

# Characterization of p53 in rainbow trout cell lines

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.

I understand that my thesis may be made electronically available to the public.

## **Abstract**

The tumour suppressor protein p53 is a critical protein in the DNA damage checkpoint pathway. It acts as a transcription factor that is involved in initiating the apoptotic pathway; disruption of this pathway can lead to various forms of cancer. Due to its importance in cancer prevention, p53 has been extensively studied, though only a small fraction of these studies have been in non-mammalian models. Some previous studies of p53 expression and regulation in lower vertebrate species have shown potential differences in its control, in comparison to the better characterized mammalian pathways. These differences emphasize the need to further investigate its mechanism of action in lower vertebrate models. To evaluate its biomarker potential for aquatic toxicology studies, two rainbow trout cell lines (RTbrain-W1 and RTgill-W1) were used in dose response experiments using DNA damage checkpoint inducing agents: bleomycin, hydroxyurea, and methyl methanesulfonate. For our studies, a rainbow trout specific polyclonal antibody was developed using purified recombinant rainbow trout p53. The purified antibody was shown to be successful in detecting p53 in rainbow trout cell lines and tissues. Our results show that rainbow trout p53 is not induced by checkpoints initiated by different types of DNA damage. These results infer the possibility of an alternate mechanism of DNA damage checkpoint reaction in these cell lines, which is independent of p53 induction.

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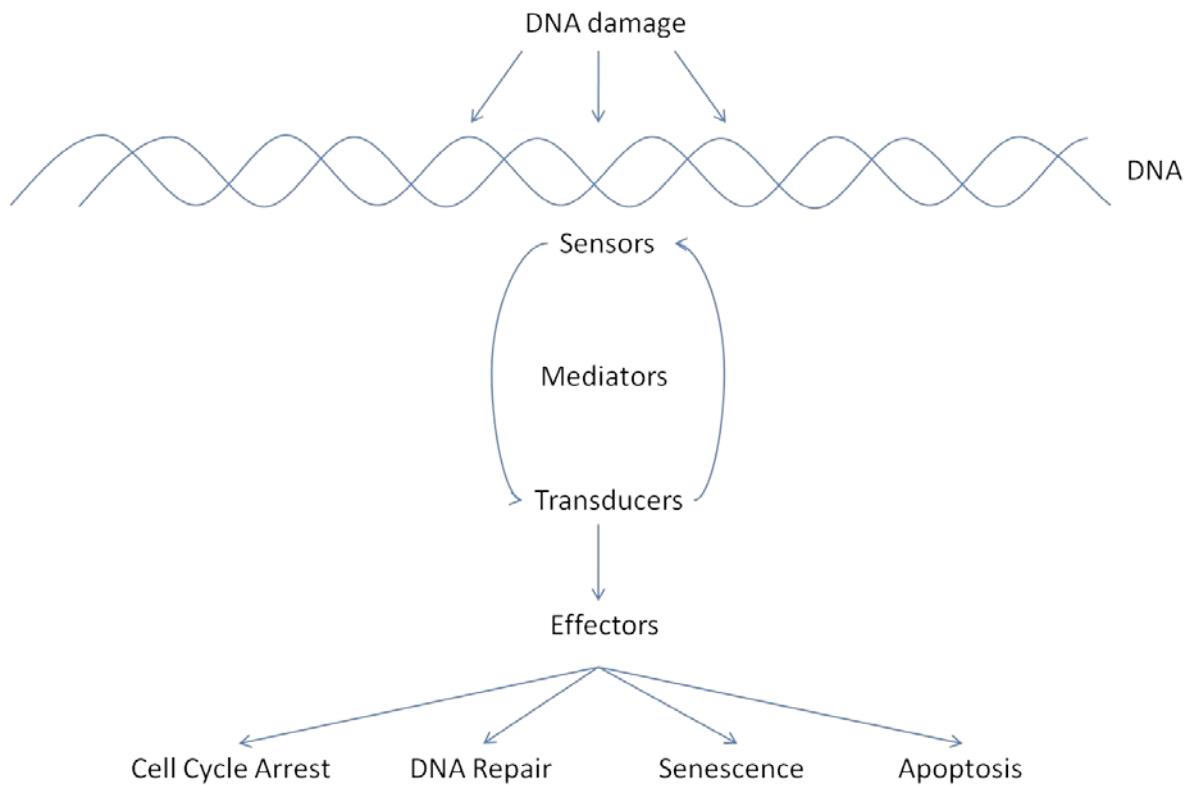
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# **Chapter 1**

## **General Introduction**

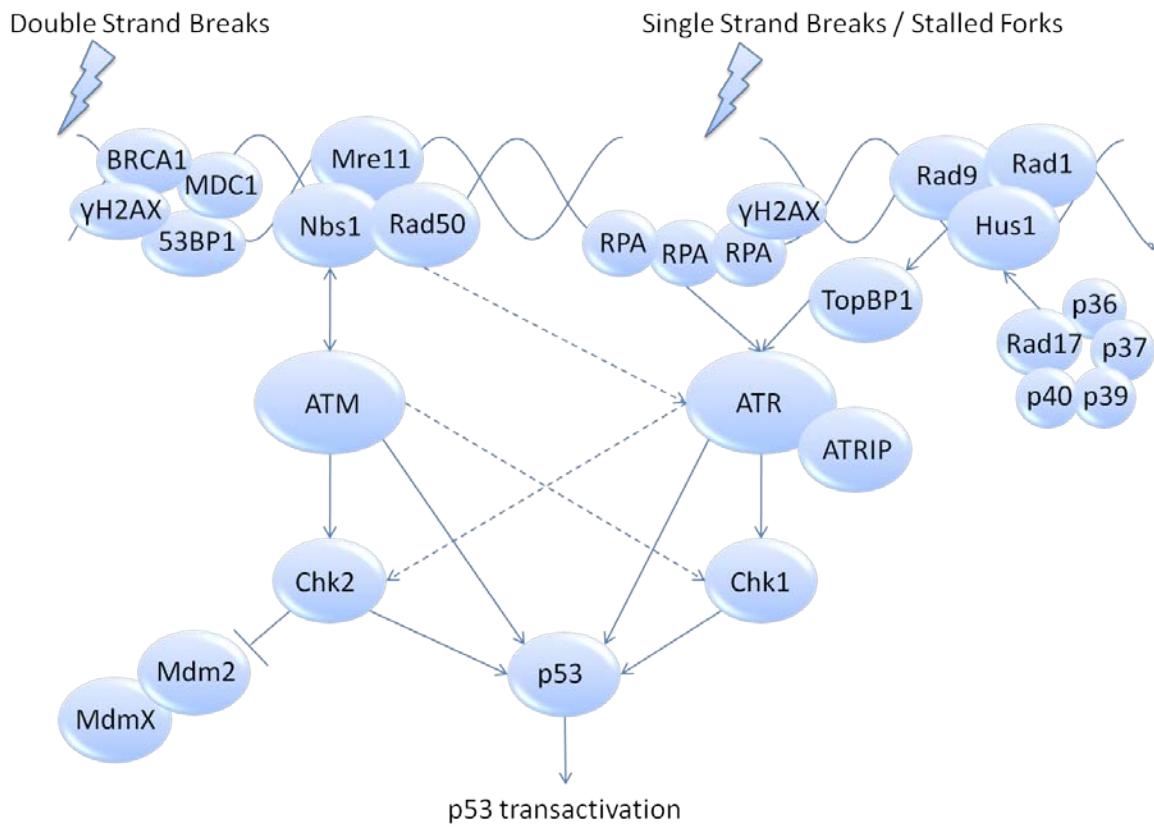
### **1.1 DNA damage checkpoint**

DNA damage is a common occurrence in the cell that can lead to mutations and cancer. To protect against the onslaught of DNA damage, eukaryotic cells have DNA damage response pathways which are composed of cell cycle checkpoints, DNA damage repair, and apoptosis (Sancar *et al.*, 2004). When cell cycle checkpoints are activated, cell cycle arrest is initiated, allowing time for DNA damage repair. In situations where the damage is too extensive to be repaired efficiently, cells can undergo apoptosis. In the DNA damage response pathways, there are several main types of proteins that are involved in leading the cells to the desired outcome. Due to the complexity of these pathways, various proteins function at multiple points in the pathways. Based on their function, proteins in the DNA damage checkpoint are loosely grouped into the categories of sensors, transducers, mediators, and effectors (Niida and Nakanishi, 2006), representing the flow of signals in the DNA damage checkpoint (Figure 1.1). There are several lines of thought that exist for categorizing these proteins that can be based on chronological activation, as well as function. It seems that many proteins in the pathway have multiple roles, and depending on the stage of DNA damage checkpoint activation, the proteins could have roles that transition from one role to the next. The main network of these proteins is shown in Figure 1.2, and their roles are discussed below.



**Figure 1.1 The DNA damage response pathway**

The DNA damage response pathway is composed of many proteins that are grouped loosely into the categories of sensors, mediators, transducers, and effectors. Sensors are localized to sites of DNA damage and are able to activate downstream transducers with the assistance of mediator proteins. The transducers are then able to activate downstream effector proteins. Arrows represent interactions between the proteins in each category and the bottom row represents the potential end results of the pathway.



**Figure 1.2 The DNA damage checkpoint**

The DNA damage checkpoint is composed of many proteins. The diagram here represents the main interactions in the mammalian pathway, which are initiated upon DNA damage. Solid arrows represent positive interactions between the proteins. Dotted arrows represent “cross-talk”. Perpendicularly ended lines indicate an inhibitory effect.

### **1.1.1 Sensors**

During the cell cycle, there are several types of changes in DNA that could initiate a DNA damage checkpoint. These changes could include double strand breaks, single strand breaks, and stalled replication forks. Depending on the type of change, different sensor proteins can be activated and localized to the site of damage (Bartek and Lukas, 2007). The 9-1-1 complex which is composed of Rad9, Hus1, and Rad1 is essential for the detection of DNA damage (Qin and Li, 2003). The 9-1-1 complex is similar in structure to the homotrimeric sliding clamp protein, proliferating nuclear antigen (PCNA) (Venclovas and Thelen, 2000). While both the 9-1-1 complex and PCNA have similar roles as sliding clamps, they are active under different circumstances. The PCNA complex is loaded onto DNA by the RFC (replication factor C) complex during regular DNA replication and serves as a sliding scaffold for the tethering of replication proteins (Moldovan *et al.*, 2007). This RFC complex is composed of 5 subunits (p36, p37, p39, p40, and p140). Alternatively, the 9-1-1 complex is loaded onto the DNA in response to DNA damage with the help of the Rad17-RFC (Rad17-replication factor C) checkpoint clamp loader complex which contains Rad17 in place of p140 (Parrilla-Castellar *et al.*, 2004). The activation of the 9-1-1 complex has been shown in the presence of alkylation, ultraviolet light, ionizing radiation, and stalled replication (Parrilla-Castellar *et al.*, 2004). This formation of the Rad17-RFC/9-1-1 checkpoint complex is one of the early steps in the initiation of the DNA damage checkpoint response.

For detection of double strand breaks (DSBs), the MRN complex (Mre11-Rad50-Nbs1) is localized to regions of DSBs, through the recognition of DNA by the Rad50 subunit

(Van Den Bosch *et al.*, 2003). The MRN complex is thought to be an upstream sensor complex because of its early localization to DSBs. Its recruitment to DSBs is also independent of other proteins in the DNA damage response checkpoint, which is another factor contributing to its categorization as a sensor complex. The localization of the Nbs1 subunit to sites of DSBs is also required for the downstream recruitment of ATM (ataxia telangiectasia mutated). ATM is a component of the PIKK (phosphoinositide 3-kinase related kinases) family of kinases, which also includes ATR (ATM and Rad3 related) and DNA-PK (DNA-dependent protein kinase) (Ackermann and El-Deiry, 2008). ATM and ATR are known as both sensors and transducers in the DNA damage checkpoint pathway. Once ATM has been recruited to the DSBs by the MRN complex, several events are initiated that result in local conformational changes in the DNA. This change in the structure of DNA is thought to facilitate the subsequent DNA checkpoint signalling and DNA repair events. To begin this process, the PIKK kinases phosphorylate serine139 of the histone protein H2AX, which acts as a scaffold for the mediator protein MDC1 (mediator of damage checkpoint protein 1). While bound directly to  $\gamma$ H2AX, MDC1 will then also interact with the Nbs1 subunit of the MRN complex. Together, these processes allow for continued H2AX phosphorylation. These changes in chromatin conformation and foci formation also lead to the autophosphorylation of ATM.

Along with the recruitment of ATM, ATR is also recruited to sites of DNA damage through the presence of ssDNA. When genotoxic stress is present, large stretches of single-stranded DNA can be generated which are coated in RPA (replication protein A) (Zou and Elledge, 2003). ATR senses stretches of RPA coated ssDNA that may be a result of single-

stranded breaks (SSBs), replication fork stalling, or DNA lesions caused by DSB resection.

ATR is localized to sites of ssDNA through forming a complex with ATRIP (ATR-interacting protein). After recruitment to the ssDNA, it is bound to the DNA through ATR-ATRIP oligomerization and interaction with RPA (Ackermann and El-Deiry, 2008), though this localization is not sufficient for activation of the complex (MacDougall *et al.*, 2007). It has been shown that the interaction of the ATR-ATRIP complex with TopBP1 (DNA topoisomerase II $\beta$  binding protein I) is necessary for the activation of ATR once it is localized. Once activated, ATR is able to phosphorylate downstream proteins to propagate the kinase signal cascade.

While the 9-1-1 complex and the ATR-ATRIP complex are recruited to DNA independently, the presence of the 9-1-1 complex facilitates the ATR-mediated phosphorylation of the downstream transducer kinase Chk1 (Weiss *et al.*, 2002). These results indicate that while the initiation events of each pathway may be separate, interaction between the pathways may still be required.

### **1.1.2 Mediators**

Mediators are proteins that act as recruiters and scaffold components for other components in the DNA damage checkpoint pathway and can modulate the activity of ATM/ATR, as well as facilitate interactions between kinases and their substrates (Kastan and Bartek, 2004). The MRN complex mentioned above is also occasionally thought of as a mediator protein, due to its function as a scaffold for other proteins, though its early recruitment to the sites of DNA damage indicate that it functions mainly as a sensor. MDC1 is one of the main mediator proteins, and was briefly mentioned above. The phosphorylation of H2AX is thought to be

sustained by MDC1, possibly through retention of ATM. Together, these proteins act as a scaffold to help form foci with other mediators such as 53BP1 (p53 binding protein 1), Claspin, and BRCA1 (Kastan and Bartek, 2004). Along with phosphorylation, ubiquitination also plays a role in generating mediator foci at sites of DSBs. The E3 ubiquitin ligase Ubc-13-Rnf8 is recruited by the phosphorylated form of MDC1, and is then able to ubiquitinate H2AX. Along with the E3 ubiquitin ligase, BRCA1 also plays a role in the ubiquitination of proteins at the foci (Starita and Parvin, 2003). Through phosphorylation, ubiquitination, and the formation of foci, the mediator group of proteins help to facilitate the interactions between the other proteins and substrates in the DNA damage pathway.

### **1.1.3 Transducers**

The transducer category consists of protein kinases that are activated in the presence of DNA damage, which then participate in the signalling cascades that result in an amplification of the original DNA damage signal. Though both ATM and ATR were mentioned earlier in the sensor category, they are both also commonly characterized as transducer proteins.

Temporally, they respond early in the DNA damage response, indicative of many sensor proteins. However, their critical role in perpetuating the kinase cascade is considered as part of their role as transducers. While ATM and ATR are structurally similar large proteins, they carry out distinct roles in the DNA damage response pathway. ATM is mainly activated in response to DSBs, whereas ATR is activated in response to DNA replication stress that results in abnormally long stretches of ssDNA (Ackermann and El-Deiry, 2008). ATR can be activated in response to DSBs as well, but through an ATM dependent mechanism (Yoo *et al.*, 2007).

ATM is activated through phosphorylation at several sites in response to DSBs. Its inactive form is found as dimers or higher-ordered oligomers, which prevents it from binding to downstream substrates. Autophosphorylation of Ser1981 allows the deoligomerization of ATM oligomers, releasing monomers of ATM which can now interact with downstream substrates (Bakkenist and Kastan, 2003). This autophosphorylation was seen in HEK-293T cells but the site is also conserved in both mouse and *Xenopus* ATM. Other sites have also been implicated in phosphorylation events involved with ATM signalling, such as the autophosphorylation of Ser367 and Ser1893 in human lymphoblastoid cell lines (Kozlov *et al.*, 2006). While phosphorylation of these sites has been shown to monomerize ATM and aid in signalling, other research has shown that these phosphorylation events may not be necessary. ATM has been found in monomeric states, without phosphorylation at Ser1981 (Dupré *et al.*, 2006) and mutational studies in mice have shown that mutation of Ser1987 (mouse equivalent of Ser1981) does not abolish the response of ATM-dependent pathways (Pellegrini *et al.*, 2006). While these phosphorylation events do not appear to be essential for ATM activation, induction of these events have been shown in response to DNA damage induced by ionizing radiation (Bakkenist and Kastan, 2003). Once ATM has been recruited to sites of DNA damage and has been activated through phosphorylation, it can initiate the kinase cascade of downstream pathways through phosphorylation of various substrates including H2AX, Nbs1, BRCA1, MDC1, Chk2, Mdm2, and p53 (Ackermann and El-Deiry, 2008). H2AX, Nbs1, BRCA1, and MDC1 were mentioned above as early responders to DNA damage. These proteins help to form the initial scaffold for foci formation which facilitates the interactions between transducers and their substrates. Chk2, another transducer protein,

can also be phosphorylated by ATM, and is discussed in further detail below. ATM is also able to phosphorylate p53, which is a downstream effector protein. Phosphorylation of p53 and its negative regulator Mdm2 results in an inhibition of interaction between the two proteins, which in turn, stabilizes and activates p53 (Hock and Vousden, 2010). The mechanisms of this will be discussed in further detail later in this chapter. Mutations that compromise the function of ATM result in defective responses to specific types of DNA damage, which is a characteristic of the disease ataxia telangiectasia (McKinnon, 2004). Though ATM is a key part of the DNA damage response pathway, knockdown of ATM in mice has shown that ATM is not essential for viability in this organism (Barlow *et al.*, 1996).

Unlike ATM which functions in a monomeric state, ATR exists in a complex with ATRIP and is activated through interaction with TopBP1 (Mordes and Cortez, 2008). ATRIP is essential for ATR signalling and aids in the localization of ATR to site of DNA damage (Cortez *et al.*, 2001). TopBP1, which is necessary for ATR activation, is localized to sites of DNA damage through the 9-1-1 complex independently of ATR (Delacroix *et al.*, 2007). Cimprich and Cortez (Cimprich and Cortez, 2008) hypothesized that this independent recruitment of TopBP1 and ATR-ATRIP to sites of DNA-damage reduces the probability of inappropriate initiation of checkpoints, due to the requirement of two independent recruitment events. With both TopBP1 and ATR-ATRIP localized to the site of damage, TopBP1 is then able to activate the kinase activity of the ATR-ATRIP complex (Kumagai *et al.*, 2006). Since TopBP1 is a substrate of ATM, phosphorylation of TopBP1 by ATM can activate ATR in response to DSBs (Yoo *et al.*, 2007). While it is known that TopBP1 is required for ATR-ATRIP activation, the exact mechanisms are not as clear. It has been

shown that TopBP1 activates the ATR-ATRIP complex primarily through binding with ATRIP, though there are specific amino acids within ATR that are necessary for activation by TopBP1 (Cimprich and Cortez, 2008). Once activated, ATR is able to phosphorylate many substrates including ATRIP and TopBP1, which increases the kinase activity of ATR (Itakura *et al.*, 2004; Kumagai *et al.*, 2006). ATR is also involved in the phosphorylation of Nbs1 and H2AX for amplification of signal at the site of DNA damage, as well as phosphorylation of the transducer kinase Chk1 (Liu *et al.*, 2006). ATR is clearly important in the DNA damage response pathway and mutations in ATR have been associated with the congenital growth disorder Seckels syndrome. Unlike with ataxia telangiectasia patients, patients with Seckels syndrome display severe mental disabilities without displaying sensitivity to DNA damaging agents or a predisposition to cancer (O'Driscoll *et al.*, 2004). Also, differing from studies with ATM, knockdown of ATR resulted in embryonic lethality in mice (De Klein *et al.*, 2000). The embryonic lethality of ATR knockdown in mice indicates that ATR is an essential gene for viability in this organism.

