibiting DNA replication and translation is far less than other histone stem-loop mRNAs (Bonner et al., 1993). Comparisons of histone regulation amongst different species, let alone H2AX in particular, have not been made in the same study, though subtle differences between cell lines from mice and humans have been documented, such as slight differences in maximal H2AX levels during S-phase between murine and human cells (Delisle et al., 1983; Heintz et al., 1983; Sariban et al., 1985). Unfortunately, such studies of H2AX regulation in fish (even zebrafish and medaka) do not exist, posing a difficulty in extrapolating from mammalian literature. However, since H2AX is well conserved across most multicellular animals it is likely that the cell-cycle dynamics of H2AX are similar in fish species. Thus, it is possible that equivalent cell lines of species with similar genome sizes may produce more or less H2AX relative to another; however this is probably a minor consideration and negligible regarding the extent of the differences seen in **Figures 3.2** and **3.3**. If this was a factor responsible for the results from **Section 3.1**, one would expect that cells with more H2AX would be more readily induced at lower concentrations of DSB-forming genotoxicant.

Genome size and nucleotide bias may also present reasons why one may see differences in  $\gamma$ H2AX induction between cell lines of different species. One would expect that cells with a larger genome size would inherently contain more H2AX protein (and possibly more copy numbers of the H2AX gene). For example, the *Acipenser* genus appears



to be in the middle-late stages of genome reduction, with species varying greatly in chromosomal content and ploidy (Ludwig et al., 2001). *Acipenser fulvescens*, the origin of SB3, is a diploid sturgeon with ~262 chromosomes (D. S. Kim et al., 2005). Cells from fish with these relatively large genomes may be more susceptible to genetic damage, such as by a rather non-specific genotoxicant like bleomycin. In terms of biases, it is known that different organisms have differences in GC content, which has not been explored across fish but has been demonstrated between humans, *Drosophila*, and *Caenorhabditis elegans* (Ho et al., 2014). The difference in GC content between fish species, although unexplored, may increase hotspots that may be sensitive targets for adduct formation. In relation to the genotoxicants used in this thesis, it would be interesting to see if a correlation exists between GC content and MMS/4-NQO-induced  $\gamma$ H2AX induction. However, the mean genome size of salmonids (including Atlantic salmon and rainbow trout) is much smaller in genomic size compared to sturgeon (Hardie & Hebert, 2004). Following this logic one would expect that SB3 cells would be significantly more sensitive than RTbrain-W1 cells, which was not evident. Thus, it is likely that it is a combination of the many aforementioned factors that contribute to the responses that were observed.

In addition to genetic composition, the extent of chromatin condensation (ie. euchromatin or heterochromatin) may be different in the same tissues amongst different species and play a role in differences in H2AX levels between different species. In chinese hamster ovary (CHO) cells, it has been demonstrated that in regions of low chromatin density, H2AX phosphorylation is higher compared to regions of high density (Firsanov et al., 2011), which is due to the inability of repair proteins and upstream kinases in accessing heterochromatin-bound H2AX (Falk et al., 2008). Interestingly, a high-throughput

bioinformatics study has revealed that there are indeed differences in chromatin densities across specific metazoans, in particular humans, *Drosophila*, and *Caenorhabditis elegans* (Ho et al., 2014), although these are not necessarily representative of the differences that may exist between more closely related animals. Thus, it is likely that fish species differ from one another in the proportion of euchromatin versus heterochromatin, albeit probably less than what one would expect between humans, *Drosophila* and *C. elegans*. It would be interesting to perhaps examine the degree of heterochromatin abundance in the cell lines tested from **Section 3.1** by also probing for heterochromatin markers, such as HP1 (heterochromatic adaptor protein), or H3K9me3 (histone H3 trimethylated at lysine 9) (Bartkova et al., 2011).

A final speculation as to justifying the differences observed in  $\gamma$ H2AX induction across fish species is that these may be due to differences in the H2AX sequence itself. Although the anti- $\gamma$ H2AX antibody from Cell Signaling yielded a band at the expected size (of human  $\gamma$ H2AX), and it was significantly higher after treatment of cells with DNA-damaging agents, there are two major considerations that must be mentioned. First, H2AX has not been sequenced and annotated in rainbow trout (although H2A has), sturgeon, European eel, or walleye, so it is impossible to say with absolute certainty that the antibody is binding to H2AX in these species, despite the signal similar to mammalian literature. Second, the exact epitope sequence that the antibody is specific to is not publicly available, as the manufacturer states that it is specific to the “residues surrounding the phosphorylated serine 139 and SQE motif”. Thus, it is possible that variations in amino acids within the recognized epitope, but outside of the conserved SQE motif, may alter the binding affinity of the antibody to  $\gamma$ H2AX of different fish species and artificially skew the results. Unfortunately, the epitopes of the four previously mentioned fish species are unknown, but the H2AX

sequences of Atlantic salmon, zebrafish, humans, and other relevant fish species are presented in a multiple sequence alignment in **Appendix A**. All of the fish species shown share identical sequences in the last 7 amino acids that include the SQE motif, but interestingly, the first three amino acids are different in humans, the organism that the antibody is specific to. The highlighted fish species are different, but in the same way, suggesting that perhaps the three amino acids preceding the SQE motif may even be dispensable for antibody binding.

#### *4.1.2 Differences in $\gamma$ H2AX Response Between Cell Lines of the Same Species*

The ability of different cell types from the same species to phosphorylate H2AX has also been shown to differ in a tissue-specific manner, and cellular sensitivity can depend on the organs that genotoxicants may target. Similar to the arguments posed in **Section 4.1.1**, there are several factors that may contribute to the different levels of H2AX phosphorylation observed in cell lines from the same organism (**Figure 3.1**). These include tissue-specific differences in molecular defenses, repair activity, cell cycle progression, abundance of H2AX, and chromatin organisation.

