THE CHARACTERIZATION OF CHECKPOINT KINASE 2 IN *Oncorhynchus mykiss*: TISSUE SPECIFIC EXPRESSION SUGGESTS BIOMARKER POTENTIAL.

By

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Abstract

Chk2 is a cell cycle checkpoint kinase that is essential for initiating the DNA damage response in the presence of genetic damage. Its role is highly conserved from budding yeast (where it is named Rad53) to humans. Very few cell cycle checkpoint proteins have ever been studied in fish and the role of Chk2 has never been characterized. *Oncorhynchus mykiss* (Rainbow trout) was chosen for this project due to its importance in the commercial aquaculture industry and the availability of rainbow trout cell cultures at the University of Waterloo. This study was the first to clone the *CHK2* gene in a teleost species, verified through both genomic and cDNA cloning. A section of the *CHK2* gene, specifically the forkhead associated domain (FHA), was used to express recombinant Chk2 protein and generate polyclonal anti-Chk2 antibodies. A southern blot was performed and *CHK2* was found to exist as a single copy number in the rainbow trout genome. The tissue specificity of Chk2 was also examined both at the mRNA transcript and protein level. Interesting tissue specific differences were discovered with transcript levels moderately low in gill and higher in brain, while protein levels were extremely high in gill and lower in brain tissues. Protein levels were verified in both whole fish tissue samples and in cell culture suggesting that cell cultures accurately reflect the state of checkpoint proteins *in vivo*. These tissue specific differences suggest that in gill, Chk2 is maintained at a high protein level to combat any toxins in the water attempting to transverse this barrier tissue and gain access to the fish's circulatory system. Meanwhile, the blood brain barrier offers protection to the highly sensitive brain tissue, suggesting that high levels of Chk2 protein are not constitutively required, but instead remain in a transcript reservoir able to be quickly translated in the event of DNA

damage. To determine whether Chk2's checkpoint role is conserved in *O. mykiss,* both gill and brain cell cultures were treated with low and high doses of bleocin (a commercially available form of bleomycin) known to cause high levels of double-strand breaks, the most deleterious type of DNA damage and a specific activator of the Chk2 DNA damage response (DSB). Results showed that bleocin had no effect on levels of Chk2 in gill cells, confirming that the protein is constitutively active in this tissue always on alert against potential genetic insult. In contrast, brain cells were able to upregulate Chk2 in a dose-dependent manner to bleocin induced DNA damage demonstrating that Chk2 can act as a biomarker for genetic damage in brain cells. In conclusion, the tissue specific expression of Chk2 and its ability to respond to DNA damage suggests that checkpoint proteins may serve as suitable biomarkers for DNA damage in *O. mykiss* and other fish species.

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CHAPTER 1

General Introduction

1.1 The Cell Cycle

The eukaryotic cell cycle is a series of tightly controlled events that results in the faithful transmission of genetic information through generations of cells. It is comprised of four phases: G1, G2, S (synthesis) and M (mitosis) as illustrated in Figure 1.1. The G1 and G2 gap phases can be extremely short in unicellular organisms but are much longer and easier to observe in mammalian cells versus other cell cycle stages. There are two major events that occur within the cell cycle, the S phase encompasses the replication of cellular DNA and the M phase is when the chromosomes become equally divided between two identical daughter cells (2). Before entering S phase, a multi-protein complex called the pre-replicative complex (Pre-RC) is assembled on DNA at replication origins scattered throughout the nuclear chromosomes (3). During S phase, pre-RCs initiate replication by promoting origin unwinding and facilitating the recruitment of DNA polymerases. DNA strands are then synthesized at the astonishing rate of 500 nucleotides per minute with an error rate of only one nucleotide in a billion (3). In M phase (mitosis), cells partition their newly replicated nuclear DNA into two identical daughter cells, ending with an event called cytokinesis where the cytoplasm and cell membrane are also divided (4). In the G1 phase of the cell cycle, the cell increases in size and starts synthesizing RNA and proteins in preparation for S phase, such as DNA polymerases and topoisomerases. In the G2 phase, the cell continues to grow and produce mitotic proteins to ensure proper chromosomal segregation and the viability of the newly formed daughter cells (5). In order to correctly duplicate the genome during DNA replication these distinct processes must be finely coordinated.

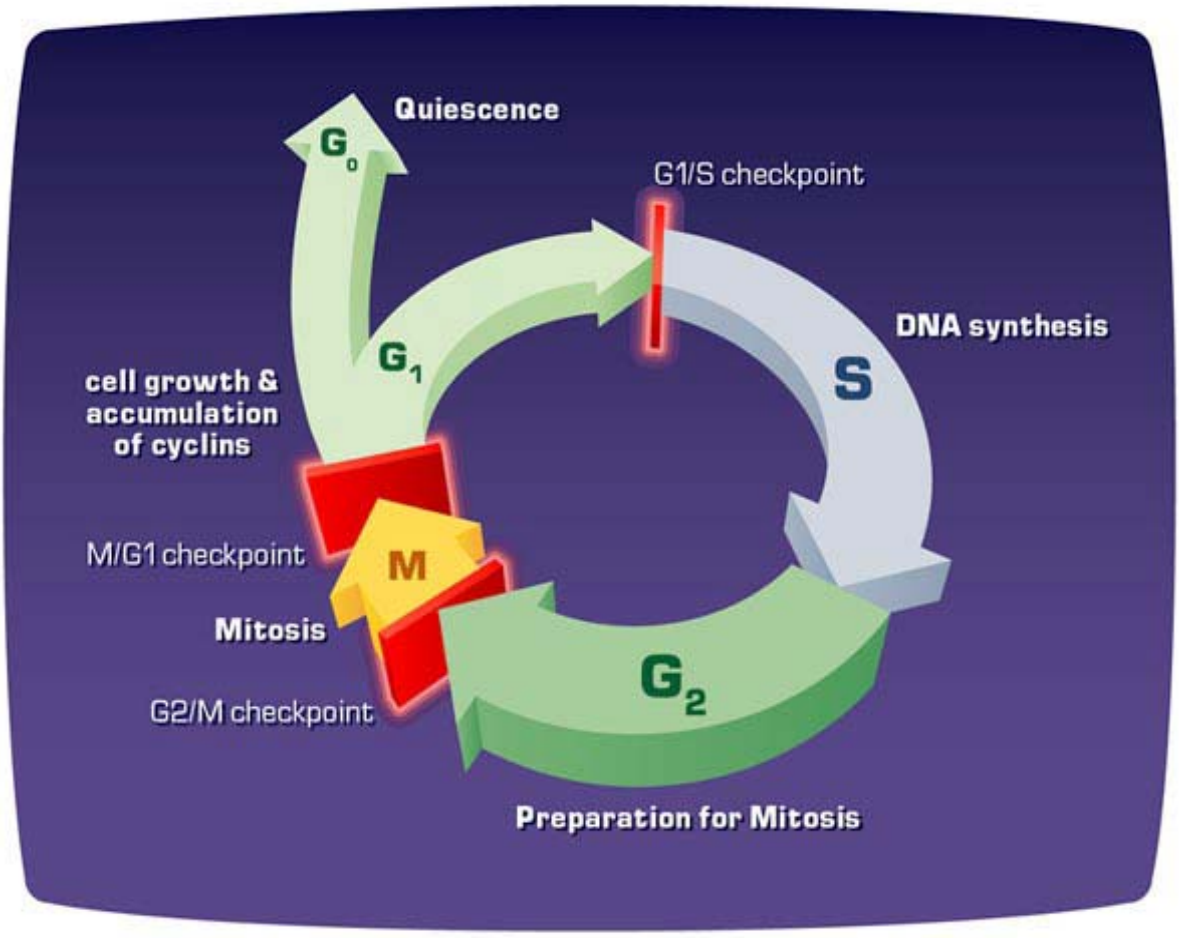


Figure 1.1: The Eukaryotic Cell Cycle. During the G1 gap phase, the cell grows in size and synthesizes the proteins it will need to replicate its genome. In S (synthesis) phase, the chromosomes duplicate as a result of DNA replication. In the G2 gap phase, the cell continues to grow in size and synthesize the proteins it will need for proper cell division. During the M (mitosis) phase the chromosomes separate in the nucleus and the division of the cytoplasm (cytokinesis) occurs. There are checkpoints in the cycle at the end of G1 and G2 that can prevent the cell form entering the S or M phases of the cycle. Cells that are not in the process of dividing are in the G0 stage. Adapted from Houtgraaf *et al*. (5).

Maintenance of genomic integrity is vital for the survival of eukaryotic cells, while failure to do so may result in the accumulation of mutations, oncogenesis, and/or cell death(6).

1.2 Cell Cycle Checkpoints

Within the nucleus DNA is constantly under threat from exogenous damaging agents and harmful cellular metabolites, such as reactive oxygen species (2;7). DNA damaging agents such as ionizing radiation, UV light and chemotherapeutic agents are being used more and more to treat serious diseases such as cancer and arterial stenosis in an effort to kill cells that are undergoing aberrant cell division (5) . In order for proliferating cells to move successfully through all stages of the cell cycle, they have developed cell cycle checkpoints which serve to control the rate of progression, to monitor the integrity of the DNA being copied, and to initiate DNA repair when necessary (8). Checkpoints act by arresting cell cycle progression until the DNA damage can be repaired or alternatively can activate an apoptotic pathway if the damage is too severe to be repaired successfully. Many proto-oncogenes have been implicated in regulation of the cell cycle and in the DNA damage response (9).

The first checkpoint genes were discovered in 1976 by Hannan and Nasim. They identified fission yeast strains with mutations in *RAD1, RAD3* or *RAD9* that were unable to delay cell cycle progression after treatment with UV radiation (10). From this study they concluded that cell cycle delay is an active part of the DNA repair process and not simply due to damage induced by UV exposure. In the early 1980s, it was discovered that cells from patients with ataxia telengiectasia (A-T) failed to elicit proper cell cycle delay after irradiation exposure. A-T is an inherited disease associated with an increased risk of sporadic tumor development. This research provided the first link between failed cell cycle delay and cancer (11).

The events that occur within the DNA damage response form a signal transduction cascade, with sensor proteins detecting the damage and relaying the signal to mediator or transducer proteins that ultimately act on effector proteins, determining the specific outcome of each checkpoint event (12;13). Within the cell nucleus the amount of undamaged DNA vastly exceeds that of damaged DNA, yet sensor proteins are still able to carry out damage recognition although discrimination is not always absolute. Sensor proteins are in constant contact with DNA always searching for areas that might require repair, therefore checkpoints are not really an "on or off" type of event, but are more accurately viewed as a steady presence always surveying for damaged genetic material (12;14). Stalled replication forks, DNA strand nicks, base-pair mismatches, RPA-coated single stranded DNA, double strand breaks, and DNA cross-links are all indications that DNA damage has occurred (15). Once the DNA strand has been exposed to a DNA damaging agent DNA lesions, adducts or abasic sites typically form providing higher-affinity binding for sensor proteins (14). The proteins involved in the DNA damage response are also involved in regulating the transition points between G1/S, G2/M and intra-S phase providing further evidence to the theory that checkpoint pathways are operative under normal growth conditions and simply become amplified once an increase in DNA damage is detected (14).

1.2.1 Sensor Proteins

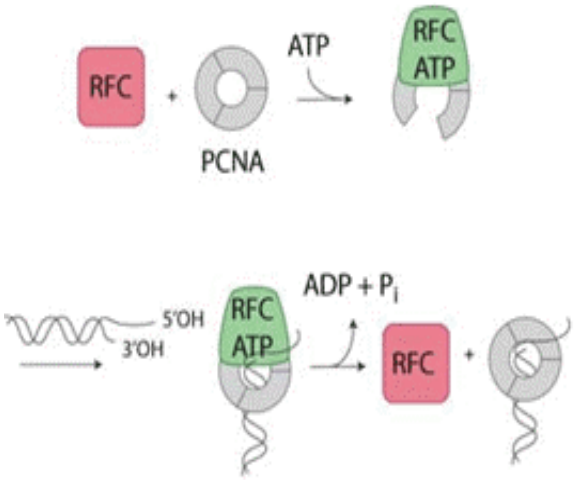
There are two main groups of checkpoint damage sensors, the phosphoinositide 3-kinase-like kinase (PIKK) family members, ataxia-telangiectasia-mutated (ATM) and ATM and Rad-3 related (ATR) and the RFC/PCNA (clamp loader, polymerase clamp)-related Rad17-RFC/9-1-1 complex (12;16). The first step in the DNA damage response is the recognition within the cell that DNA damage has occurred. In both mammals and yeast, Rad9, Rad1, Hus1 and Rad17 are all essential for sensing damage and activating

checkpoint responses (17). The Rad17-Replication Factor C (RFC) complex is globular shaped with a deep grove running down its length which allows it to bind along the DNA strand (18), while the 9-1-1 (Rad9-Rad1-Hus1) complex has a similar homotrimer ring-like structure to that of the PCNA replication complex, a clamp-like structure that encircles DNA (19).

The Rad17-RFC complex is activated only during a checkpoint response, but is a structural homolog of the RFC replication factor, which is active throughout S phase. RFC is a heteropentamer composed of p140, p40, p39, p37 and p36 involved specifically in DNA replication. ATP-dependent structural changes in RFC promote the loading of the PCNA clamp onto DNA, as shown in Figure 1.2 A), which is required for efficient strand synthesis by DNA polymerase δ, a eukaryotic replicative polymerase. Both PCNA and RFC are essential for the functional assembly of the replication fork complex (20).

The Rad17-RFC complex is very similar to RFC, only the Rad17 protein replaces the p140 subunit (21). During a checkpoint response Rad17-RFC binds to the site of damaged DNA and recruits the 9-1-1 complex, as shown in Figure 1.2 B). Rad17-RFC then acts as a DNA-stimulated ATPase binding to both DNA and the 9-1-1 complex simultaneously (22). It then loads the 9-1-1 complex onto DNA in a clamp-like manner which has been effectively demonstrated in both budding yeast and humans (23;24). The chromatin-bound 9-1-1 complex then facilitates phosphorylation mediated by ATM and ATR (23;25). ATM and ATR share partially redundant functions within a cell cycle checkpoint, but are preferentially activated by different types of DNA damage as shown

A)



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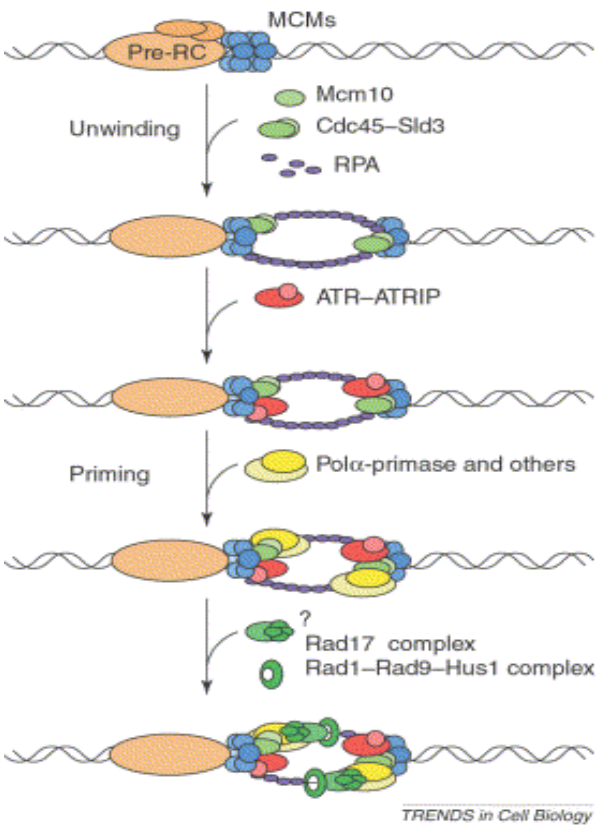

Figure 1.2: Schematic of RFC and PCNA in DNA Replication.

A) RFC loads PCNA clamp onto DNA, hydrolysis of ATP dissociates the complex.

B) Proteins involved in the initiation of DNA replication under stressed conditions in *Xenopus* including the RFC-Rad17 and 9-1-1 complex. Adapted from Osborn *et al.* (1).

in Figure 1.3 (26). ATM is an oligomeric protein approximately 350 kDa in size and exhibits protein kinase activity in response to double-strand breaks (DSB) *in vivo* (27) and to linear DNA *in vitro* (28). Mutations in ATM (ataxia telangiectasia mutated) cause A-T in humans as described above. Cells lacking ATM are viable, suggesting that ATM is not essential for normal cell cycle progression (23;29). Once activated in response to DNA damage, ATM monomerizes and becomes capable of phosphorylating many downstream targets including Chk2, p53, NBS1, BRCA1 and itself (30). ATM autophosphorylation is regulated by PP2A which it binds constitutively in the absence of DNA damage, but rapidly dissociates from once DNA damage has been detected (31). ATM's kinase activity is monitored in part by MRE11, which enhances its ability to phosphorylate downstream targets, and within the MRN complex helps to localize ATM to sites of DNA damage (32). The MRE11–RAD50–NBS1 (MRN) protein complex has been linked to many DNA metabolic events that involve DNA double-stranded breaks. MRN is one of the first factors to be localized to the DNA lesion, where it might initially have a structural role by tethering together, and therefore stabilizing, broken chromosomes (33). ATM is also regulated through a single major damaged induced phosphorylation site at Ser198 (humans), which permits a rapid and sensitive switch for checkpoint activation (30). The monomerization and autophosphorylation of ATM does not require the protein to bind to damaged DNA, but seems to be orchestrated instead by changes in higher-order chromatin structure which ATM can sense from a distance (30) .

ATR was discovered by Cimprich *et al.* through the human genome database with sequence homology to ATM and *S. pombe* Rad3, giving it the name ATR (ATM and Rad3 related) (34). ATR is approximately 303 kDA in size and is embryonic lethal in

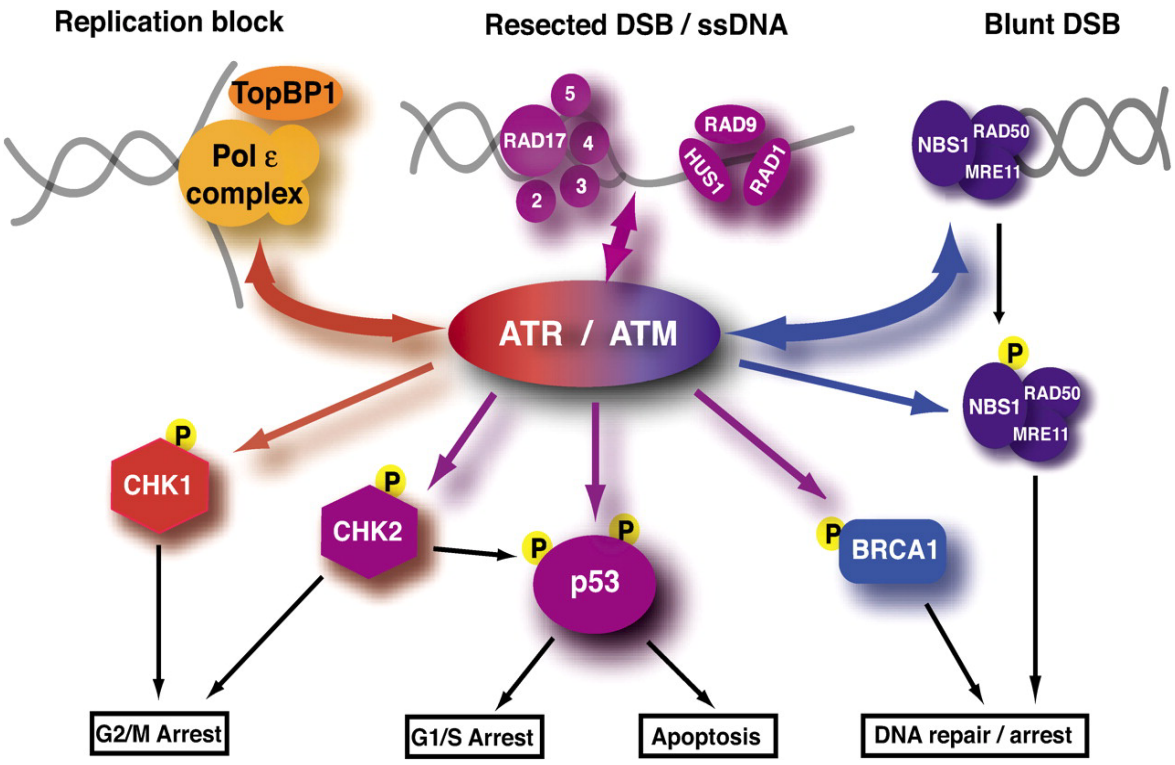

Figure 1.3: Schematic of DNA Damage Proteins. ATR and ATM are the central proteins regulating checkpoint responses to various forms of DNA damage. Activation of ATR and its downstream effects are indicated in red, whereas activation and downstream effects of ATM are indicated in blue (not absolute; some exceptions of overlapping function do occur). Signals and downstream effects common to both kinases are designated in purple. Adapted from Nyberg *et al.* (35).

mammals (36). Partial loss of ATR activity in humans is associated with the inherited disorder Seckel disease, which has many shared characteristics with A-T (37). ATR does not recognize DSB, but is activated preferentially by UV-induced DNA damage and by DNA damaging agents that result in inhibition of DNA replication (ex. hydroxyurea – a ribonucleotide reductase inhibitor that depletes dNTP pools) (38). ATR is recruited to DNA through an intermediate DNA-binding partner known as ATRIP (ATR interacting protein), which allows the complex to bind to RPA coated single-stranded DNA rather than naked DNA (39). When damage does occur, single-stranded DNA (ssDNA) becomes coated with replication protein A (RPA), which stimulates the binding of ATRIP to RPA. ATR is also critical for cellular responses to stalled replication fork progression caused by DNA damage directly or indirectly through other stresses (29;38). It shows no measurable change in kinase activity in the event of DNA damage, suggesting it may be constitutively ready to phosphorylate its downstream targets (38). Instead, its cellular functions seem to be largely controlled by its subcellular localization. Since ATRIP is complexed with ATR, this binding demonstrates a way that the subcellular localization of ATR can be changed in response to DNA damage (39). In response to many genomic stresses both ATM and ATR are eventually activated working in unison to elicit an appropriate checkpoint response.