Chk1 and Chk2 represent the other two main transducer kinases that are required for cell cycle arrest in the response to DNA damage. Unlike ATM and ATR, Chk1 and Chk2 are structurally different but appear to have overlapping functions in the DNA damage response pathway (Bartek *et al.*, 2001). The role of Chk1 and Chk2 is mainly focused on the relay of phosphorylation signals from the foci situated ATR and ATM to downstream effector proteins (Abraham, 2001). While both Chk1 and Chk2 are activated as part of the DNA damage response pathway, their basal expression and activation differ greatly. Chk1 is present in S phase and G2 phase, and though further induction of its activation has been

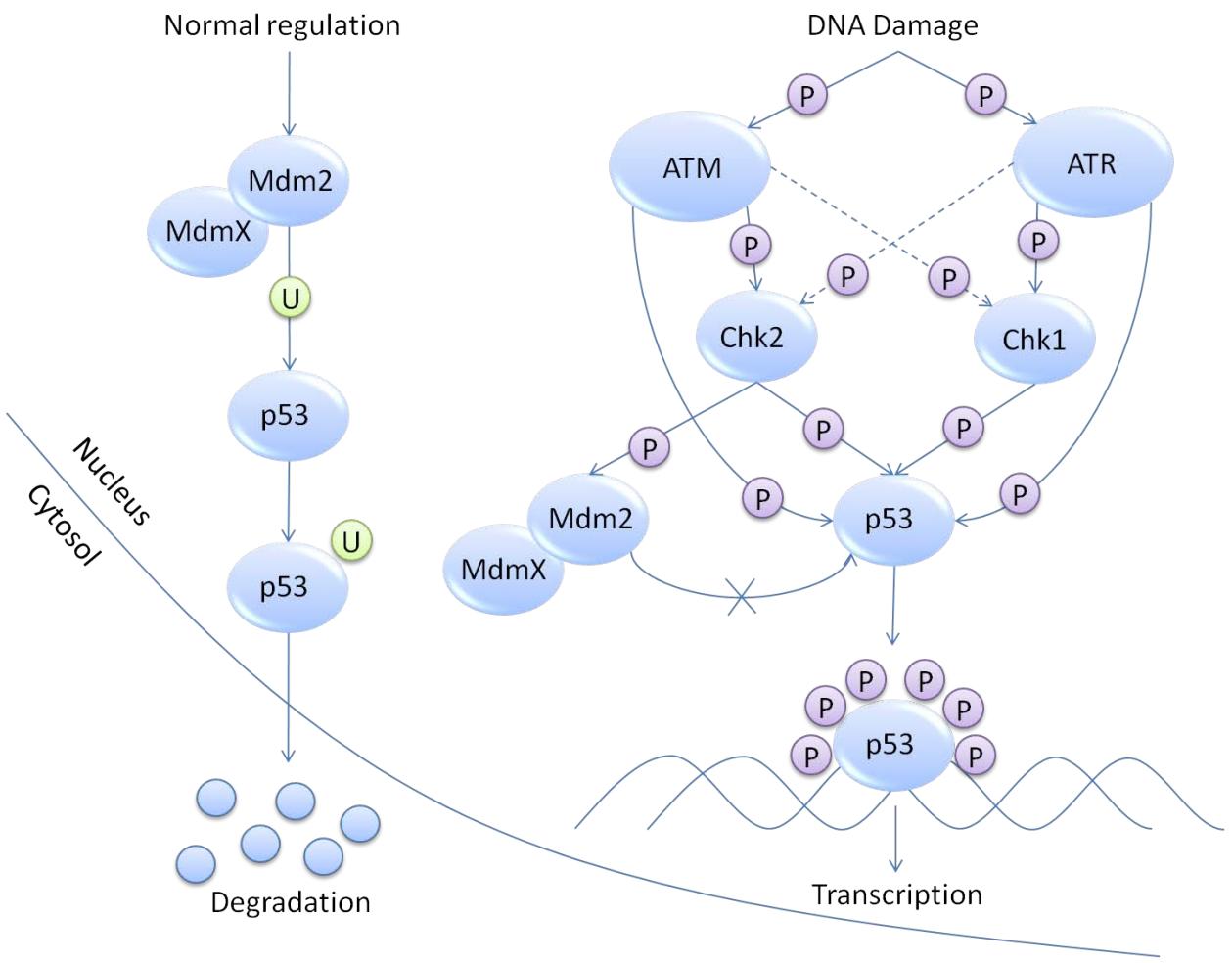
shown in response to DNA damage, it has also been shown in an active state in unperturbed cell cycles (Bartek and Lukas, 2003). Chk2 on the other hand, is stably expressed in an inactive form throughout the cell cycle, and it is activated in response to DSBs through phosphorylation mainly by ATM (Chaturvedi *et al.*, 1999). Activation of Chk2 requires dimerization and autophosphorylation, whereas Chk1 does not require either of these for activation (Bartek and Lukas, 2003). It was originally thought that Chk1 was activated through ATR and Chk2 was activated through ATM, representing two different pathways. Studies showing “cross-talk” between the two pathways have demonstrated that the phosphorylation of Chk1 and Chk2 by ATR and ATM are not exclusive. ATM-independent activation of Chk2 has been shown (Hirao *et al.*, 2002), along with ATM-dependent phosphorylation of Chk1 in response to ionizing radiation (Gatei *et al.*, 2003). Along with direct activation by transducer kinases, the activation of Chk1 and Chk2 is also modulated by sensors and mediators such as 53BP1, BRCA1, and MDC1, which share a BRCT domain in common (Bartek and Lukas, 2003). Once active in the pathway, both Chk1 and Chk2 can phosphorylate p53 on Ser20, disrupting its association with its negative regulator Mdm2 (mouse double minute 2) (Shieh *et al.*, 2000). MdmX, which forms an active heterodimer with Mdm2, has also displayed phosphorylation by Chk2, leading to further inhibition of interaction between p53 and Mdm2/MdmX (Chen *et al.*, 2005b). Both of these events lead to an accumulation of p53, which can then transactivate downstream proteins.

#### **1.1.4 Effectors**

Chk1 and Chk2 are occasionally categorized as effector kinases. While Chk1 and Chk2 primarily act as transducers in the DNA damage checkpoint pathway, they help to regulate

proteins involved in DNA repair, replication fork maintenance, transcriptional regulation and apoptosis (Stracker *et al.*, 2009). The regulation of these proteins is more characteristic of proteins in the effector category. Both Chk1 and Chk2 have been shown to target the Cdc25 family of phosphatases (Cdc25A, B, and C), which are involved in cell cycle progression, both during normal division, and after recover from DNA damage (Boutros *et al.*, 2006). The Cdc25 family of proteins normally dephosphorylate cyclin dependent kinases (Cdks) to regulate cell cycle progression, but when phosphorylated, their phosphatase activity is downregulated by direct inhibition, nuclear exclusion, ubiquitination, and degradation (Niida and Nakanishi, 2006).

Another prominent effector protein is the tumour suppressor protein p53, which is an important part of the DNA damage checkpoint. It is known as a tumour suppressor protein because loss-of-function mutations in the p53 gene are present in greater than 50% of cancers. It is a transcription factor that can initiate DNA repair, cell-cycle arrest, senescence and apoptosis in the checkpoint pathway, aiding the suppression of cancer (Vazquez *et al.*, 2008). In normally dividing cells, p53 levels are kept low by targeted nuclear export and degradation by its negative regulator Mdm2, although other research has shown that there may be other factors (MdmX, ARF, COP1, PirH2) which are also involved in the control of p53 ubiquitination and degradation (Brooks and Gu, 2006). In the event of DNA damage, p53 is phosphorylated by the signal transducers in the checkpoint pathway, which prevents its ubiquitin-mediated protein degradation (Helton and Chen, 2007). The regulation, activation and functions of p53 are shown in Figure 1.3 and will be discussed in detail in the following section.



**Figure 1.3 p53 regulation and activation**

Under normal conditions, p53 is negatively regulated and kept at low levels through degradation. In the presence of DNA damage, p53 is activated, mainly through phosphorylation. This diagram highlights the main pathways of p53 regulation and activation in mammalian models. Solid arrows represent protein interactions. Dotted arrows represent “cross-talk”. Purple Ps represent phosphorylation. Green Us represent ubiquitination. Solid arrows with an X represent an inhibited interaction.

## 1.2 p53

### 1.2.1 Regulation

p53 can be controlled in several ways but the main mode of regulation is through changes in protein stability. The levels of p53 protein are normally kept at low levels in the cell through proteasomal degradation. Mdm2, a well known negative regulator of p53, is one of the key proteins responsible for targeting p53 for degradation. Mdm2 is an E3 ubiquitin ligase that can ubiquitinate p53, targeting it for export from the nucleus and subsequent degradation by proteasomes (Michael and Oren, 2002). It was shown in 1997 to be highly specific for p53 (Haupt *et al.*, 1997; Honda *et al.*, 1997; Kubbutat *et al.*, 1997) and loss of Mdm2 *in vivo* can lead to p53-mediated apoptosis. Along with Mdm2, the related protein MdmX is also important for p53 regulation. Unlike Mdm2, MdmX is an essential negative regulator of p53 (Marine and Jochemsen, 2005), though MdmX does not possess any E3-ligase ability. Instead, it regulates p53 through its interaction and binding with Mdm2. It has also been shown to repress the transcription factor functions of p53 (Marine and Jochemsen, 2005). Although Mdm2 is the main ubiquitin ligase for p53 regulation, other studies have shown the involvement of other ubiquitin ligases such as COP1 (Dornan *et al.*, 2004), Pirh2 (Leng *et al.*, 2003), and Arf-BP1 (Chen *et al.*, 2005a) in the degradation of p53, displaying the complexity of p53 regulation. While these proteins are involved in the control of p53, their presence cannot compensate for loss of Mdm2 *in vivo* (Hock and Vousden, 2010). While ubiquitination is the main method of targeting p53 for degradation, it has also been shown that ubiquitination of p53 can be independent of degradation. The proteins Msl2 and Wwp1 are able to ubiquitinate p53, localizing it to the cytoplasm, without targeting it for

degradation. It is hypothesized that these mechanisms of localization may be involved in the transcription-independent functions of p53, which require its localization to the cytoplasm (Kruse and Gu, 2009).

While ubiquitination is the main method of p53 regulation, p53 can also be regulated by other other ubiquitin-like protein (UBL) modifications such as SUMOylation and NEDDylation. SUMOylation involves the modification of proteins through the addition of SUMO proteins (Small Ubiquitin-like Modifier), whereas NEDDylation involves the addition of the ubiquitin-like protein Nedd8. These UBL modifications also act on lysine residues at the carboxy terminal end of p53, similar to ubiquitination (Hock and Vousden, 2010). Both SUMOylation and NEDDylation can be promoted by Mdm2, as well as several other proteins. For SUMOylation, the PIAS family (protein inhibitors of activated STAT) of SUMO E3 ligases has to been shown to target p53 for SUMOylation (Rytinki *et al.*, 2009). SUMOylation of p53 results in reduced acetylation and reduced chromatin binding (Wu and Chiang, 2009), which is likely to inhibit the function of p53 as a transcription factor. SUMOylation mainly affects lysine 386 of human p53 and it has also been hypothesized that it can aid in the nuclear localization of p53 (Stehmeier and Muller, 2009). NEDDylation on the other hand, mainly affects lysines 320 and 321 of human p53. Aside from Mdm2, the F-box protein FBXO11 can also NEDDylate p53 (Abida *et al.*, 2007). NEDDylation of p53 shows similar outcomes to SUMOylation, with decreased transcriptional activity, and inhibition of nuclear export (Xirodimas, 2007). Together, these results emphasize the importance of ubiquitination and of other UBL protein modifications which are crucial in the regulation of p53 protein stability and function.

### **1.2.2 Activation**

Since Mdm2 is an important regulator of p53, changes in Mdm2 itself are important for control of p53. Under normal conditions, Mdm2 forms either a homodimeric complex with itself, or a heterodimeric complex with the related protein MdmX (Linke *et al.*, 2008).

Though MdmX lacks E3 ligase activity, there is increasing evidence for the importance of the Mdm2/MdmX heterodimer as the more active regulator of p53 (Okamoto *et al.*, 2005).

Independently, both Mdm2 and MdmX have the ability to bind p53 and, despite their sequence homology, they carry out non-redundant roles in the negative regulation of p53.

Since ubiquitin ligases play a large role in p53 regulation, the role of deubiquitinating proteins have also been examined. Certain proteins that function as deubiquitinating enzymes, such as HAUSP (Herpesvirus-associated ubiquitin-specific protease), can deubiquitinate both p53 and Mdm2, leading to stabilization of both proteins in a competitive feedback loop (Li *et al.*, 2004). Mdm2 and MdmX can also be phosphorylated by ATM (Cheng and Chen, 2010), resulting in the inhibition of dimerization, as well as prevention of binding with p53. Chk1 and Chk2 are also able to phosphorylate MdmX, promoting its own inactivation and degradation (Chen *et al.*, 2005b). Under conditions of genotoxic stress, Mdm2 can also act as a negative regulator of MdmX, targeting it for degradation through ubiquitination (Cheng and Chen, 2010). In addition to these mechanisms, there are other proteins that can interact with Mdm2 to regulate its activity. p14ARF (Khan *et al.*, 2004), Numb (Colaluca *et al.*, 2008), and Pin1 (Siepe and Jentsch, 2009), have all been shown to inhibit the Mdm2 directed degradation of p53 through interaction with the Mdm2/p53 complex.

In addition to inhibiting negative regulators of p53, several protein kinases in the DNA damage checkpoint pathway are also able to stabilize and activate p53 through direct phosphorylation of its serine and threonine residues at several different sites, mainly located in the N-terminal region (Joerger and Fersht, 2008). ATM and ATR have both been shown to activate p53 through phosphorylation, mainly of Ser 15 (Abraham, 2001). Chk1 and Chk2 have also been shown to phosphorylate p53 at several sites (Ou *et al.*, 2005). These phosphorylation events are induced by DNA damage, leading to an activation of p53. Phosphorylation of p53 at certain sites also facilitates acetylation of p53 lysine residues, a result which is seen during genotoxic stress. It is hypothesized that the acetylation of p53 inhibits its ubiquitination by Mdm2, due to competition for the same carboxy terminal residues. Phosphorylation of human p53 has been detected at 17 different serine (6, 9, 15, 20, 33, 37, 46, 315, 376, 378, 392) and threonine (18, 55, 81, 150, 155) sites after DNA damage induced by irradiation, with 3 of these sites (Thr55, Ser376, Ser378) being constitutively phosphorylated (Bode and Dong, 2004). Of these sites, some are phosphorylated by multiple kinases, some of which can phosphorylate multiple sites, generating a complex network of regulation through phosphorylation. While research in p53 phosphorylation is mainly focused on phosphorylation as a consequence of DNA damage, one study has examined the phosphorylation of p53 at various stages throughout the cell cycle in untreated human fibroblasts (Buschmann *et al.*, 2000). Their study revealed that p53 phosphorylation is dynamic, and that phosphorylation at several sites were transient events throughout the cell cycle of normal cells. Of the known sites of phosphorylation, Ser15 phosphorylation is one of the most frequently described and has been shown in response to genotoxic stress signals.

One of the main proteins responsible for phosphorylation of Ser15 of p53 is ATM (Khanna *et al.*, 1998).

The studies summarized here show that the modifications of p53 through ubiquitination, acetylation, and phosphorylation form a complicated map of interactions, which function together to regulate and activate p53.

### **1.2.3 Mechanisms of action**

p53 is an important tumour suppressor protein that is involved in the transactivation of various proteins involved in cell cycle arrest, DNA repair, senescence, or apoptosis (Levine *et al.*, 2006). Upon examining the structure of p53, several functional domains have been defined. The N-terminal regional contributes to the transactivation domain, followed by a proline-rich domain which is required for the mediation of apoptosis (Venot *et al.*, 1998). The central core of the protein contains the DNA-binding domain and a tetramerization domain (Joerger and Fersht, 2008). This DNA-binding domain can target specific sequences of DNA for binding and the tetramerization domain regulates the oligomerization of p53 in the formation of its active state. The C-terminal region of the protein contains a regulatory domain which is thought to bind DNA in a non-specific manner. Once it has been activated through post translational modifications, p53 forms a tetramer and is able to act as a transcription factor for downstream proteins through its DNA-binding and transactivation domains (Joerger and Fersht, 2008). The first discovery of the sequence specific DNA-binding activity of p53 was shown in 1991 (Bargonetti *et al.*, 1991) (Kern *et al.*, 1991), and the DNA consensus sequence of p53 response elements was published soon after in 1992 (El-Deiry *et al.*, 1992). The determination of the sequence specific DNA-binding activity of p53

was an important discovery in defining the importance of p53 in tumour suppression. Many tumor-associated p53 mutations exist in the DNA-binding domain (Olivier *et al.*, 2002), indicating that the sequence specificity is of great importance in p53 function. When p53 is stabilized and activated, it can interact with the promoter region of target genes to regulate their rates of transcription. Since p53 is involved in the regulation of many genes, Riley *et al.* (2008) proposed a set of four experimental criteria in their study to find p53-response genes. The putative p53-response gene must contain a p53 response element, it must also show a p53-dependent upregulation or downregulation at the RNA and protein levels, it must demonstrate direct control of gene expression by p53 through a luciferase assay, and it must demonstrate direct binding of p53 to the p53 response element through chromatin immunoprecipitation (ChIP) (Riley *et al.*, 2008). They were able to identify over 100 human genes that are candidates for regulation by p53, mostly involved in apoptosis, senescence, or cell-cycle arrest. These results further emphasize the importance of p53 in the control and maintenance of genome integrity and cell cycle regulation, through its transactivation of downstream genes.

#### **1.2.4 Non-mammalian models of p53 regulation**

While p53 has been studied to great depth in mammalian models, the research for p53 in non-mammalian models represents only a small portion of the published data on p53. From the data that is available for non-mammalian p53, similarities between p53 regulation, activation, and function can be found, though there is also strong evidence of unique mechanisms which differ from the mammalian models.

Studies on the Drosophila p53 orthologue have shown that like mammalian p53, it is able to bind specific regions of DNA and act as a transcriptional activator (Steller, 2000). These studies also demonstrated that p53 overexpression can induce apoptosis, and dominant-negative mutations can block IR-induced apoptosis (Steller, 2000). Despite these similarities, several key differences exist between Drosophila p53 and mammalian p53. As mentioned earlier, in mammalian models, Mdm2 is the key negative regulator of p53. However, in Drosophila, the essential residues for Mdm2 binding are not conserved in p53 (Brodsky *et al.*, 2004) and a homologue for Mdm2 has not been found (Rubin *et al.*, 2000). Activation of p53 in Drosophila also appears to differ from established mammalian models. In a study of apoptotic pathways in Drosophila, it was found that, like mammalian p53, Drosophila p53 was responsible for the transactivation of many genes in the apoptotic pathway (Brodsky *et al.*, 2004). However, the same study also showed that while p53 was necessary, it was activated without changing total p53 protein levels. This differs from mammalian models which usually display a several fold increase in p53 levels once it has been activated. Along with differences in Drosophila p53 regulation and activation, differences in p53 function have also been shown. Mammalian p53 is known to be involved in cell cycle arrest, DNA repair, and apoptosis, and while Drosophila p53 was shown to be involved in apoptosis, it does not seem to be involved with cell cycle arrest (Steller, 2000). Although these results are novel for p53, they are not unique to Drosophila, as other studies have shown similar findings in additional non-mammalian organisms.