As previously mentioned, cells are equipped with an arsenal of molecular defenses to protect against oxidative stress. Not only have these antioxidants been found to differ in expression and occurrence across the same tissue types between different species, but they also differ between tissues of the same species. Evident in one example, the common carp and Nile tilapia were dosed with two pesticides (2-4D and azinphosmethyl) and tissue-specific antioxidant expression was analyzed (Ozcan Oruc et al., 2004). The two fish exhibited different expressional profiles of antioxidants, however a common interesting trend

emerged amongst the two fish. The overall highest antioxidant response was highest in gill tissue, followed by metabolically active tissues including the kidney and liver, and the lowest in brain tissues. This pattern appears to occur in studies concerning other fish (Martinez-Lvarez et al., 2005), and supports the data shown in **Figure 3.1** with rainbow trout cell lines exposed to bleomycin. It is possible that the cell lines I studied follow this same trend, and thus, the order of susceptibility to oxidation-mediated DNA damage is evident by  $\gamma$ H2AX induction. It would be interesting to confirm this by assaying for GST, CAT, SOD, and GPX activity in parallel with  $\gamma$ H2AX induction. In addition, antioxidants serve no purpose in defending against alkylating agents such as MMS and 4-NQO, and it would be of interest to examine  $\gamma$ H2AX induction following exposure of RTliver-W1 and RTgill-W1 to these agents. It is possible that RTbrain-W1 may not be the most sensitive to adduct formation between these three cell lines, reinforcing the role of antioxidants in influencing bleomycin-mediated DNA damage.

Tissues also differ in their repair activity within the same organism. In a comprehensive review by Kienzler and colleagues (2013), strong evidence shows that liver cells in particular possess remarkably inefficient NER machinery in rainbow trout (Weimer et al., 2000), zebrafish (Troxel et al., 1997), and several species of catfish (Willett et al., 2001), where no detectable adduct repair was found. This is also evident in a study that treated medaka, guppy, Siamese fighting fish, blonde cave fish, and the Oriental weatherfish with 4-aminohydroxyaminoquinoline-1-oxide and MMS, revealing that the brain has much higher NER capacity than liver and intestinal tissues (Ishikawa et al., 1984). The other commonly studied repair pathway, BER, has been found to operate weakly in liver tissue as well and show a similar trend. This has been observed in both rainbow trout (Kienzler,

Tronchère, et al., 2013) and *Xiphophorus* (Walter et al., 2001). In the rainbow trout study, gills showed higher BER activity in comparison to liver, while in the *Xiphophorus* study the brain, testis, and gill were also studied. Interestingly, the brain showed the highest BER activity, followed by the testis and gills, and again the liver was the least active. In speculation, the presence of an elevated repair system may have two possible outcomes on the state of  $\gamma$ H2AX induction in a cell. In one scenario, it is plausible that a robust adduct repair system may quickly repair damage across the genome, in which the damage is overwhelmed by repair and higher doses of genotoxicant are required in order to induce H2AX phosphorylation. Alternatively, one might argue that the presence of a robust repair system also results in heightened signal transduction and presence of upstream sensor kinases that phosphorylate H2AX. In this case, although repair activity may be high, a greater abundance of sensor kinases may result in greater  $\gamma$ H2AX induction at lower concentrations of genotoxicant. To investigate the relative repair activities of our rainbow trout cell lines; again, it would have been useful to compare  $\gamma$ H2AX induction in response to 4-NQO and MMS across RTbrain-W1, RTliver-W1, and RTgill-W1.

Tissues and cell types within an organism exhibit differences in cell cycle progression, and thus H2AX levels may differ given the time frame of the experiment. As described in **Section 4.1.1**, the abundance of H2AX is highest in S-phase of cycling cells and there is a subset of H2AX that is constitutively expressed (Bonner et al., 1993). There are no instances in the literature to my knowledge that explore the effects of speed in cell cycle progression may have on H2AX persistence or activation, however, I speculate that a cell with slow growth may be more sensitive to H2AX activation. A reason could be that these cells progress through S-phase slower, and may present a larger window in which H2AX is

being upregulated. This could also mean that at an arbitrary time point for protein extract collection, there is a greater chance that a population of slow-growing cells may still be in S-phase compared to a quickly growing cell, which may have replicated to maximum density and remained in G<sub>1</sub>. One must also consider that it may not just be simply cell-cycle dependent control that influences H2AX levels; tissue-specific chromatin silencing could be contributing as well. Studies of this sort in fish species have been made in zebrafish, and interestingly, many xenobiotic compounds appear to have an influence on epigenetic modifications in the cell, regardless of whether genotoxicity is occurring (Williams et al., 2014). Methylation is important to the process of silencing chromatin, and is executed by DNA methyltransferases (DNMT). One study of DNMTs involved in *de novo* methylation measured mRNA abundance in zebrafish and revealed high levels of *dnmt6* and *dnmt8* transcripts in the brain and eye compared to muscle (Smith et al., 2011; Thisse & Thisse, 2004). However, epigenetic modifications play a crucial role in development and maturation, and their tissue-specific levels fluctuate with age (Metzger & Schulte, 2016). As such, tissue-specific differences in H2AX accessibility due to silencing, or whether the H2AX locus itself is silenced, has not been explored yet in fish species, but could play a role in regulating  $\gamma$ H2AX formation.

In summary, a good fish cell line for use in this bioassay involving the surveillance of  $\gamma$ H2AX may be in part because of a lack of antioxidant defense systems, especially when assaying oxidative genotoxicity by compounds such as bleomycin. In order to examine the genotoxicity of adduct-forming compounds, a cell line should have an active repair system that will generate detectable DSBs as a secondary event of trying to repair DNA. Conveniently, these two points support the use of a fish brain cell line for studying  $\gamma$ H2AX,

as they seem to follow these criteria in the limited literature concerning DNA damage and repair in fish. A cell line should also be actively dividing and in the cell cycle, since S-phase is the main driver of H2AX upregulation. Finally, a cell line for use should have a large proportion of euchromatin, and possibly a relatively larger genome, so that there is not only a greater abundance of accessible H2AX, but of H2AX itself.

#### 4.2 The Relationship of H2AX Phosphorylation with Bleomycin Treatment

Surprisingly,  $\gamma$ H2AX decreased with increasing concentrations of bleomycin up to 100  $\mu\text{g}/\text{mL}$  (**Figure 3.5A**). This was unexpected as literature could not be found that reported a decline in H2AX phosphorylation over the concentrations we assayed in **Figure 3.5A**, and because the results from confocal microscopy in **Figure 3.6** did not show a strong dose-dependent decline either. It should be noted that after the submission of this thesis for defense, two more replicates of this experiment were performed with a larger range of concentrations (0.39 – 400  $\mu\text{g}/\text{mL}$ ) which yielded contrasting results, showing a definitive dose-dependent increase in  $\gamma$ H2AX starting at the lowest concentration and increasing gradually through to 400  $\mu\text{g}/\text{mL}$ . Positive dose-response relationships of  $\gamma$ H2AX induction following bleomycin treatment have been shown in the literature with many cell types (Banáth & Olive, 2003; Watters et al., 2009), with an example including human peripheral lymphocytes (0 – 6  $\mu\text{M}$ ; Scarpato et al., 2013) Thus, the results from **Figure 3.5A** must be treated as preliminary, however, possible reasons for pattern in  $\gamma$ H2AX induction will be discussed below.