In human cells, an alternative mechanism for DNA damage recognition has also been elucidated. It has been shown that the interaction of checkpoint sensors with DNA repair molecules may help to recruit them to sites of DNA damage. In a study by Wang and Qin in 2003, it was shown that ATR can associate with Msh2, a protein involved in mismatch repair (40). In a subsequent study by Giannattasio *et al.*, it was also shown that Rad9, a subunit of the 9-1-1 clamp, can interact with nuclear excision repair protein,

Rad14 in response to UV-induced DNA damage (41). Further research is required to confirm whether the interaction between checkpoint sensor proteins and DNA repair proteins is an essential component of the DNA damage response.

1.2.2 Mediator Proteins

Once DNA damage has been sensed the signal must be relayed to appropriate downstream targets. Mediator proteins associate with damage sensing proteins and signal transducer proteins to help provide specificity within the signal transduction cascade (14). A classic example of a mediator protein from *S. cerevisiae* is Rad9, which is specifically required to mediate the signal between Mec1 (ATR) and Rad53 (Chk2) (42). In humans four specific ATM mediator proteins have been characterized: the p53 binding protein, 53BP1; the topoisomerase binding protein, topBP1; the mediator of DNA damage checkpoint 1, MDC1, and the breast cancer susceptibility gene 1, BRCA1. All four proteins contain BRCT protein-protein interaction domains (43). MDC1 has been shown to function as a bridge between histone H2A isoform γ (γ H2AX) and Nijmegen breakage syndrome 1 (NBS1) in the MRE11-Rad50-NBS1 (MRN) complex (44). Mediator proteins are recruited to sites of DNA damage following the phosphorylation of γH2AX by ATM and become microscopically visible as foci on the DNA strand (45). These mediators can then promote sustained multi-protein interactions and facilitate ATM's downstream signaling (45). ATR checkpoint signaling is mediated primarily through Claspin, which is an adaptor protein that is structurally unrelated to the other mediator proteins. Claspin interacts with chromatin in active replication forks and is required for ATR-mediated phosphorylation of Chk1 (46). The importance of mediator proteins is highlighted by the fact that mammalian cells that lack any of the above-mentioned mediators show enhanced sensitivity to DNA damaging agents and impaired intra-S phase and G2/M checkpoints (47).

1.2.3 Transducer Proteins

In order to relay the checkpoint signal, two signal transducer kinases, Chk1 and Chk2, become activated. These serine/threonine kinases were first identified in mammals based on sequence similarity with yeast Chk1 and Rad53/Cds1, respectively (12). In general, Chk2 is activated by ATM in response to double strand breaks and Chk1 is activated by ATR after UV-induced damage (38), although some overlap in their actions does exist and will be discussed in more detail below. Chk1 is not essential for viability in fission yeast, but is required for viability in mammals suggesting that it may have acquired other functional roles. A Chk1 deletion in mice results in early embryonic death due to loss of microtubule integrity and gross chromosomal abnormalities within the cell nucleus (48). Chk1 is phosphorylated in an ATR-dependent manner at two serine residues, Ser317 and Ser345 (mammalian numbering), in response to DNA damage in both yeast and mammals (49). Chk1 activity is also greatly reduced in cells with decreased levels of Rad17 or lacking Hus1 (50). Chk1 has been shown to phosphorylate Cdc25A, Cdc25B and Cdc25C, which downregulates their phosphatase activity causing cell cycle arrests within the DNA damage response.

Chk2 is not required for prenatal development in mice, but is an integral component of the DNA damage signaling pathway(51). Chk2 is activated through either upregulation or phosphorylation of the threonine residue T68 in mammals in an ATM-dependent manner in response to ionizing radiation (IR) treatment (52). Mutations in human Chk2 are responsible for Li-Fraumeni syndrome which causes an increase in sporadic tumor incidence (53). Chk2 phosphorylates many downstream targets including Cdc25A, Cdc25C, BRCA1, and p53, as shown in Figure 1.4 (13). Chk2 seems to be primarily active in the G2/M and intra-S phase checkpoints (54).

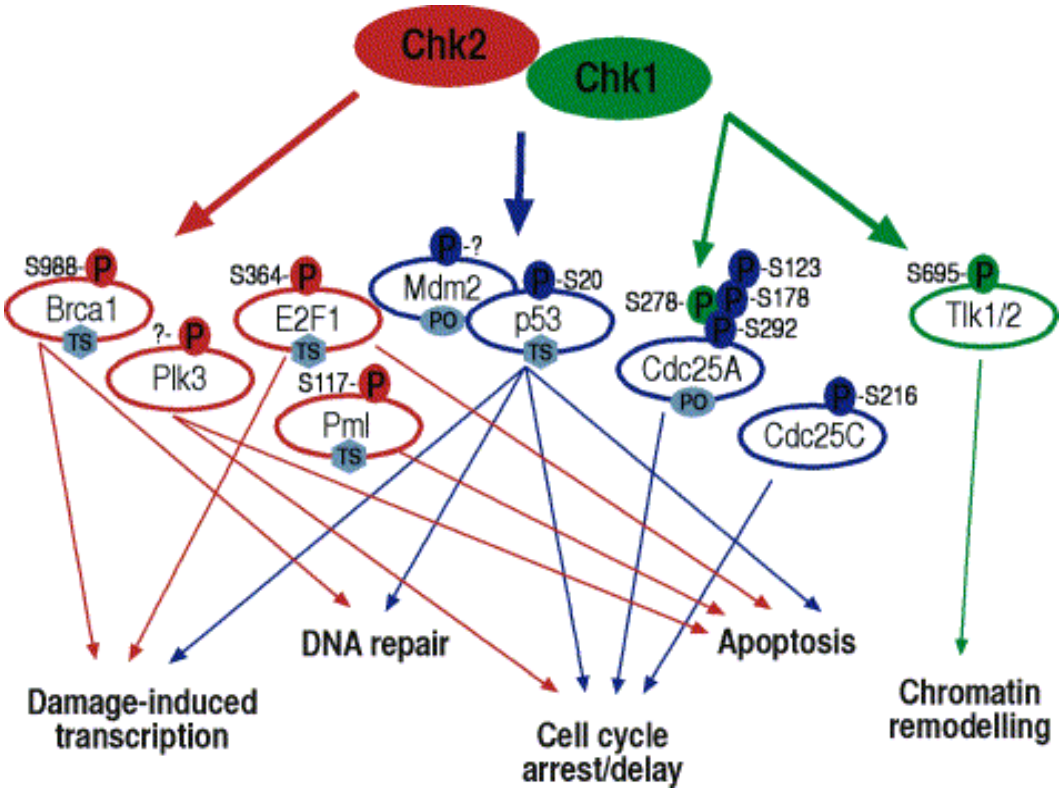

Figure 1.4: Targets of Chk1 and Chk2 in Human Cells. Following their activation, Chk1 and Chk2 phosphorylate unique (green and red, respectively) and overlapping (blue) downstream effectors that further propagate the checkpoint signaling. The known target sites of Chk1 (green), Chk2 (red), and both Chk1 and Chk2 (blue) on the individual substrates are shown. Some of the Chk1/Chk2 downstream effectors are classified as protooncogenes (PO) or tumor suppressors (TS), as indicated. Adapted from Bartek & Lukas (55).

1.2.4 Effector Proteins

Cell cycle effector proteins are ultimately responsible for instigating cell cycle arrest, DNA repair or apoptosis. In humans, the Cdc25 proteins (A,B,C), are phosphatases that de-phosphorylate cyclins to promote down cell cycle transitions (56). When the Cdc25 proteins are phosphorylated in response to DNA damage they become inactivated either by nuclear exclusion or proteolytic degradation (56). Studies in mammals and in *Xenopus* have shown that phosphorylation of the Cdc25 family results in the creation of binding sites for 14-3-3 proteins (highly conserved cellular proteins that are known to regulate various oncogenes and tumor suppressors), which ultimately causes their phophatase activity to be downregulated (57). For example, within the intra-S phase response activated Chk1 phosphorylates Cdc25A causing it to be degraded. Without Cdc25A, cyclin Cdk2 does not become activated which blocks the loading of Cdc45 onto chromatin. Cdc45 is required for the initiation of DNA replication through recruitment of DNA polymerases to the pre-replicative complex (58). The p53 tumor suppressor protein is another major effector protein and can direct the cell to undergo cell cycle arrest or apoptosis in response to DNA stressors (59). p53 activity can be modified through phosphorylation, sumorylation, neddylation and acetylation (60). p53 is negatively regulated by Mdm2 which causes any excess p53 within the cell to be targeted for degradation or to be exported from the nucleus (61). Upon DNA damage, p53 can be phosphorylated by ATM, ATR or Chk2 which inhibits the interaction between p53 and Mdm2 resulting in the accumulation of stable p53 (62). To help reinforce this stabilization, ATM also phosphorylates Mdm2 reducing its ability to successfully bind p53 and promote its degradation (63).

1.3 Checkpoint Events

1.3.1 The G1/S Checkpoint

The G1/S checkpoint inhibits the initiation of DNA replication at the G1/S transition in the presence of DNA damage as shown in Figure 1.5. Under normal conditions, cells commit to enter S phase at a stage called "start" in *S. cerevisiae* or the "restriction point" in mammalian cells (64). In the presence of DNA damage, entry into S phase is prevented regardless of whether or not this restriction stage has been reached. In the event of double strand breaks caused by IR or radiomimetic agents, ATM becomes phosphorylated and activates both p53 and Chk2. ATM's actions initiate two distinct signal transduction cascades, one to initiate and one to maintain the G1/S arrest (56). To initiate the arrest, Chk2 phosphorylates Cdc25A causing it to be exported from the nucleus and degraded (65). As described above, lack of Cdc25A results in the accumulation of phosphorylated (inactive) Cdk2 which can no longer phosphorylate Cdc45 to initiate DNA replication (58). If the damage is caused by UV radiation the signal is sensed by ATR, Rad17-RFC and the 9-1-1 complex leading to ATR dependent Chk1 phosphorylation. Chk1 can then phosphorylate Cdc25A resulting in G1 arrest as described above (14). Irrespective of the initial response pathway, the maintenance of the G1/S arrest is p53 dependent and only becomes fully sustained well after the damage is initially detected (56). In the maintenance stage, p53 is directly phosphorylated by either ATM (or ATR) which inhibits its nuclear export and degradation, resulting in the accumulation of p53 within the nucleus (66). Additionally, the ubiquitin ligase Mdm2 that normally binds p53 and targets it for degradation is inhibited by both ATM and ATR (63). p53 acts on its target protein $p21^{WAF\text{-}1/Cip1}$ which binds to and inhibits the Cdk2-

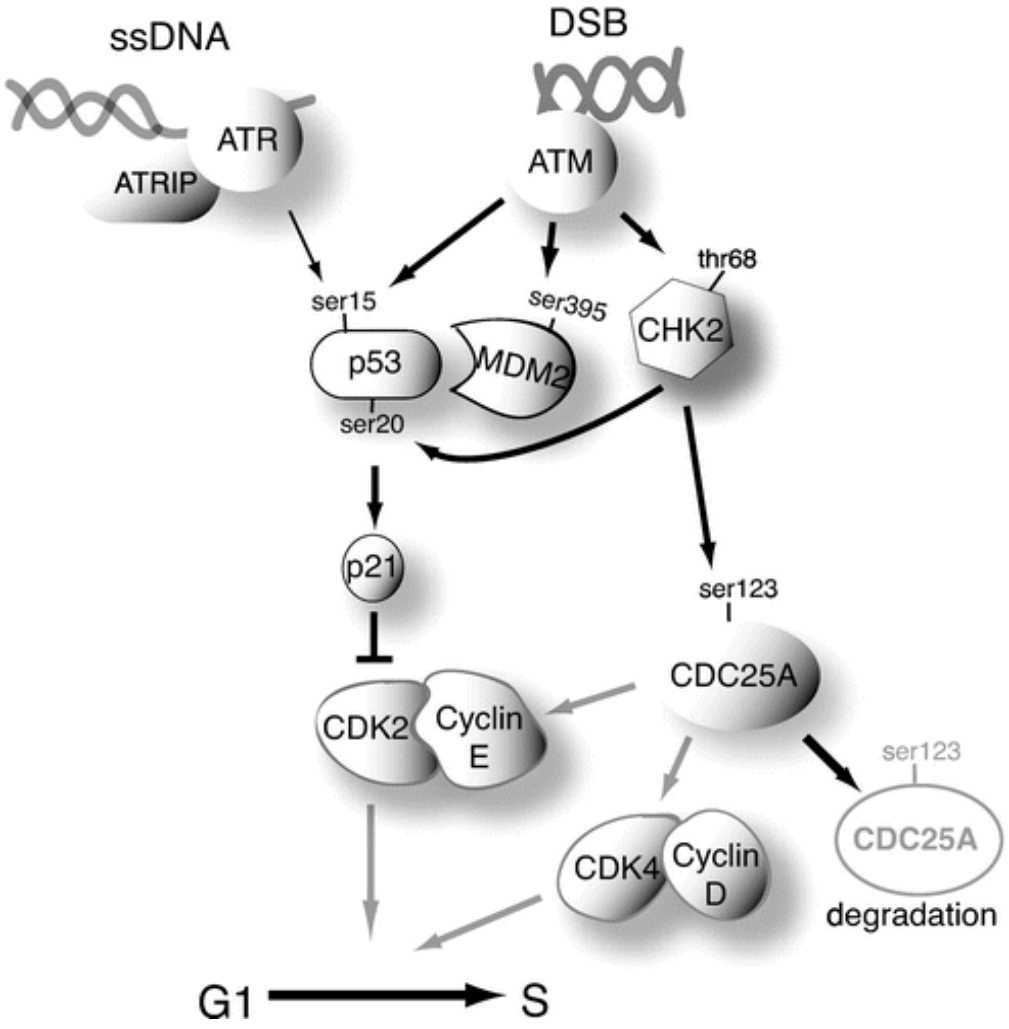

Figure 1.5: The G1/S Checkpoint in Mammals. The proteins involved in the G1/S checkpoint work together to block Cdk2-cyclin E activity. This is achieved by stabilizing p53 and degrading Cdc25A to maintain Cdk2 inhibitory phosphorylation. Gray arrows denote functions that are lost upon activation of the checkpoint cascade, and labeled amino acids on proteins indicate sites of phosphorylation. Adapted from Nyberg *et al.* (35).

cyclinE S-phase promoting complex, effectively maintaining the G1/S arrest. $p21^{WAF-1/Cip1}$ also binds to the Cdk4-cyclinD complex, preventing it from phosphorylating Rb. In order for the transcription of S phase genes to proceed the transcription factor E2F needs to be released from Rb, which can only occur once Rb has been phosphorylated (67) thereby providing a sustained G1 block. The p53 pathway and the Rb pathway are arguably the most commonly deregulated pathways in human cancers (47).

1.3.2 The Intra-S-Phase Checkpoint

The intra-s-phase checkpoint can be activated either by damage encountered during replication or by earlier damage that was not initially detected at the G1/S transition. It is typically characterized by largely transient, reversible inhibition of origins of DNA replication that have not yet fired. The replication fork itself can be slowed down actively through the binding and sequestration of PCNA by $p21^{WAF-1/Cip1}$ or by the degradation of PCNA by Rad6 in budding yeast (67). There are many checkpoint and repair proteins involved in the intra-s phase checkpoint as shown in Figure 1.6. In the event of a double strand break ATM, the MRN complex and BRCA1 are all required for checkpoint activation (68) and the initiation of the signal transduction cascade. The ATM-Chk2-Cdc25A-Cdk2 pathway acts as described above, with the inhibition of Cdk2, preventing the loading of Cdc45 onto chromatin, which in turn prevents the recruitment of DNA polymerases and late origin firing (69). There is also a second pathway that is activated in the intra-S phase response. SMC1 is phosphorylated by ATM in conjunction with BRCA1, FANCD2 (Fanconi anaemia complementation group D2), and NBS1, and acts to help sustain the cell cycle arrest. SMC1 is a cohesin and may act with SMC3 to

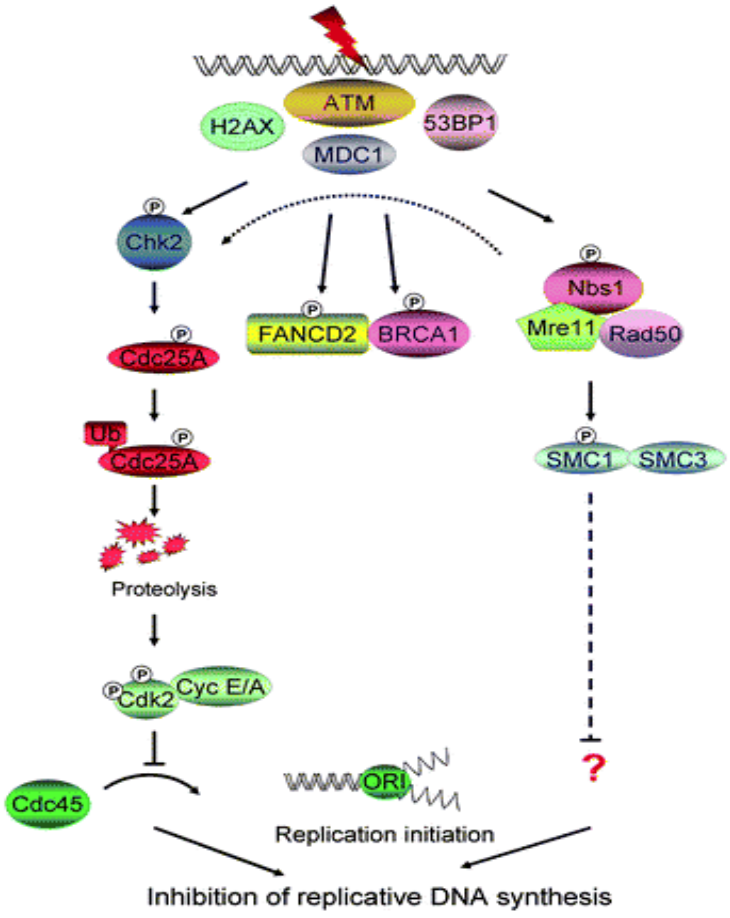

Figure 1.6: The Intra-S Phase Checkpoint in Mammals in response to DSB. In response to double-strand breaks induced by ionizing radiation, ATM triggers two cooperating parallel cascades to inhibit replicative DNA synthesis. ATM, through the intermediacy of MDC1, H2AX, and 53BP1, phosphorylates Chk2 on Thr68 to induce ubiquitin-mediated degradation of Cdc25A phosphatase. The degradation locks the S phase–promoting Cyclin E/Cdk2 in its inactive, phosphorylated form and prevents the loading of Cdc45 on the replication origin. ATM also initiates a second pathway by phosphorylating NBS1 of the MRN complex, as well as SMC1, BRCA1, and FANCD2. Adapted from Sancar *et al.* (14).

activate the S-phase recovery process (70). Cell cycle checkpoint mediators, γH2AX, 53BP1, BRCA1, Mre11, and SMC1 colocalize through MDC1 at sites of DSB to form ionizing radiation induced foci (IRIF) initiating DNA repair mechanisms as required (44).