Like Drosophila p53, zebrafish p53 also displays similarities and differences to mammalian p53, though the differences are fewer. Unlike in Drosophila, a zebrafish Mdm2

protein has been found that has conserved functional domains and displays interaction with p53 (Thisse *et al.*, 2000). The importance of the p53 DNA-binding domain is also apparent in zebrafish, as studies into mutations in the DNA-binding domain have shown that they result in higher susceptibility of these zebrafish to neuronal tumors (Berghmans *et al.*, 2005b).

While the mechanisms of zebrafish p53 activity appear very similar to mammalian p53, a study by Thisse *et al.* in 2000 showed that overexpression of Mdm2 in zebrafish embryos did not lead to spontaneous tumour formation, though similar studies in mouse models did display a high rate of tumour formation (Jones *et al.*, 1998).

In contrast to the results seen with zebrafish, studies of p53 in the medaka fish displayed several distinct differences in activation and function in comparison with mammalian p53. It has been noted that mutations in the p53 gene have rarely been found in naturally occurring or induced tumours in fish and studies of induced tumours in medaka fish have shown similar results, with no mutations found in p53 mutational hotspots of several induced tumours (Krause *et al.*, 1997). This result differs greatly from the mammalian model, as mutations in p53 are found in many mammalian tumours. In terms of p53 activation, it has been shown that p53 transcript levels can be induced in mammalian cells after exposure to UV radiation, but this induction of p53 transcripts was absent in medaka based experiments (Chen *et al.*, 2001).

Comparable differences in p53 have also been found in several other non-mammalian models. Similar to the results discussed for drosophila and zebrafish, rainbow trout cells have shown a lack of p53 induction when exposed to agents that are known to induce p53 in mammalian models (Embry *et al.*, 2006). Dosing with various chemotherapeutic agents

showed a lack of induction for rainbow trout p53, in comparison to the obvious increase seen in mammalian control cells (Embry *et al.*, 2006). Similarly, a study in ayu showed a lack of total p53 induction under apoptotic conditions induced by aging related oxidative stress (Nagasaki *et al.*, 2006). However, they did observe an induction of p53 phosphorylation on Ser15, an event which commonly accompanies total p53 induction in mammalian models (Canman *et al.*, 1998). A study of urodele p53 generated similar results to those described in the ayu study. After treatment of urodele cells with UV, a lack of induction in p53 after treatment was seen, along with an induction of Ser15 phosphorylation (Villiard *et al.*, 2007). This study also showed interesting results upon sequencing of urodele p53. In a comparison of urodele p53 and human p53, it was found that the urodele p53 had multiple amino acid differences that were found in human tumours. This result indicated that the urodele p53 was able to tolerate amino acid changes that are predicted to inactivate human p53.

When viewing p53 from an evolutionary point of view, the differences in p53 between the different animal models can be partially supported by the ancestry of p53. In a study of p53 ancestry, it was determined that the appearance of cancer was predicated by primordial p53 genes, inferring that p53-like proteins were not originally selected for based on tumour suppressive properties (Lu *et al.*, 2009). This change in function of p53 could explain the differences between mammalian models of p53 versus the non-mammalian models. Well known mechanisms of regulation, activation and function of mammalian p53 may not be a result of ancestral p53 mechanisms, but rather represent newer developments in p53 evolution. Given the important role of p53 in tumour suppression and the high frequency of p53 mutations in human cancers, the current link between mammalian p53 and cancer is

indisputable, but this link may not be found in lower vertebrates. By examining the early mechanisms of p53 regulation, activation, and function, new insights into the evolution of p53 can be gained, possibly leading to potential uses in the development of novel cancer therapeutics.

### **1.3 Aquatic toxicology**

#### **1.3.1 Rainbow trout as a model organism**

Environmental toxicology is an important area of research that deals with the effects of toxic substances on the environment. These toxicants are usually released into the environment by human actions and can negatively affect the environment. For research into aquatic toxicology, teleost species are commonly used as model organisms. Due to a genome duplication event at the base of teleost radiation, the evolution of fish species was facilitated, leading to fish composing more than half of all vertebrate species (Furutani-Seiki and Wittbrodt, 2004). They are also useful due to their presence in most aquatic environments around the world, and their importance in the diet of diverse populations. *Oncorhynchus mykiss* (rainbow trout) in particular, is a commonly cultivated freshwater fish in North America and it is one of the best studied fish species due to its usefulness as a model organism for environmental carcinogenesis, toxicology, comparative immunology, physiology, and disease ecology (Thorgaard *et al.*, 2002). It also has a high sequence availability, in terms of expressed sequence tags (ESTs), with *Danio rerio* (zebrafish) being the only fish species to have a higher availability than the rainbow trout (Govoroun *et al.*, 2006). In terms of aquatic toxicology research into the effects of toxicants, the use of rainbow trout as a model organism is also advantageous due to the large number of cell lines

available for use. Currently, a search of the American Type Culture Collection catalogue retrieves 5 rainbow trout derived cell lines (RTG-2, RTH-149, SOB-15, RTgill-W1, RTG-P1), though these represent only a fraction of the total number of rainbow trout cell lines that have been available for research. A list of fish cell lines compiled in 1994 consisted of 125 cell lines from 52 species of freshwater fish in 21 families, with 13 of those cell lines originating from rainbow trout (Fryer and Lannan, 1994). The use of cell lines can also be advantageous to whole fish studies in regards to constraints of experimental design. With cell lines, a large amount of data can be produced in a shorter amount of time with less resources, which is useful in generating initial discoveries that can then be examined in whole fish studies. For these reasons, the rainbow trout represents a great model organism for a variety of studies regarding the effects of environmental contaminants.

### **1.3.2 Biomarkers**

Due to the constant increase of urban communities and industries, many aquatic environments are increasingly exposed to xenobiotics and other pollutants being released into the environment. Normally when assessing water quality, a number of physical, chemical, and biological parameters are experimentally assessed to generate a water quality index (WQI), which can be compared to existing guidelines (Boyacioglu, 2009). These parameters can be evaluated through environmental risk assessment (ERA), which attempts to determine the likely or actual adverse effects of pollutants on ecosystems using scientific methodologies (Depledge and Fossi, 1994). While many chemicals are not directly toxic to humans, they can have deleterious effects on the natural resources we rely on, emphasizing the importance of ERA in environmental analysis. Though the determination of water quality through

detection of environmental levels of pollutants is useful, this does not provide a full picture of their effects on the environment, as various toxicants can have a combined consequence that is greater than the sum of their individual effects (Calabrese, 1995). The deleterious effects of long term exposure to compounding pollutants may not be apparent initially in the endogenous fish population, and by the time the effects manifest, it may be too late to reverse the damage. It is due to these concerns that recent research has highlighted the use of biomarkers in the assessment of changes within a biological system. Biomarkers can be defined as measurements in body fluids, cells, or tissues, which can indicate biochemical or cellular changes (NRC, Committee on Biological Markers, 1987). In terms of toxicology, they can also be defined as xenobiotically induced changes in cellular or biochemical components or processes, structures or functions, which can infer exposure and be used to assess the biological effects (Shugart, 1992). Ideally, a biomarker should meet several requirements. The method of collection should be simple, reliable and ethical; it should be indicative of a specific type of exposure; and it should detect a reversible subclinical change (Gil and Pla, 2001). While biomarkers can be used to assess exposure between an organism and potential hazards in any environment, a good proportion of the research has been aquatically based, focusing on fish studies. Both transcript levels and protein levels have been used as environmental biomarkers in a variety of fish studies for possible use in diagnostic tools (Tom and Auslander, 2005). Metallothioneins (MT), a family of cysteine-rich proteins, has been examined for biomarker use due to its metal binding capacity. They have been studied in many aquatic vertebrates and have shown clear induction in several of these marine species after exposure to metal contaminants (Monserrat *et al.*, 2007). Another

well studied biomarker for environmental contamination in aquatic environments is cytochrome P450. It has shown sensitive dose dependent induction in many marine species after exposure to contaminants (Bucheli and Fent, 1995). Currently, the search for new biomarkers has involved the use of new technology for large screening tests in an effort to identify possible candidates for further testing. Due to the use of tissue microarrays, many potential biomarkers have been found for many areas of research (Giltinan and Rimm, 2004). Many of these studies are clinically based, but the use of tissue microarrays for screening could be extrapolated to aquatic biomarker studies as well. Though the use of biomarkers is not a recent development, the increasing use of biomarkers in aquatic toxicology requires increased research into characterizing DNA damage response pathways in fish, as these pathways may differ from the expected mammalian mechanisms.

#### **1.4 Research Objectives**

The main purpose of this thesis was to generate a rainbow trout specific p53 antibody for use in rainbow trout cell line based studies to determine the potential biomarker application of p53. This thesis is a small component of a larger project, with the goal of generating a checkpoint biomarker assay for potential use in environmental testing.

## Chapter 2

### Materials and Methods

#### **2.1 Expression construct**

Rainbow trout p53 was cloned from rainbow trout gill cell line (RTgill-W1) cDNA using forward (5' GACTTCTCGAGCTGGCGGAGAACGTGTCTCTTC) and reverse primers (5' GGACTTAAGCTTCACTCCGAAGTCCCCTTGGC) with XhoI and HindIII restriction sites respectively. These primers are targeted to amplify a 1125bp region of the rainbow trout p53 cDNA sequence (Caron de Fromentel *et al.*, 1992) that encodes amino acids 4-378 of the 396 aa long protein. The amplified fragment was gel purified and then inserted into a pRSET A expression vector (Invitrogen) through digestion with XhoI and HindIII restriction enzymes. The ligation reaction was performed using T4 DNA ligase (Promega) which results in the expression of a fusion protein with a polyhistidine tag and an Xpress epitope at the N-terminal end of the recombinant protein. This construct, pRSETA-RTp53, was then transformed into competent (DE3)pLysS *E. coli* cells (Promega) for inducible expression of recombinant rainbow trout p53 according to the manufacturer suggested protocol. The construct was also sequenced to confirm that the insert was in frame. The sequencing revealed a point mutation at base 1096, which causes an amino acid substitution of a cysteine to an arginine. This mutation is located in a non-conserved region of the gene.

#### **2.2 Protein expression and purification**

Cells transformed with the expression construct were grown in liquid SOB media supplemented with 100 $\mu$ g/ml ampicillin, shaking at 250 rpm at 37°C to an optical density of

0.4-0.6, before a 4 hour induction of recombinant protein expression with IPTG (in a final concentration of 1mM). After the 4 hour induction, the *E. coli* cells were harvested by centrifugation at 5 000 x g for 10 min and were lysed overnight at room temperature with a denaturing 8M urea buffer (100mM NaH<sub>2</sub>PO<sub>4</sub>, 10mM Tris-HCl, 8M urea, pH 8). The lysate was then centrifuged at 10 000 x g for 6 mins at 4°C before saving the supernatant and discarding the pellet. To isolate the recombinant p53 from the supernatant, affinity chromatography was performed in an econo-column (Bio-Rad) filled with Ni-NTA resin (Qiagen). This resin was allowed to bind the polyhistidine tag at the N-terminus of the recombinant protein during a 2 hour incubation with the lysate on a rotator at 4°C. After incubation, the flowthrough was discarded. The protein was then refolded on the column using a decreasing gradient of urea (6M-0M) before eluting in its native form with a 250mM imidazole elution buffer in 8 elution fractions of 1mL each. A 10µL sample of each fraction was then run on a 12% SDS-PAGE gel and analyzed by Coomassie blue staining to determine the fraction with the highest protein yield. This elution fraction (~1mL) was then dialyzed overnight at 4°C into 200x buffer volume of 1x PBS. The dialyzed protein sample was then analyzed through SDS-PAGE and Coomassie blue staining once again to verify that the protein of interest was still intact and present in the sample. A Bradford protein assay was then performed to determine protein yield of the samples. The dialyzed recombinant protein sample fractions were then combined and stored at -20°C in 1ml aliquots of 0.7mg/ml for future use as immunogen injections in rabbits.

### **2.3 Production of polyclonal antibodies to recombinant proteins**

The purified protein was injected into 2 rabbits over a period of three months to produce a high titre of antibodies to the purified protein. Rabbits were immunized subcutaneously with 200µL of recombinant p53 (~0.7mg/mL) mixed with 200µL of Freund's complete adjuvant (Sigma), at four different injection sites. The rabbits were then given booster shots of a similar emulsion (but with Freund's incomplete adjuvant) at three week intervals, for three additional boosts. Blood samples were obtained before each boost through the marginal ear vein of each rabbit. To separate the serum from the blood, samples were left at room temperature for 2 h and then overnight at 4°C to allow the blood to clot. Samples were then centrifuged at 5 000 x g for 10 min at 4°C to pellet the blood cells, and the serum was collected and assessed for antibody titre through ELISA analysis with purified recombinant p53 (described below). At the end of the twelfth week, the rabbits were exanguinated by carotid cannulation for final collection of total blood.

### **2.4 Determination of antibody titre through Enzyme-Linked Immunosorbent Assay (ELISA)**

Before each boost, and after the final bleed, serum antibody titers were determined through ELISA. Microtiter plates were coated with 100µl of 10µg/ml purified recombinant p53 protein diluted in coating buffer (15mM Na<sub>2</sub>CO<sub>3</sub>, 34mM NaHCO<sub>3</sub>, 0.02% NaN<sub>3</sub>, pH 9.3) and incubated for 2 h at room temperature. The wells were then blocked with 300µl of blocking buffer (1% BSA and 0.02% NaN<sub>3</sub> in 1X TBST) for 2 h at room temperature. The collected serum was then serially diluted and added to the wells in replicates at 100µl per well in blocking buffer and incubated for 2 h at room temperature. An alkaline phosphatase

conjugated anti-rabbit IgG antibody (Bio-Rad) was diluted 1:5000 in blocking buffer and added at 100 $\mu$ l per well and incubated for 2 h at room temperature. The plates were then detected with SigmaFAST p-Nitrophenyl phosphate tablets (Sigma) according to the manufacturer suggested protocol, and incubated for 30 min in the dark before stopping the reaction with 3N NaOH by adding 50 $\mu$ l directly to the wells. Absorbance was measured at 405 nm using a microplate reader (VERSAmax microplate reader, Molecular Devices). Readings were obtained after background correction through the SOFTmax PRO 2.6.1 program.

## **2.5 SulfoLink purification of RTp53 antibody from serum**

Antibodies specific to recombinant rainbow trout p53 were purified from crude serum with a SulfoLink Immobilization Kit for Proteins (Pierce). The SulfoLink column was generated by binding recombinant rainbow trout p53 to the resin to produce an affinity column specific for antibodies against this protein. This was done according to the manufacturer's suggested protocol with minor changes. To prepare the protein for coupling, the protein was reduced with 2-mercaptoethylamine (2-MEA) and run through the provided desalting column to remove remaining 2-MEA. The recombinant p53 had a concentration of 0.7mg/ml and 1ml of this was used directly in the reducing reaction before running through the desalting column. All steps for binding of the recombinant protein to the SulfoLink column were done according to the manufacturer's protocol. For affinity purification of p53 specific antibodies from the serum, 2ml of pure serum was used for each run. Each wash was performed the maximum amount of suggested times. The bound antibodies were then eluted into 4 aliquots, 1mL at a time with elution buffer. These aliquots were then evaluated using a Bradford

protein assay to determine the aliquots with the highest concentration of antibody. These aliquots were then stored for future use and the SulfoLink column was prepared for storage. A prepared SulfoLink column can be reused for multiple purifications when properly stored according to the manufacturer's suggested protocol.

## **2.6 Cell culture and treatment regimes**

The RTbrain-W1 and RTgill-W1 cell lines were obtained from Dr. Niels C. Bols (University of Waterloo). The RTbrain-W1 cell line was derived from rainbow trout brain glial cells. The RTgill-W1 cell line was derived from rainbow trout gill epithelium and is available from the American Type Culture Collection (ATCC CRL 2523). The cell lines were maintained in 75cm<sup>2</sup> flasks at 18°C in L-15 media (Sigma) supplemented with 15% FBS (Sigma) and 10% FBS respectively. Media was also supplemented with 1% penicillin-streptomycin solution (Sigma) to inhibit bacterial growth. For dosing studies, cells were seeded in 25cm<sup>2</sup> flasks at either 1.5 x 10<sup>6</sup> (RTbrain-W1) or 3 x 10<sup>6</sup> (RTgill-W1) cells per flask, 24 hours prior to treatment. Bleomycin (Calbiochem), hydroxyurea (HU)(Sigma), and methyl methanesulfonate (MMS)(Sigma) were all readily dissolved in media and were added to the cells at the concentrations indicated for each experiment. At the time of dosing, fresh media was also added to untreated control flasks to mimic dosing. Flasks were then incubated in the dark at room temperature for the indicated time points of exposure.

## **2.7 Protein extraction from tissue culture**

Cells were harvested by scraping the cells with a cell scraper and pelleted through centrifugation at 1000 x g before lysis with 50µL of RIPA lysis buffer (50mM Tris-Hcl pH 8.0, 150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium

dodecyl sulphate), with 1x Complete Mini Protease Inhibitor Cocktail Tablets (Roche) and 1x PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche) per 25cm<sup>2</sup> flask of confluent cells. Lysate was then spun at 16 000 x g for 10 min to remove cell debris before determining protein concentration using a Bradford reagent (Bio-Rad) according to the manufacturer's suggested protocol.

## **2.8 Protein extraction from tissues**

Rainbow trout were obtained from Alma Research Station (in association with the University of Guelph) and were acclimated for at least two weeks at the University of Waterloo prior to any experimentation in 200 L aerated tanks with constant water flow at 13°C. Fish were maintained on a 12 hr light:12 hr dark photoperiod. Organs were harvested from the rainbow trout and frozen on dry ice before storage at -80°C. Lysates were prepared through homogenization and sonication of frozen tissues in 50mM Tris-HCl buffer (pH 7.5) plus 1x Complete Mini Protease Inhibitor Cocktail Tablets (Roche). Lysates were centrifuged at 16 000 x g for 10 min to remove insoluble proteins. The supernatant was retained and protein concentrations were determined through Bradford protein assay (Bio-Rad) according to the manufacturer suggested protocol.

## **2.9 Western blotting**

Protein extracts were run at 20μg per sample (unless otherwise specified) on 12% SDS-PAGE gels and transferred to 0.2μM nitrocellulose membranes (Bio-Rad). Membranes were blocked in 5% dry milk in 1X TBST (50mM Tris-HCl pH 7.4, 150mM NaCl, 0.1% Tween 20) for 1 h at room temperature. The membranes were then probed with the appropriate primary antibodies at the following concentrations: anti-Xpress antibody (Invitrogen) 1:5000,

p53 (FL-393) antibody (Santa Cruz Biotech) 1:200, purified rainbow trout p53 antibody 1:200, Phospho-p53(Ser9) antibody (Cell Signalling) 1:1000, Phospho-p53(Ser293) antibody (Cell Signalling) 1:1000, p-Histone H2A.X (Ser139) antibody (Santa Cruz Biotech) 1:200, Phospho-(Ser/Thr) ATM/ATR Substrate Antibody (Cell Signalling) 1:1000, Monoclonal anti-actin-Cy3 (Sigma) 1:1000. For all primary antibodies except the anti-actin-cy3, membranes were probed with either an Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (1:3000) or an anti-rabbit-HRP secondary antibody (Bio-Rad) 1:3000, before detection with ECL Plus detection kit (Amersham) as per the manufacturer's suggested protocol. The anti-actin-Cy3 did not require a secondary antibody for detection due to its conjugation to a fluorescent Cy3 tag. For the phospho specific antibodies, incubations were done in 5% BSA in 1X TBST rocking overnight at 4°C. All other antibodies were incubated for an hour at room temperature in 5% dry milk in 1X TBST. The membranes were then imaged using a Typhoon 9400 Variable Image Scanner (GE Healthcare Life Sciences).