A similar phenomenon has been observed in a zebrafish cell line treated with uranium. Interestingly, a paper studying the genotoxicity of uranyl nitrate reported a significant decline in  $\gamma$ H2AX phosphorylation at high concentrations after first observing an

increase with low doses (Pereira et al., 2012). When embryonic zebrafish (ZF4) cells were exposed for 24h, an almost linear increase in  $\gamma$ H2AX induction was observed over a range of 1-100  $\mu$ M, while a linear decrease to near-background levels was seen at higher concentrations of 250  $\mu$ M and 500  $\mu$ M. The authors concluded that at higher concentrations uranium was no longer genotoxic despite showing a definite dose-dependent increase in micronuclei formation, even at the highest concentrations. Another interesting result of this study was the formation of uranium precipitates within cells at one of the higher concentrations, 250  $\mu$ M, as revealed by transmission electron microscopy. In drawing parallels to the phenomenon observed in **Figure 3.5A**, it is possible that as concentrations of bleomycin increase past a certain threshold, its solubility decreases within the cell. This could impact the ability of the drug to interact with DNA.

Aside from the conclusions drawn from the uranium study, one can speculate several other reasons why this might be occurring. The possibility exists that regulation of the PIKKs involved in the DDR is altered at higher concentrations, either causing less H2AX phosphorylation or stimulating repair mechanisms to catch up to the ensuing DNA damage. This is possible since HR repair, the mechanism responsible for repairing DSBs, occurs relatively quickly compared to other repair mechanisms. In addition it is known that bleomycin-induced DSBs occur due to reactive oxygen species formation, a process that is known to induce gene expression of antioxidants. It is possible that with the increase in bleomycin concentration, both repair and antioxidant proteins are upregulated to have a synergistic effect in protecting the cell. Finally, and purely speculatively, it could be that DSBs accumulate so frequently with higher bleomycin concentrations that DNA is practically “shredded” into many smaller free-floating fragments. These fragments of DNA,



and the H2AX bound to them, could be dislocated from repair foci if the amount and rate of damage is greater than the repair capacity of the cell. It is known that any H2AX within a megabase distance of DSBs is phosphorylated bilaterally (Savic et al., 2009), thus suggesting a stoichiometric explanation to this problem if H2AX protein levels decrease in nearby damaged chromatin.

#### 4.3 MMS Weakly Induces H2AX Phosphorylation

An interesting inference from the information gathered in the blots of **Figure 3.5** is that  $\gamma$ H2AX is more heavily induced in RTbrain-W1 by bleomycin than MMS or 4-NQO in the concentrations tested. In a study by Watters *et al.* (2009),  $\gamma$ H2AX induction in mouse lymphoma cells (L5178Y) was two times higher in cells treated with 0.0065% MMS compared to those treated with 10  $\mu$ g/mL bleomycin. In the results demonstrated with RTbrain-W1, induction by MMS is undetectable at 0.005%, and barely visible at 0.01% (**Figure 3.5B**). Additionally, at all of the concentrations tested, induction of  $\gamma$ H2AX in response to bleomycin was much higher. When comparing the concentrations of MMS that we studied to this, it is possible that there is a fundamental difference between rainbow trout and mouse cells in susceptibility to these drugs, that concentration differences have an impact, or that treatment time has an effect, since exposures in our assay from **Figure 3.5** were for 24h and exposures in the mouse cells were for 4h. However, when interpreting time-course data from **Figure 3.8** at the 4h mark,  $\gamma$ H2AX induction was undetectable from MMS treatment and unambiguously strong from bleomycin treatment. Of course, this could be specific to the cell lines in question and not indicative of evolutionary differences between trout and mice per se. Nevertheless, this difference was striking and the notion that functional

differences between repair pathways exist across higher and lower vertebrates is a possibility that has been suggested before while studying other DDR proteins (Embry et al., 2006).

#### 4.4 4-NQO Causes a Subtle Increase in H2AX Phosphorylation

Data from 24h exposures of RTbrain-W1 to 4-NQO showed a subtle but reproducible increase of  $\gamma$ H2AX at the highest tested concentrations, where  $\gamma$ H2AX levels were 1.5x and 2x relative to the control in 500 nM and 1000 nM treatment groups, respectively (**Figure 3.5C, D**). Though 4-NQO-induced increases in  $\gamma$ H2AX levels have been reported in mouse cells (Watters et al., 2009) and human cells (Banáth & Olive, 2003), such a response has not yet been explored in rainbow trout. The most comparable study from the literature is that of Smart *et al.* (2011), which showed a 3.5x and 4x increase in  $\gamma$ H2AX induction compared to a negative control after a 3h exposure of mouse lymphoma (L5178Y) cells to 500 nM and 1000 nM doses, respectively. Differences could be due to the exposure time, and perhaps that damage to DNA occurs quicker than the 24h time point we are assaying. This is a possibility, but in addition, the competency of repair mechanisms may play a large role in the genotoxicity of 4-NQO as well. A study on 4-NQO mutagenicity and clastogenicity using human cell lines in comparison with L5178Y explored these factors (Brüschäfer et al., 2016) and it was shown that genotoxicity of 4-NQO in the human lymphoblastoid (TK6) cell line was highest when exposure times were short (4h), as well as recovery times were long, the latter being a parameter that was not addressed in our studies. Interestingly, after the same 4h exposure and a 24h recovery, L5178Y cells showed significantly more MN than TK6 cells. The main difference between these cell lines is that TK6 possesses wild-type p53 activity, while L5178Y does not, meaning that the presence of an active p53 (and functional DDR) helps to lower 4-NQO genotoxicity by coordinating repair efforts. Thus, when drawing

conclusions regarding sensitivity of induction by 4-NQO in our study with that of the study by Smart *et al.*, one must take into account the possibility of compromised repair systems, in addition to parameters of exposure (i.e. duration, concentration, any recovery times). In addition, technical issues in densitometric analysis could contribute to the differences observed. The representative blot in **Figure 3.5C** is the first of two independent trials used to calculate induction and was overexposed in order to capture the faint signal. As a result, the bleomycin-treated positive control was heavily saturated and the signal bled into the adjacent mock-treated lane, artificially increasing the intensity of mean  $\gamma$ H2AX induction between both trials (**Figure 3.5D**). This could not be completely corrected for using the densitometry software; as a result, any inferences made regarding induction changes when comparing to the control are artificially smaller.