When DNA is damaged by UV-radiation or by chemicals that cause bulky DNA adducts, ATR acts as the main damage sensor. In conjunction with its chaperone protein, ATRIP, ATR binds either directly to chromatin or to RPA-coated single stranded DNA where it becomes activated (39). Similar to the G1/S checkpoint, the ATR-Chk1-Cdc25A-Cdk2 pathway becomes activated and origins are prevented from firing (71). There is also evidence from *Xenopus* studies that ATR-dependent checkpoints may also downregulate Cdc7-Dbf4 kinase activity, which is required for Cdc45 to bind chromatin (72). ATR also promotes recruitment of checkpoint mediator proteins such as MDC1, BRCA1, and SMC1, which are essential for orchestrating DNA repair and reinitiating DNA replication (73).

1.3.3 The G2/M Checkpoint

In the presence of DNA damage the G2/M checkpoint prevents cells from undergoing mitosis as shown in Figure 1.7. Again, the initial response pathway is dependent on the type of DNA damage. The ATR-Chk1-Cdc25 pathway is activated in response to UV-damage and the ATM-Chk2-Cdc25 pathway is activated in response to IR. Upon phosphorylation, the Cdc25 phosphatase binds to the 14-3-3 proteins, becomes sequestered in the cytoplasm and is degraded by the ubiquitin-proteasome pathway (74). Next, Wee1 becomes upregulated, which in combination with the degradation of Cdc25 inhibits Cdc2/Cyclin B activity and prevents the cell from entering mitosis (75). Initially, it was believed that Cdc25C was the primary effector of the G2/M checkpoint. However, a study using Cdc25C (-/-) murine cell lines was found to have a normal G2/M response,

while disruption of the Chk1-Cdc25A pathway completely abrogated the checkpoint, pointing to Cdc25A as the major G2/M effector (76;77).

1.4 Checkpoints and Cancer

DNA damage from both endogenous and exogenous sources is a major contributor to the development of human cancers, thus malfunctioning cell cycle checkpoints may be linked to cancer incidence and progression. When cells have an intact DNA damage response they are able to arrest progression through the cell cycle, initiate repair, or promote apoptosis in response to genetic insult. Mutations in these checkpoint proteins permits cells to divide uncontrollably and allow the accumulation of cells with genetic mutations (47). Many of the above-mentioned proteins have been classified as either tumor-suppressors or proto-oncogenes, but determining exactly which proteins would be useful targets in chemotherapeutics remains an ongoing challenge. Mutations in ATM lead to an increased rate of lymphomas in both humans and mice (29). Studies in ATM-deficient mice have found that excessive recombination, normally mediated by ATM, may be an important contributor to tumorigenesis in ataxia-telangiectasia patients (A-T) (78). Low levels of ATR expression have been linked to Seckel syndrome which causes growth retardation, dwarfism, microcephaly, mental retardation and chromosomal instability (79).

In mice that are defective in DNA mismatch repair, low levels of ATR expression have also been associated with increased tumorigenesis (80). Mice lacking either γH2AX or 52BP1 show defective cell cycle checkpoints and cancer predisposition, although no link has been clearly established in humans (81;82). Chk1 (+/-) mice also show a modest increase in cancer predisposition with cells showing inappropriate S- phase entry,

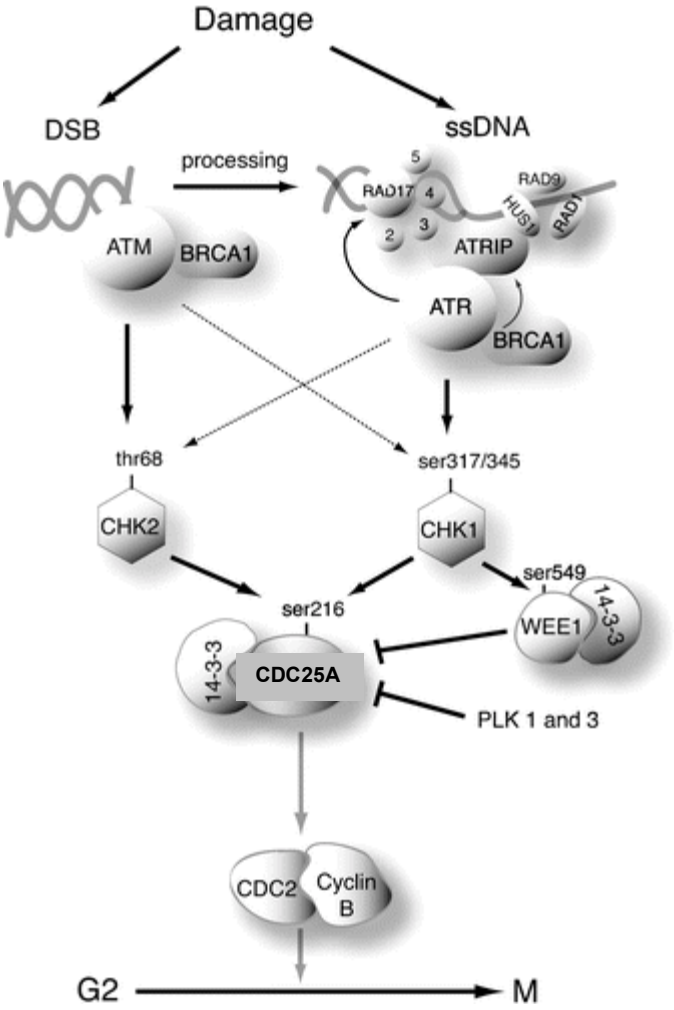

Figure 1.7: The G2/M Checkpoint in Mammals. The G2 checkpoint in mammalian cells primarily functions to block Cdc2-cyclin B activity. The common means of maintaining Cdc2 inhibitory phosphorylation is by blocking Cdc25A phosphatase activity, namely by promoting its association with 14-3-3-proteins. Gray arrows denote functions that are lost upon activation of the checkpoint cascade, and labeled amino acids on proteins indicate sites of phosphorylation. Adapted from Nyberg *et al.* (35).

accumulation of DNA damage, and inappropriate mitotic entry (83). Chk2's relationship to cancer will be discussed below in section 1.5.5.

1.5 Characterization of Checkpoint Kinase 2

Chk2 is a protein kinase – an enzyme that transfers the phosphate group from ATP to a given substrate target, causing a covalent modification. Phosphorylation is a way for cellular proteins to become activated or inactivated, for them to interact with different protein partners, and a way for their subcellular localization to be changed. This modification is used to initiate or spread a wide range of cellular signals in many different fundamental cellular processes (84). Double strand breaks (DSB) are among the most severe type of DNA damage and can be caused experimentally through ionizing radiation, through radiomimetic drugs that bind to DNA (such as bleomycin and neocarcinostatin), or through topoisomerase II inhibitors (such as etoposide and doxorubicin) (85). The importance of Chk2 in the DNA damage response is indicated by its conservation across all eukaryotes studied to date, such as yeast, mice and humans. It is not required for viability in vertebrate cells, but lack of Chk2 results in defective cell cycle checkpoints and apoptosis after induction of DSB (84). Chk2 is preferentially activated in response to DSB by ATM, but in some cases can be activated by ATR depending on the exact type of DNA lesion. In such cases where both ATR and ATM are activated, the ATM-Chk2 response is initiated first and appears to be more transient, where as the ATR response occurs after and is often more sustained (86). Chk2 is expressed throughout the cell cycle due to its broad role in responding to DSB independent of whether the cell is in the process of DNA replication or not (87).

1.5.1 Domains of Chk2

The Chk2 protein is composed of three distinct domains, an SQ/TQ cluster domain (SCD), a forkhead-associated domain (FHA), and a Ser/Thr kinase domain as shown in Figure 1.8. The SCD domain is the primary substrate target of ATM/ATR, and in humans the T68 residue has been shown to be a key phosphorylation site (88). In fission yeast, the phosphorylation of T11 in the SCD domain by Rad3 is required for Cds1 (Chk2) activation (89). The FHA domain is an 80-100 amino acid phosphopeptide-binding region and is highly conserved from yeast to humans (90). Structural analysis of the FHA domain in combination with x-ray crystallography has shown that it consists of eleven beta-pleated sheets forming a structural core with the connecting loops exposed for phosphopeptide recognition (91). The kinase domain occupies almost the entire carboxy-terminal half of Chk2, and is similar to other Ser/Thr kinases with a glycine rich region near a section of lysine residues towards the N-terminal region and an aspartic acid acting as a catalytic residue at the active site (92). Mutating one of the key residues in the human Chk2 kinase domain, Asp345, results in a kinase-defective mutant which has proven to be a valuable research tool (57). The budding yeast orthologue of Chk2 (named Rad53) is the only member of the Chk2 family to have a large carboxy-terminal extension that includes a second forkhead associated (FHA) domain (84). A feature unique to mammalian Chk2 is a c-Abl SRC homology-3 (SH3) domain-consensus binding domain, which is located just upstream of the FHA domain. The functional significance of this domain is unknown, but Chk2 and c-Abl are both phosphorylated and activated by ATM in response to DNA damage (93).

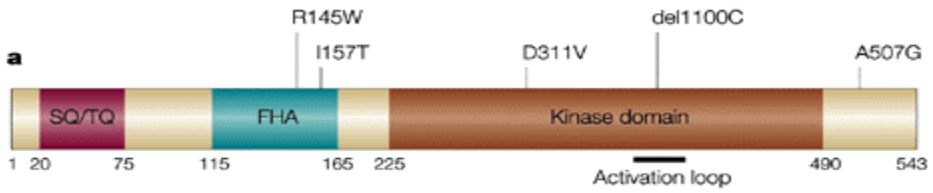

Figure 1.8: Domains of Chk2. Schematic of the Chk2 domains in humans, including the SQ/TQ, FHA and Kinase regions. This figure also shows the mutations that have been identified so far in human cancers. Adapted from Takai *et al.* (55).

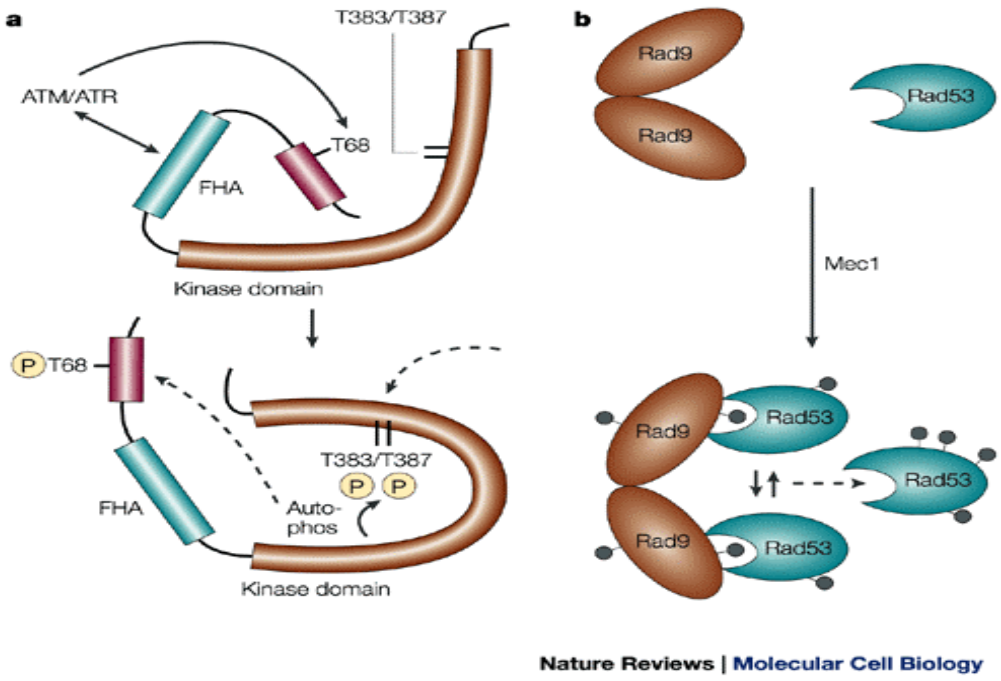

Figure 1.9: Activation of Chk2. A) In mammals Chk2 is phosphorylated by ATM/ATR on T68 in the SCD domain inducing a conformational change (dashed arrows) which allows Chk2 to autophosphorylate. **B)** Model of Rad53 activation in *S. cerevisiae*, in which Mec1-dependent, Rad9-mediated *in trans* autophosphorylation of two Rad53 molecules leads to release of activated Rad53. Pinheads indicate phosphorylation. Adapted from Takai *et al.* (55).

1.5.2 Details of Chk2 Activation and Regulation

Chk2 kinase activity is increased following DNA damage caused by IR and chemotherapeutic agents as described above. More recently, telomere erosion leading to senescence has also been implicated as an activating signal for Chk2 (94). In A-T cells that lacked a functional ATM protein, Chk2 failed to be activated and only after ectopic ATM expression could a Chk2-dependent intra-S phase checkpoint be restored (95). ATM has been shown to phosphorylate Chk2 specifically at T68 in human cell lines, and an alanine mutation of this residue prevented Chk2 activation after IR *in vivo* (96). ATR has also been shown to activate Chk2 after high levels of UV exposure or after treatment with the ribonucleotide reductase inhibitor, hydroxyurea (HU) (97). Chk2 normally resides in the cell as a monomer, but after DNA damage undergoes dimerization, with the SCD domain (containing the phosphorylated T68 residue) binding to the FHA domain in a neighbouring Chk2 protein (98). Chk2 then undergoes multiple autophosphorylation steps ultimately resulting in kinase activation as shown in Figure 1.9 A) (99). The T68 phosphorylation event is only required for the initial dimerization and autophosphorylation of Chk2, and is not required for sustained Chk2 kinase activity (100). Phosphorylation of various residues within the Chk2 protein may allow for stress specific differences in Chk2 kinase activity (57).

In budding yeast, DNA damage leads to the rapid binding of Rad53 to a dimer of Rad9 (part of the 9-1-1 damage sensing complex). This interaction between Rad53 and Rad9 is dependent on Rad9 first being phosphorylated by Mec1 (the budding yeast homologue of ATR) (101). This event allows two Rad53 molecules to be brought into close proximity, and promotes their *in trans* autophosphorylation, as shown in Figure 1.9

B). This event in turn releases Rad53 from Rad9, allowing it to act on subsequent downstream targets (101).

Analyses of Chk2 in cultured mammalian cells have shown that it is primarily a nuclear protein, which would be expected for a protein involved in the DNA damage response (102). However, in human neuronal cells Chk2 has a predominately cytoplasmic localization, which is also true of ATM in neuronal cells (87). This indicates that ATM and Chk2 may have an additional cytoplasmic role in the protection of sensitive neuronal cells perhaps in guarding against oxidative stress (38). This hypothesis is supported by the fact that when neurons are exposed to reactive oxygen species they undergo apoptosis and patients who suffer from A-T suffer from severe neurodegeneration (103). In humans, Chk2 is expressed to varying degrees in cells of proliferating and terminally differentiated, non-proliferating tissues and since Chk1 is restricted to primarily proliferating cells, the DNA damage response may rely more heavily on Chk2 in quiescent cells (87).

1.5.3 Targets of Chk2

Chk2 can act on a variety of downstream effector targets, which in turn mediate the cell cycle arrest or apoptosis that characterize the DNA damage response as shown in Figure 1.10. In vitro studies were able to show that Chk2 seems to preferentially phosphorylate the L-X-R-X-X-S/T consensus motif (104). One of the most important of Chk2's downstream targets is the Cdc25 family of phosphatases, which in unperturbed cells promote cell cycle progression by activating the cyclin-dependent kinases Cdk2 and Cdk1 (65). By phosphorylating Cdc25, Chk2 causes it to either be degraded or exported from the nucleus efficiently causing a cell cycle arrest (92). The identification of the ATM-Chk2-Cdc25A-Cdk2 axis in response to ionizing radiation offers an explanation for

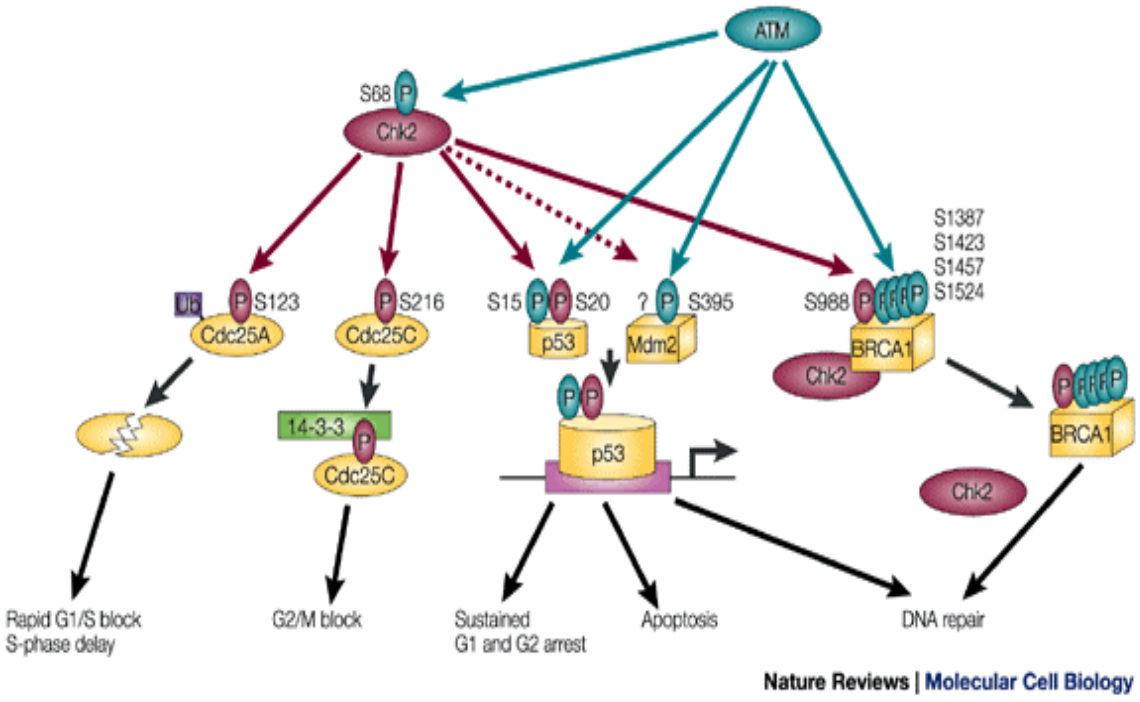

Figure 1.10: Downstream Targets of Chk2. Activated Chk2 induces rapid G1/S and G2/M cell cycle arrest and/or S-phase delay by phosphorylating Cdc25A. Chk2 also phosphorylates the p53 tumour suppressor, which results in stabilization of p53 and transactivation of p53's target genes. The p53-specific ubiquitin ligase Mdm2 might also be a substrate for the Chk2 kinase. Finally, Chk2 and ATM jointly phosphorylate the BRCA1 tumour suppressor. Chk2-dependent phosphorylation leads to dissociation of Chk2 from BRCA1, an event that is required for efficient repair of DSBs and survival of cells that are exposed to ionizing radiation. Adapted from Bartek & Lukas (55).

the clinically important radioresistant DNA synthesis (RDS) phenotype which allows the replication of DNA even in the presence of significant DNA damage. When any of the components of this axis are disrupted RDS is the result, showing that this checkpoint is extremely important for maintaining genomic integrity (65).