## **2.10 Cell proliferation assay**

RTbrain-W1 and RTgill-W1 cells are plated into 24 well plates at a density of  $0.6 \times 10^5$  cell per well or  $1.2 \times 10^5$  cells per well respectively, 24hr prior to treatment. The media and incubation conditions are the standard conditioned outlined above for cell culture and treatment regimes. After allowing cells to attach for 24hr, the cells were then dosed for the specified concentrations and incubation times. At each time point specified, cells are trypsinized and collected for cell counting using a Coulter Counter Z2 (Beckman Coulter), set for counting cells between 8 $\mu$ m and 20  $\mu$ m.

## **Chapter 3**

### **Production of a rainbow trout specific polyclonal p53 antibody**

#### **3.1 Introduction**

The DNA damage checkpoint is an important part of cell cycle regulation. When cells are exposed to agents such as reactive oxygen species, UV radiation, and environmental pollutants, DNA within the cells can be damaged. The various types of damage caused to the DNA signals the DNA damage checkpoint response. This initiates pathways that lead to cell cycle arrest, DNA repair, or apoptosis (Niida and Nakanishi, 2006). Different pathways are initiated, depending on the type of DNA damage, but there are 4 main types of checkpoint proteins: sensors, transducers, mediators, and effectors (Houtgraaf *et al.*, 2006). p53 is a main effector in the pathway and plays an important role as a tumour suppressor.

The rainbow trout has been suggested as a good candidate for a model organism of aquatic environmental carcinogenesis due to its sensitivity to an array of human carcinogens (Bailey *et al.*, 1996), low spontaneous tumour incidence and similar gene expression to mammalian hepatocarcinoma models (Williams *et al.*, 2008). There are also a variety of rainbow trout cell lines available (Fryer and Lannan, 1994) for use in *in vitro* studies of DNA damage.

Biomarker-based research is increasingly being used in the area of environmental carcinogenesis for the development of new tools and methods for early detection of potential disease-related changes in the cell (Kyrtopoulos, 2006). For aquatic studies, fish biomarkers of genotoxic damage are a valuable part of environmental risk assessment (ERA) of aquatic

ecosystems and environmental pollutants (van der Oost *et al.*, 2003). Changes in gene expression caused by DNA damage can be used to determine the effects of pollutants in the environment (Liyan *et al.*, 2005). RNA transcripts and proteins are gaining use as environmental biomarkers in the development of detection tools for changes caused by environmental contaminants (Tom and Auslander, 2005). In a recent study from the Duncker, Dixon, and Bols labs, rainbow trout transcript and protein levels for Chk2 were determined in tissue and cell line samples respectively, for potential biomarker use (Steinmoeller *et al.*, 2008). Another checkpoint protein, fish p53, has also been suggested as a possible biomarker for genotoxins in aquatic environments (Bhaskaran *et al.*, 1999). A variety of fish p53 genes have been sequenced, including that of rainbow trout (Caron de Fromentel *et al.*, 1992). Sequence data is useful in determining the degree of conservation between species, which can be used to infer functional similarities. Also, given the important role p53 plays in the checkpoint pathway, and the presence of its mutated form in many cancers, extensive studies have been published concerning the mechanisms and pathways of p53 (Vousden and Lane, 2007).

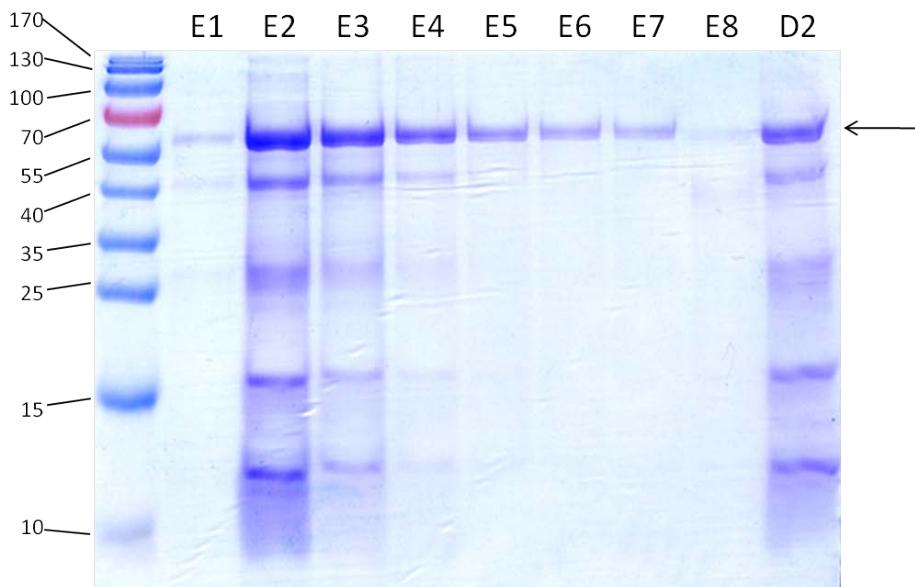
To evaluate the usefulness of p53 as a biomarker of DNA damage in rainbow trout, a polyclonal antibody to rainbow trout p53 was generated as described below. The potential applications of this antibody were investigated and outlined in Chapter 4. This antibody could be used in combination with other rainbow trout specific checkpoint protein antibodies to generate an ELISA or western blot based checkpoint biomarker assay for use in environmental testing. This method would represent a quick and efficient method of testing the biological effects of environmental pollutants on rainbow trout. To generate the assay,

rainbow trout specific antibodies will be generated against an array of checkpoint proteins to be used as biomarkers. These antibodies will be provided as part of a kit to be used for sensitive and specific detection of changes in gene expression caused by DNA damage.

## 3.2 Results

### 3.2.1 Preparation of rainbow trout p53 immunogen

Catherine Tee, a previous member of the Duncker lab, cloned rainbow trout p53 from RTgill-W1 cDNA using primers which amplified a 1125 base pair region of rainbow trout p53 cDNA, coding for amino acids 4-378 of the 396 aa protein. An expression construct was then generated using the pRSET A expression vector system (Invitrogen). Sequencing of this construct, pRSETA-RTp53, revealed the presence of one point mutation at base 1096, causing an amino acid substitution from a cysteine to an arginine. This mutation is located in a non-conserved region of the gene and was determined to be acceptable for the purposes of antibody generation. The construct was then transformed into competent BL21 (DE3) pLySS *E. coli* cells. From this point, the recombinant protein was purified using a modified method of affinity purification with nickel resin (Qiagen) as described in Chapter 2. Figure 3.1 shows a Coomassie stain of the elution fractions that were obtained. The recombinant p53 has a theoretical molecular weight of 48 kDa based on protein sequence. This is slightly heavier than the theoretical molecular weight of 44 kDa for endogenous rainbow trout p53, which is expected, due to the addition of an Xpress epitope tag and a polyhistidine tag in the recombinant p53. The lone band at the top of the gel in all elution fractions appears at a molecular weight of ~60 kDa, which is not unexpected based on previously reported gel



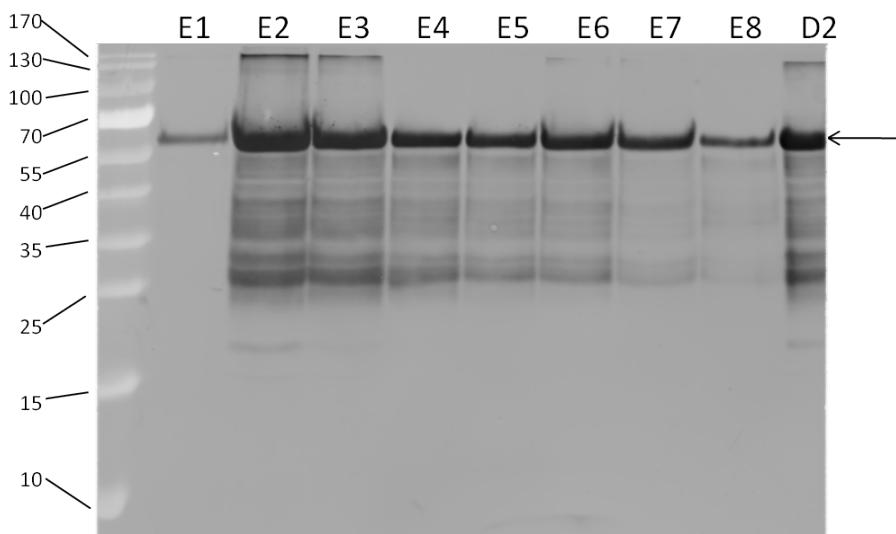
**Figure 3.1 Coomassie staining of recombinant protein elution fractions**

Elution fractions were collected in 1mL aliquots and 10 $\mu$ L of each fraction was run on a 12% SDS-PAGE gel before Coomassie staining to determine the fraction with the highest concentration of the protein of interest (indicated by the arrow). The molecular weights of the marker bands are indicated in kDa to the left of the image (Fermentas). The elution fractions are labelled E1-E8 in order of elution. The last lane (D2) is 10 $\mu$ L of elution fraction 2 after dialysis, showing that the protein of interest is retained in the dialyzed fraction. Background bands are also present but may be breakdown products of the recombinant protein.

migration properties of p53. Human p53 has a theoretical molecular weight of around 43 kDa, though it runs on a gel at around 53 kDa. This has also been observed in p53 proteins of other species and is thought to be due to a high number of proline residues in the protein (Oren, 1985; Soussi and May, 1996). Lane D2 shows that the protein is retained after dialysis in 1x PBS. There are several background bands visible in the high concentration fractions but these may be degradation products from the recombinant protein, due to the many steps during purification. Another possibility is the presence of histidine rich regions in other proteins, which could be binding to the affinity resin.

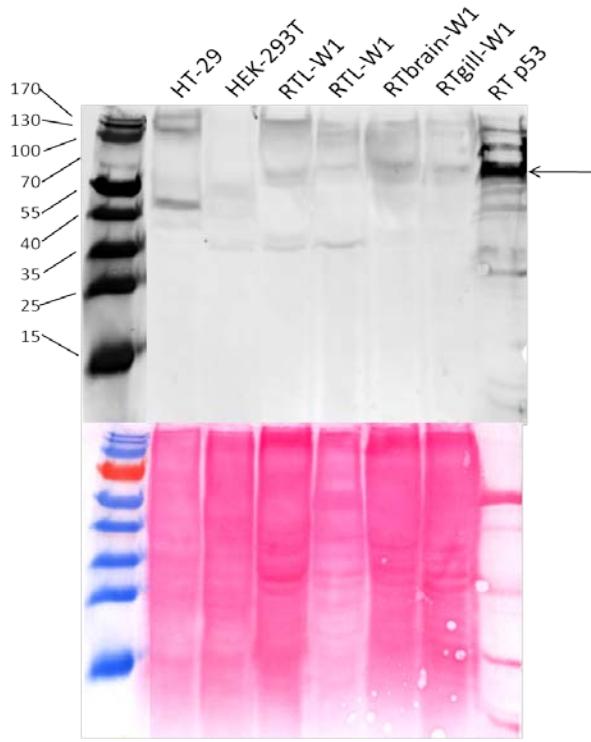
To confirm that the protein in the Coomassie stain is the recombinant protein of interest, a western blot was done, detecting for the N-terminal Xpress epitope of the recombinant protein (Figure 3.2), using an anti-Xpress antibody (Invitrogen). From the Coomassie stain in Figure 3.1, the background bands are visible, but they are absent from the western blot. This indicates that the bands do not contain the XpressTM epitope, but it is still possible that they are degradation products of the original protein.

To further confirm that the purified protein is recombinant p53, a commercial anti-human p53 antibody (Santa Cruz) was used for western blot detection of the recombinant protein. In addition, human and rainbow trout cell lysate samples were used to determine the specificity of the antibody to endogenous human and rainbow trout p53. In Figure 3.3, maximum sample volume was loaded per well to facilitate detection of p53. The recombinant protein was run with human positive controls (HEK 293 T, HT-29) and various rainbow trout lysates to test specificity. HEK 293 T (human embryonic kidney cell line) lysates do not show a clear detection of p53. This result is unexpected for the HEK 293 T lysates because it



**Figure 3.2 Detection of Xpress epitope in elution fractions.**

Western blot showing clear detection of the Xpress epitope in the elution fraction, confirming that the prominent band indicated by the arrow is the recombinant protein. Elution fractions were loaded at  $10\mu\text{L}$  per well in a 12% SDS-PAGE gel. The molecular weights of the marker bands are indicated in kDa to the left of the image (Fermentas). The elution fractions are labelled E1-E8 in chronological order. The last lane (D2) is  $10\mu\text{L}$  of elution fraction 2 after dialysis, showing that the Xpress epitope tagged recombinant protein is retained in the dialyzed fraction. The bands appearing beneath the prominent band are probably degraded forms of the protein. The Xpress epitope was detected using a primary antibody specific for the peptide sequence of the epitope (Invitrogen). An Alexa Fluor 488 goat anti-rabbit IgG secondary antibody was used for fluorescent detection (Invitrogen).



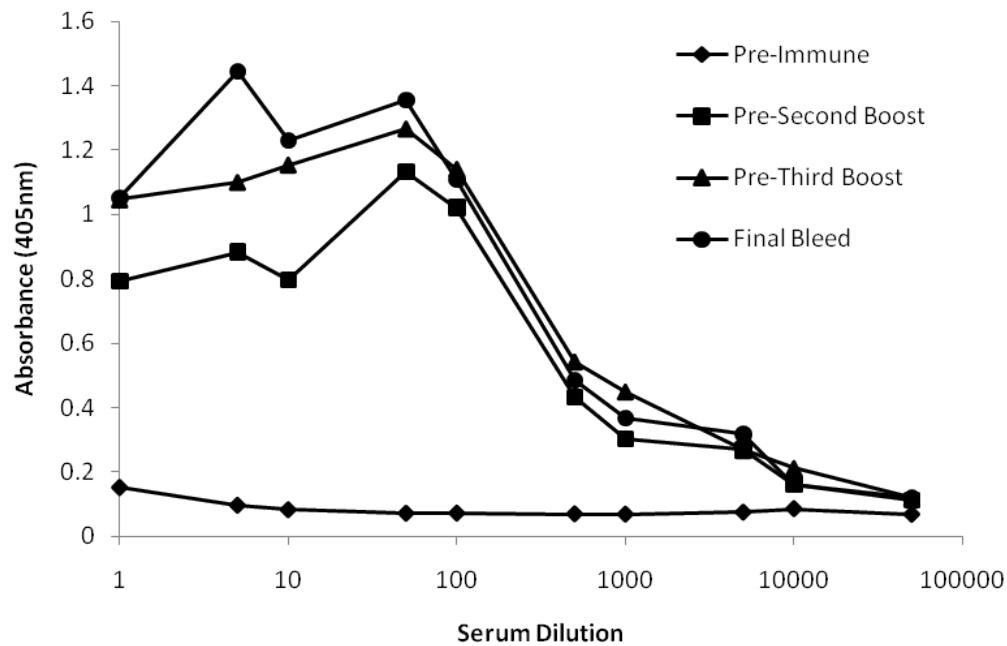
**Figure 3.3 Western blot of recombinant protein and various lysates detected with a human p53 antibody.**

Western blot showing detection of p53 in various samples using an antibody specific for human p53. Recombinant protein was loaded at 10 $\mu$ L and protein lysates were loaded at maximum volume (30 $\mu$ L) per well in a 12% SDS-PAGE gel. The molecular weights of the marker bands are indicated in kDa to the left of the image (Fermentas). The last lane is the recombinant protein, which is clearly detected by the p53 antibody, confirming that our recombinant protein is p53. The human and rainbow trout lysates were run to assess antibody specificity. Bands are detected for p53 in each sample except HEK-293T but the detection is not always clear. Ponceau S staining is provided as a loading control. p53 was detected using a polyclonal antibody to human p53 (Santa Cruz Biotech). An Alexa Fluor 488 goat anti-rabbit IgG secondary antibody was used for fluorescent detection (Invitrogen).

has been previously shown that p53 basal levels can be detected on a western blot of HEK-293 cell lysates (Evdokiou *et al.*, 1999). This difference in detection could be due to the use of different commercial p53 antibodies, protein concentrations, and detection method. In contrast, the HT-29 (colon cancer cell line), does show a clear band between ~40 kDa, which is lower than expected for human p53. However, this result is consistent with previous literature that has shown the detection of p53 in HT-29 cell lysates (O'Connor, 1997). For the rainbow trout cell lines, RTL-W1 (liver cell line) was selected because p53 was detected by western in a previous study using the same commercial anti-human p53 antibody (Embry *et al.*, 2006). The RTgill-W1 and RTbrain-W1 cell lines were chosen based on their anticipated use for the study outlined in Chapter 4, as well as their use in a previous study which determined Chk2 protein levels in these cell lines (Steinmoeller *et al.*, 2008). There are detectable bands in the rainbow trout samples that are visible at the same level as the recombinant p53 band, but they are less distinct. This provided further evidence that the purified recombinant protein is indeed rainbow trout p53, since it has similar migration for SDS-PAGE as endogenous rainbow trout p53 and is easily detected by the human polyclonal antibody. This result also validates the need for a rainbow trout specific antibody for the detection of p53, as the detection from the rainbow trout samples was less than ideal.

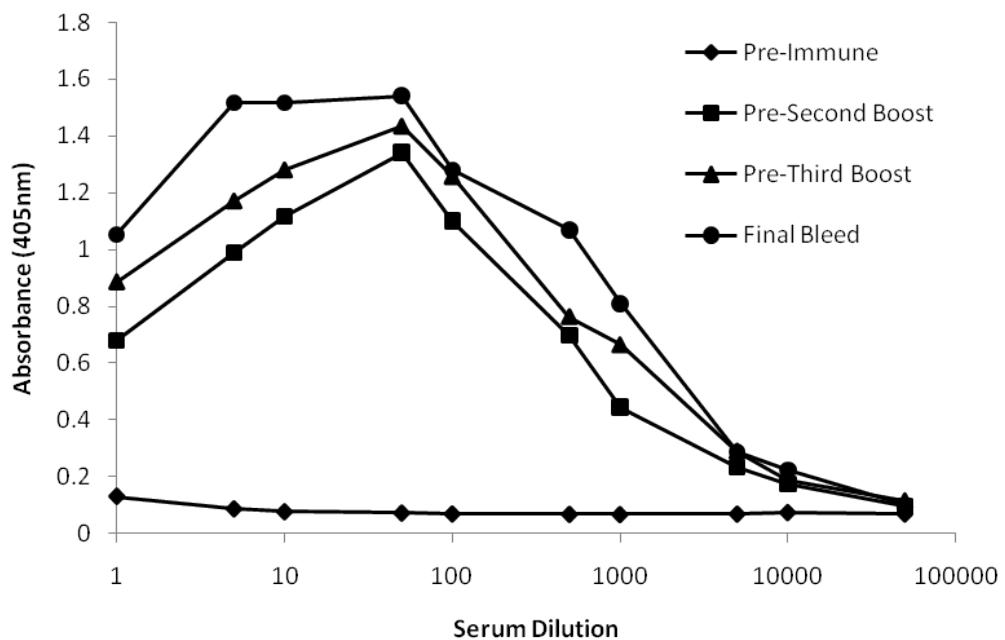
### **3.2.2 Analysis of anti-serum quality**

Once the quality of the purified protein had been checked, it was used for polyclonal antibody production in rabbits. Rabbits were immunized with aliquots of the purified protein over a period of 3 months. To monitor the production of antibodies, serum was tested before each booster shot through an ELISA. The results of the assay showed that a high titre of



**Figure 3.4 ELISA of serum samples from Rabbit 1**

Serum samples were taken from rabbit 1 before the initial immunization and before each booster shot. The serum from the final bleed was also tested. The ELISA shows that a high titre of antibodies to recombinant p53 was being produced, starting before the second boost.