Another interesting comparison that can be made is with a study by Nehls & Segner from 2001, which reported comet assay results of RTG-2 and RTliver-W1 cells treated within a lower range of 4-NQO concentrations than we used for our experiments. After a 2h exposure of cells to doses in the 6.5-210 nM range of 4-NQO, single-fold and three-fold increases in comet assay tail scores were observed for RTliver-W1 and RTG-2 cells, respectively. Again, the time of exposure is inconsistent between their study and ours, but it was also shown that 4-NQO had varying genotoxicity amongst cell types, even from the same organism, an observation that was echoed in the above study by Brüsehafer *et al.* (2016). Cell type-specific  $\gamma$ H2AX sensitivity in rainbow trout cell lines is evident in **Figure 3.1**, specifically showing that  $\gamma$ H2AX induction is higher in RTbrain-W1 compared to RTliver-W1. It would be interesting to see if identical exposure conditions in RTbrain-W1 as the study by Nehls & Segner (2001) would elicit a comparable response to 4-NQO as RTG-2

via the comet assay. Likewise, it may also be of interest to assay for  $\gamma$ H2AX induction in RTG-2 cells and compare its sensitivity to RTbrain-W1. It may be possible that tissue-specific sensitivities of  $\gamma$ H2AX induction exist within rainbow trout to 4-NQO, an observation that has been reported previously in this lab while studying p53 and Chk2 with other genotoxicants (Liu et al., 2011; Steinmoeller et al., 2009). This may give insight as to mechanistic differences in regulation of repair mechanisms across different cell types.

#### 4.5 Differences in $\gamma$ H2AX Localization and Dose Response

To further characterize  $\gamma$ H2AX induction, cells exposed to bleomycin and MMS were analyzed via confocal microscopy to observe focus formation as shown in **Figures 3.6 & 3.7**. In a previous study, mouse embryonic fibroblasts (MEFs) were exposed to bleomycin and  $\gamma$ H2AX levels were analyzed via manual foci scoring using confocal microscopy and by quantitating immunofluorescence using flow cytometry (Watters et al., 2009). It was reported by Watters and colleagues that 1.7x, 1.4x, 1.9x, 2.1x, and 2.4x increases in  $\gamma$ H2AX induction corresponded to respective bleomycin concentrations of 0.1, 0.5, 1.0, 5.0, and 10.0  $\mu$ g/mL over a 4h exposure. In comparison with our results,  $\gamma$ H2AX was undetectable in any cells at 1.56  $\mu$ g/mL for 24h, suggesting that RTbrain-W1 cells may not be as sensitive to bleomycin as MEF cells. This is a possibility, since robustness of H2AX phosphorylation differs across cell lines, as is shown in **Section 3.1**. In addition, because of the nature of dealing with very small concentrations of chemicals, it is possible that differences in experimentation accuracy may be part of the explanation. However, in cultures that were treated with low concentrations not present in **Figure 3.5A**, a dose-dependent increase in  $\gamma$ H2AX across the cell population was observed.

Interestingly, the distribution of  $\gamma$ H2AX induction was not uniform across the population of cells, meaning that not all cells elicited the same intensity of response, or sometimes even a response at all. This was especially evident in response to bleomycin (**Figure 3.6A**). We speculate several possible explanations for this phenomenon. First, the cells were asynchronous prior to exposure and it is possible that not all cells were in the same phase of the cell cycle. Though the genotoxicity of bleomycin occurs independent of the cell cycle, unlike model drugs such as hydroxyurea, which depletes cellular dNTP pools and subsequently inhibits S-phase, it is possible that the abundance of available H2AX to be phosphorylated varies according to cell cycle stage. Indeed in humans, *Arabidopsis*, and *Drosophila*, nucleosomes are modified at the level of their subunits; subunit abundance may be specific to the phase of the cell cycle (Millar, 2013) and indeed this is observed in yeast with an S-phase specific upregulation in H2A mRNA (Hereford et al., 1981), which serves an analogous function to H2AX. However, these mechanisms are largely uncharacterized in higher eukaryotes and are unknown in fish (Millar, 2013). It would be interesting from a perspective of basic research to repeat this experiment by synchronizing the cells in the various phases of the cell cycle and examining when H2AX is phosphorylated the most, however, the purpose of the confocal microscopy was to validate results obtained from blots and was not pursued.

A second reason could be technical issues that arose concerning antibody penetrance into the nucleus, as focussed on in the high-magnification image shown in **Figure 3.6B**. Typical high-resolution nuclear images of  $\gamma$ H2AX immunofluorescence in the literature reveal punctate and discrete foci of  $\gamma$ H2AX around DSBs rather than a uniform distribution across the nucleus. Though it may be possible that the DNA within a nucleus may be

damaged severely enough that discrete foci cannot be resolved from one another, it is possible that a technical error with nuclear permeabilization occurred, possibly preventing antibody entry into the nucleus. Although protocols were followed strictly from the original characterization of  $\gamma$ H2AX foci (Paull et al., 2000), the study using RTH-149 and RTliver-W1 from rainbow trout (Krumschnabel et al., 2010), and routine parameters used by the Bols lab, this phenomenon reoccurred and was confirmed by Z-stacking, a scanning technique. By capturing images at nanometer intervals while scanning through the depths of the nucleus, it was revealed that the anti- $\gamma$ H2AX antibody localized with DAPI predominantly on the periphery of the nucleus (data not shown). Alternatively, it is possible that this is an activity associated with DSB repair by HR or NHEJ. During repair of DSBs in heterochromatin by HR in *Drosophila* during S/G<sub>2</sub> phase, damaged fragments of DNA are relocalized to the nuclear periphery in order to ensure safe strand invasion prior to HR (Ryu et al., 2015). DSBs are also tethered to the nuclear periphery during NHEJ repair outside of S/G<sub>2</sub> phase, especially for repair of subtelomeric regions, as shown in yeast (Therizols et al., 2006). For this reason, a quantitative comparison of foci counts with western blot densitometry could not be made, but the qualitative observation of  $\gamma$ H2AX intensities between treatments could. In the future, it could be interesting to perform confocal microscopy of synchronous cells that are treated with genotoxicants in different phases and observing  $\gamma$ H2AX. This could be done by arresting cells in G1 with lovastatin, before S-phase entry with hydroxyurea, in mitosis with nocodazole, and in quiescence with serum starvation (Rosner et al., 2013). Further to discussion in **Section 4.1**, it would be interesting to see if any changes in  $\gamma$ H2AX induction occur, especially in S-phase, where H2AX is intensely upregulated.