Chk2 can also phosphorylate E2F-1 in response to the DNA damaging agent etoposide, regulating both its stability and its transcriptional activity (105). E2F-1 has been shown to be important in initiating etoposide-induced apoptosis and when over expressed can induce cell death (106). In modulating E2F-1, Chk2 may have an important role in the DNA damage induced apoptosis pathway. Chk2 can also interact with the checkpoint proteins BRCA1 and Promyelocytic Leukemia (PML) in the absence of DNA damage. Following IR treatment both BRCA1 and PML are phosphorylated in a Chk2-dependent manner resulting in the loss of their abilities to bind Chk2 (73), an event which is important for cell survival after DNA damage. Exactly how this modification impacts the function of these proteins remains unknown, although it has been hypothesized that these proteins may actively inhibit Chk2 activity in unperturbed cells (92).

Chk2 does not become immobilized at sites of DNA damage but instead acts as a signal spreader, relaying the checkpoint signal to its downstream targets (107). It initially interacts with 53BP1, γH2AX, and NBS1 in nuclear foci, but once activated it disengages from the complex (possibly by phosphorylation of PML or BRCA1) to seek out and activate its various substrates (108). In patients with Nijmegen Breakage Syndrome who carry inactivating mutations in Nbs1, Chk2 fails to be activated showing that the MRN complex plays a central role in ATM/Chk2 activation after DNA damage (109). Defects in the MRN complex have also been associated with radioresistant DNA synthesis (RDS) another link in the Chk2-DNA damage response pathway (110).

1.5.4 Chk2 and p53

Both Chk2 and p53 are tumor suppressors that play central roles in cell cycle arrest and in apoptosis, although conflicting results have made it difficult to ascertain exactly how these two proteins interact. Chk2 was originally hypothesized to act upstream of p53 in mice, phosphorylating it at SER20 causing p53 stabilization and its dissociation from the p53-Mdm2 complex (62). It was also shown that immunoprecipitated Chk2 is activated by IR to phosphorylate p53 and overexpression of Chk2 increased p53's transcriptional activity as demonstrated via a p53 responsive reporter plasmid (111). However, other studies suggest that the interaction between Chk2 and p53 may not be so straight-forward. p53 does not have the Chk2 "L-X-R-X-X-S/T" substrate consensus motif at SER20, but other regions of the protein may instead act as substitute Chk2 docking sites (112). Several *in vivo* studies have failed to show the Chk2-p53 interation. When Chk2 siRNA was introduced into human tumor cell lines p53 stabilization was not affected (113) and when Chk2 was purified from tumor cell lines it was unable to phosphorylate either truncated or full-length versions of p53 (114). These conflicting results suggest that the relationship between p53 and Chk2 may be stimuli and cell-type specific, and it remains to be determined exactly when Chk2 regulates p53 and what factors might compensate for the Chk2 role in cases where it is not required. The downstream mediators of these p53-regulated cellular effects include GADD45 and the $p21^{CIP1/WAF1}$ inhibitor of cyclin-dependent kinases and apoptosis promoting factors, Bax and Fas (115).

1.5.5 Conservation of Chk2

Chk2 appears to be highly conserved across a wide range of species, which is not surprising since all species need to be able to react to DNA damage or replication blocks. The first member of the Chk2 family to be discovered was the *Saccharomyces cerevisiae* homologue, Rad53 in 1994 (116). It was subsequently discovered in fission yeast, *Saccharyomyces pombe* (named cds1) and in higher eukaryotes (117). The human homologue was discovered in 1998 and was named Chk2 by the Elledge lab (57). While Chk2 does show high levels of structural similarity it does have functional differences that are species specific. In yeast, Rad53 and cds1 can respond to a wide variety of DNA damage, while in mammals Chk2 activity seems to respond mostly to the most serious form of DNA damage, double strand breaks (118). Work in *D. melanogaster* and *C.elegans* on Chk2 found new functions not documented in either mice or humans. Mnk, which is the fruit fly homologue of Chk2, is highly expressed in the ovaries during embryogenesis suggesting a possible developmental role (119). Loss of Chk2 in fly embryos has also been linked to an abnormal centrosome function wherein lethally damaged cells cannot be efficiently eliminated from the developing fly embryo (120). Loss of Chk2 also causes fly embryos to become resistant to radiation-induced apoptosis and prevents cell-death caused by an over-expression of p53 (121). An interesting study done in *C.elegans* found that RNA interference of Cds1 (Chk2 homolog) resulted in failed meiotic recombination, a function not yet shown in other organisms (122). In budding yeast, a distantly related kinase to Rad53 called Mek1 monitors meiotic recombination and it remains unknown which kinase is responsible for monitoring this process in mammals (123). While the overall function of Chk2 does show some species specific variation, its general role seems to be conserved with the human Chk2 gene able

to complement checkpoint defects in both fission and budding yeast strains that are deficient in Cds1 and Rad53 respectively (57;124).

1.5.6 Chk2 and Cancer

The first indication that Chk2 may act as a tumor suppressor came from a subset of patients with Li-Fraumeni syndrome, a disease that is typically associated with a germline p53 mutation. These patients were atypical in that they still had two wildtype copies of p53 but a mutant form of Chk2, suggesting that mutations in the Chk2 gene may cause phenotypic consequences equal to TP53 inactivation (53). A second indication that Chk2 may have a role in cancer development came when a Chk2 allele containing the mutation 1100delC, which results in a truncated C-terminus through the kinase domain, was found to cause an increased risk of breast cancer. This result was independent of mutations in the BRCA1, breast cancer susceptibility gene (125). Further analyses have confirmed that truncated and mutated forms of Chk2 are unable to interact with and/or phosphorylate its primary downstream targets, p53 and Cdc25A (126). Mutations in Chk2 have since been associated with an increased cancer risk in prostate, lung, brain and lymphoid tissues (127). Mutations in the kinase domain of Chk2 have been associated with faulty checkpoint activation after IR treatment and mutations within the FHA domain have been associated with accelerated Chk2 degradation and defects in its protein-protein interactions (90). When Chk2 and p53 are both mutated, tumor cells have been shown to have a selective viability advantage over cells that have either mutation on its own. This cooperative effect is probably due to the fact that p53 and Chk2 operate in the same DNA damage response pathway but also have independent functions (111).

A recent study by Bao *et al.* in 2006 showed that a subset of glioma tumor cells was able to become highly radioresistent and invasive due to a hyperactive DNA damage

response, which could only be attenuated by specifically inhibiting Chk2. They concluded from this work that targeting the DNA damage checkpoint response in cancer cells could help overcome their radioresistance and provide a therapeutic model for malignant brain cancers (128).

In the future, Chk2 may become an attractive target in chemotherapeutic treatments. Cancer cells often lack at least one cell cycle checkpoint and inhibition of the remaining checkpoints could make tumor cells more sensitive to anticancer therapies such as gamma-irradiation or DNA-damaging pharmaceuticals (84). Normal cells could activate their other functional checkpoints and recover from the cell cycle arrest, while cancer cells effectively deprived of all checkpoints would undergo cell death. Some success has already been obtained in this pursuit such as ATM/ATR activity being blocked by caffeine (129) and inhibition of Chk1 by the anti-cancer drug UCN-01 (130), both of which resulted in decreased checkpoint activation and preferential cancer cell death after exposure to DNA damaging agents.

1.6 Rainbow Trout (*Oncorhynchus mykiss*) as a Model Organism

There are numerous advantages to using rainbow trout as a model organism for carcinogenesis, including its known sensitivity to cancer-causing aflatoxins, nitrosamines and polycyclic aromatic hydrocarbons (PAHs) (131). Rainbow trout are much less expensive to purchase and maintain than rodents and have an extremely low incidence of spontaneous tumors. It has also been shown that many mechanisms involved in carcinogenesis are conserved between trout and mammals (132). Rainbow trout are relatively portable, have wide-ranging body size, and are easily maintained in laboratory culture. For these reasons rainbow trout has proven useful for statistically challenging studies, such as those studying low-dose response that require large numbers of animals to be statistically significant (132).

The first study to link rainbow trout with carcinogenesis occurred in the early 1960s when liver cancer was found in Pacific Northwest rainbow trout hatcheries. This work eventually identified aflatoxin B1 as a human hepatocytic carcinogen (133). After this discovery a major research effort was undertaken using rainbow trout as a model for cancer research at Oregon State University which primarily studies *in vivo* whole-animal responses to carcinogens (132). Since then a variety of carcinogens have been tested on rainbow trout, although an understanding of the molecular basis for cancer initiation, and progression in rainbow trout has yet to be investigated. It is currently unknown whether the DNA damage response pathway remains conserved relative to other eukaryotes, and there is little expectation that rainbow trout will surpass traditional rodent models in human cancer research. The biggest limitation in using a teleost model is the lack of organ similarity to prostate and breast cancer. Trout also have late sexual maturity (2-3 years) and a long life span concurrent with somatic growth, which would make waiting for fish to finish development a considerable disadvantage (132). In conclusion, a rainbow trout model offers distinct advantages and disadvantages relative to other organisms in the study of cell cycle control and carcinogenesis.

1.7 Checkpoint Proteins in Fish

There have only been a handful of studies published that have examined the roles of checkpoint and DNA replication proteins in fish. They have primarily centered on p53 and a brief summary of the results to date are outlined below.

1.7.1 p53

It has been previously shown that p53 is mutated in more than 50% of human cancers resulting in loss of p53 mediated apoptosis and cell cycle control (134). p53 is highly conserved and has been cloned in a wide variety of fish species including Japanese medaka (*Oryzias latipes*) (135), coho salmon *(Oncorhynchus kisutch),* chum salmon

(*Oncorhynchus keta),* Chinook salmon (*Oncorhynchus tshawytscha),* puffer fish (*Tetraodon miurus),* barbel (*Barbus barbus*) (136), flounder (*Platichthys flesus*) (137), and zebra fish (*Danio* rerio) (138). In 2002, a study by Langheinrich *et al.* examined the roles of p53 and Mdm2 in zebrafish by generating separate p53 and Mdm2 deficient embryos. Unperturbed p53 mutant embryos were indistinguishable from control embryos, while Mdm2 mutant embryos suffered high levels of cell death and arrested very early in development. p53 deficiency did however result in decreased DNA-damaged induced apoptosis after UV and camptothecin treatment suggesting a defective DNA-damage response (139). This study concluded that zebrafish could act as a model organism for the development of anticancer drugs and treatments in cell cycle control.

A study by Berghmans *et al.* in 2005 further examined the phenotype associated with a p53 defective zebrafish mutant. They verified the failure of embryos to undergo apoptosis after IR exposure and discovered that the mutants also failed to upregulate $p21^{CIP1/WAF1}$ and to arrest at the G1/S checkpoint. They also found that a third of the mutant embryos began to develop malignant peripheral nerve sheath tumors beginning at 8.5 months of age (140). In combination these two studies support the usefulness of teleost models in understanding the role of p53 in the DNA damage response.

1.7.2 Separase, DTL/CDT2, mps1 and RB

Separase is a known tumor suppressor and mitotic regulator in vertebrates. It was successfully cloned in zebrafish in 2007. Separase was found to be the gene responsible for the improper segregation and genomic instability that was previously characterized in the *cease&desist (cds)* zebrafish mutant. These mutants showed high levels of aneuploidy, polyploidy, spinal defects, and mitotic exit delays with an eight-fold increase in epithelial tumors (141).

DTL/CDT2 is required for normal cell cycle control, specifically preventing rereplication, a role which is also conserved in humans. A study using a zebrafish DTL mutant in 2006 showed that DTL acts by regulating CDT1, which is a protein required for pre-replication complex formation. DTL associates with the CUL4-DDB1 E3 ubiquitin ligase and is required for CDT1 downregulation through degradation after entry into S-phase. This study also showed that DTL is required for the early G2/M checkpoint response (142).

Mps1 is a kinase that is required for the mitotic checkpoint. Aneuploidy from chromosomal missegregation is a major cause of human birth defects and is caused by meiotic error. A study by Poss *et al.* in 2004 found that Mps1 is a critical regulator of proper chromosome segregation in zebrafish and was able to demonstrate that even a slight disruption of the mitotic checkpoint can reduce the accuracy of chromosome segregation during nuclear division (143).

The RB gene has been cloned and characterized in a *Xiphophorus* interspecies hybrid of platyfish (*Xiphophorus maculates)* and swordtail (*Xiphophorus helleri)*. This study found that RB mRNA is expressed at a two-fold higher level in melanized skin and skin tumors versus muscle tissue, with corresponding higher levels of RB protein, suggesting that there is an change in expression level in RB in melanized cells (144).

1.8 Molecular Biomarkers

There are currently a wide range of biomarkers being used worldwide to assess the impact of highly persistent pollutants on marine ecosystems. Some of the more serious pollutants being monitored include polychlorinated biphenyls (PCBs), polychlorinated dibenzo-dioxins (PCDDs), polychlorinated dibenzo-furans (PCDFs), polynuclear aromatic hydrocarbons (PAHs), tributyltin (TBT) and other toxic metals which have been shown to accumulate in the tissues of various marine organisms (145).

Biomarkers at the cellular level act as an early warning system of the toxic effect of these pollutants before they can become concentrated through successive trophic levels of the food chain (146). Biomarkers have been traditionally defined as "the measurements of body fluids, cells, or tissues that indicate in biochemical or cellular terms the presence of contaminants or the magnitude of the host response" (147). Biomarkers can be molecular, cellular or whole animal, and change in response to both natural stressors and pollutants. In general, they should offer a specific and sensitive method for assaying the effect of pollution on a wide range of organisms (145). Biological monitoring offers many advantages over environmental monitoring including being able to assess the internal dose of a compound, taking into account differences in absorption, excretion, and rates of DNA repair (148). There are numerous criteria that have been suggested when evaluating a potential biomarker such as its potential usefulness in laboratory and field studies, the ability to determine routes of exposure, and the ability to provide temporal and spatial measures of pollutants (145). The ideal biomarker should have a collection method that is simple and reliable, it should be specific for a particular type of exposure, it should reflect a subclinical and reversible change, and be ethically acceptable to use (148).

In marine systems several biomarkers have already been classified including cytochrome P4501A induction, acytelcholinesterase activity and metallothionein induction (145). Cytochrome P4501A plays a key role in the breakdown of organic contaminants and is used as an indicator of exposure to organic contaminants such as PAHs, PCBs, and PCDDs. Its enzymatic activity is measured using either fluorometric or spectrophotometric methods and the amount of enzyme can be characterized through ELISA (enzyme-linked immunosorbent assays) (149). Acetylcholinesterase (AChE) hydrolyzes the neurotransmitter acetylcholine into choline and acetic acid. AChE is

inhibited by organophosphorus and carbamate insecticides through irreversible binding of its catalytic site. Its enzymatic activity can be measured using either spectrophotometric or delta pH metric measurements (150). Lastly, metallothionein (MT) is a non-enzymatic protein which specifically binds to heavy metals including zinc, copper, cadmium and mercury in both vertebrates and invertebrates. MT levels are quantified using liquid chromatography and absorption spectroscopy (146).

1.9 Research Aims

The overall purpose of this research project was to characterize Chk2 in *Oncorhynchus mykiss* and determine whether it could serve as a biomarker of DNA damage in fish. Chk2 was chosen for this study because of its prominent role in the DNA damage response and because it has shown high levels of conservation in the metazoans studied to date. The specific goals are outlined below.

1. Characterize the *CHK2* gene in rainbow trout.
2. Purify recombinant Chk2 protein.
3. Generate anti-Chk2 polyclonal antibodies.
4. Study the evolutionary conservation of Chk2 across metazoans.
5. Characterize Chk2 at the transcript and protein level to determine tissue specific differences.
6. Examine whether Chk2 can be induced in response to DNA damage in rainbow trout cell lines and determine its potential as a biomarker in fish.

CHAPTER 2:

The Characterization of Chk2 in *Oncorhynchus mykiss*: Tissue Specific Expression Suggests Biomarker Potential

This chapter will be submitted for publication in the Journal of Biological Chemistry as Steinmoeller, J.D., Fujiki, K., Arya, A., Muller, K., Bols, N., Dixon, B., and Duncker B.P. Figure 3 was contributed by K. Fujiki.

2.1 Introduction

Cell cycle checkpoints are critical for maintaining genomic stability, and function by monitoring DNA integrity and the successful completion of cell cycle events (151). Numerous checkpoint proteins work together to sense DNA damage and activate the DNA damage response, resulting in either DNA repair or programmed cell death depending on the severity and duration of the genetic injury. The expression level and/or phosphorylation state of these proteins changes in response to DNA damage, which provides a means of identifying cells that have activated a checkpoint response (152).

DNA damage is initially detected in eukaryotes by Rad9, Rad1, Hus1 (the heterotrimeric 9-1-1 complex), and Rad17 (12). These proteins form a PCNA-like sliding clamp, which binds DNA and activates the PIKK-like transducer kinases, ATM and ATR (25). ATM resides in the cell as a homodimer, and responds primarily to DNA damage in the form of double strand breaks (DSB). It is activated through phosphorylation by the 9-1-1 complex, which causes its dissociation into monomers and initiates extensive autophosphorylation (153). ATR normally resides in the cell as a complex with its accessory protein, ATRIP. This complex confers stability to ATR and is conserved in all eukaryotes, from budding yeast to humans. ATR is activated by DNA adducts or stalled forks, which result in changes in its subcellular localization allowing the ATR/ATRIP complex to bind to RPA-coated single-stranded DNA (39). Both ATM and ATR are capable of phosphorylating serine or threonine residues in SQ/TQ sequences of the checkpoint kinases, Chk1 and Chk2. Chk1 plays an important role in the DNA damage response and is phosphorylated in an ATR-dependent manner (154). It was first identified as an essential protein in fission yeast and later in mammalian cells, but is not

essential for viability in budding yeast (155). Chk1 can be activated throughout the cell cycle and loss of Chk1 leads to mitotic failure and cell death (156). Chk2 is not an essential protein in mammalian cells, but its yeast homologue, Rad53, is essential for cell viability. It is activated in an ATM-dependent manner in response to DSB and plays an important role in DNA-damage signaling. DNA double strand breaks can be generated by exposure to ionizing radiation or through contact with radiomimetic chemicals such as bleomycin. They can also arise as by-products of oxidative metabolism or through replication of damaged DNA. If misrepaired, DSBs have the potential to lead to genomic instability and in higher eukaryotes, cancer predisposition or cell death (157). Once activated, Chk2 phosphorylates a variety of downstream targets including Cdc25A, Cdc25C, BRCA1 and p53. Chk2 plays an important role in all the major checkpoints (G1/S, intra-S, G2/M) of the cell cycle by acting on the above-mentioned downstream targets causing cell cycle arrest, DNA repair or apoptosis (51).

The link between *CHK2* mutations and inherited cancer susceptibility was originally discovered by Bell *et al.* in 1999 (53) when three *CHK2* germline mutations were found in families with classic Li-Fraumeni syndrome, a disease which is characterized by an increase in sporadic tumor risk in a variety of organ systems. A subsequent study, looking specifically at the *CHK2* 1100delC mutation, which results in a truncated inactive form of the Chk2 protein, found that it increased the risk of breast cancer twofold and was associated with an earlier age of onset (158). Infrequent somatic *CHK2* mutations have also been found in lung cancer, ovarian tumor, vulval tumors and osteosarcomas (159-161). The first evidence for the presence of *CHK2* mutations in brain tumors was reported in 2005 by Sallinen *et al.* (162), who found the 1100delC mutation in a subset of human glioblastoma cases. A more recent study of murine primary brain tumors by Bao *et al.* in 2006 (128) showed that that the DNA damage

response, and Chk2 activity specifically, was upregulated in a subset of radioresistant tumors. This increased activity enabled CD133+ glioblastomas exposed to ionizing radiation to efficiently repair DNA damage, increasing the survival and proliferation of these invasive tumor cells.