**Figure 3.5 ELISA of serum samples from Rabbit 2**

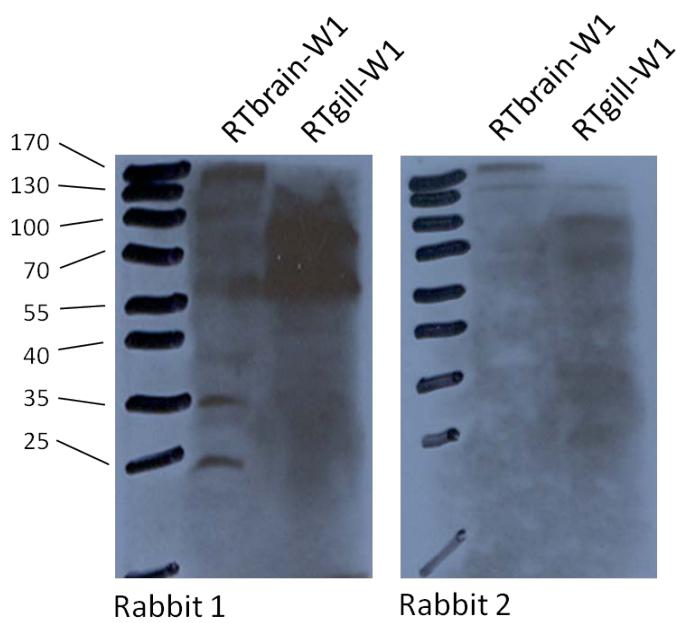
Serum samples were taken from rabbit 2 before the initial immunization and before each booster shot. The serum from the final bleed was also tested. The ELISA shows that a high titre of antibodies to recombinant p53 was being produced, starting before the second boost.

antibodies was being produced to the recombinant protein in both rabbits starting before the second boost (Figure 3.4 and Figure 3.5).

When the final bleed serum was obtained, it was tested for specificity by western blotting. The pre-immune serum from each rabbit was also tested to determine if any initial background binding was present before immunization. Figure 3.6 shows that rabbit 1 displayed higher initial background binding of pre-immune serum in comparison to the serum of rabbit 2. The background binding also seemed to be stronger for proteins around the size of p53. It was also determined that the final bleed serum from both rabbits produced a high amount of background binding (data not shown). To reduce this background, the serum was purified using a SulfoLink column as described in Chapter 2. The purified antibody was then compared to the final bleed serum (Figure 3.7). Serum from rabbit 2 was chosen for purification based on the lower amount of initial background in the pre-immune detection. Figure 3.7 shows that the SulfoLink purified antibody detects a clean band for p53, though some background binding is still present.

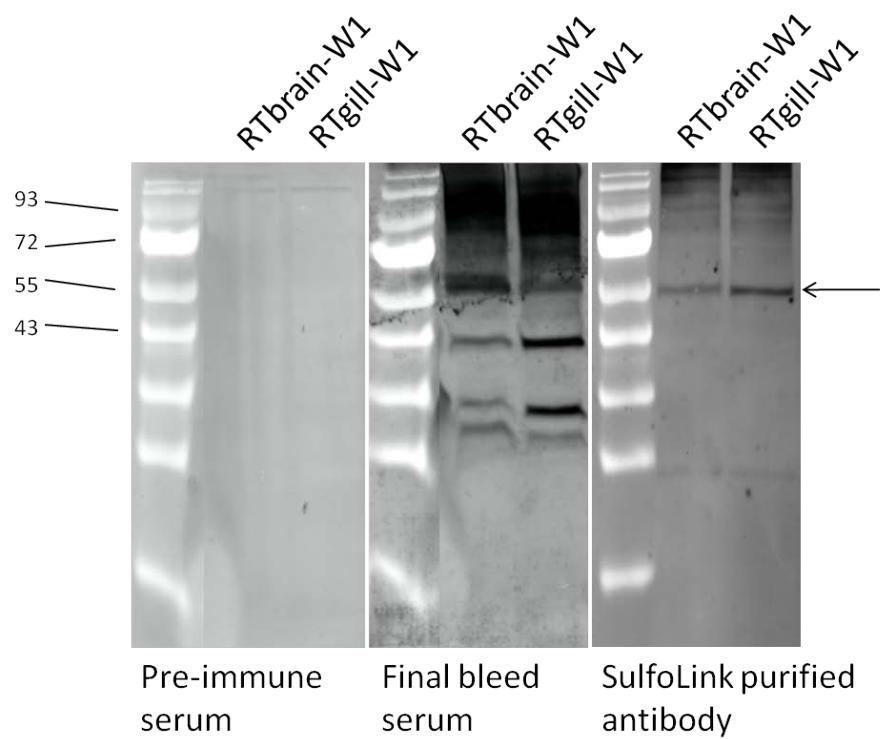
### **3.2.3 Detection of rainbow trout p53 in cell lines and tissues**

The purified antibody was then tested for specificity in basal expression panels of various rainbow trout cell lines (Figure 3.8) and tissues (Figure 3.9). From these panels, it can be seen that p53 expression varies between tissue type and cell line type. For the cell line panel, the human antibody does not seem to be detecting p53 in all samples. Only the RTgut-GC sample shows a clear detection of the p53 at the correct size. The SulfoLink purified antibody shows clear detection of p53 bands, indicating that it is highly specific for p53 in rainbow trout cell lines, though the levels of background vary between the different tissues. For the



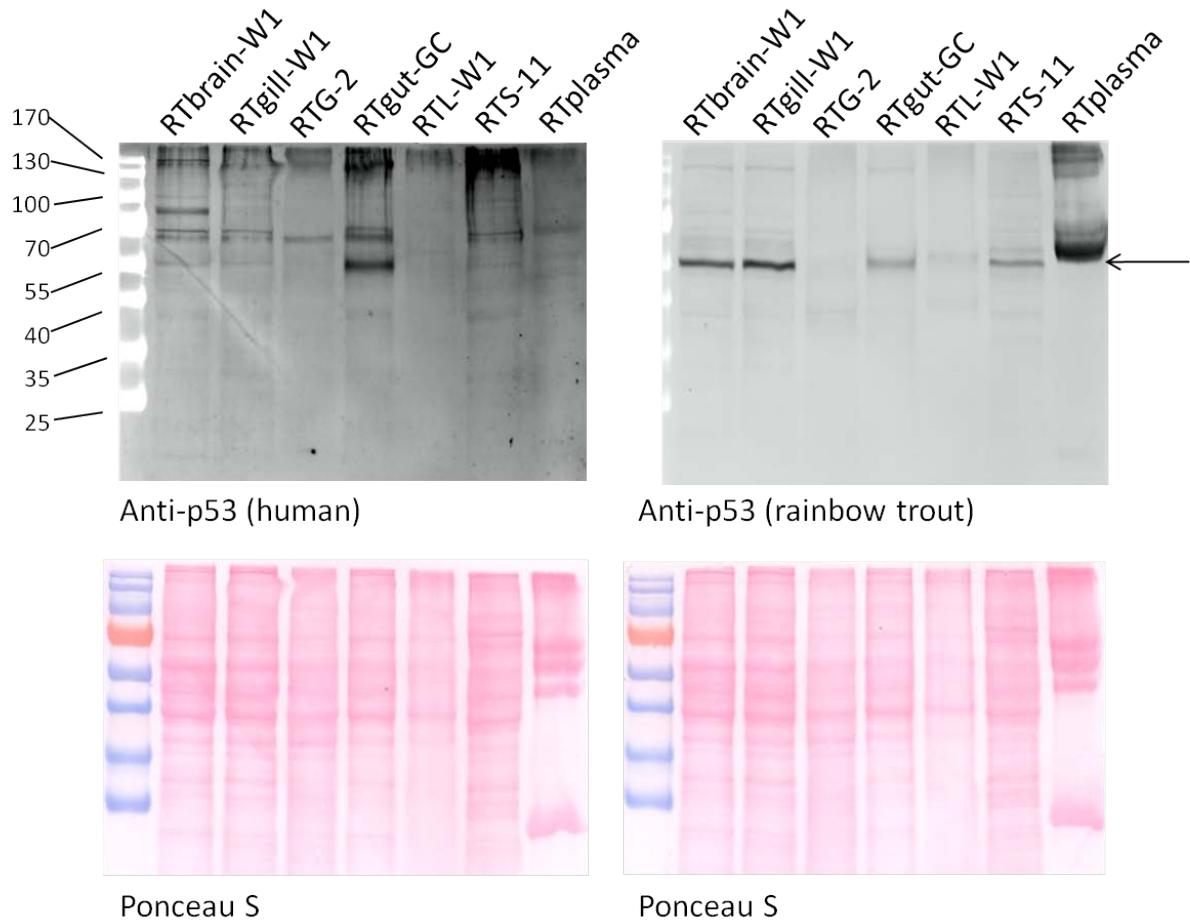
**Figure 3.6 Western blot of RTbrain-W1 and RTgill-W1 lysates using pre-immune serum.**

Western blots using pre-immune serum as primary antibody for detection (1:200). Proteins were loaded at 20 µg per well in a 12% SDS-PAGE gel. The molecular weights of the marker bands are indicated in kDa to the left of the image (Fermentas). Rabbit 1 shows a higher amount of cross reactivity in the pre-immune serum than Rabbit 2. A goat anti-rabbit IgG HRP conjugated secondary was used for detection (Bio-Rad).



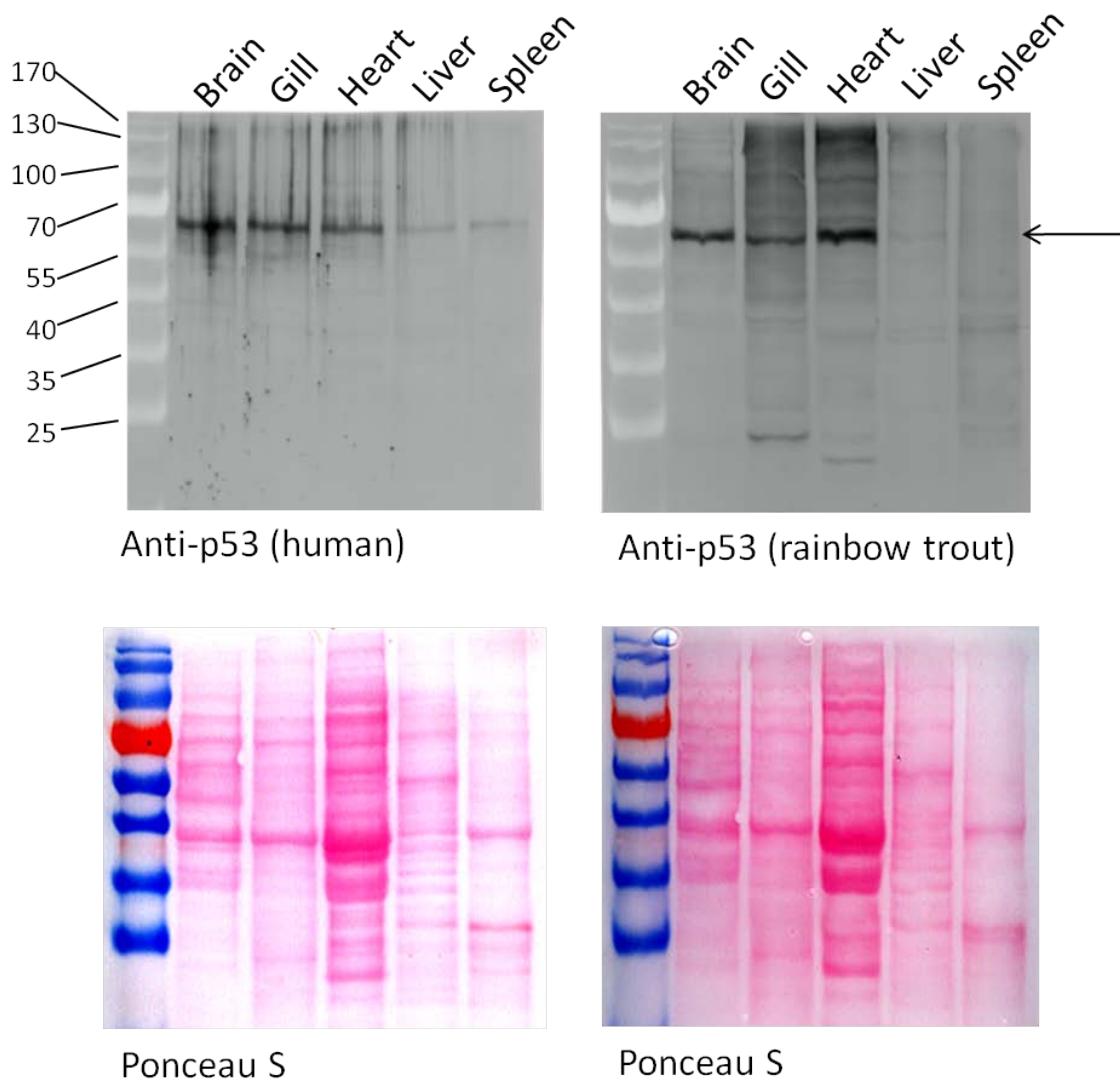
**Figure 3.7 Western blot of RTbrain-W1 and RTgill-W1 lysates showing specificity of the SulfoLink purified antibody.**

Western blots using either pre-immune serum, final bleed serum, or SulfoLink purified antibody as primary antibodies for detection (1:200). Proteins were loaded at 20 $\mu$ g per well in a 12% SDS-PAGE gel. The molecular weights of the marker bands are indicated in kDa to the left of the image (Fermentas). Serum from rabbit 2 was chosen for purification due to the lower background reactivity of the pre-immune serum. The SulfoLink purified antibody shows a clear detection of the p53 band, with most of the background binding from the final bleed serum removed. A goat anti-rabbit IgG HRP conjugated secondary was used for detection (Bio-Rad).



**Figure 3.8 Panel of rainbow trout cell line samples detected for p53, comparing anti-rainbow trout and anti-human antibodies.**

Western blots using either an anti-human p53 antibody (Santa Cruz Biotech) or the SulfoLink purified antibody, specific for rainbow trout p53. Proteins were loaded at 20 $\mu$ g per well in a 12% SDS-PAGE gel. Ponceau S staining is provided as loading controls. The samples represent cell lines derived from the following tissues: brain, gill, gonad, gut, liver, spleen. RTplasma is part of the panel though it is not a cell line. A goat anti-rabbit IgG HRP conjugated secondary was used for detection (Bio-Rad).



**Figure 3.9 Panel of rainbow trout tissue samples detected for p53, comparing anti-rainbow trout and anti-human antibodies**

Western blots using either an anti-human p53 antibody (Santa Cruz Biotech) or the SulfoLink purified antibody, specific for rainbow trout p53. Proteins were loaded at 20 $\mu$ g per well in a 12% SDS-PAGE gel. Ponceau S staining is provided as loading controls. A goat anti-rabbit IgG HRP conjugated secondary was used for detection (Bio-Rad). These results are representative of samples from three fish.

tissue panel, the detection of p53 with both antibodies shows a clear band for the first four tissues. For the spleen sample, a band is only detected with the human antibody. The SulfoLink purified antibody also shows a higher degree of background binding that is not seen in the cell line panel.

### **3.3 Discussion**

In this study, I aimed to generate a polyclonal antibody that is specific for rainbow trout p53. This antibody would be used in conjunction with other rainbow trout checkpoint antibodies (that are currently being developed) to generate a biomarker assay for environmental toxicology by detecting DNA damage caused by environmental toxins. Currently, a commonly used technique for assessing DNA damage in the cell is through the comet assay (single cell microgel electrophoresis). Cells are embedded in agarose and lysed, before gel electrophoresis in alkaline conditions. The amount of DNA damage is assessed through fluorescence microscopy to determine the extent of DNA migration from the core of intact DNA (Singh *et al.*, 1988), forming a comet-like appearance. Unfortunately, this method cannot be used as a standard bio-indicator in aquatic environments due to lack of standardization of methods and measurements of DNA damage (Lee and Steinert, 2003). By generating an ELISA based assay, analysis of results will be more absolute, based on comparisons with a given standard.

To generate antibodies specific to rainbow trout p53, the recombinant p53 had to be purified from the bacterial host cells in a form that is acceptable for use as an immunogen. Purification of recombinant p53 from the BL21(DE3)pLysS E. coli was initially attempted using affinity chromatography with Ni-NTA agarose resin (Qiagen) and the manufacturer's

denaturing purification protocol for cell lysis with 8M urea. Unfortunately, the purified protein from this method aggregated and formed a precipitate when the solvent (urea) was removed by dialysis into 1x PBS in preparation for injections. Previous research has documented that pure p53 protein can oligomerize and precipitate at high concentrations due to the oligomerization domain of p53 (Chalkley *et al.*, 1994). It is also possible that in the fully denatured state, hydrophobic regions become accessible and form aggregates with the other denatured p53 proteins. The manufacturer's suggested method of native purification was also attempted but Coomassie staining of the elution samples on a 12% SDS-PAGE gel showed that p53 protein yield was extremely low, possibly due to incomplete lysis of the cells. After sonication of the E. coli cells, a large pellet was still present after lysis, more so than with the denaturing method, indicating the presence of intact cells with the cell debris. To circumvent these issues, a modified method of affinity purification was created. Cells were lysed using the denaturing method with 8M urea buffer and incubated with the affinity resin for protein binding. The protein was then refolded on the column using a decreasing gradient of urea (6M-0M), before elution with a 250mM imidazole buffer and dialysis into 1x PBS. This method was an optimal balance between quality and quantity of protein necessary for use in antibody production. Different antibodies were then used to confirm that the protein in the elution fractions was actually Xpress epitope tagged p53 (Figure 3.2 and Figure 3.3).

Throughout the process of immunization, the serum of the rabbits was monitored for antibody titre through ELISA. The serum from both rabbits showed high reactivity to recombinant p53, even before the second boost (Figure 3.4 and Figure 3.5). Oddly, for both

ELISAs, there seemed to be an increase in reactivity, relative to the undiluted serum, as the dilution of the serum increased to 100. This could possibly be due problems with the preparation of the plates or interference of antibody binding from other substances in the serum. In either case, the ELISAs showed a definite increase in reactivity of serums to the purified recombinant p53 protein, relative to the pre-immune controls. This indicated that the immunization of the rabbits was working in generating antibodies to the recombinant p53 protein.

To determine the specificity of the final bleed anti-serum, western blots were performed. When using the pre-immune serum as the primary antibody, there seemed to be a higher amount of background reactivity in rabbit 1 (Figure 3.6). The final bleed serum from both rabbits showed a high amount of background binding in the initial detection, indicating the need for purification of the antibody from the serum. The serum from rabbit 2 was chosen for purification, as there was less initial background reactivity in the pre-immune serum. Since the banding pattern of detection with the pre-immune serum of rabbit 1 appeared around the size of p53, it would be harder to determine the specificity of the serum after purification due to lack of differentiation between initial background and final p53 detection. In Figure 3.7, a western blot with the purified serum shows that the background binding is greatly decreased and the p53 band can be clearly seen. There are still background bands present but these may represent detection of oligomers of p53 or possibly isoforms of p53.