Another interesting observation from the confocal images surfaced in **Figure 3.7** from cells treated with MMS. Very few cells actually showed nuclear localization of  $\gamma$ H2AX, but rather  $\gamma$ H2AX was seen in the cytosol of severely sick and dead cells. This could be due to nuclear fragmentation caused by apoptosis and subsequent lysis of the nuclear membrane, releasing histones into the cytosol. Alternatively, it could be an unintended cross-reaction of the primary or secondary antibody. However, the possibility that the secondary antibody reacted non-specifically was considered by treating a parallel culture with bleomycin and incubating without an anti- $\gamma$ H2AX primary antibody, though one would expect to see this in **Figure 3.6** as well. Despite this oddity, the levels of  $\gamma$ H2AX in cells of each treatment appeared dose-dependent, which agreed with data shown by western blot in **Figure 3.5B**. In contrast, the dose-dependent decrease in  $\gamma$ H2AX levels with higher concentrations from the western blot in **Figure 3.5A** was not nearly as obvious in the confocal images from **Figure 3.6**, though it is important to mention that these images are small representations of the entire culture. As previously mentioned, cells were different from one another in terms of relative  $\gamma$ H2AX levels, despite being in the same treatment conditions. It is possible that the cumulative amount of  $\gamma$ H2AX present in a culture may not be as easy to interpret qualitatively as a cell lysate that is visualized by western blotting.

#### 4.6 Temporal Differences Between MMS- and Bleomycin-induced $\gamma$ H2AX

In our study, RTbrain-W1 exhibited a robust and rapid induction of  $\gamma$ H2AX expression within 4h in response to treatment with 100  $\mu$ g/mL of bleomycin (**Figure 3.8A**), similar to results seen in bleomycin-treated mouse lymphoma cells subjected to 10  $\mu$ g/mL (Watters et al., 2009). However, treatment with 0.015% of MMS was unable to induce H2AX phosphorylation until 12h, as shown in **Figure 3.8B**. This could be due in part to the

differences in DNA damage nature, respective repair responses, and time necessary to form DSBs. The relative speeds of repair responses to counter ionizing radiation (IR) and monoalkylation have been previously reported in mammalian cells (Regan & Setlow, 1974). IR, and radiomimetic agents such as bleomycin, immediately cause discrete DSBs that are quickly repaired by HR mechanisms; however, alkylation caused by drugs such as MMS is wide-spread over many nucleotides and results in the BER pathway (Sobol et al., 1996). The latter takes longer and the processing and resection of ssDNA lesions is what leads to DSB formation, potentially explaining the temporal difference in  $\gamma$ H2AX emergence between drug treatments. It could also be due to the very purpose that  $\gamma$ H2AX serves in the DDR. As mentioned in Sections 1.3 and 1.4, the phosphorylation of  $\gamma$ H2AX is due to the PIKKs ATM, ATR, and DNA-PK. Activation of the DDR is also reliant on ubiquitylation. These two modifications have a critical role in recruiting more H2AX-phosphorylating PIKKs to damaged sites in a positive feedback loop and amplifying the signal. It is possible that since MMS-induced DSBs form more slowly, the threshold of activation is not reached as quickly to initiate this positive feedback loop; thus, the response is slower to initiate, takes longer, and is weaker during the 24h time frame used in this study.

## **CHAPTER FIVE: CONCLUSIONS**

From the present study, the phosphorylation of H2AX as a biomarker of genotoxic stress in RTbrain-W1 has been characterized in response to parallel experimentation with various DNA damaging agents including bleomycin, MMS, and 4-NQO. Each chemical presented a different mechanism of producing DSBs capable of triggering signalling events



involving H2AX phosphorylation. These treatments have been widely employed using mammalian models to study the DDR, but their effects in rainbow trout are understudied, especially those of MMS and 4-NQO. These data represent essential steps in understanding the sensitivity and function of  $\gamma$ H2AX in rainbow trout, as well as filling holes in the literature regarding its sensitivity in rainbow trout. These steps are important and necessary in order to apply it as a biomarker in environmental testing.

Our study suggests that  $\gamma$ H2AX is a sensitive biomarker that could be added to existing protocols for a more detailed explanation of the genetic responses that rainbow trout exhibit during severe genotoxic stress. Its usefulness lies in the fact that its very presence indicates DSB formation. Likewise, the dynamics of its induction in response to a chemical can be used to understand how DSBs may be occurring (i.e. discrete DSB formation versus adduct formation or other types of DNA damage). However, this is also a disadvantage of  $\gamma$ H2AX as a biomarker; the specificity of H2AX phosphorylation is uninformative when assaying for other types of damage. Nevertheless, it could be a useful addition to the comet assay or MN test, which are routinely employed to assay the broad genotoxic effects that environmentally relevant DNA damaging agents possess. It may be an appealing augmentation to current freshwater toxicity analysis protocols involving rainbow trout, such as those used by regulatory bodies in Canada, and other members of the OECD.

## 5.1 Future Considerations

In direct continuation from the blots in **Figure 3.5**, future work to validate the use of  $\gamma$ H2AX in rainbow trout as a biomarker would include performing exposures with broader ranges of bleomycin to quantify the  $\gamma$ H2AX levels at low concentrations. This was performed

by confocal microscopy, but analysis by western blot would help illustrate a more complete dose-response. It would also be interesting to perform time-course experiments of the model compounds used at different concentrations, in parallel, to observe the effects of dose on the temporal properties of  $\gamma$ H2AX response. In addition, it might be beneficial to compare the relative sensitivity of different methods in detecting  $\gamma$ H2AX in RTbrain-W1, as the work in this thesis was confined to western blotting and confocal microscopy. By running the same experiments in parallel and measuring  $\gamma$ H2AX levels using western blotting, flow cytometry, and automated foci counting, one could address this problem. One could also adopt an in-cell western approach, which was shown to work for characterizing genotoxicity of PAHs in human cells (Audebert et al., 2010). Finally, it would be interesting to directly compare H2AX phosphorylation with conventional methods using RTbrain-W1, such as the MN test and comet assay.  $\gamma$ H2AX could conveniently be used in a panel of diagnostic tests, which would make a combinatorial approach useful for diagnosing genotoxicity of unknown compounds.