Owing to its important role in cell cycle control and tumorigenesis, Chk2 has been studied in a wide range of species such as budding yeast, mice and humans. However, few checkpoint proteins have ever been studied in fish, therefore the conservation of the DNA damage response across teleost species remains largely unknown. Rainbow trout (*Oncorhynchus mykiss)* was selected for this study because of its prominence as a sentinel for assessing water quality and ecosystem health, as well as its importance to the commercial aquaculture industry. Rainbow trout is one of many species that is exposed to aquatic pollution and is well-suited as a model for checkpoint proteins in fish. Of the checkpoint proteins that have been cloned and characterized the majority have been from zebrafish, including *TP53* (138;140), *MPS1* (143), *DTL* (142) and *separase* (141). *TP53* has also been cloned in Japanese medaka (135) and European flounder (137) and the *RB* gene has recently been isolated in platyfish (144). A DNA sequence analysis done by Krause *et al.* in 1997 (135) demonstrated a high degree of sequence similarity between the functional domains of Japanese medaka *TP53* and other vertebrate taxa such as rainbow trout (*Oncorhynchus mykiss*), frog (*Xenopus laevis*), chicken (*Gallus gallus*), rat (*Rattus norvegicus*), mouse (*Mus musculus*), hamster (*Mesocricetus auratus*), green monkey (*Ceropithecus aethiops*) and human (*Homo sapiens*), suggesting that checkpoint genes are indeed highly conserved. The function of the TP53 protein was also shown to be conserved across teleosts, in a study by Berghmans *et al.* (140), where *tp53*-deficient zebrafish lines spontaneously developed malignant peripheral nerve sheath tumors due to a deficient cell-cycle arrest response to DNA damage.

In the present report, we have isolated the *CHK2* gene for the first time in fish, specifically *Oncorhynchus mykiss,* assessed its evolutionary conservation, characterized its tissue specific expression and examined its induction in response to DNA damage. This initial work will help to determine whether checkpoint proteins can serve as accurate biomarkers of genotoxic exposure in fragile aquatic ecosystems.

2.2 Materials and Methods

2.2.1 Whole Fish

Rainbow trout (*Oncorhynchus mykiss*) weighing approximately 500 g were obtained from Rainbow Springs Hatchery, Thamesford, Ontario and held in well-water flow-through tanks at the University of Waterloo. Fish were maintained at 13°C on a daily diet of Classic Floating Trout Grower 5pt Regular pellets (Martin Mills, Elmira ON) and then euthanized by overdose with ethyl-3-aminobenzoate methanesulphonate salt (MS222; Sigma, St. Louis MO) followed by caudal vein exsanguinations. Tissues were extracted and flash frozen in liquid nitrogen prior to storage at -80˚C.

2.2.2 Fish Cell Lines

RTgill-W1 and RTbrain-W1 cell lines used in this study were derived from rainbow trout gill epithelium and brain respectively. RTgill-W1 is available from the American Type Culture Collection (ATCC # CRL 2523). The cell lines were routinely cultured in 25 cm^2 culture flasks at 18 °C in Leibovitz's L-15 culture medium supplemented with 1% penicillin-streptomycin solution (100 μg/ml streptomycin, 100 IU/ml penicillin, Sigma, St. Louis, MO) and 10% fetal bovine serum (FBS, Sigma, St. Louis, MO) for RTgill-W1, 15% FBS for RTbrain-W1.

2.2.3 Isolation of Fish Liver Genomic DNA

Approximately 50 mg of adult rainbow trout liver tissue was weighed out and kept on ice in a sterile 1.5 mL microfuge tube. 550 µL of lysis buffer (50 mM Tris pH 8.0, 20 mM EDTA pH 8.0, 1% SDS, 200mM NaCl, 1% 2-ME), 11 µL of 20 mg/mL proteinase K (Sigma, St. Louis, MO) and 5.5 µL of 10 mg/mL RNAse A (Sigma, St. Louis, MO) were added to the tube and it was incubated at 55˚C for one hour. The tube was then centrifuged at 13 500 rpm for 10 minutes to pellet the debris, and the supernatant was transferred to a fresh microcentrifuge tube. 350 µL of 5M NaCl was then added and after vortexing, the tube was centrifuged at 13 500 rpm for 30 minutes at room temperature. In a fresh tube, the supernatant was combined with 900 µL of ice-cold absolute ethanol and the DNA was allowed to precipitate out of solution. The visible DNA was then removed from the ethanol and combined with 100 µL ddH_2O in a fresh 1.5 mL microcentrifuge tube and incubated at room temperature for 2 hours. The concentration of the genomic DNA was determined by spectrophotometry and the integrity was assured by agarose gel electrophoresis.

2.2.4 Isolation of Rainbow Trout *CHK2*

A pair of degenerate primers was designed from two conserved amino acid regions (sp2 - 5'ACGTAYAGCAARAARCAYTTYMG in the forkhead associated [FHA] domain and sp3 - 5'AGCATYTGRTARAARTAGARYTT in the kinase domain) based on the alignment of human [AJ783839.1], mouse [BC056617.1] and zebrafish [AF265346.1] *CHK2* orthologues obtained from Genbank (see Figure 2.1 for all genomic primer locations). PCR was performed using 17 µl LA Taq mix (TaKaRa, Shiga, Japan), 1 µl each of the degenerate primers (10 µM) and 1 µl of rainbow trout genomic DNA (50

ng/μl) with the conditions of 95°C 5 min, 35 cycles (95°C 30 sec, 47°C 30 sec, 72°C 3 min) and 72°C 5 min using a PTC-100 thermocycler (MJ Research, Watertown, MA). Following agarose gel electrophoresis, the expected 2 kb band was purified using an Ultrafree-DA spin column (Millipore, Billerica, MA), subcloned into pGEM-T Easy (Promega, Madison, WI) and fully sequenced using a Long-Read Tower automated sequencer (Visible Genetics, Toronto, Canada). Based on this sequence, pairs of primers were designed for inverse PCR. Aliquots of rainbow trout genomic DNA (5 μg) prepared by the method of Sambrook *et al*. 1989 (163), were digested in separate reactions with a restriction enzyme (*Bam*HI, *Hinc*II, *Hind*III, *Pdm* I or *Xba* I) and purified by phenol chloroform extraction and ethanol precipitation. A tenth of each digest (0.5 μg) was then subjected to self ligation using LigaFast System (Promega, Madison, WI) in a 10 μl reaction volume for 2 weeks at 4°C, and then heated at 70°C for 10 min after which the separate digests were pooled. Inverse PCR was carried out with 17 μl LA Taq mix, 1 μl of each primer (10 μM): sp25 (5'-TTGCTTTGATGACCCCATACT) /sp26 (5'-GTTGAGGACGACACTTGGTT), sp5 (5'-TGGCGGATGAACAGTCCAAC) /sp26, sp29 (5'-TCCAAATGGGACGGCATCTC)/sp28 (5'-TGATATGCCACAACTGTCAG), sp29/sp30 (5'-CTATCCATGCTGGCTTTTGC) and 1 μl of the template with the conditions of 95°C 5 min, 35 cycles (95°C 30 sec, Tm of primers - 5º 30 sec, 72°C 6 min) and 72°C 5 min using PTC-100 thermocycler (MJ Research, Watertown, MA). Nested inverse PCR was done using the same conditions with 1 μl of the first PCR product as the template. Two sets of inverse PCR to the 5' end, using primers sp27 (5'-ACTGAAGAAATCGCCCAGAT)/sp24 (5'-CAACTGGTCATTCAGAACAG), sp1 (5'-TCGTTTGAATAGCCAAAAGA /sp24 and three sets to the 3' end, using primers sp31 (5'-TGAGGAGGCTTTACACCATC/sp28, sp31/sp30, sp18 (5'-

TTCAAATAACTTGGGCCAAG/sp31, covered the *CHK2* gene. Three clones from each nested inverse PCR were subcloned into pGEM-T Easy (Promega, Madison, WI) and sequenced. There was good sequence agreement between all three clones.

2.2.5 cDNA Isolation of Rainbow Trout *CHK2*

PCR was used to obtain a rainbow trout *CHK2* cDNA fragment (see Figure 2.1 for primer locations). One microgram of DNAse I treated total brain RNA from an adult rainbow trout was reverse transcribed with an antisense primer sp82 (5' TAATGGAAACACTTAATGTT) corresponding to the region immediately downstream of the putative *CHK2* polyadenylation signal (AATAAA), using the First Strand cDNA Synthesis Kit (MBI Fermentas, Burlington, Canada) in a 10 µl reaction. The first PCR was performed with 17 µl LA Taq mix (TaKaRa), 1 µl each of sp82 primer (10 µM), sense primer sp51 (5'ATGCCATGATAACCTGAATG) and the first strand cDNA under the conditions of 95°C for 5 min, 35 cycles for (95°C 30 sec, 45°C 30 sec, 72°C 3 min) and 72°C for 5 min. The nested PCR was performed under the same conditions as the first PCR except that nested sense primer sp53 (5'GACCATGTCCCAGGAGAAGC) was used, with the first PCR product as the template. A PCR band of the expected size (1.9 kb) was gel-purified, subcloned into pGEM-T Easy (Promega, Madison, WI) and fully sequenced for both strands of six clones. There was complete sequence agreement with no variability between all six clones.

2.2.6 Isolation of CHK2 FHA fragment from a cDNA Library

Based on the sequence obtained above, the following sense primer, sp61 (5′-GGCTGCGGATCCAGCCATGGGGTCGCCTG) and antisense primer, sp62 (5′-GGCTGCAAGCTT GCACTTTATGGCGTTCCTCAGCTAGG) were employed to amplify the 300 bp forkhead-associated domain of the *CHK2* gene from a rainbow trout

cDNA library (see Figure 2.1 for primer locations). These primers respectively incorporated *Bam*HI and *Hind*III restriction enzyme sites (underlined). The following PCR conditions were used for a 50µl reaction: 94°C 5 min, 35 cycles at (95°C 1 min, 53°C 1 min, 72°C 1 min) and a final step of 72°C 5 min in an Eppendorf thermocycler (Mastercycler, Westbury, NY). The PCR product was run on a 0.8% agarose gel, purified and subcloned in pGEM-T easy (Promega, Madison, WI) for sequencing. Sequencing showed complete agreement with expected results based on genomic and cDNA cloning of the *CHK2* gene as described above.

2.2.7 Multiple Sequence Alignment

The Chk2 sequences of the nine species used in the amino acid multiple sequence alignment and their Genbank accession numbers are listed in Table 2.1. The *Oncorhynchus mykiss* amino acid sequence was obtained experimentally as described above. Sequences were corrected and assembled using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The amino acid sequences were aligned using Clustal W on the basis of sequence similarity (164), and the alignment was finalized manually. Sequences were only included in the alignment if the sequence could be reliably aligned. An identity matrix that computes the quantitative degree of similarity between the individual amino acid sequences was also generated using BioEdit (Supplementary Figure 2.1). A multiple sequence alignment of the same species comparing nucleotide sequence was also constructed (data not shown) and was used in the subsequent phylogenetic analysis of the dataset.

Table 2.1: Species names and Genbank accession numbers of amino acid sequences used in the multiple sequence alignment.

Chk2 Species	Protein Accession number
H. Sapiens	AAD_48504
T. Nigroviridis	CAF92381.1
R. Norvegicus	NP_446129
M. Musculus	AAH_56617
B. Taurus	NP_001029703
C. Familiaris	XP_543464
X. Laevis	AAG_59884.1
D. Rerio	AAK52419.1
P. Troglodytes	XP_515051.1

	RN	MM	HS	CF	BT	PT	XL	TN	DR	OM
R.norv	ID	0.925	0.854	0.843	0.843	0.793	0.631	0.514	0.583	0.597
M.musc	0.925	ID	0.852	0.849	0.841	0.791	0.633	0.510	0.577	0.593
H.sapi	0.854	0.852	ID	0.904	0.894	0.931	0.651	0.496	0.576	0.577
C.famil	0.843	0.849	0.904	ID	0.922	0.839	0.658	0.508	0.573	0.578
B.taur	0.843	0.841	0.894	0.922	ID	0.828	0.643	0.504	0.575	0.584
P.trogl	0.793	0.791	0.931	0.839	0.828	ID	0.602	0.457	0.536	0.538
X.laevi	0.631	0.633	0.651	0.658	0.643	0.602	ID	0.495	0.569	0.572
T.nigro	0.514	0.510	0.496	0.508	0.504	0.457	0.495	ID	0.584	0.600
D.rerio	0.583	0.577	0.576	0.573	0.575	0.536	0.569	0.584	ID	0.656
O.mykis	0.597	0.593	0.577	0.578	0.584	0.538	0.572	0.600	0.656	ID

Supplementary Figure 2.1: Amino acid identity matrix generated by Bioedit. Shows sequence similarity between taxa from the Chk2 multiple sequence alignment.

2.2.8 Phylogenetic Analysis

To determine the appropriate evolutionary model for analysis of the data, the finished nucleotide alignment was run through ModelTest®, which established the process of DNA base-pair substitution that best fit the dataset (165). The data was next analyzed using PAUP 4.0 (166) using maximum parsimony, likelihood, and neighbour-joining methods. For the analysis of the *CHK2* dataset no outgroup was specified, as no

suitable outgroup could be found in the literature. In the maximum parsimony and neighbour-joining analyses the dataset was subjected to exhaustive searches and the trees were midpoint rooted. In the maximum likelihood analysis the dataset was put through a heuristic analysis due to limited computational capacity and the tree was again midpoint rooted. The maximum parsimony and neighbour-joining tree datasets were then subjected to bootstrap sampling (1,000 replicates) and the consensus tree was retained. The results of all three analyses generated trees with identical topology.

2.2.9 Southern Blot Analysis

Southern blot analysis was performed according to the protocol described by Fujiki *et al*. 2001 (167) except that *Dra* I, *Hinc*II, *Pdm* I and *Pst* I were used for digestion and CDPstar (Roche, Mississauga ON) was used for detection of the signals. The DNA probe (201 bp) was prepared by PCR-amplifying the rainbow trout genomic DNA with the primers sp9 (5'-TAGGGTCAAAAGCAAGAACC), /sp16 (5'-CCAAGATACTGGAGGAGTCT), followed by subcloning and labeling with DIG using PCR DIG Probe Synthesis Kit (Roche, Mississauga ON). The authenticity of the probe was confirmed by sequencing as described above.

2.2.10 RT-PCR

Total RNA was extracted from rainbow trout peripheral blood leukocytes (PBL), head kidney, posterior kidney, spleen, liver, heart, gill, brain, intestine and muscle using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA was reverse-transcribed with Moloney mouse leukemia virus (MMLV) reverse transcriptase (RT) using a First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON). The synthesized first strand cDNA was diluted 10-fold in dH_2O. The PCR reaction was conducted in a total volume of 20 µl, including 1 µl of the diluted first strand cDNA, 1 µl

of each PCR primer and 17 µl of LA Taq master mixture (TaKaRa). PCR was performed in a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA), using the following parameters: 95°C 5 min; 22-35 cycles at (95°C 30 s, 53-55°C 30 s, 72°C 1 min) and a final extension at 72°C for 5 min. Oligonucleotides used as PCR primers are as follows: sp41 (5'-CTTTGACAACAGTGGCAATG), and sp42 (5'-TTGACTCCTCTATCTGGTTC) to give a 481 bp product (primer locations are shown in Figure 2.1). A primer pair for the ribosomal protein S11 transcript was prepared to be used as an internal control using 5′-AGCAGCCAACCATCTTCCAG and 5′-ACTCTCCGACGGTAACAATG, as described in Nath et al (2006). An aliquot (5 µl) of the PCR product was electrophoresed on a 1.3% agarose gel containing 1 µg/ml of ethidium bromide and photographed on a UV illuminator. The intensities of the PCR amplicons in the gel were measured by NIH Image version 1.52, and relative intensity for each tissue was calculated by dividing the intensity of the *CHK2* amplicon at 35 cycles by that of the S11 amplicon at 22 cycles.

2.2.11 Expression Vector Construction

Following digestion with the appropriate restriction enzymes, the *CHK2* FHA fragment was ligated into the *BamHI* and *HindIII* sites of expression vector pRSET A (Invitrogen, Carlsbad, CA) using T4 DNA ligase (Promega, Madison, WI) according to manufacturer's protocol, transformed into the *E. coli* bacterial strain, DH5α (Life Technologies, Rockville, MD) and sequenced to verify that the insert was in frame with the N-terminal 6x His-tag. For expression of the recombinant protein, the construct was transformed into the *E. coli* bacterial strain, BL21 (DE3) pLysS (Promega, Madison, WI).

2.2.12 Protein Expression and Purification

A 5-ml overnight culture grown at 37°C with 200 rpm shaking and was used to inoculate 2 l of LB culture, containing 100 µg/ml of ampicillin. Cultures were induced by the addition of isopropyl-beta-D-thiogalactopyranoside to 1mM at an OD_{600} of 0.4–0.6. Cells were harvested 5 h after induction by centrifugation at 5,000×*g* for 10 min and lysed with 100 mM NaH_2PO_4, 10 mM Tris–HCl, 8 M urea, pH 8. The supernatant was collected after centrifugation of cells in a Sorvall RC-5B ultracentrifuge (Du Pont, Newtown, CT) at 30,000 rpm for 30 min. Protein was purified using a Ni-resin column (Qiagen, Valencia, CA) as per the manufacturer's instructions. In brief, 10 ml of crude cell lysate was incubated with 1 ml of Ni resin for 2 h at room temperature and then allowed to flow through an econo-column (Bio–Rad Laboratories, Hercules, CA). The column was subjected to several rounds of washes each time using 10–20 ml of urea buffer (100 mM NaH_2PO_4, 10 mM Tris–Cl, 8 M urea) at pH 8, 6.3, and 5.9. The Chk2 recombinant protein was eluted in 1 ml aliquots using urea buffer at a final pH of 4.5, see Appendix I for immunoblot of purified recombinant protein. A Bradford assay (168) was performed concurrently with the elution steps to determine protein concentration. The Ni column washes and eluted fractions were separated on a 15% sodium dodecyl sulfate (SDS) gel and stained with Coomassie blue to assess purity. Purified recombinant protein was concentrated by combining 10 ml of the appropriate eluted fractions in dialysis tubing (Fisher Scientific, Ottawa, ON), and incubating for 2.5h in excess polyethylene glycol (PEG 4000; Qiagen, Valencia, CA). The sample was then dialyzed for 3 hours at 4°C in 1L of 4M urea buffer, which effectively reduced the volume inside the dialysis tubing by half. Subsequent sequential overnight dialysis steps at 4°C were performed

with 2M and 1M urea buffers. A final overnight dialysis step at 4°C was performed in 500 ml of 1xPBS. The recombinant protein was then stored in 1xPBS; 0.02% NaN_3 pH 9.3, at 4°C at a concentration of 1mg/ml prior to use for antibody production.

2.2.13 Immunization of Rabbits

Rabbits were immunized subcutaneously with an emulsion of 500 µl recombinant Chk2 protein (1 mg/ml) and 500 µl of Freund's incomplete adjuvant (Sigma, St. Louis, MO, USA). The rabbits were subsequently boosted three times with the same emulsion, at 3 week intervals. Blood samples were collected from the marginal ear vein of rabbits before every boost to assess antibody titers. Blood was allowed to clot at room temperature for 2 h and at 4°C overnight. Serum was separated by centrifugation at 5,000×*g* for 8 min at 4°C (IEC 21000R). After the twelfth week, the rabbits were exsanguinated by carotid cannulation.