In terms of sample specificity, it can be seen that the SulfoLink purified antibody is better for detection of p53 in rainbow trout cell lines versus detection of p53 in rainbow trout tissues (Figure 3.8 and Figure 3.9). The detection in tissues shows a higher degree of

background binding. This is possibly due to the fact that cell lines are generated from one cell type, whereas tissues samples are a mass of various cell types, leading to more non-specific binding of the antibody. For the cell lines, the SulfoLink purified antibody appears to detect p53 consistently, whereas the human p53 antibody does not. In using the human p53 antibody, it has shown inconsistency in p53 detection, depending on the quality of the particular lot of antibody.

Upon detection of p53 in various cell lines and tissues, a variability in basal protein levels can be seen. Normally, p53 is kept at a low level in mammalian cells until a checkpoint is activated, which in turn stabilizes p53. Protein levels of p53 may differ between cell lines and tissues due to efficiency and frequency of checkpoint activation. It has been shown in the past that checkpoint activation in mammalian cells after IR can differ between cell types (Gudkov and Komarova, 2003). These differences could be attributed to the tissue type, and the need for an active checkpoint in tissues that are exposed to a higher level of DNA damage. These differences in activation can also translate to differences in basal checkpoint protein levels, due to checkpoints that may be activated during DNA replication. For cell lines and tissues, basal p53 protein levels may also differ due to cell and tissue type specific differences in total protein expression. Differentiated tissue types have displayed different profiles of gene expression and alternative splicing when analyzed through microarray analysis (Johnson *et al.*, 2003). These basal differences in gene expression could account for differences in p53 expression levels. For the tissue samples, the external environment could have also affected the levels of p53 prior to sample collection. Samples obtained from gill tissue were directly exposed to the external environment, which

could have increased p53 due to constant activation. From these blots, it appears that brain and gill p53 is easily detected by the SulfoLink purified antibody in both cell lines and tissues. This high expression of p53 in the gill cells could be due to the exposure to the external environment. For the brain samples, the high levels of p53 could be attributed to an active checkpoint in the brain cells, due to the importance of this organ. In contrast, liver shows low expression of p53 in both cell line and tissues. This is an unexpected result, since one of the main liver functions is detoxification. A high level of p53 would be expected for a tissue that is more frequently exposed to toxicants. On the other hand, an unstressed liver may have constitutively lower levels of p53. For the spleen tissue, p53 is not detected by the SulfoLink purified antibody, but the RTS-11 cell line showed clear detection of p53. This difference could be due to the specific cell type that RTS-11 is derived from. RTS-11 is a macrophage-like cell line which was isolated from a long-term spleen haemopoietic culture, which is not representative of all the components of the spleen. The lack of p53 detection in the spleen tissue could be explained by the results of an early study into p53 expression in various human tissues (Rogel *et al.*, 1985). They determined that p53 mRNA expression was heightened in the spleen, though the protein levels were barely detectable, due to high turnover of p53 in the spleen from rapid degradation. For the remaining cell lines, RTgut-GC also shows a clear detection of p53 whereas RTG-2 does not appear to show detection of p53 with the SulfoLink purified antibody. For the RTgut-GC cell line, a higher level of p53 can be possibly explained by the origin of the cell line, as it was derived from gut epithelial cells. The constant exposure of this tissue type to the external elements, brought in through the digestive tract, may have lead to elevated basal p53 levels. The lack of p53 in the gonad-

derived cell line, RTG-2, is unexpected but could possibly be due to the developmental stage of the original rainbow trout from which the cell line was derived. RTplasma is not a cell line but was added to the panel based on the fact that p53 levels in plasma have been used in studies pertaining to DNA damage and cancer (Soussi, 2000). The SulfoLink purified antibody shows a good detection of p53 in RTplasma, indicating that plasma extraction may be an easy and non-lethal method of monitoring p53 levels in rainbow trout. The remaining tissue, heart, also shows a clear detection of p53. Together, these panels indicate that the expression pattern of p53 is similar between cell lines and tissues, which further supports the use of cell lines for future studies into p53 expression. It can also be seen that the SulfoLink purified antibody is better used for detection of p53 in cell lines, rather than in tissues, where a higher amount of background is present.

### **3.4 Conclusion**

For this study, the objective was to generate a rainbow trout specific polyclonal p53 antibody that could be used in future studies of p53 expression. This antibody could also be used as part of a panel of antibodies for use in the larger goal of generating an environmental biomarker assay. From the results shown above, it is clear that the final SulfoLink purified antibody product is specific for detection of p53 in rainbow trout, though it is more compatible for detection of p53 in cell lines rather than tissues. In comparison with the human antibody, the SulfoLink purified antibody appears to be more consistent in its quality of detection and can be used in future studies into p53 expression in rainbow trout.

## **Chapter 4**

### **p53 protein expression is not induced in rainbow trout cell lines after exposure to DNA damage checkpoint inducing agents**

#### **4.1 Introduction**

Eukaryotic cells grow and divide through a series of steps known as the cell cycle. The DNA in the original cell must be replicated to properly complete the cell cycle and produce two daughter cells. To ensure proper DNA replication and division of cells, mechanisms known as checkpoints are in place throughout the cell cycle. Specific cell cycle checkpoints can be activated by DNA damage or stalled replication forks to ensure high fidelity of DNA replication. DNA maintenance checkpoints are composed of a network of proteins that are activated to preserve genomic integrity in the presence of genotoxic stress. Genotoxic stress can be present in the form of double strand breaks (DSBs), single strand breaks (SSBs), or stalled replication forks, which can alert certain sensor proteins to activate the DNA damage checkpoint (Nyberg *et al.*, 2002). This usually involves the activation of several groups of proteins through a kinase cascade. The pathway begins with the detection of the initial DNA damage or replication stress and leads to the activation of downstream effector proteins, which will result in cell cycle arrest, DNA repair, or apoptosis (Sancar *et al.*, 2004).

The tumour suppressor protein p53 is an important factor in the DNA damage checkpoint pathway. It is a transcription factor that is involved in the transactivation of downstream factors which can help lead the cells to resolution of the checkpoint (Levine *et al.*, 2004). As one of the main effector proteins in the checkpoint pathway, mutations in its

functional domains can lead to tumourigenesis and cancer. In over 50% of human tumours, mutations in p53 can be found, which lead to its inactivation. Due to its importance in cancer research, it has been extensively studied and well characterized in mammalian models; unfortunately, its regulation and mechanisms of action are not as well characterized for lower vertebrate species.

Since p53 is a well known protein that is involved in cancer prevention, it has been suggested for use as a biomarker in aquatic toxicology studies, based on the assumption that the well characterized mammalian pathways are conserved in teleost species. While only a fraction of p53 studies are done in lower vertebrate species, these studies have been very important in highlighting some key differences in p53 function. Some of these studies do show that p53 is involved in pathways that are analogous to mammalian pathways, based on knockdown studies in fish models. Several of these studies have shown that p53 is necessary in the irradiation-induced apoptosis of cells in developing zebrafish (Berghmans *et al.*, 2005a; Inohara and Nunez, 2000). Other studies in zebrafish have shown that reduced p53 levels in embryos lead to lowered apoptotic response when exposed to DNA damaging agents (Stern and Zon, 2003). These studies display the importance of p53 in apoptosis, but they do not explore the mechanisms of activation and regulation of p53 for comparison with mammalian models. Certain studies, however, have highlighted some differences in the function and regulation of lower vertebrate p53, when compared to mammalian p53.

In the mammalian model of p53 regulation, it is kept at low levels in the cell by its negative regulator mdm2. It has been shown in mice that overexpression of mdm2 can lead to a high rate of spontaneous tumour formation, possibly due to the down regulation of p53

(Jones *et al.*, 1998). However, when a similar study was performed with zebrafish embryos, an increased rate of spontaneous tumour formation did not occur as a consequence of mdm2 overexpression (Thisse *et al.*, 2000). In terms of transcript regulation, it has been shown in mammalian models that p53 transcripts are upregulated after UV exposure but this same result was not seen in medaka cell lines or whole fish experiments, even at lethal doses of UV exposure (Chen *et al.*, 2001). Instead, p53 transcript levels remained at a constant level after treatment in both *in vitro* and *in vivo* experiments. Following the cloning of the medaka p53 gene, it was also shown that N-methyl-N'-nitro-N-nitrosoguanidine (MMNG) induced tumors in medaka failed to show mutations in p53 mutational hotspots (Krause *et al.*, 1997). Several studies in fish have also shown a lack of p53 mutation in a variety of tumours, which is unexpected due to a high prevalence of p53 mutations in mammalian tumours. In mammalian models, most studies focus on activation of p53 at the protein level, showing an induction of p53 protein after DNA damage, which is a well established mechanism. In 2006, this induction was shown to be absent in a rainbow trout liver cell line and in primary hepatocytes after treatment with model chemotherapeutics (Embry *et al.*, 2006). Other studies have shown that p53 phosphorylation is increased at serine 15, but that total p53 levels remained unchanged after DNA damage caused by hydroxyurea in urodele (Villiard *et al.*, 2007) or aging related oxidative stress in ayu (Nagasaki *et al.*, 2006). These studies highlight the need for further investigations into p53 regulation in lower vertebrate models, before drawing conclusions concerning its potential as an aquatic biomarker.

The study presented here was performed for the purpose of further characterizing lower vertebrate p53 after exposure to genotoxic stress inducing agents. The rainbow trout

was chosen for this study due to its abundance in North American waters, its commercial importance, and the availability of rainbow trout cell lines. For this study, rainbow trout brain (RTbrain-W1) and rainbow trout gill (RTgill-W1) cell lines were treated with several types of DNA checkpoint inducing agents. The results obtained show that total p53 protein is not induced after treatments, even in the event of DNA damage, cell cycle arrest, or apoptosis.

## 4.2 Results

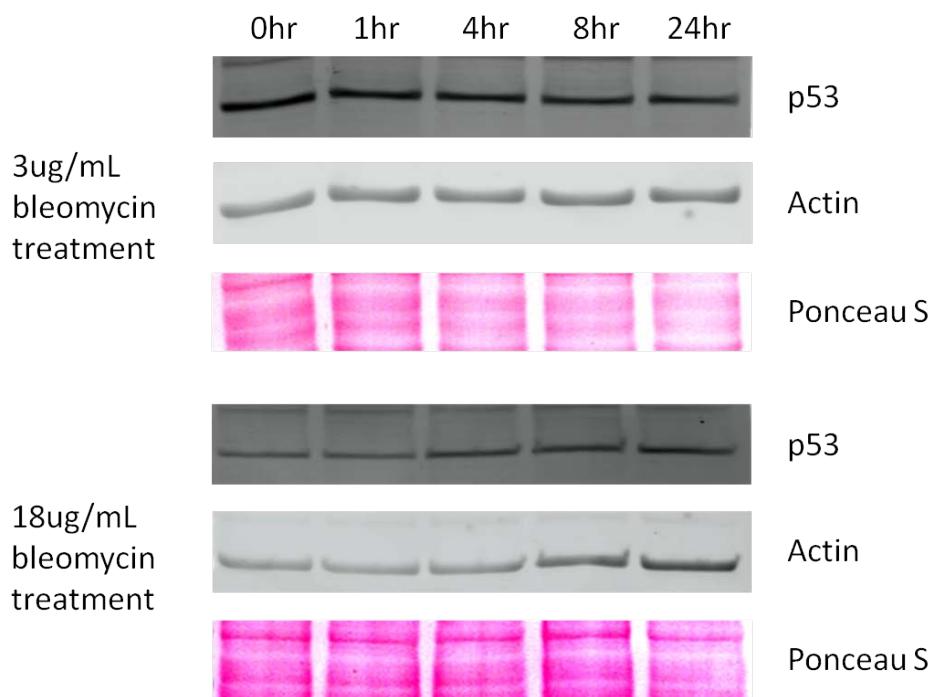
### 4.2.1 Rainbow trout p53 is not induced despite the presence of DNA damage caused by bleomycin

Bleomycin is a radiomimetic agent that damages DNA by predominantly producing double stranded breaks (Povirk *et al.*, 1977). It passed clinical trials in the 1970s (Blum *et al.*, 1973) and it is still commonly used as a cancer therapeutic due to its ability to cause apoptosis in cells (Tounekki *et al.*, 1993). In mammalian models, it has been shown that bleomycin induced apoptosis is p53 dependent (Araki *et al.*, 1998) and that p53 is induced as a result of bleomycin treatment (Canman *et al.*, 1994; Nelson and Kastan, 1994; Okudela *et al.*, 1999). However, a study in 2006 showed that p53 was not induced in bleomycin treated primary hepatocytes from rainbow trout (Embry *et al.*, 2006) despite its effect on mammalian cell lines. For the purposes of this study, two rainbow trout cell lines were used: RTbrain-W1 and RTgill-W1. Previously published work in our lab has shown that these two cell lines displayed distinct differing responses of the checkpoint protein Chk2 as a consequence of treatment with bleomycin (Steinmoeller *et al.*, 2008). It was shown that Chk2 was induced in RTbrain-W1 cells as a result of bleomycin treatment. However, Chk2 was not induced in RTgill-W1 cells which showed constitutive expression of Chk2. These results indicated a

possible difference of checkpoint activation between cell types. As shown in Figures 4.1 and 4.2, cells were treated with bleomycin for 0h, 1h, 4h, 8h, and 24h at concentrations of 3 $\mu$ g/ml or 18 $\mu$ g/ml. At both treatment concentrations, no induction of p53 is seen with either the RTbrain-W1 or RTgill-W1 cell line. To determine whether bleomycin was affecting the cells and inducing a checkpoint response, the detection of phosphorylated histone H2A ( $\gamma$ H2AX) on serine 139 was added as a marker for DNA damage. Double stranded breaks have been shown to induce H2AX phosphorylation on serine 139 in several mammalian models (Rogakou *et al.*, 1998) and this result has been replicated in a rainbow trout hepatoma cell line (RTH-149), specifically after IR treatment (Krumbschnabel *et al.*, 2010). The initial maximum concentration of bleomycin (18 $\mu$ g/ml) was near the amount necessary to cause ~30% apoptosis in cultured cerebellar granule neurons of mice with wild type p53 treated for 36 h with bleomycin (Araki *et al.*, 1998). At this concentration, p53 was not induced and the rainbow trout cells did not undergo apoptosis (results not shown). To determine if the cells were more resistant to DNA damage by bleomycin, the dosing concentrations were increased up to 200 $\mu$ g/ml. With these new conditions, it can be seen through the increase in  $\gamma$ H2AX that DNA is being damaged by treatment with bleomycin in a dose dependent manner, even though levels of p53 are not induced (Figure 4.3). This lack of induction differs from the expected result, based on mammalian models.

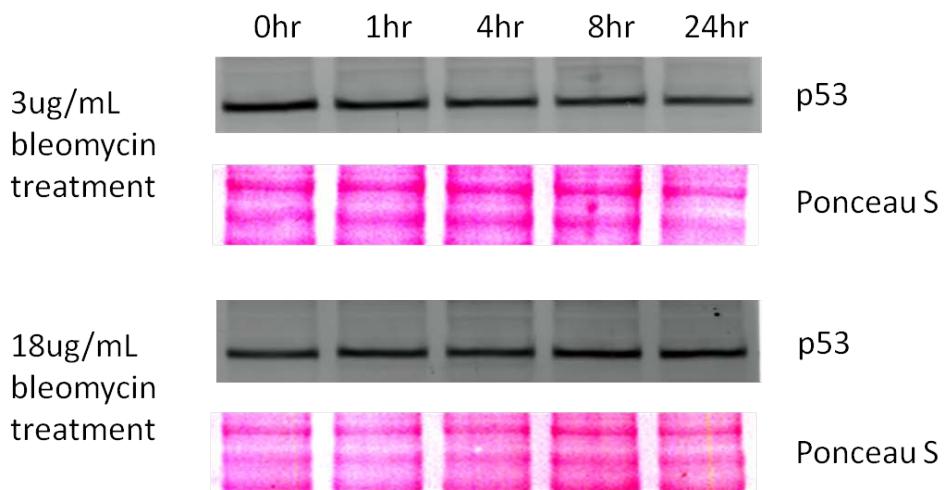
#### **4.2.2 Hydroxyurea causes cell cycle arrest without induction of p53**

Hydroxyurea is a antineoplastic agent that is commonly used to induce cell cycle arrest at the onset of S phase. It depletes deoxyribonucleotide pools in cells through inhibition of the



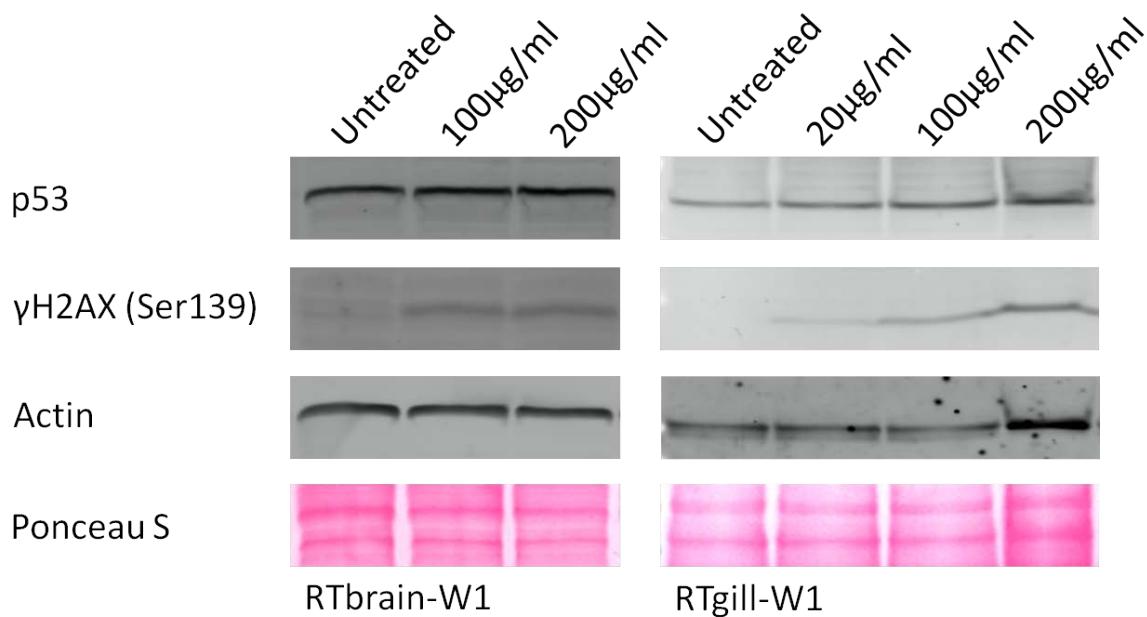
**Figure 4.1 Detection of p53 in a time course of RTbrain-W1 cells treated with bleomycin for 24h.**

Western blots showing no time dependent induction of p53 over a 24h period after treatment with bleomycin at 3 $\mu$ g/ml and 18 $\mu$ g/ml. Protein extracts from RTbrains-W1 cells were loaded at 20 $\mu$ g per well in a 12% SDS-PAGE gel. Ponceau S staining and actin detection are provided as loading controls. p53 was detected using a polyclonal antibody to rainbow trout p53 that I produced (Chapter 3). Actin was detected with a monoclonal commercial antibody, specific to the conserved c-terminal region in the actin family and generated against a synthetic peptide, designed to be specific to multiple species and tissues (Sigma). A goat anti-rabbit IgG HRP conjugated secondary was used for detection (Bio-Rad).