In terms of broad future directions for developing rainbow trout biomarkers, the upstream inducers of  $\gamma$ H2AX such as DNA-PK, ATM, and ATR would be of interest to study in rainbow trout, since  $\gamma$ H2AX is downstream of all three. If one was able to assay the signalling events that these PIKKs are part of during different types of DNA damage, it would put  $\gamma$ H2AX induction into greater context, show similarities or differences in DDR signal transduction between rainbow trout and other animals, and also give greater resolution to the genetic responses exhibited by fish challenged with genotoxicity. For instance, one could assay for changes in expression or phosphorylation levels of sensor complexes such as MRN or the 9-1-1 clamp. Of these complexes, only the Rad1 component of the 9-1-1 clamp

has been cloned in rainbow trout (Bozdarov et al., 2013). In addition, one could simultaneously look downstream of the DNA-PK, ATM, and ATR signal transducers and  $\gamma$ H2AX to assay the expression and phosphorylation levels of the main DDR effector kinases, Chk1 and Chk2. Post-modification events of these proteins in response to DNA damage would be equally interesting as biomarkers, since they are indicative of the type of signal transduction that is occurring. For example, although Chk2 has been characterized in rainbow trout (Steinmoeller et al., 2009), it would be of interest to identify the residues that, when phosphorylated, indicate activation (ie. T68 of human Chk2). The modifications of one particular protein, p53, would be very interesting to characterize since it is such a central transducer of critical cellular responses. However, as mentioned earlier, these mechanistic aspects of DDR regulation and signalling in rainbow trout are unknown. Extensive basic characterization needs to occur first, as well as generation of antibodies directed towards post-translational modifications.

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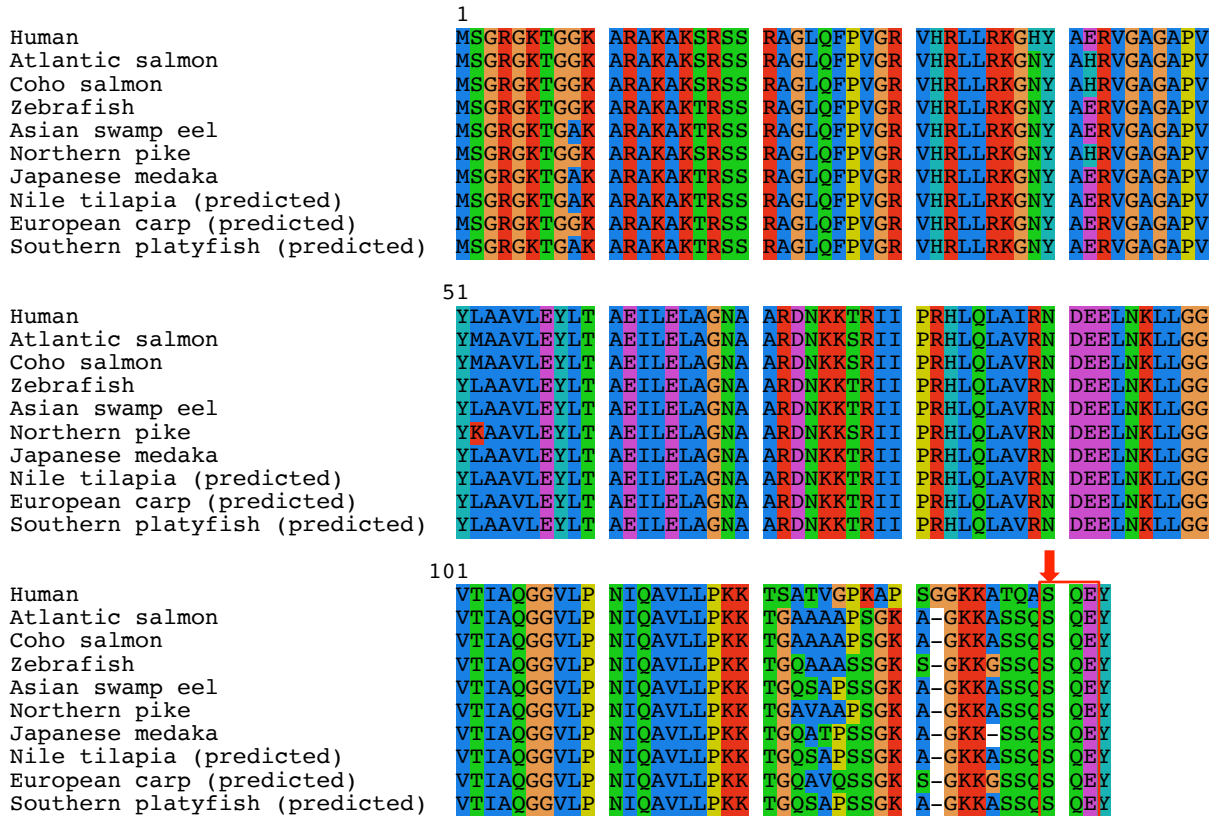
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## APPENDIX A: Multiple Sequence Alignment of the H2AX Peptide