2.2.14 Monitoring Antibody Titers

After each boost, serum antibody titers were evaluated by performing enzyme-linked immunosorbent assay. Ninety-six well plates were coated with 100 µl of 10 µg/ml recombinant Chk2 protein diluted in coating buffer (15 mM Na_2CO_3, 34 mM $NaHCO_3$, 0.02% NaN_3, pH9.3) and left overnight at room temperature. Each well was then blocked with 300 µl blocking buffer (1% BSA in TBS-T) at 37°C for 1 h, and then washed three times with T-TBS. The primary rabbit antiserum (100 µl) was then added at different serial dilutions to the wells in four replicates and incubated for 1h at room temperature. Goat anti-rabbit IgG antibody conjugated to alkaline phosphatase (Sigma, St. Louis, MO) was used as the secondary antibody (1:5000) and after incubation for 1 h at room temperature, *p*-nitrophenyl phosphate tablets (Sigma, St. Louis, MO) dissolved in 20 ml distilled water was added as the substrate. The plates were incubated in the dark at room

temperature for 30 min then 50 µl of 0.03M NaOH was added to stop the reaction. Absorbance was measured at 405 nm using the SOFTmax PRO 2.6.1 program with a microplate reader (VERSAmax microplate reader, Molecular Devices). Readings were obtained after background correction.

2.2.15 Whole Fish Protein Lysate Preparation

Brain and gill tissues were obtained from two adult rainbow trout as described above. Tissues were measured out into 200 mg aliquots and each of these was combined with 500 µl NP-40 lysis buffer (10% Non idet P40, 1M Tris-HCL; pH 7.5, 5M NaCl) and 50 µl protease inhibitor cocktail (Sigma, St. Louis, MO). Samples were then sonicated using a Microson ultrasonic cell disruptor (Misonix, Farmingdale, NY) at medium intensity for 30 sec or until tissue was lysed, and centrifuged at 13000 rpm for 10 minutes at 4°C in a Biofuge-Pico centrifuge (Heraeus, Chandler, AZ). Cleared lysate was transferred to 1.5 ml microcentrifuge tubes and stored at -20° C.

2.2.16 Cell Culture Protein Lysate Preparation

RTgill-W1 and RTbrain-W1 cells were washed with 1 ml Versene (Gibco-Invitrogen, Carlsbad, CA) solution and removed from the growing surface of the 25 cm^2 flat-bottomed flasks using 1 ml trypsin. Cells were resuspended in 10 ml fresh Leibovitz's L-15 culture medium supplemented with 1% penicillin-streptomycin solution (100 µg/ml streptomycin, 100 IU/ml penicillin) (Sigma, St. Louis, MO), 10% fetal bovine serum (Sigma, St. Louis, MO) for RTgill-W1, 15% FBS for RT-BR, and then spun at 440 x g for 5 minutes in an Eppendorf 5801R centrifuge (Hamburg, Germany). Pelleted cells were washed in 1 x PBS and centrifuged at 13000 rpm for an additional 5 minutes in an

Eppendorf 5415 centrifuge (Hamburg, Germany). The pelleted cells were then combined with 150 µl NP-40 lysis buffer (10% Non-idet P40, 1M Tris-HCL; pH 7.5, 5M NaCl) and protease inhibitor cocktail (Sigma, St. Louis, MO) and stored on ice for 30 minutes. Cells were then sonicated using a Microson ultrasonic cell disruptor (Misonix, Farmingdale, NY) at a medium intensity for 30 sec and centrifuged 13000 rpm for 10 mins at 4°C in a Biofuge-Pico centrifuge (Heraeus, Chandler, AZ). Cleared protein lysates were transferred to 1.5 ml microcentrifuge tubes and stored at -20° C.

2.2.17 Determining Chk2 Antibody Specificity

RTgill-W1 protein lysates (45 µg) were boiled with 2x sample buffer (62.5 mM Tris–HCl, pH 6.8, 20% glycerol, 2% SDS, 250 mM DTT, 10 µl saturated bromophenol blue) run in duplicate on a 15% SDS-PAGE gel according to standard protocols (163) and then transferred to 0.2 µm nitrocellulose membrane (Pall Life Science, Pensacola, FL). The first set of samples was detected with anti-Chk2 primary antibody (1:50), while the second set of samples was detected with final bleed anti-Chk2 primary antibody (1:50) that had been pre-incubated in excess recombinant Chk2 purified protein (1 mg/ml). The blots were then washed three times for ten minutes and probed using anti-rabbit secondary fluorescent antibody (1:3000) (W647; Invitrogen, Missisauga, ON), and visualized using the Typhoon 8600 scanner (Amersham Pharmacia Biotech).

2.2.18 Tissue Distribution – Cell Lines and Whole Fish

Protein lysates were harvested, as described above, for RTgill-W1 and RTbrain-W1 cell lines. 45 µg of each sample was run on a 15% SDS-PAGE gel and transferred to a 0.2 µm nitrocellulose membrane, as described above. The samples were detected using

final bleed anti-Chk2 primary antibody (1:50) and anti-rabbit IgG secondary fluorescent antibody (1:3000) (W647; Invitrogen, Missisauga, ON). Chk2 was visualized using the Typhoon 8600 scanner (Amersham Pharmacia Biotech).

Similarly, gill and brain protein lysates obtained from two adult rainbow trout were prepared as described above. 45 µg gill and brain samples from the two fish were run on a 7.5% SDS-PAGE gel and transferred to a 0.2 µm nitrocellulose membrane. Detection was performed as described above for the cell line protein samples.

2.2.19 Bleocin Treatment to Induce Double Strand Breaks

RTgill-W1 and RTbrain-W1 cell lines were treated with either 3 mg/ml (low dose) or 18 mg/ml (high dose) (169) of Bleocin (commercial bleomycin), a radiomimetic agent that specifically causes a high proportion of double strand breaks (Calbiochem, San Diego, CA). 10 µl of each Bleocin stock solution was added to 10 ml Leibovitz's L-15 culture medium supplemented with 1% penicillin-streptomycin solution (100 µg/ml streptomycin, 100 IU/ml penicillin; Sigma, St. Louis, MO), with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO) for RTgill-W1 and 15% FBS for RTbrain-W1. Cells were incubated at room temperature in the dark (Bleocin is photosensitive) for 1, 4, 8 or 24 hours. Protein lysates were harvested as described above. 35 µg of each protein sample was run as described above on a 10% SDS-PAGE gel and transferred to 0.2 µm nitrocellulose membrane. The samples were detected using anti-Chk2 primary antibody (1:50) and anti-rabbit IgG secondary fluorescent antibody (1:3000) (W647; Invitrogen, Missisauga, ON). Chk2 was visualized using the Typhoon 8600 scanner (Amersham Pharmacia Biotech).

2.3 Results

2.3.1 The initial isolation of the rainbow trout *CHK2* gene.

To design primers to obtain the full-length *Oncorhynchus mykiss CHK2* sequence, a preliminary comparison between nucleotide human, mouse and zebrafish *CHK2* orthologues was conducted as described in *materials and methods*. PCR was performed using a rainbow trout genomic DNA template, which produced an initial 2kb PCR product that was subcloned and sequenced. Based on this initial sequence, pairs of primers were designed for inverse PCR reactions in an attempt to obtain the remaining *CHK2* gene sequence. The genomic DNA template was digested in separate reactions with a restriction enzyme (*Bam*HI, *Hinc*II, *Hind*III, *Pdm* I or *Xba* I), purified, allowed to self-ligate and then pooled as described in *materials and methods*. Inverse PCR was done following the self-ligation reactions, and two sets of nested inverse PCR to the 5'end and three sets to the 3'end allowed for the complete sequence to be obtained for the *CHK2* gene. Three clones from each nested inverse PCR were subcloned into pGEM-T Easy (Promega, Madison, WI) for sequencing and in each case an identical *CHK2* sequence was obtained.

cDNA cloning was also performed, using total brain RNA from a healthy adult rainbow trout. An initial PCR antisense primer (sp82) was designed to bind just downstream of the putative polyadenylation signal and was used with a sense primer (sp51) in the initial PCR reaction. Using the first PCR product as a template a nested PCR using primer (sp53) was done to obtain the full *CHK2* coding sequence (see Figure 2.1 for primer locations). A PCR band of the expected size (1.9 kb) was obtained, gel-

Figure 2.1: Rainbow Trout *CHK2* Sequence and Location of Primers Used for Isolation and Characterization. Exons and introns are written in upper case and lower case, respectively. Putative start and stop codons are in bold and in bold italics, respectively. The number on the right side indicates the nucleotide position. PCR primers used in the initial genomic cloning are highlighted in yellow above the genomic sequence they correspond to. PCR Primers used in the cloning of *CHK2* cDNA are highlighted in pink. PCR primers used for the cDNA probe in the *CHK2* southern blot analysis are highlighted in green. PCR primers used in the *CHK2* RT-PCR analysis are highlighted blue. PCR primers used to isolate the sequence encoding the FHA domain are highlighted in red. The forkhead-associated domain is indicated in bold, and the kinase domain is indicated by italics based on sequence comparison with other metazoans.

purified and sub-cloned into pGEM-T Easy (Promega, Madison, WI) and fully sequenced for both strands of six clones. Consensus was obtained between the six sequences. Figure 2.1 shows the *O. mykiss CHK2* gene sequence with introns and exons indicated. The obtained *CHK2* DNA sequence in *O.mykiss* was 6448 bp encoding a protein of 508 amino acids.

2.3.2 The *CHK2* gene sequence is well conserved and its phylogenetic evolution matches known speciation events.

Having obtained a putative rainbow trout Chk2 sequence, a multiple sequence alignment was performed to assess the conservation of the Chk2 protein sequence among metazoans. The taxa names and Genbank accession numbers for the amino acid sequences used in the alignment are listed in *materials and methods,* Table 2.1.

The amino acid sequences were initially aligned using ClustalW (164), then loaded into Bioedit® where the alignment was finalized manually and unalignable sequences were removed. Other eukaryotes such as *S. cerevisiae, C. elegans* and *D. melanogaster* were not included due to lower sequence similarity with the metazoan sequences. Figure 2.2A shows the finalized amino acid multiple sequence alignment, with the experimentally derived rainbow trout Chk2 sequence showing a very high level of sequence similarity with the other metazoan species. Based on the finished alignment, an identity matrix was created, which provides a quantification of the degree of similarity between the aligned amino acid sequences (see Supplementary Figure 2.1). This data suggests that the Chk2 protein is very highly conserved, consistent with a central role in the DNA damage response.

Next, a phylogenetic analysis was performed to assess the evolutionary relationship of the *CHK2* gene among the same metazoans used in the multiple sequence

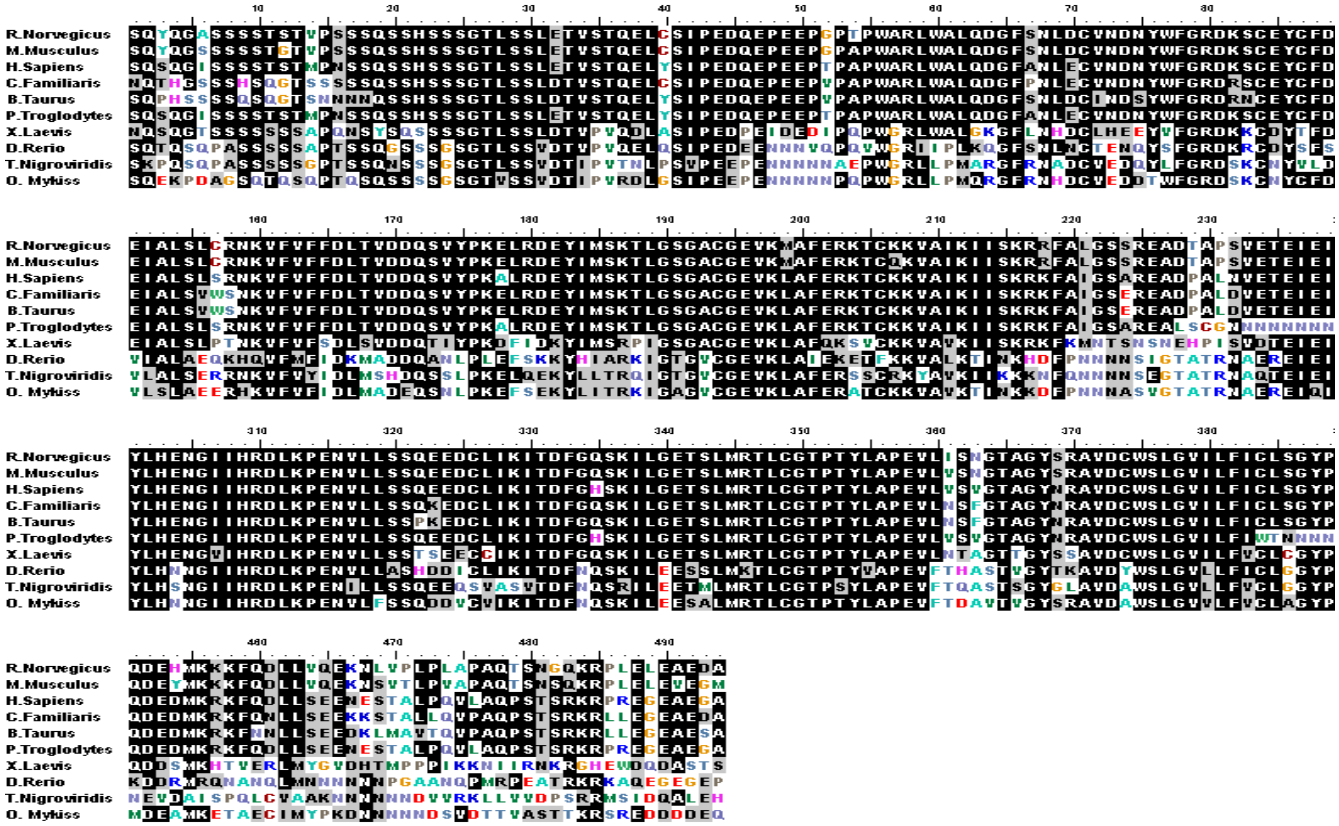

Figure 2.2A: Finalized multiple sequence alignment of Chk2 protein sequences. A solid black background indicates conserved amino acids. Full species names and Genbank accessions numbers are listed in Table 2.1 (*materials and methods).*

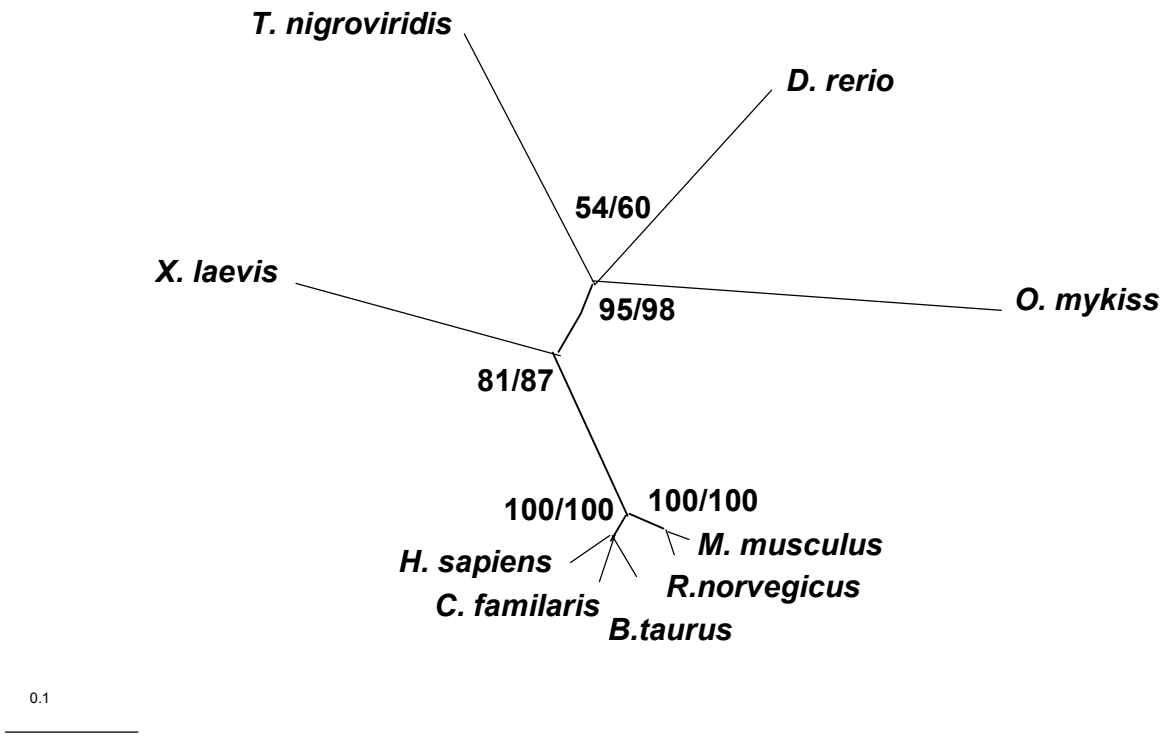

Figure 2.2B: Representative neighbour-joining phylogenetic tree of the *CHK2* gene. Maximum parsimony and maximum likelihood trees were also generated and resulted in the same topology (data not shown). Common species names are: human (*H. sapiens)*, domestic dog *(C. familiaris)*, cow *(B. taurus)*, house mouse *(M. musculus)*, Norway rat *(R. norvegicus)*, African clawed frog *(X. laevis)*, zebrafish *(D. rerio)*, pufferfish *(T. nigroviridis)* and rainbow trout *(O. mykiss)*. Full species names and Genbank accession numbers are listed in Table 2.1 *(materials and methods)*. Bootstrap values were obtained after 1000 replicates for both neighbour-joining and maximum parsimony as indicated by nj/mp values given.

alignment. Based on the evolutionary model, GTR+I+G (general time reversible + proportion invariate + gamma), the dataset was analyzed by PAUP 4.0 using maximum parsimony, maximum likelihood, and neighbour-joining distance measures. Bootstrapping was done on both the maximum parsimony and neighbour-joining analyses. All of the above analyses generated a phylogenetic tree with the same topology, and a representative neighbour-joining tree with bootstrap values (both neighbour joining and maximum parsimony) obtained after 1000 replicates is represented in Figure 2.2B. This tree conforms to known speciation events, and shows the taxa grouping in their expected clades with relative distance measures indictated. All species used in this analysis form part of the Gnathostomata, or jawed vertebrate group (170). The ray-finned fish, or Actinopterygii group (171), which contains the pufferfish, zebrafish, and rainbow trout sequences, group together away from the mammalian species. Within the mammals, the *CHK2* sequences again cluster as expected, with the rodentia family grouping together apart from the larger mammals and primates (172). This analysis confirms that the evolution of the *CHK2* gene matches known phylogenetic relationships.