**Figure 4.2 Detection of p53 in a time course of RTgill-W1 cells treated with bleomycin for 24h.**

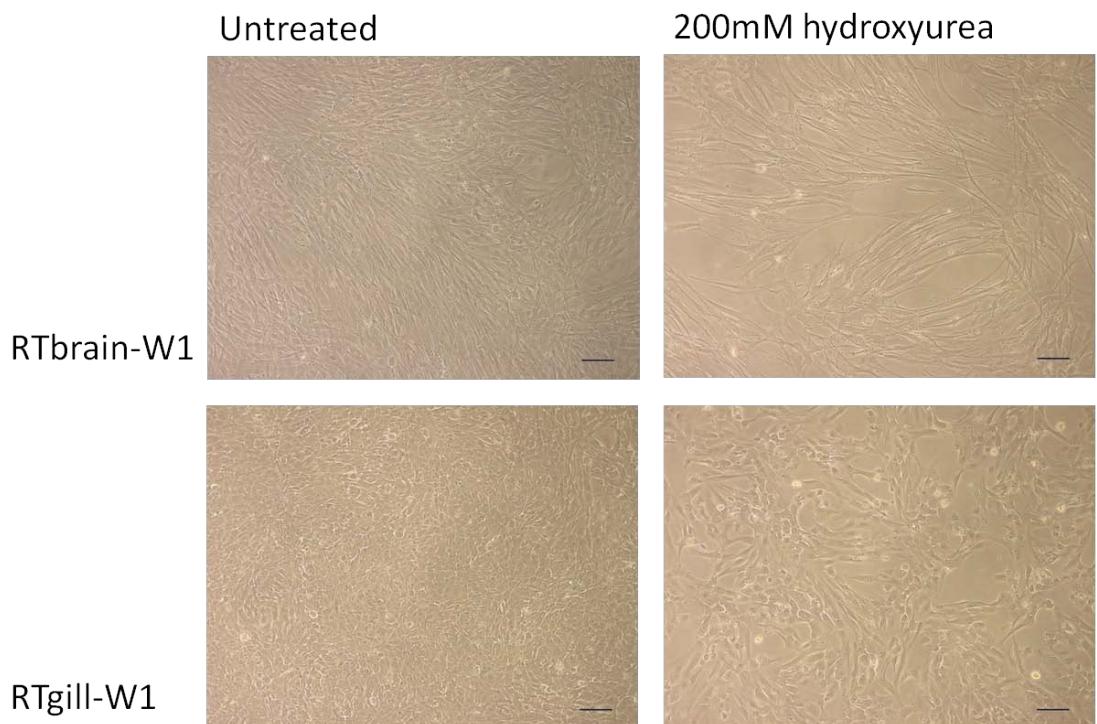
Western blots showing no time dependent induction of p53 over a 24h period after treatment with bleomycin at 3 $\mu$ g/ml and 18 $\mu$ g/ml. Protein extracts from RTgill-W1 cells were loaded at 20 $\mu$ g per well in a 12% SDS-PAGE gel. Ponceau S staining and actin detection are provided as loading controls. p53 was detected using a polyclonal antibody to rainbow trout p53 that I produced (Chapter 3). Actin was detected with a monoclonal commercial antibody, specific to the conserved c-terminal region in the actin family and generated against a synthetic peptide, designed to be specific to multiple species and tissues (Sigma). A goat anti-rabbit IgG HRP conjugated secondary was used for detection (Bio-Rad).



**Figure 4.3 Detection of p53 and  $\gamma$ H2AX in cells treated with high dose bleomycin for 24h.**

Western blots showing no induction of p53 in both RTbrain and RTgill cells after treatment with bleomycin at various concentrations for 24h. The presence of DNA damage is shown by the induction of phosphorylated histone H2AX. Protein extracts were loaded at 20 $\mu$ g per well in a 12% SDS-PAGE gel. Ponceau S staining and actin detection are provided as loading controls. p53 was detected using a polyclonal antibody to rainbow trout p53 that I produced (Chapter 3).  $\gamma$ H2AX was detected with a commercial antibody specific for the phosphorylated form of H2A at ser139 (Santa Cruz Biotech). Actin was detected with a monoclonal commercial antibody, specific to the conserved c-terminal region in the actin family and generated against a synthetic peptide, designed to be specific to multiple species and tissues (Sigma). A goat anti-rabbit IgG HRP conjugated secondary was used for detection (Bio-Rad).

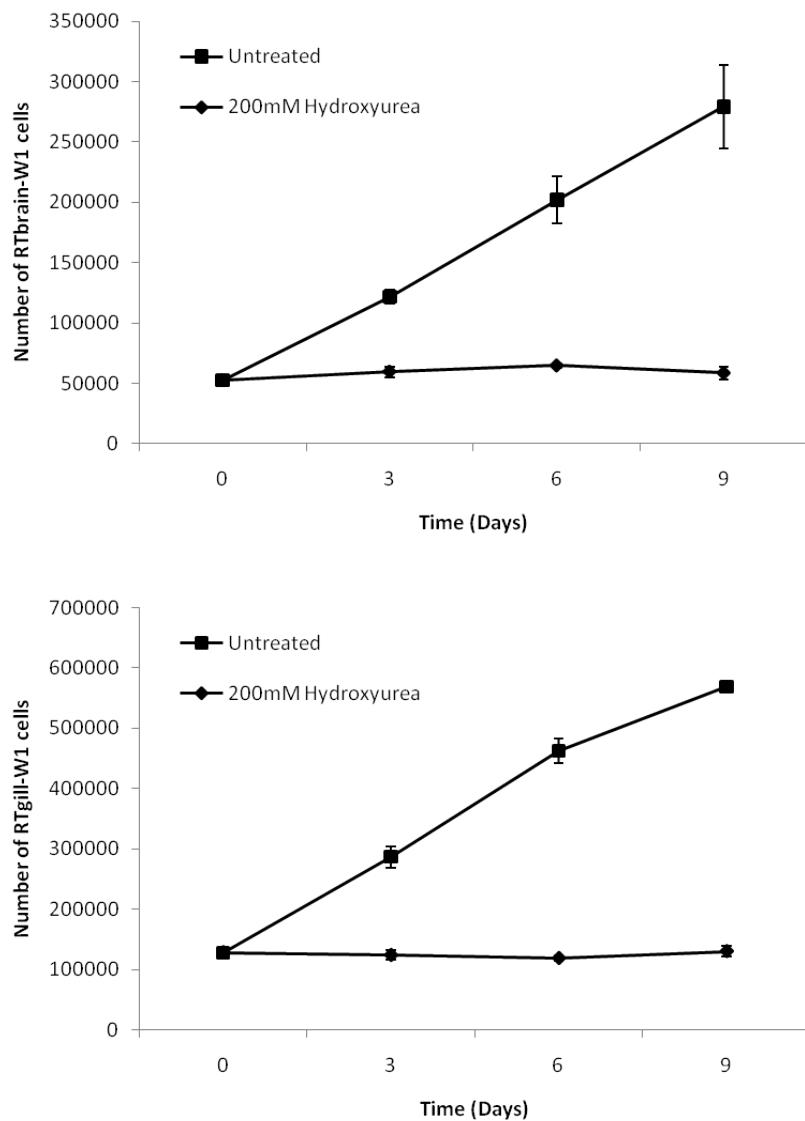
enzyme ribonucleotide reductase, which causes replication forks to stall due to lack of deoxyribonucleotides. In mammalian cell lines, treatment with hydroxyurea has shown a time dependent induction of p53 up to 24 h (Ho *et al.*, 2006b). For the dosing studies, several concentrations of hydroxyurea were used to produce cell cycle arrest in the cells. In Figure 4.4, cell images taken after 3 days of treatment with 200mM hydroxyurea show that treated cultures are less confluent when compared to the untreated control. A proliferation assay, performed by Fanxing Zeng of Dr. Niels Bols' lab, was done for both the gill and brain cell lines (Figure 4.5), which show that cell proliferation is stably arrested up to 9 days after treatment with 200mM hydroxyurea. These results show that the hydroxyurea treatment succeeded in arresting the cells. However, as with the bleomycin treatment, Figure 4.6 shows that levels of p53 do not increase after treatment with hydroxyurea even though cells are clearly growth arrested. It was next decided to look into p53 activation by phosphorylation. In the data sheet provided by Cell Signalling with their phosphorylated p53 antibody (specific for phosphorylated serine 392 of human p53), they showed that treatment of mink lung cells (MvLu) with 20mM hydroxyurea for 16 h or 30 h was able to induce phosphorylation of p53 at serine 392. In our study, it was seen that treatment with hydroxyurea did not induce p53 phosphorylation on serine 392 (Figure 4.6). It is also seen that this phosphorylation on serine 392 is present in control cells, indicating that it is constitutively phosphorylated at that site.



**Figure 4.4 Appearance of cells treated with 200mM hydroxyurea for 3 days.**

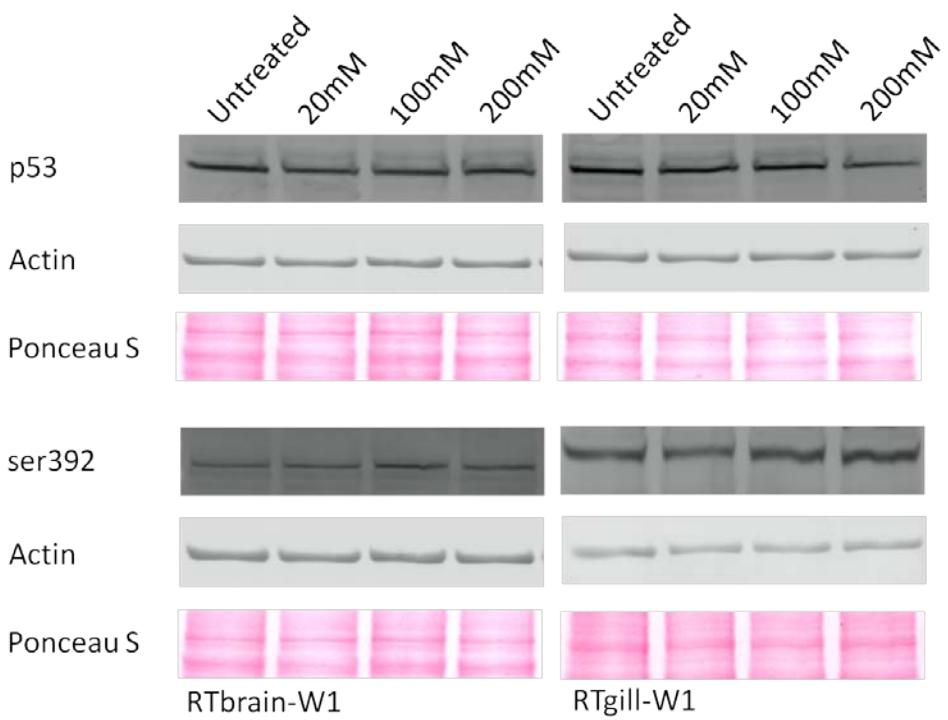
Phase-contrast microscopy appearance of RTbrain-W1 and RTgill-W1 cultures.

Photomicrographs were taken of cultures treated with 200mM hydroxyurea for 3 days for comparison with untreated control cultures at a magnification of 100x. These images show that the treated cultures are less confluent, when compared to control cultures. Cells were seeded in 25cm<sup>2</sup> flasks at either 1.5 x 10<sup>6</sup> (RTbrain-W1) or 3 x 10<sup>6</sup> (RTgill-W1) cells per flask, which is half of the usual plating densities to allow for obvious differences in cell growth after 3 days. Scale bar indicates 100 μm.



**Figure 4.5 Growth curves for cells treated with 200mM hydroxyurea.**

Growth curves showing a growth arrest for both RTbrain-W1 and RTgill-W1 cells treated with 200mM hydroxyurea when compared to untreated control cultures. Cells were counted at the indicated time points using a Coulter Counter (Beckman Coulter). Error bars represent standard deviation of 3 replicate counts of cells.

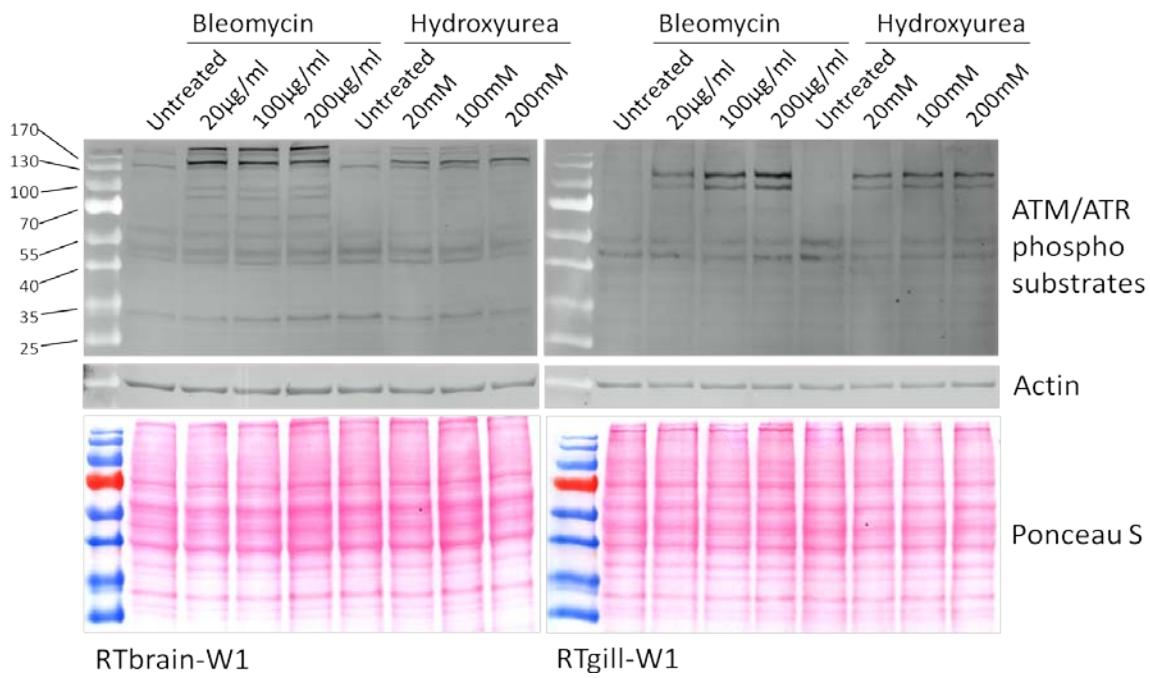


**Figure 4.6 Detection of p53 and p-p53 (ser392) in cells treated with hydroxyurea for 24h.**

Western blots showing no induction of total p53 or phosphorylated p53 (Ser392) in both RTbrain and RTgill cells after treatment with hydroxyurea at various concentrations for 24h. Protein extracts were loaded at 20 $\mu$ g per well in a 12% SDS-PAGE gel. Ponceau S staining and actin detection are provided as loading controls. p53 was detected using a polyclonal antibody to rainbow trout p53 that I produced (Chapter 3). Phosphorylated p53 was detected with a commercial antibody specific for the phosphorylated form of p53 at ser392 (Cell Signaling). Actin was detected with a monoclonal commercial antibody, specific to the conserved c-terminal region in the actin family and generated against a synthetic peptide, designed to be specific to multiple species and tissues (Sigma). A goat anti-rabbit IgG HRP conjugated secondary was used for detection (Bio-Rad).

#### **4.2.3 ATM/ATR activation**

Given that  $\gamma$ H2AX is being phosphorylated as a result of DNA damage by bleomycin in the rainbow trout cells, I also wanted to determine if other components of the DNA damage pathway were also being activated. This could help determine if the pathway is being activated similarly to mammalian models, which would help characterize the differences seen thus far for rainbow trout p53. Determining if proteins are active in the DNA damage checkpoint could also be useful in finding potential biomarkers for the generation of an environmental biomarker assay, which was discussed in Chapter 3. Two of the major kinases in the DNA damage pathway are ATM (Ataxia Telangiectesia Mutated) and ATR (ATM and Rad3 related). Activation of these kinases plays a major role in the DNA damage checkpoint pathway (Stokes *et al.*, 2007). In the mammalian pathway, ATM and ATR are activated upstream of p53. By looking at phosphorylation of its downstream targets, activation of the ATM/ATR pathways can be inferred. For the bleomycin and hydroxyurea treated samples, it can be seen that downstream substrates of ATM/ATR are being phosphorylated upon treatment with either bleomycin or hydroxyurea (Figure 4.7), though the effect does not appear to be dose dependent at the concentrations tested in RTbrain-W1 cells. For the treatments in the brain cell line, a difference in profile can be seen between bleomycin treated samples and hydroxyurea treated samples; this difference in profile between the two treatments is not seen in the gill cell line. Differences in basal profiles, as well as dosed profiles, also exist between the two cell lines, indicating cell line specific differences in phosphorylation states before and after treatments. These differences display the importance of cell line selection for different treatments since the phosphorylation of proteins differs



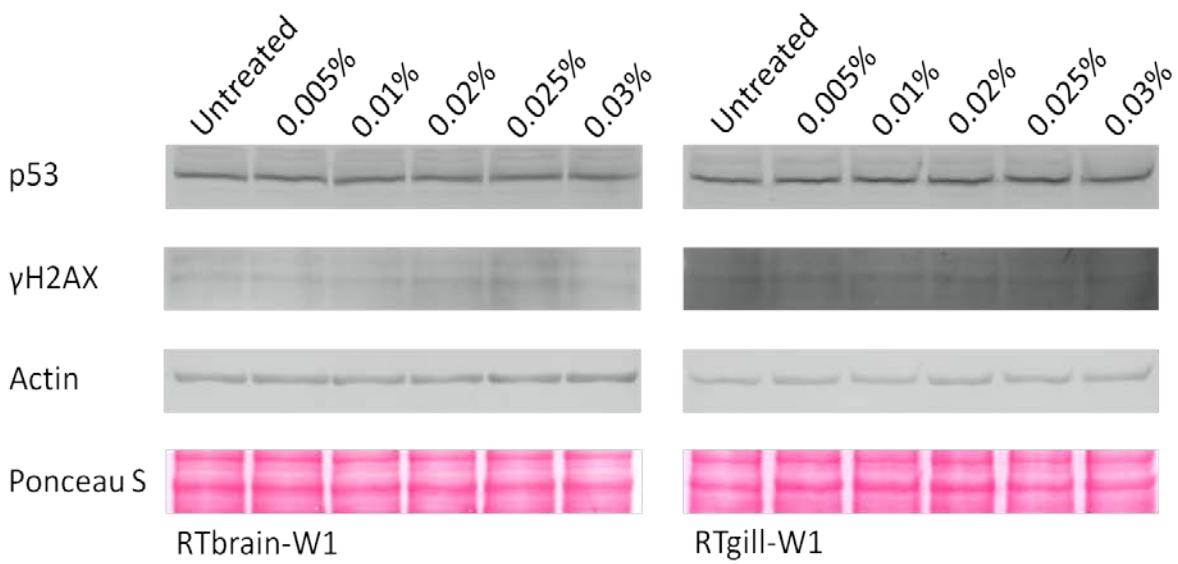
**Figure 4.7 Detection of phosphorylated downstream substrates of ATM/ATR after treatment with bleomycin or hydroxyurea for 24h.**

Cells were treated with different doses of bleomycin or hydroxyurea for 24h. Proteins were loaded at 20 $\mu$ g per well in a 12% SDS-PAGE gel. Ponceau S staining and actin detection are provided as loading controls. The treatments caused a distinct change in phosphorylation profile for the ATM/ATR substrates. These treatments also seem to affect phosphorylation in a manner that is dependent on both the reagent and cell type used. The blots were detected with Phospho-(Ser/Thr) ATM/ATR Substrate antibody (Cell Signalling) which detects endogenous levels of proteins containing the phosphorylated ATM/ATR substrate motif. Actin was detected with a monoclonal commercial antibody, specific to the conserved c-terminal region in the actin family and generated against a synthetic peptide, designed to be specific to multiple species and tissues (Sigma). A goat anti-rabbit IgG HRP conjugated secondary was used for detection (Bio-Rad).

between cell types. Depending on the protein of interest, certain cell lines may not show activation of the protein through phosphorylation. For the environmental biomarker project outlined in Chapter 3, the assay should have the capacity to examine several tissue types, to account for differences in activation between tissues. The use of one tissue type does not generate a profile specific enough to determine the type and severity of DNA damage. These results also confirm that the DNA damage pathway is being activated by both treatments and in both cell types, even though p53 is not being induced.