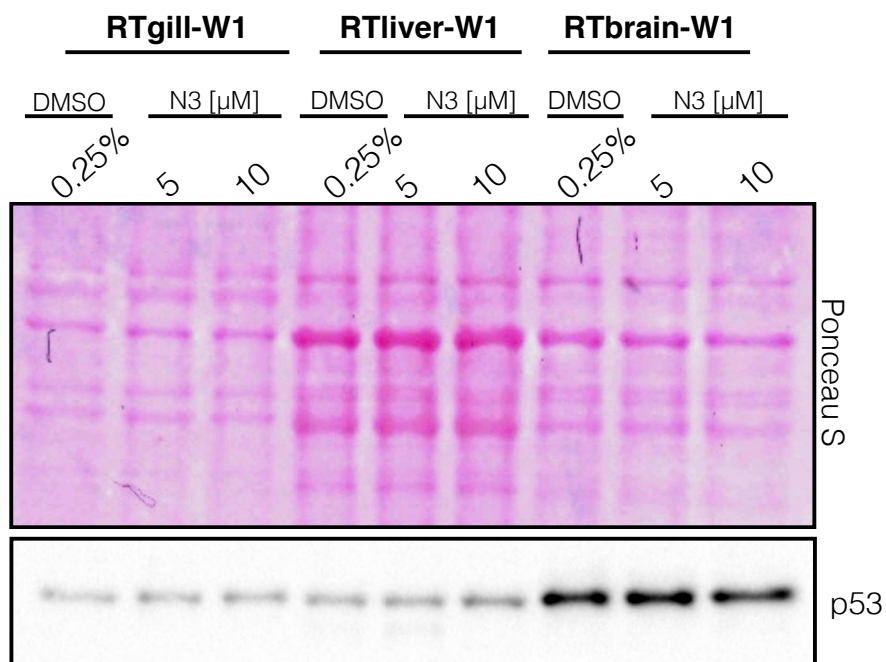


**Figure A1: COBALT Alignment of H2AX in Humans and Fish**

Multiple sequence alignment using the Constraint-Based Alignment Tool (COBALT) of H2AX peptides extracted from GenBank. The red box highlights the conserved SQE motif in H2AX, and the red arrow shows the phosphorylated serine residue of  $\gamma$ H2AX. The colour scheme used organizes amino acids with similar structural traits: blue – hydrophobic aliphatic side chains, teal – hydrophobic aromatic side chains, green – polar side chains, yellow – proline, orange – glycine, red – positively charged, purple – negatively charged.

## APPENDIX B: p53 Expression in Response to MMS and Bleomycin

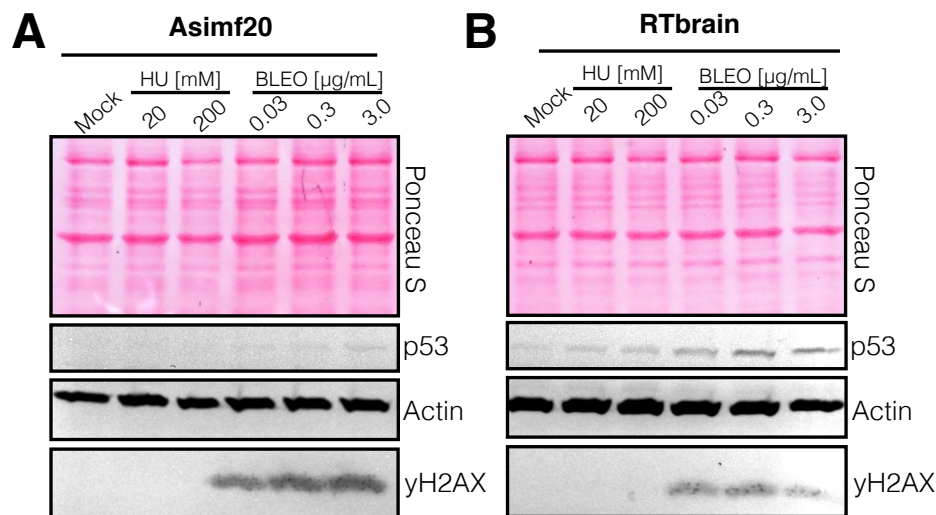
Initially, we wanted to examine the potential of both p53 and  $\gamma$ H2AX as biomarkers of genotoxicity in rainbow trout. Unfortunately, only bleomycin treatment was able to induce an increase in p53 expression of all the compounds tested in this thesis; thus,  $\gamma$ H2AX became the focus. However, some interesting data emerged from these early studies including possible conservation of p53 between Atlantic salmon and rainbow trout (**Figure B1**) and possible contradiction to reported activities of p53 in rainbow trout with regards to drug response in the literature (**Figure B2**).



**Figure B1: Comparison of Baseline p53 levels in Rainbow Trout Cell Lines**

Rainbow trout cell lines were exposed to different concentrations of an Mdm2 inhibitor, N3, and analyzed for p53 expression after a 24h exposure. Protein extracts were subjected to SDS-PAGE electrophoresis and western blotting. Ponceau S staining of protein extracts is shown at the top of the figures followed by detection of p53. The primary antibody is a rabbit anti-p53 polyclonal antibody developed in this laboratory. The secondary antibody was an HRP-conjugated goat anti-rabbit IgG.

During the preliminary screening experiments shown in **Section 3.1**, cell lines were also probed with the anti-p53 polyclonal antibody (Liu et al., 2011) generated by Michelle Liu, a previous MSc student of the Bols and Duncker laboratories. First RTgill-W1, RTliver-W1, and RTbrain-W1 were compared directly for expression of p53 during normal, unperturbed conditions (**Figure B1**). Interestingly, RTbrain-W1 showed a significantly higher level of p53 expression compared to the other rainbow trout cell lines, even when taking into account the differences in Ponceau stain intensities. The use of Nutlin-3, a known Mdm2 inhibitor, was examined as an aside to see if it had an effect on p53 regulation. The result shown is unexpected, since p53 is under constitutive negative regulation by Mdm2, and