2.3.3 Characterization of the *CHK2* gene: Copy number and mRNA tissue distribution.

To further characterize the *CHK2* gene in rainbow trout, a southern blot was performed to determine its copy number in the *O. mykiss* genome. The total genomic DNA was digested using various restriction enzymes (*Dra*I, *Hinc*II, *Pdm*I, and *Pst*I), separated on a 1% agarose gel and transferred to a nylon membrane, as described in Fujiki *et al* 2001 (167). A labeled DNA fragment corresponding to a 201 bp region (see

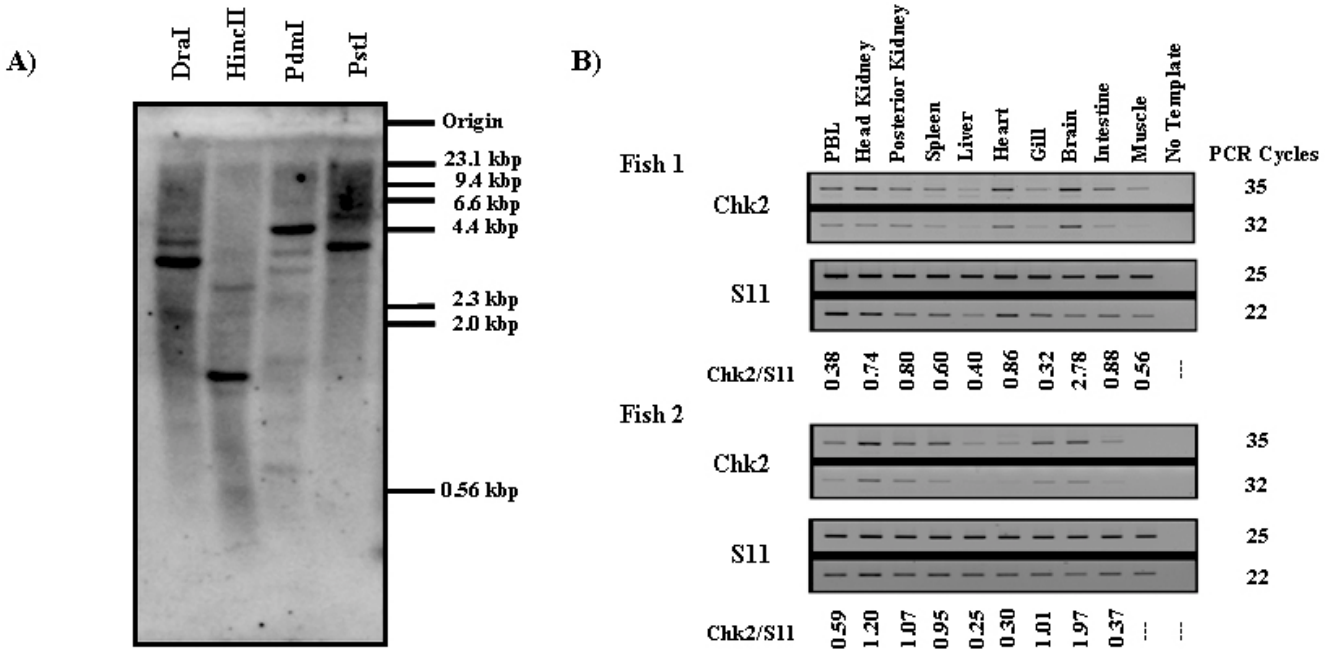

Figure 2.3: *CHK2* gene copy number and mRNA tissue distribution. A) Southern blot of genomic DNA from *O. mykiss* probed with a 201 bp *CHK2* fragment that overlaps the FHA domain (see Figure 2.1 for primers). Separate digests were done with DraI, HincII, PdmI, and PstI. Results indicate that *CHK2* is present as a single copy. **B)** RT-PCR gels showing the transcript expression levels of *CHK2* in various rainbow trout tissues, independently verified from two fish. Primers were designed based on highly conserved regions of the *CHK2* gene. Expression level results were normalized using an S11 gene transcript control. PCR results were examined after 35 and 32 cycles for the CHK2 gene, and after 25 and 22 cycles for the S11 control. *CHK2* shows the highest level of expression in brain tissue.

Figure 2.1 for primer locations) of the *CHK2* gene overlapping the FHA domain was allowed to hybridize to the membrane bound genomic DNA. One prominent hybridizing band was detected for each of four single restriction digests, indicating a single copy of the *CHK2* gene in the rainbow trout genome as shown in Figure 2.3A. The faint banding pattern indicates that the probe may be binding to other genes containing an FHA domain.

RT-PCR was then performed to determine *CHK2* mRNA levels, using RNA extracted from head kidney, posterior kidney, spleen, liver, heart, gill, brain, intestine, and muscle, as described in *materials and methods*. *CHK2* mRNA was found to be expressed most strongly in brain, posterior kidney, head kidney, spleen, and gill, although there was some variability in expression between the two fish analyzed. The *CHK2* mRNA levels were generally lower in muscle, peripheral blood leukocytes, heart, liver, and intestine (Figure 2.3B).

2.3.4 Production of recombinant Chk2 protein and specificity of polyclonal anti-Chk2 antibodies.

We next wanted to analyze the Chk2 protein in *O. mykiss* by creating a sensitive and reliable anti-Chk2 polyclonal antibody. A new set of primers, sp61/sp62 (see Figure 2.1 for primer locations), were designed to amplify a 300 bp region of the forkhead-associated domain of the *CHK2* gene. The forkhead-associated domain was chosen because it is a highly conserved region of the *CHK2* gene that could potentially result in the generated antibodies being able to detect the Chk2 protein in other fish species

through cross-reactivity. PCR was performed as described in *materials and* methods, and the PCR product was sub-cloned into pGEM-T Easy (Promega, Madison, WI). After

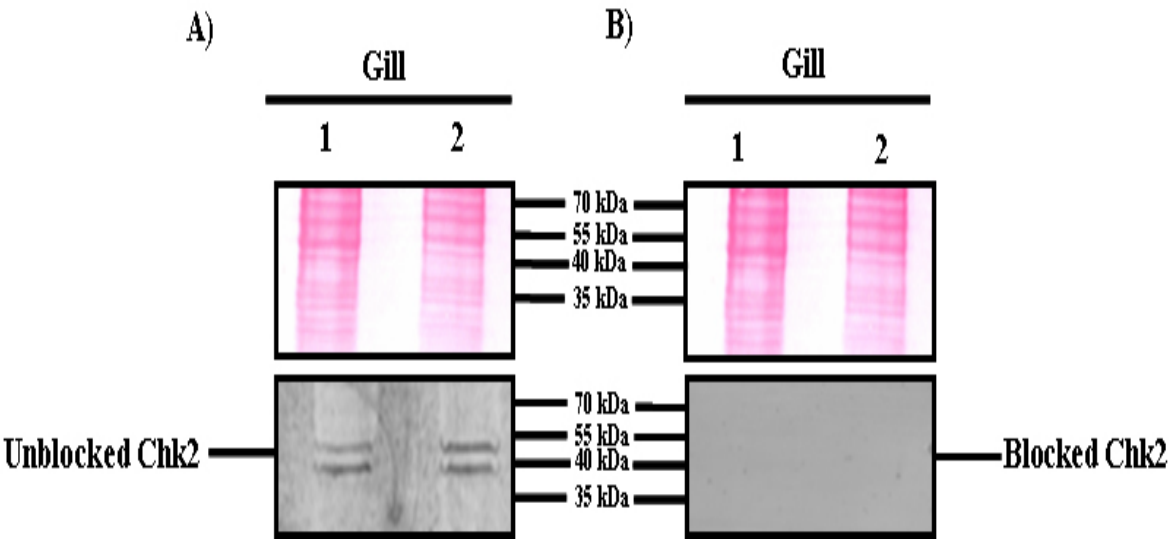

Figure 2.4: Verification of Chk2 antibody specificity. A) Western blot showing detection of Chk2 polypeptide (49 kDa) using anti-Chk2 polyclonal antiserum (1:50). **B)** Western blot showing that these bands fail to be detected when the antiserum is pre-incubated with recombinant Chk2 protein. 45 µg of two gill protein lysates from the rainbow trout RTgill-W1 (173) cell line were used to illustrate this specificity. Ponceau S staining is shown as a loading control.

confirming its identity through DNA sequencing, the *CHK2* FHA fragment was cloned into the *E. coli* expression vector pRSET-A (Invitrogen, Carlsbad, CA), and used to generate recombinant protein, see Appendix I for immunoblot of purified recombinant protein. Rabbit antisera generated against the recombinant Chk2 protein strongly recognized the recombinant and native forms of rainbow trout Chk2. To confirm the specificity of the polyclonal antibodies, a western blot was performed using either untreated final bleed anti-Chk2 antiserum or anti-Chk2 antiserum that had been pre-incubated with excess recombinant Chk2 protein. As shown in Figure 2.4, when the polyclonal anti-Chk2 antibodies were blocked with excess recombinant Chk2 protein they were no longer able to detect the Chk2 protein (49 kDa) in two samples of RTgill-W1 rainbow trout lysates. Due to the excess recombinant protein binding up all the available anti-Chk2 antibodies, the non-specific binding shown in Figure 2.4 A) was lost in Figure 2.4 B). Ponceau S staining served as an indicator of protein loading and integrity. These results indicate that the anti-Chk2 antibodies are highly specific to the Chk2 protein in rainbow trout. Chk2 often appears as a doublet throughout these analyses, which may indicate differentially phosphorylated forms of the Chk2 protein.

2.3.5 Tissue specificity of Chk2 protein expression.

Since our initial RT-PCR analysis showed that *CHK2* mRNA levels vary based on tissue type (Figure 2.3B), western blots were performed to determine if Chk2 protein expression levels were similarly variable. In contrast to what was observed for mRNA, the level of Chk2 protein was more abundant in gill tissue versus brain tissue (Figure 2.5A). To confirm these observations, Chk2 levels were assessed using rainbow trout gill (RTgill-W1) and brain (RTbrain-W1) cell lines (173). Comparable to the results obtained

with the whole fish protein lysates, Chk2 protein levels were found to be much higher in the RTgill-W1 cell line relative to the RTbrain-W1 cell line (Figure 2.5B). Gills serve as

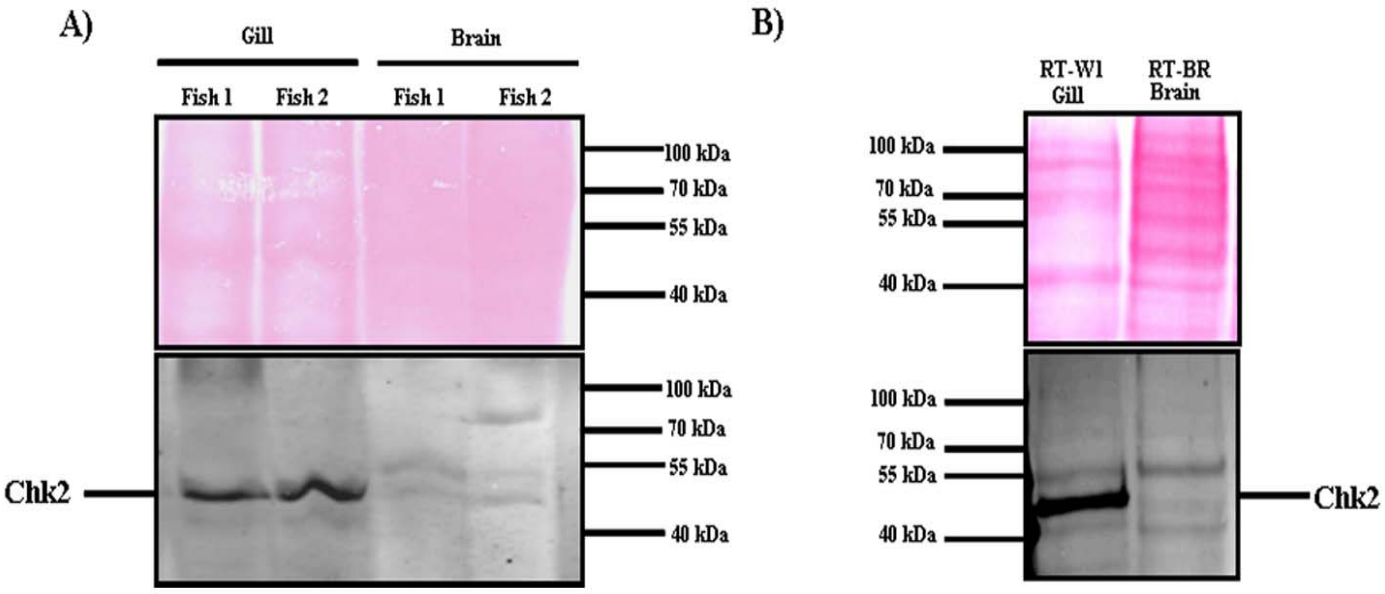

Figure 2.5: Assessment of Chk2 protein levels in adult rainbow trout and rainbow trout cell lines.

A) Whole fish: Western blot of gill and brain tissue from two adult rainbow trout. Protein lysates (45 µg) were probed with anti-Chk2 polyclonal antiserum (1:150 dilution). **B)** Cell lines: Western blot of RTgill-W1 and RTbrain-W1 (173) protein lysates (45 µg) probed with anti-Chk2 polyclonal antiserum (1:100 dilution). Detection

confirms presence of Chk2 protein (49 kDa). Ponceau S staining is shown as a loading control.

the initial barrier between fish and their external aquatic environment, and are the first tissues to come into contact with any toxins that might be present in the water. The high levels of Chk2 protein may indicate that the DNA damage response is constitutively active or "on alert" in gill tissue, as cells respond to genotoxic agents present in the water.

2.3.6 Effect of Bleocin induced DNA damage on Chk2 protein expression in RTgill-W1 and RT-Brain.

Upregulation and/or phosphorylation of Chk2 in response to DNA damage has been shown in numerous species; specifically in response to double-strand breaks (DSB) (92). RTgill-W1 cells were treated with either a low dose (3mg/ml) or a high dose (18mg/ml) of Bleocin, a commercial form of bleomycin, which is a radiomimetic drug that causes a high-percentage of DSBs in proliferating cells (169). Cells were harvested at 1, 4, 8 or 24 hours post treatment and protein lysates were obtained for immunoblot analysis with anti-Chk2 antiserum. As shown in Figure 2.6A and B, at both dosages, Bleocin has no effect on expression levels of Chk2 in RTgill-W1. Given our previous observations that Chk2 levels were constitutively high in untreated gill samples obtained from both cell lines and whole fish (Figure 2.5), it is possible that no upregulation or modification of Chk2 may be required in gill to respond to the double strand breaks caused by Bleocin under these treatment conditions.

Since levels of Chk2 protein are considerably lower in rainbow trout brain than in gill for both whole fish and tissue culture cells, we hypothesized that the levels of Chk2 protein in rainbow trout brain might be upregulated or phosphorylated in response to DNA damage. To investigate this possibility RTbrain-W1 cells were also treated with

Bleocin and protein extracts were analyzed by immunoblot. As seen in Figure 2.6C, untreated RTbrain-W1 cells show relatively low levels of Chk2. When exposed to a low

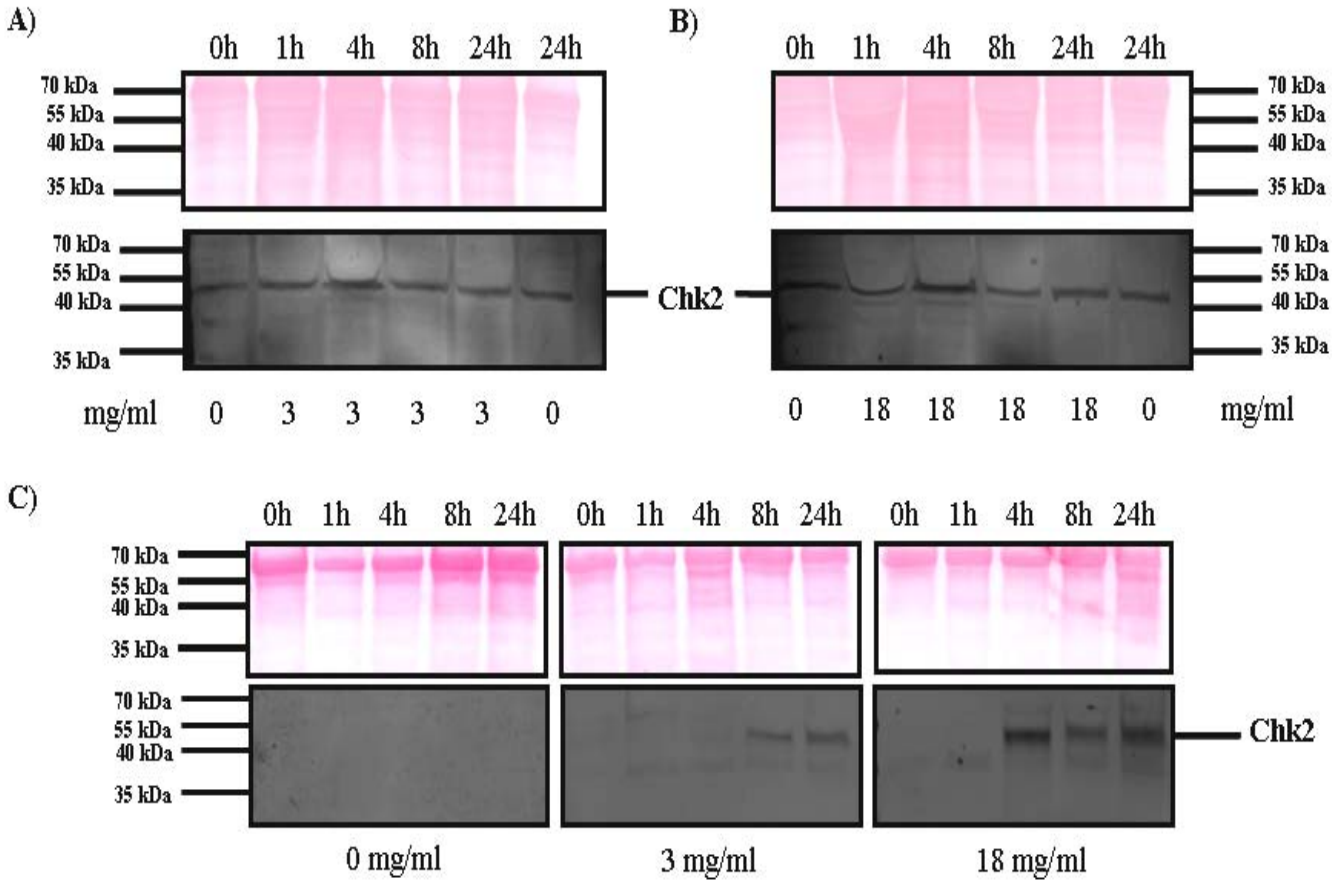

Figure 2.6: Effects of Bleocin treatment on the expression of Chk2. A) Western blot of RTgill-W1 protein lysates (45 µg) after exposure to a low dose (3 mg/ml) of Bleocin at indicated time points. **B)** Western blot of RTgill-W1 protein lysates (45 µg) after exposure to a high dose (18 mg/ml) of Bleocin at indicated time points. Untreated control samples are shown at 0h and at 24h. **C)** Western blot of RTbrain-W1 brain protein lysates (35 µg) after exposure to either a low dose (3 mg/ml) or high dose (18 mg/ml) of Bleocin at indicated time points. Untreated control samples for all time points are also shown. Samples were detected using anti-Chk2 polyclonal antiserum (1:100 dilution). Detection

confirms presence of Chk2 (49 kDa). Ponceau S staining of the membrane is shown as a loading control.

dose of Bleocin (3mg/ml), Chk2 levels begin to increase after approximately 8 hours and remain high up to 24 hours after the initial exposure. At the higher dose of Bleocin (18 mg/ml), Chk2 levels begin to increase earlier, at approximately 4 hours, and again remain high up to 24 hours after the initial exposure. There also appears to be a modest upward shift consistent with increased phosphorylation of the Chk2 protein.

2.4 Discussion

The *CHK2* gene has been successfully cloned for the first time in fish, specifically *Oncorhynchus mykiss*. The predicted peptide sequence of 508 amino acids is similar in length to the sequences from other teleost and mammalian species, which have an average length of approximately 520 amino acids. The sequence also shows high levels of conservation in the three distinct functional domains of Chk2: the SQ/TQ cluster domain (SCD), the forkhead-associated domain (FHA) and the Ser/Thr kinase domain (92). The deduced amino acid sequence for the rainbow trout Chk2 protein is 66% identical to the zebrafish Chk2 sequence and 57% identical to that of human Chk2 (Figure 2.2A and Supplementary Figure 2.1) showing a high level of sequence similarity across metazoans. Likewise, a study by Cheng *et al.* in 1997 found that the tumor-suppressor TP53 amino acid sequence in zebrafish was 63% identical to the predicted TP53 sequence in trout and 48% identical to that of human TP53 (138). Therefore, the rainbow trout Chk2 sequence makes an important contribution to the growing body of knowledge showing the high level of conservation in checkpoint proteins and their roles in the DNA damage response.

To further characterize their evolutionary relationship, complete Chk2 amino acid sequences from rainbow trout and other metazoans were used to create a neighbour-joining phylogenetic tree (Figure 2.2B). As expected, teleost sequences clustered together with high confidence, while mammalian sequences formed their own distinct clusters. Interestingly, most of the bootstrap values are very high, which indicates that the branching order has a strong level of confidence. The one exception is the branch that encompasses the trout and pufferfish sequences, indicating that the analysis can not distinguish with a high level of certainty which of these two species has the highest sequence similarity to the zebrafish sequence, as more teleost sequences become available this grouping should become more definitively resolved.