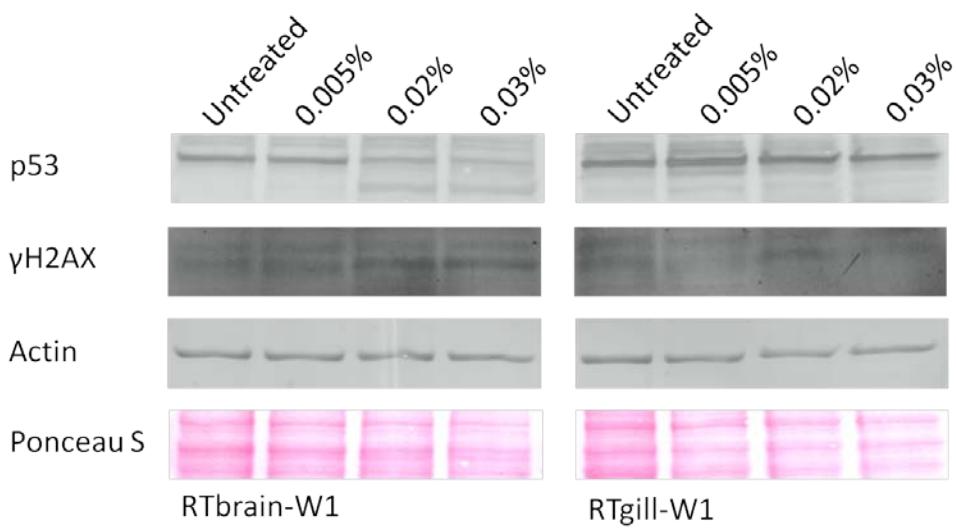
#### **4.2.4 Methyl methane sulfonate (MMS) induces cell death without induction of p53**

MMS is a DNA-alkylating agent which methylates DNA. It has been shown to cause apoptosis and formation of  $\gamma$ H2AX foci in mammalian cell lines (Zhou *et al.*, 2006). In a mammalian hepatoblastoma cell line, both lethal and non-lethal doses of MMS caused an induction in total p53 (Jiang *et al.*, 1999). For this study, cells were dosed for two different time points: 4h and 24h. The 4h incubation represented a non-lethal treatment as cell death was not observed. The 24h treatment represented a lethal treatment condition as dead cells were clearly visible in the treated cultures. In contrast to the mammalian model, my preliminary results show that p53 in both brain and gill cell lines was not induced by either non-lethal (Figure 4.8) or lethal treatments with MMS (Figure 4.9). An induction of  $\gamma$ H2AX is detected in the lethal treatments for RTbrain-W1 cells, though no increase is seen in the non-lethal treatments. In a study by Burma *et al.* in 2001, they showed that MMS treatment did not induce phosphorylation of H2AX on serine 139 in spontaneously immortalized mouse fibroblasts. Only double strand break inducing treatments caused a noticeable increased in  $\gamma$ H2AX through phosphorylation by ATM. MMS damage is usually repaired



**Figure 4.8 Detection of p53 and γH2AX in cells treated with methyl methanesulfonate (MMS) for 4 h, representing non-lethal treatment.**

Western blots showing no induction of p53 in both RTbrain and RTgill cells after treatment with methyl methanesulfonate at various concentrations for 4h. This treatment represents a non-lethal treatment of cells. There is no induction of γH2AX. Proteins were loaded at 20μg per well in a 12% SDS-PAGE gel. Ponceau S staining and actin detection are provided as loading controls. p53 was detected using a polyclonal antibody to rainbow trout p53 that I produced (Chapter 3). γH2AX was detected with a commercial antibody specific for the phosphorylated form of H2A at ser139 (Santa Cruz Biotech). Actin was detected with a monoclonal commercial antibody, specific to the conserved c-terminal region in the actin family and generated against a synthetic peptide, designed to be specific to multiple species and tissues (Sigma). A goat anti-rabbit IgG HRP conjugated secondary was used for detection (Bio-Rad).



**Figure 4.9 Detection of p53 and  $\gamma$ H2AX in cells treated with methyl methanesulfonate (MMS) for 24h, representing lethal treatment.**

Western blots showing a no induction of p53 in both RTbrain and RTgill cells after treatment with methyl methanesulfonate at various concentrations for 24h. The higher doses represent a lethal dosing treatment of the cells. The presence of DNA damage is shown by the induction of phosphorylated histone H2AX in the RTbrain-W1 cells. Proteins were loaded at 20 $\mu$ g per well in a 12% SDS-PAGE gel. Ponceau S staining and actin detection are provided as loading controls. p53 was detected using a polyclonal antibody to rainbow trout p53 that I produced (Chapter 3).  $\gamma$ H2AX was detected with a commercial antibody specific for the phosphorylated form of H2A at ser139 (Santa Cruz Biotech). Actin was detected with a monoclonal commercial antibody, specific to the conserved c-terminal region in the actin family and generated against a synthetic peptide, designed to be specific to multiple species and tissues (Sigma). A goat anti-rabbit IgG HRP conjugated secondary was used for detection (Bio-Rad).

through base excision repair and since MMS does not predominantly produce DSBs, it is possible that the slight appearance of  $\gamma$ H2AX in the lethal treatment of RTbrain-W1 cells may be due to transiently appearing double strand breaks that are occurring due to the cells inability to keep up with repairs. The lack of  $\gamma$ H2AX induction in the RTgill-W1 cells could indicate a more efficient DNA damage response, which is repairing the DNA at a quicker rate, leading to less detection of DSBs. These initial results also show that the lethal doses of MMS seem to generate a different profile of p53 detection in the rainbow trout brain cell line (Figure 4.9). For doses above 0.01% for 24h treatments of the RTbrain-W1 cell line, the main p53 band is decreased, while an extra band appears approximately 10 kDa lower. This initial result may represent modifications that are happening to p53 in dying cells. The absence of this result in the RTgill-W1 cells may be caused by a difference in the pathways activated by MMS, as the RTbrain-W1 cells appear to have an activated DSB response that is absent in the RTgill-W1 cells.

### **4.3 Discussion**

In mammalian species, p53 is a highly conserved protein that plays a key role in tumour suppression and is an important factor in research towards cancer prevention and treatment. In lower vertebrates however, the mechanisms behind its regulation and activation are mostly unexplored. It has been suggested in the past that p53 would make a good molecular biomarker for aquatic toxicology studies based on sequence comparison of fish p53 and mammalian p53 sequences (Bhaskaran *et al.*, 1999), though actual functional data for p53 in fish is limited in contrast to mammalian models. Previous studies have highlighted some differences in the function and regulation of p53 in lower vertebrate species, compared to

mammalian models (Krause *et al.*, 1997; Chen *et al.*, 2001; Embry *et al.*, 2006), which emphasizes the need for more research into p53 and its use as a potential biomarker for environmental carcinogenesis.

To study p53 protein expression, a polyclonal p53 antibody was generated from recombinantly expressed rainbow trout p53. This antibody was shown to detect p53 in both human and rainbow trout cells lines (Chapter 3). To examine p53 activation, two different cell lines were dosed with the chemotherapeutic agent bleomycin. In mammalian models, bleomycin has been shown to produce double strand breaks in DNA, which then lead to induction of p53 (Canman *et al.*, 1994; Nelson and Kastan, 1994; Okudela *et al.*, 1999). In contrast, a study in rainbow trout has shown that primary hepatocytes do not exhibit an induction in p53 after treatment with bleomycin after 24 hours (Embry *et al.*, 2006). To examine this further, two rainbow trout cell lines that were unrelated to the liver were chosen for study. These two cell lines have shown interesting results regarding Chk2, which acts upstream of p53 in the DNA damage checkpoint pathway, that indicated different cell line specific mechanisms of activation and control for Chk2 in the presence of bleomycin (Steinmoeller *et al.*, 2008). The use of these cell lines would help determine if the lack of p53 induction in primary hepatocytes was a tissue specific phenomenon. For both RTbrain-W1 and RTgill-W1 cell lines, the results show that p53 is not induced up to 24 hours after treatment with either 3 $\mu$ g/ml or 18  $\mu$ g/ml bleomycin (Figure 4.1 and Figure 4.2). Increased concentrations of bleomycin (up to 200  $\mu$ g/ml) also did not induce p53, but histone H2A was shown to be phosphorylated on serine 139 in a dose dependent manner (Figure 4.3). This indicated that the treatment of the cells was inducing DNA damage and initiating the DNA

damage checkpoint pathway. The lack of increase in p53 infers that this pathway acts independently of p53, or that p53 is activated through a different mechanism. Post translational modifications such as phosphorylation or acetylation of p53 may be the main method of control, independent of total p53 induction.

To further explore the differences between rainbow trout p53 and mammalian p53, the cells were dosed with hydroxyurea, which initiates a checkpoint through DNA replication fork stalling. Hydroxyurea treatment has shown induction of total p53 and cell cycle arrest in mammals (Ho *et al.*, 2006a; Taylor *et al.*, 1999). In human and mouse fibroblasts, cell cycle arrest caused by hydroxyurea has been shown to be p53 dependent. In wild type cells, p53 was induced by the hydroxyurea treatment and cell cycle arrest was achieved, whereas p53 null cells were able to undergo mitosis despite treatment with hydroxyurea (Taylor *et al.*, 1999). According to the data sheet that accompanied the phospho-specific p53 antibody for serine 392 (Cell Signalling), treatment of mink lung cells (MvLu) with 20mM hydroxyurea also showed a strong induction of p53 phosphorylation on serine 392 after either 16 or 30 hours of treatment. Unlike the mammalian models, hydroxyurea treatments did not increase total p53 or phosphorylated p53 at serine 392 for treatments up to 200mM (Figure 4.6). The phosphorylation of p53 at serine 392 also appeared in the untreated control samples, indicating a constant level of basal phosphorylation at that site for both RTbrain-W1 and RTgill-W1 cell lines. Despite this lack of induction and phosphorylation of p53, cells were still visibly arrested after treatment (Figure 4.4), even with low concentrations of hydroxyurea. Through a cell proliferation assay, it was determined that cells remain arrested up to 9 days after treatment with 200mM hydroxyurea, even though p53 is not being induced

(Figure 4.5). These results indicate that the cells were arrested, independent of p53 induction, which differs from the mammalian model of cell cycle arrest after treatment with hydroxyurea.

To further examine the effects of treatment with bleomycin and hydroxyurea, an ATM/ATR phosphorylated substrate antibody was used to detect changes in phosphorylation states of downstream substrates of ATM/ATR. The results show an obvious difference in phosphorylation profiles between control and treated samples. There is also a difference in profile between the two cell types, for both control and treated samples. This indicates that there are cell line specific differences in basal phosphorylation states of proteins, as well as differences in phosphorylation substrates when a checkpoint is induced. The difference between the basal profiles is to be expected, as each cell line is generated from a specific cell type from a particular tissue. Different tissues are known to have different basal protein expression profiles which could affect the basal phosphorylation states of proteins due to upregulation of certain kinases in different cell types. As for the differences in phosphorylated substrates between the two treatments, this is likely due to the different types of checkpoints that become activated. Whereas bleomycin can act anytime during the cell cycle to damage the DNA and produce double-stranded breaks, hydroxyurea usually generates replication blocks during S-phase. It has also been shown that hydroxyurea induced p53 accumulation can be generated through mechanisms that are distinct from the DNA damage checkpoint (Ho *et al.*, 2006a). This would support the hypothesis that both treatments are activating their respective pathways, possibly independent of p53 or through a mechanism separate from induction of total p53 accumulation.

For a third type of DNA damage, MMS was used to generate replication blocks caused by methylation of the DNA. It has been shown in the past that MMS was able to induce total p53 in the Hep G2 human hepatoblastoma cell line after treatment with both lethal and non-lethal doses (Jiang *et al.*, 1999). In this preliminary study, dosing studies were performed under two types of conditions: non-lethal and lethal. For the non-lethal treatment, cells were incubated with MMS for only 4 h, whereas the lethal treatment lasted for 24h, resulting in visible dose dependent cell death. Initial results show that both non-lethal (Figure 4.8) and lethal treatment (Figure 4.9) conditions produced no induction of p53 in either cell line. Under the lethal treatment conditions, a very mild increase of  $\gamma$ H2AX can be seen in RTbrain-W1, though this increase is lacking in the non-lethal treatment samples. Though foci of  $\gamma$ H2AX have been detected after MMS treatment in human amnion epithelial cells (Zhou *et al.*, 2006), it has also been shown that  $\gamma$ H2AX is only phosphorylated after treatment with double strand break inducing agents (Burma *et al.*, 2001). MMS is a DNA alkylating agent and DSBs are not the main type of damage produced but the appearance of  $\gamma$ H2AX in RTbrain-W1 cells could be a by-product of repair. It can also be seen that lethal treatments produced a slight decrease in p53 in the brain cells (Figure 4.9), but this decrease is accompanied by the appearance of a lower band in the 0.02% and 0.03% treatments that coincides with the decrease in the main p53 band. It has been previously shown that p53 is cleaved by caspases in apoptotic cells, which generates fragments that localize to the mitochondria to induce transcription-independent changes that aid in apoptosis of the cells (Sayan *et al.*, 2006). Based on the profiles of fragmentation seen in the Sayan *et al.* study, this lower band could be due to fragmentation of the original p53 protein. One of the main

fragments that appeared in the fragmented profile of the mitochondrial study appears to be similar in size to the lower band seen in the brain cells after lethal MMS treatments. These preliminary results could possibly indicate that p53 is still being activated for apoptosis through fragmentation, but not for its well known function as a transcription factor. These initial results with MMS treatment indicate some cell line specific differences pertaining to p53 fragmentation, as well as  $\gamma$ H2AX induction. These differences may be a result of variation in the efficiency of DNA repair and the initiation of apoptosis in different cell types. As mentioned earlier, a more efficient DNA damage response could account for quicker repair in the RTgill-W1 cells, which could decrease the amounts of DSBs. This more efficient DNA damage response could have evolved for the gill cells due to their constant direct exposure to the external environment. This constant exposure could have aided in selecting for a highly efficient DNA damage response, to deal with the constant onslaught of DNA damage. These results pertaining to MMS treatments are preliminary, and need to be repeated further for confirmation of the results before any definite conclusions can be drawn.

#### **4.4 Conclusions**

From this study, it can be seen that the commonly observed induction of p53 in mammalian models is absent from the rainbow trout model after checkpoint induction with bleomycin, hydroxyurea, and MMS. These treatments have been shown to induce p53 in mammalian models, leading to either cell cycle arrest or apoptosis but the induction of p53 is not seen in this study, even though the effects of the treatments can be seen. In the case of bleomycin, the DNA damage being produced is evident in the dose dependent accumulation of  $\gamma$ H2AX. For hydroxyurea treatment, cells are clearly arrested and fail to proliferate in comparison to

their untreated counterparts. With MMS, treatments for 24 hours produced a high degree of apoptosis at the higher doses, along with a slight induction of  $\gamma$ H2AX in the case of RTbrain-W1. All of these treatments produced results indicating activation of their respective checkpoints yet p53 levels remain constant. This result indicates that the DNA checkpoints may function independently of p53, or that p53 is regulated in a manner that does not result in accumulation of total p53 protein. The activation of p53 may be occurring through phosphorylation, acetylation, or fragmentation, similar to mammalian models, but does not result in an accumulation of total p53. A difference in p53 regulation and function between mammalian models and lower vertebrate models is also supported by a study regarding the phylogenetic analysis of p53 (Pintus *et al.*, 2007). The study showed that the rate of fixation of nonsynonymous substitutions over synonymous substitutions significantly increased when the ancestors of amphibians started moving onto land. They hypothesized that the more hostile land environment required an increased role for p53 as a tumour suppressor, in comparison to their previous aquatic environment. Whether it is a difference in activation, regulation or function of p53, the results of this study show that p53 may not be an ideal biomarker for genotoxic stress in lower vertebrate species. However, the dose dependent increase of  $\gamma$ H2AX was apparent in both RTbrain-W1 and RTgill-W1 cells after treatment with bleomycin, indicating its potential as a possible biomarker of DNA damage in rainbow trout. While lack of induction in total p53 indicates a lack of biomarker potential, its differences from the mammalian model are definitely a point of interest for future studies. It is evident that further study needs to be done in order to elucidate the mechanisms behind the

regulation and function of p53 in rainbow trout, as it appears to function differently than predicted based on mammalian models.

## **Chapter 5**

### **General Conclusions**

The study outlined in Chapter 3, is the first to generate a rainbow trout specific polyclonal antibody and it has proved useful in the studies outlined in Chapter 4, that have brought to light interesting results regarding the potential regulation, activation, and function of p53 in rainbow trout.

The rainbow trout specific p53 antibody was originally generated for use in dosing studies of rainbow trout cell lines, with the intent of subsequent use in the development of a potential environmental biomarker assay. However, the results of our dosing studies indicate that rainbow trout p53 may not be an ideal biomarker of DNA damage. With the study outlined in Chapter 4, it is apparent that rainbow trout p53 is not induced after treatment with agents that are known to cause a DNA damage response due to various different mechanisms. Under treatment conditions causing double-stranded breaks, cell cycle arrest, and apoptosis, the levels of p53 were not induced, contrary to the known mechanisms of p53 activation in mammalian models. However, these studies have revealed interesting results that could be useful in the future directions of this project. For the detection of treatment induced double stranded breaks in the rainbow trout cells, the induction of  $\gamma$ H2AX could clearly be seen, indicating that  $\gamma$ H2AX shows good promise as a biomarker of DNA damage in rainbow trout. Along with the clear induction,  $\gamma$ H2AX would also be useful as a biomarker due to its specific induction in the presence of DSBs, allowing for determination of the possible origins of damage induction. Along with the determination of  $\gamma$ H2AX as a potential

biomarker, ATM/ATR and their downstream substrates were also revealed to be activated in the DNA damage response. As with  $\gamma$ H2AX, a clear induction in ATM/ATR activity could be seen after treatment, as judged by an induction of phosphorylation in ATM/ATR substrates that was not present in the untreated control. There were also differences in profiles of phosphorylated substrates depending on the type of treatment. These results indicate that ATM/ATR are activated and would also be good biomarkers of DNA damage. In light of the treatment dependent differential activation of downstream substrates, substrates of ATM/ATR could also be tested for biomarker potential in future studies. The preliminary results in the MMS study also displayed interesting results that could be of interest for future studies. In the apoptotic RTbrain-W1 cultures, the appearance of a band around ~10kDa below the p53 band was of interest due to the fact that it coincided with a decrease in the main p53 band. As discussed in Chapter 4, this lower band could be indication of p53 activation through fragmentation by caspases, for localization to the mitochondria.

This thesis, along with other published research into non-mammalian p53, suggests that the mechanisms involved in non-mammalian p53 regulation vary between organisms and show varying levels of differences to mammalian p53. Non-mammalian model organisms are often used as models for toxicology and carcinogenesis, and drawing conclusions based on the well known mechanisms of mammalian p53 could lead to erroneous assumptions. Further studies of p53 in non-mammalian models are required to better understand the mechanisms and pathways surrounding non-mammalian p53.

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