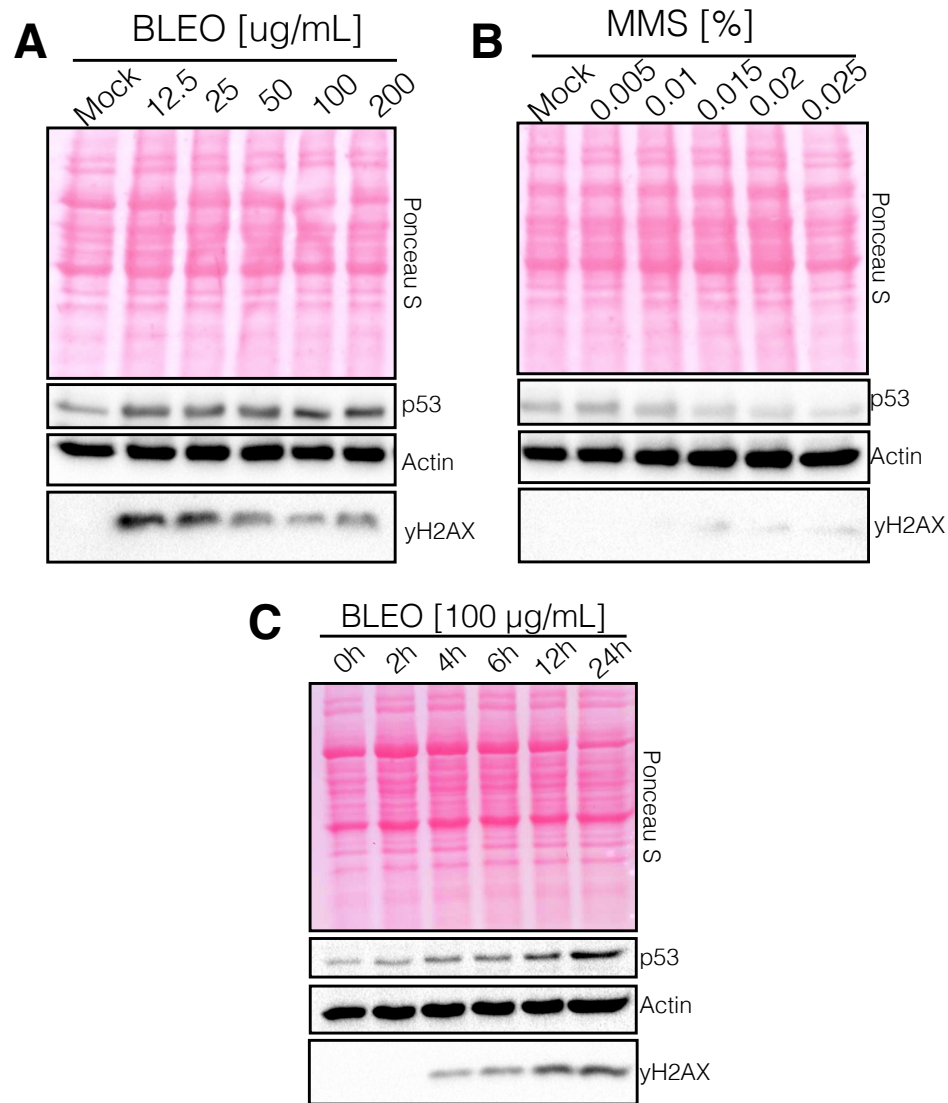
**Figure B2: Comparison of p53 levels in Asimf20 and RTbrain-W1 Treated with Bleomycin and HU**

Asimf20 and RTBrain-W1 were exposed to different concentrations of HU and Bleomycin and analyzed for p53 expression after a 24h exposure. Protein extracts were subjected to SDS-PAGE electrophoresis and western blotting. Ponceau S staining of protein extracts is shown at the top of the figures followed by detection of actin, p53, and  $\gamma$ H2AX. The primary antibodies were a rabbit anti- $\beta$ -actin polyclonal antibody (A2066, Sigma), a rabbit anti- $\gamma$ H2AX monoclonal antibody (9718, Cell Signal), and a rabbit anti-p53 polyclonal antibody developed in this laboratory. The secondary antibody was an HRP-conjugated goat anti-rabbit IgG.



these concentrations have been shown to in the literature to inhibit Mdm2 in lymphoblastic leukemia cells (Gu et al., 2008). It could be that the cancerous nature of the published cell type played a role in this, that genotoxic insult is necessary to get detectable changes in p53 expression, or it may be in support of differences in p53 activities in fish (Embry et al., 2006). Nevertheless, this reaffirmed the decision to pursue RTbrain-W1 as a candidate for studying biomarkers of genotoxicity in this thesis.

The cell lines from **Section 3.1** were also assayed for p53 expression using the same antibody, and non-specific banding was observed (data not shown). However Asimf20 showed a detectable band, as shown in **Figure B2-A**. Though very faint, it was interesting because it was the same weight as rainbow trout p53. This is plausible, since rainbow trout and Atlantic salmon are more closely related than the other species tested, but it did not appear to be inducible. The possibility of it being a non-specific still exists, which would have to be confirmed by mass-spectrometry. Another surprising observation was subtle increase in rainbow trout p53 in response to hydroxyurea, and a strong induction in expression in response to bleomycin (**Figure B2-B**). These observations were striking because bleomycin has shown in the literature to have no effect on p53 expression in RTbrain-W1 at 100 µg/mL (Liu et al., 2011), and at 100 µM in RTliver-W1 cells (Embry et al., 2006). In terms of RTbrain-W1 discrepancies, though somewhat of a mystery, I speculate that it could be due to different passages of the cell line being used, or due to the batch of bleomycin being used. In terms of null results in RTliver-W1, the results from **Figure B1** show very low basal expression of p53 in this cell line; though I did not test RTliver-W1 response to genotoxic agents, it is possible that induction of expression would be very low as well, or even non-existent.



**Figure B3: p53 Expression in RTbrain-W1 Treated with Bleomycin and MMS**

Cells were exposed to different concentrations of bleomycin (**A**) and MMS (**B**) and analyzed for p53 expression after a 24h exposure. A time-course of p53 expression is shown in (**C**) of cells treated with 100 μg/mL bleomycin. Protein extracts were subjected to SDS-PAGE electrophoresis and western blotting. Ponceau S staining of protein extracts is shown at the top of the figures followed by detection of actin, p53, and γH2AX. The primary antibodies were a rabbit anti-β-actin polyclonal antibody (A2066, Sigma), a rabbit anti-γH2AX monoclonal antibody (9718, Cell Signal), and a rabbit anti-p53 polyclonal antibody developed in this laboratory. The secondary antibody was an HRP-conjugated goat anti-rabbit IgG.

These results are echoed in **Figure B3**, the same blots as presented in **Figure 3.5A, B** but including p53 expression. An increase in p53 can clearly be seen in cells treated with increasing concentrations of bleomycin, and the opposite is observed with MMS exposure, although the latter is probably due increasing numbers of dying cells rather than a function of p53. p53 expression was also detected during the time-course from **Figure 3.8A**, as shown in **Figure B3-C**. A time-dependent increase was observed similar to  $\gamma$ H2AX, starting at 4h and increasing all the way through 24h. It is possible, since p53 is induced during starvation (Shi et al., 2012) that cells in culture for 24h may start to show molecular symptoms of starvation despite the presence of 10% FBS. A definite way to rule out this possibility would be to perform a parallel time course of cells in media lacking bleomycin, and compare p53 expression at each time point.