In characterizing the gill and brain tissue distribution of the Chk2 protein there was strong agreement between fish tissue samples and the immortalized rainbow trout cell lines maintained in the laboratory (Figure 2.5). This indicates that rainbow trout cell lines can accurately reflect the tissue-specific expression of checkpoint proteins occurring *in vivo,* making it possible to characterize checkpoint responses at the cellular level without exposing live animals to genotoxic agents. In a recent study by Kales *et al.* in 2007, a rainbow trout macrophage cell line was used to assess the effect of parasitic fungi on the immune system of rainbow trout, which showcased another example of using cell lines to look at molecular processes of fish in response to stress conditions. They were able to conclude that, similar to *in vivo* processes, rainbow trout monocyte/macrophage cell line, RTS11 was activated in response to both live and heat-killed water mold mycelium and their culture filtrates (174). The power of using cell lines in the laboratory provides a huge economic advantage over treating whole fish and facilitates the control over external variables in the continuing characterization of checkpoint proteins in fish.

In analyzing the expression levels of the *CHK2* transcript and the Chk2 protein there were interesting differences within rainbow trout gill and brain tissues. At the transcript level, gill tissue was shown to have a much lower level of *CHK2* versus rainbow trout brain tissue, while at the protein level the reverse was true. Figure 2.3B shows that gill tissue has a relatively low level of *CHK2* transcript, although this result shows some variability (relative values of 0.32, 1.01 to the S11 control); while in brain tissue, the *CHK2* transcript is higher than in any other tissue (relative values of 1.97, 2.78 to the S11 control). At the protein level, Chk2 seems to be much more strongly expressed in gill in comparison to the low levels of protein seen in brain tissue. As discussed above this result was seen in both cell culture and fish tissue samples (Figure 2.5). Constitutive expression of Chk2 protein in rainbow trout gills, may relate to the innate property of gills acting as the main barrier between the fish's internal and external environments. Previous studies have shown that the gills often show upregulated immune system proteins such as clottable protein (175), toll-like receptors (176), and IL-6 (177). The constitutive expression of checkpoint proteins, such as Chk2, may act in a similar fashion, protecting the fish's internal environment from external stresses or pollutants. Rainbow trout gill cells in culture also have a very fast growth rate and can reach confluency in 3-5 days (178). *In vivo,* this quick growth rate helps to replace cells that have been sloughed off under the constant bombardment of swiftly moving water. Consequently, Chk2 is likely maintained at high levels in gill for two reasons: to safeguard the fidelity of DNA replication in these continuously proliferating cells and to ensure that DNA damage caused by any pollutants that come into contact with the gills can be dealt with efficiently. In contrast, rainbow trout brain cells proliferate at a much slower rate than gill cells, taking approximately two weeks to reach confluency (179). The brain is relatively well protected from the external environment with genotoxic

agents only reaching it after entering the blood stream and crossing the blood-brain barrier. Under normal conditions, Chk2 may therefore be maintained in the brain in the form of a transcript reservoir, with high levels of *CHK2* mRNA (Figure 2.3B) and low levels of Chk2 protein (Figure 2.5).

The activation of Chk2 in response to DSB has been well-characterized in a variety of species including humans, mice and yeast (where it is called Rad53) (92). Typically, Chk2 is activated through phosphorylation by ATM following DNA damage and propagates the signal through phosphorylation of downstream targets involved in cell cycle progression or apoptosis (55). A recent study by Roy *et al.* in 2006 showed that Chk2 activation is not only controlled by phosphorylation but can its levels can also be upregulated in response to DNA damage as shown by Nimbolide treatment, a natural triterpenoid that causes cell cycle arrest in human colon carcinoma HT-29 cells (180). Bleocin, the radiomimetic drug used in the present study, has been shown to cause a significant number of double strand breaks and induce cell cycle arrest in proliferating human cells, while still allowing a large number (>70%) of cells to survive at least 24 hours after the initial treatment (169). As shown in Figure 2.6C, Chk2 levels clearly increase in rainbow trout brain cells in response to Bleocin treatment. This result in combination with the high-levels of brain *CHK2* transcript (Figure 2.3B), supports the notion that Chk2 is maintained in a transcript reservoir and is translated in response to DNA damage. It is also apparent from the quick response, within 1 hour, that the translation must be from existing transcript as new *CHK2* transcript could not be synthesized within that time-frame. This increase in Chk2 protein was shown to be dose-dependent with levels increasing much more quickly after a larger dose (18 mg/ml) than after a smaller dose (3 mg/ml), presumably since a higher dose would result in significantly more DNA damage, and require significantly more Chk2 activity to initiate

a successful DNA damage response. Previous work has found high levels of Chk2 protein in a subset of human and murine brain tumors exposed to ionizing radiation (128;162), the conservation of this relationship highlights the importance of the Chk2 protein in responding to DSB's in brain tissue. The rainbow trout gill tissue did not show this same response (Figure 2.6B), as Chk2 levels remained constitutively high regardless of dose or exposure time to Bleocin treatment. This supports the notion that due to their constant contact with the external environment and high rate of proliferation gills maintain a consistently higher level of protective checkpoint proteins.

In summary, Chk2 protein levels in rainbow trout brain cell lines can serve as a sensitive indicator of double strand breaks in response to genotoxic damage, while gill cell lines appear to have a constitutively high level of Chk2 expression. Our work also demonstrates that cell lines in the lab can accurately reflect the protein levels documented in samples obtained from fish tissue. Finally, this study is the first to clone and characterize Chk2 in a fish species. The characterization of additional checkpoint proteins in rainbow trout could lead to the development of a biological assay to test the quality of water samples for the presence of genotoxic agents. The nature of the checkpoint response and the subset of checkpoint proteins activated can vary considerably depending on the nature of the DNA damage and the stage of the cell cycle during which it occurs (35). Using specific cell cycle checkpoint proteins as biomarkers, a sensitive and specific system could thus be created to measure the environmental impact of water genotoxins on aquatic ecosystems and assess the potential risks for human health.

2.5 Acknowledgements

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CHAPTER 3

General Conclusions

3.1 Research Contributions

Very few checkpoint proteins have ever been studied in teleost species and this study was the first to characterize Chk2 in fish. It is also the first time a checkpoint protein has ever been characterized in *Oncorhynchus mykiss,* demonstrating the potential for a rainbow trout model in creating a checkpoint protein centered biological assay. While several genes have been characterized in other fish species as described in Chapter 1 –Section 7, this research was the first to apply several novel methods in examining checkpoint proteins in fish. This study was the first to look at the state of a checkpoint protein in whole fish tissue samples versus fish cell lines and show that cell lines accurately reflect the state of a checkpoint protein found *in vivo*. This knowledge will be useful in allowing future researchers to use cell lines instead of live animals, which are much more difficult to maintain in a laboratory setting and far less cost-effective. This study was also the first to presumably induce double strand breaks in a fish cell line and to effectively demonstrate an upregulation of a checkpoint protein in response to such treatment. Other work has compared the levels of Rb and p53 in tumor cells lines versus wildtype cell lines (139;144), but none have demonstrated an upregulation or post-translational modification in response to experimentally induced double strand breaks. This result provides the first step in showing that checkpoint proteins can be used to assess the exposure of fish to genotoxic agents present in their environment. Lastly, this study was pioneering in that it compared the tissue specific differences of the DNA

damage response in fish. This article was the first to show that checkpoint proteins appear to be consituitively active in gill, acting as a first line of defense against genotoxic agents present in the fishes external environment. This finding fits with the unique physiology of fish, where nothing in the water can enter the fish's bloodstream without first being filtered through the gill barrier. This study was also the first to show that in unperturbed brain cells, Chk2 protein levels are low but in response to genetic insult brain cells are capable of upregulating the levels of Chk2 present to mount a serious defense against DNA damage. This result was further confirmed by the initial RT-PCR finding that the Chk2 transcript is elevated in this highly sensitive tissue, and is thus prepared to initiate a DNA damage response in the event that a genotoxic agent crosses the blood brain barrier. By demonstrating that the changes in checkpoint proteins vary based on tissue type, this study was able to show that the physiology of fish has an enormous impact on how different tissues respond to genotoxic stressors.

3.2 General Discussion

The ability to detect genotoxicity in aquatic organisms caused by natural or anthropogenic stressors is an area of interest to many government and industrial parties. As human populations continue to grow and levels of pollution continue to rise the ability to understand the subtle effects of long term exposure to fish becomes increasingly important. Whether through chemicals in municipal wastewaters or through industrial or agricultural run-off very little is known about how these toxins are affecting the fish that habituate Canadian waterways. The ability to characterize the effects of such exposure on fish growth, reproduction and disease resistance will be greatly beneficial in understanding what preventative measures should be taken to protect this important natural resource. This study is the first step in creating a bioassay that would use *in vitro*

genotoxicity testing in cell lines to determine the effect of industrial effluents and contaminants on feral fish species.

The Government of Canada has designed a Water Quality Index (WQI) scale that currently assesses water quality based on the following parameters: dissolved oxygen, pH, total phophorus, total nitrogen, arsenic, lead, mercury and pesticides (181). While the guideline values for these indicators were originally derived using test organisms, the actual impact of these variables on aquatic species remains largely undetermined. Currently, there is no quick and easy way to assess biological impact on aquatic life and there is an undeniable lack of uniformity in techniques that are currently being employed (145). This presents a need for a reliable and cost-efficient method to assess the impact of a wide range of pollutants at a biologically relevant level.

As described in Chapter 1 and reviewed in Sarkar *et al.* 2006, there are currently a number of methods being used as biological indicators of pollution. Primarily, Cytochrome P450 activity, acetylcholinesterase activity, metallothionein levels and DNA integrity have all been described as methods for understanding the biological impact of aquatic pollution (145). Unfortunately these methods have serious drawbacks which limit their use and relevance as biomarkers. For example, DNA integrity is currently assessed using either a comet assay (182) or an alkaline unwinding assay (183) both of which have serious limitations. The comet assay monitors the degree of fragmentation in DNA by anchoring cells in an agarose gel, lysing them and assessing the amount of migration away from a central core of intact genomic DNA during electrophoresis (184). However, this assay would not be sensitive enough to assess the small amounts of damage that would be present in aquatic specimens since integrity of DNA can only be detected when >20% of the DNA appears in the tail (i.e. the portion that migrates away from the genomic core) (185). The alkaline unwinding assay measures the extent to

which strand breaks aid in denaturation (186), however many genotoxic agents can inhibit enzymes that are required for this process to occur naturally, such as topoisomerases and DNA polymerases (187).

In awareness of these issues, an alternative method of assessing genetic damage could be to use cell cycle checkpoint proteins as biomarkers. As described in Chapter 1, checkpoint proteins are activated when DNA damage arises and act to slow down the cell cycle, direct repair processes or initiate apoptosis (35). The DNA damage response typically causes either an upregulation or a post-translational modification of these checkpoint proteins providing a quantifiable and observable change in cells exposed to genotoxic agents. Interestingly, the subset of checkpoint proteins activated and the degree of activation varies considerably depending on the type of DNA damage and the point within the cell cycle when it occurs (35). These features of checkpoint proteins present a promising approach for assessing the quality of polluted water using a panel of relevant checkpoint biomarkers to determine the degree and type of genetic stressor present in each sample. Some examples of DNA damage that could be characterized using checkpoint proteins include single strand nicks, double strand breaks, DNA adducts and intercalation.

This study presents the first step in developing a cell cycle checkpoint protein biological assay. The checkpoint protein, Chk2 was shown to be upregulated in rainbow trout brain cells in response to double strand breaks caused by a radiomimetic agent. This result parallels findings with mammalian cells that Chk2 is a central player in the DNA damage response to double strand breaks. While these results are encouraging more work remains to be done in the creation of a biological assay using cell cycle checkpoint proteins as described below.

3.2 Future Work

In the development of the cell cycle checkpoint bioassay the first objectives will be to clone additional fish checkpoint genes, express the corresponding recombinant protein and develop highly specific polyclonal antibodies. As described in Chapter 2, genes will be initially isolated using PCR primers based on areas of high sequence similarity to genomic databases currently available, such as pufferfish and/or zebrafish. Rainbow trout templates will continue to be used in the cloning of the additional checkpoint genes as numerous high quality cDNA libraries are currently available at the University of Waterloo. The first set of checkpoint genes that should be cloned includes *TP53, MPS1, DTL* and *Separase* as they have already been identified and characterized in other fish and are central components of the DNA damage response (see section 1.7). Two separate regions of each gene will then be used to produce recombinant proteins, one of which will be used to immunize rabbits, while the other will be used to immunize chickens. All antibodies will then be assessed to determine their initial sensitivity and specificity in unperturbed rainbow trout cell lysates before being used in any future assays. Once a set of reliable antibodies has been created, the evaluation of their usefulness in detecting changes in checkpoint protein expression or modification would need to be determined in cell lines exposed to an arsenal of genotoxic agents, chemicals, and pollutants.

Several genotoxic agents will be examined in an initial assessment to determine how different checkpoint proteins respond to various forms of treatment. DNA methylating agents such as methylmethane sulfonate (MMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) have been used for decades as classical DNA damaging agents. MMS and MNNG modify DNA by adding methyl groups to a number of nucleophilic sites on the DNA bases, although MNNG produces a greater percentage of O-methyl adducts. Recent work in mice has shown that DNA methylation can be

detected by both ATM and ATR, which in turn activate downstream target genes such as BRCA1 and p53 (188). Also, DNA damaging agents involved in replication fork stalling and ATR-mediated checkpoint activation such as Aphidicolin, a DNA polymerase inhibitor, and hydroxyurea, an inhibitor of ribonucleotide reductase which depletes dNTP pools, will be studied (189). Finally, agents involved in double strand breaks such as bleomycin and ionizing radiation will continue to be examined. The induction of double strand breaks is known to be preferentially detected by ATM and results in a Chk2-mediated DNA damage response.

The rainbow trout model used in this assay was chosen because of its importance to the aquaculture industry and the availability of a number of rainbow trout cell lines at the University of Waterloo. In the initial stages of this continuing project several rainbow trout cell lines will be screened to determine which provide the most consistent and robust response in checkpoint protein changes, beginning with RTL-W1 from liver (190), RTgill-W1 from gill (173) and RTbrain-W1 from brain (under characterization). RTL-W1 has been useful in evaluating exposure to dioxin-like compounds (191) and RTgill-W1 and RTbrain-W1 have been useful in characterizing Chk2 as described in Chapter 2. The cell lines will be assayed for their sensitivity and reliability, the strength of their checkpoint response, their ease of maintenance and their tolerance to exposure manipulations.

There are many advantages to using cell lines instead of whole fish or tissue samples. Results are more reproducible and cell lines are far easier and more cost-effective to maintain versus live animals. As shown in Chapter 2 - Figure 5, RTbrain-W1 and RTgill-W1 accurately reflect the native state of checkpoint proteins in live tissue, thus demonstrating the applicability of results obtained through rainbow trout cell cultures. Cell lines provide a rapid means for harvesting protein lysates, which can than

be used to determine increases in levels of checkpoint proteins and/or reduced mobility of the checkpoint proteins, as hyperphosphorylation is often indicative of checkpoint protein activation (192). As shown in Chapter 2 – Figure 6, increased levels of Chk2 became apparent in a dose-dependent manner after exposure to the genotoxic agent, bleomycin. As additional checkpoint protein polyclonal antibodies become created rainbow trout checkpoint activation for a variety of specific DNA damage types will be assessed. Water samples will be taken from a variety of sites and following exposure to the samples, protein extracts will be subjected to immunoblot analysis and a characteristic biotoxicity scale will be developed.

Following the immunoblot analysis described above, antibodies that are able to detect changes in protein level will be used to develop a sandwich ELISA assay (193). Essentially, a sandwich ELISA involves binding a "capture" antibody to the wells of the microplate, adding a sample containing the target protein, and then adding a "detection" antibody generated in a second species that also binds the target protein and can be detected using an alkaline phosphatase-conjugated seconday antibody. The addition of p-nitrophenylphosphate results in an observable colour change which can then be quantified by optical density in a microplate reader. The use of a sandwich ELISA will permit the mass production of microplates with antibody coated wells, allowing for a more rapid screening method than if the wells had to be bound by protein extracts from the cell lines for each assay run.

Antibodies that detect checkpoint protein activation based on changes in protein mobility will not be used in the sandwich ELISA, but will instead undergo immunoblot analysis. In order to increase the efficiency of this assay protein extracts exposed to different water samples will be run in parallel lanes on the same gel (alternating with size marker), and transferred to a nitrocellulose membrane which will than be cut to separate

the lanes and placed in separate compartments on an incubation tray. Different checkpoint protein specific antibodies will then by added to each compartment and detected using the appropriate fluorescent secondary antibody. Duplicate sets of detections will be carried out using the antibodies obtained from chicken and rabbit to verify the accuracy of the results.

3.3 Closing Remarks

The use of checkpoint proteins as a means of monitoring genotoxic agents in the environment could serve as a way of early detection for harmful pollutants. In combination with cell lines an assay could be performed quickly and easily using only a small sample of polluted water and not harming any live animals. As the human population continues to grow and pollution levels continue to increase, a sensitive and specific scale could have enormous economic advantage in protecting the commercial fishing industry and maintaining clean and safe waterways. In conclusion, this research project offers preliminary evidence to support the notion that the induction of the DNA damage response and cell cycle checkpoint proteins could potentially serve as a valid and reliable biomarkers for genotoxic agents present in aquatic ecosystems.

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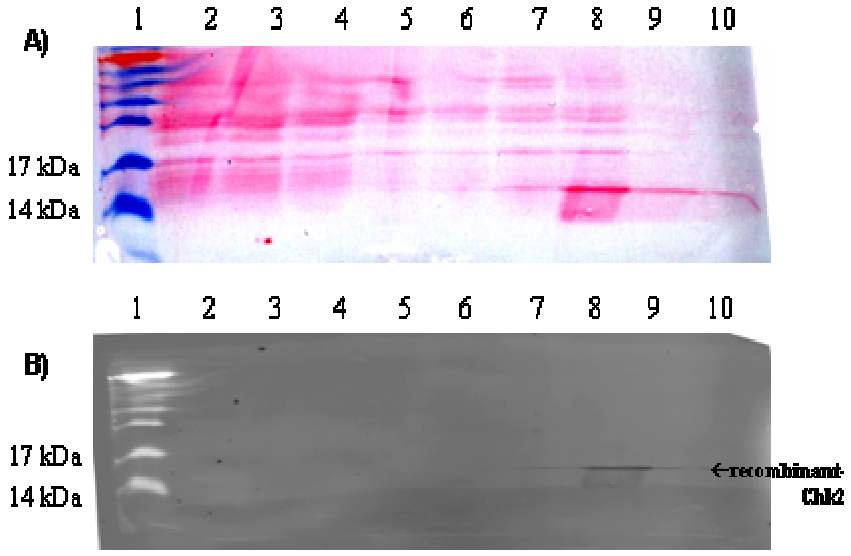
Appendix I



Protein purification of recombinant Chk2. Protein expression conditions were optimized and purification was done using an Ni-NTA agarose resin and column purification system. Washes were done with decreasing pH to elute the protein of interest. A) Ponceau S staining showing all washes. Lane 1: pre-stained high molecular weight marker. Lane 2: pH=8 unwashed lysate. Lane 3: pH=8 washed lysate. Lane 4: pH= 6.3. Lane 5: pH=5.9. Lane 6: pH=4.5(1st wash). Lane 7: pH=4.5(2nd wash). Lane 8: pH=4.5(3rd wash). Lane 9: pH=4.5(4th wash). Lane 10: pH=4.5(5th wash). Chk2 protein was eluted in the 3rd wash at pH=4.5. B) Western blot using Anti-Xpress primary antibody, specific for an epitope attached to the protein of interest by the pRSET-A expression vector. The Chk2-recombinant protein is visible in Lane 8 (eluted in the third wash at pH=4.5).