Regulation of Sox2 by Ionizing Radiation

by

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Abstract

Within a tumour, only a subset of cells are able to initiate neoplasms when transplanted or following localization to different organs. These tumour-initiating cells, often called cancer stem cells (CSCs) due to their high cellular plasticity, were found to exhibit particular resistance to genotoxic therapies. They are also thought to be the primary cause of local recurrence and metastasis. CSCs display characteristics similar to those of induced-pluripotent stem cells, namely their ability to self-renew and potential to differentiate. The transcription factor Sox2 is an essential gene involved in stem cell maintenance as well as the establishment of induced-pluripotency and cancer stem cell phenotypes. Moreover, its amplification is detected in multiple cancers and has been linked to a more invasive phenotype. Recent studies suggest that some DNA damaging cancer therapies and hypoxia can trigger dedifferentiation and an increase in cancer stem cell populations associated with elevated Sox2 levels. However the mechanism of Sox2 induction remains unclear. Contrary to previous reports, our studies indicate that ionizing radiation (IR) leads to down-regulation of Sox2 protein in cancer cell lines – this regulation occurred post-transcriptionally and is dependent on ATM activation. Using a Sox2 reporter cell line, we observed that while Sox2 protein decreases with IR, there is an induction of Sox2 activity with increasing IR dose which was further augmented when combined with hypoxia. Many cancers are characterized by hierarchical cell populations, with CSCs displaying high self renewal and differentiation capacity. Radiotherapy is a common treatment modality and has been shown to promote a CSC phenotype in surviving cells. Our studies show that Sox2 protein stability and transcriptional activity are altered by radiation treatment *in vitro*. Understanding whether regulation of Sox2 by IR has the potential to impact cancer cell fate and identifying the regulatory factors involved may provide a basis for designing novel targeted therapies and improving cancer outcome.

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LIST OF ABBREVIATIONS

ATM	Ataxia telangiectasia-mutated
ATR	ATM and RAD3-related
CAF	Cancer-associated fibroblast
CDK	Cyclin-dependent kinase
CSC	Cancer stem cell
CTC	Circulating tumour cells
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EMT	Epithelial mesenchymal transition
FBS	Fetal bovine serum
Gy	Gray, Joules/kg
HMG	High mobility group
HRP	Horseradish peroxidase
NICD	Notch intracellular domain
iPSC	induced-pluripotent stem cell
IR	Ionizing radiation
OER	Oxygen enhancement ratio
O-GlcNAc	O-linked-N-acetylglucosamine
PBS	Phosphate-buffered saline
PTM	Post-translational modification
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin ribonucleic acid
SRR	Sox2 regulatory region
TAM	Tumour-associated macrophage
TBS	Tris-buffered saline
TBST	Tris-buffered saline with tween-20
TME	Tumour microenvironment

CHAPTER 1 INTRODUCTION

1.1: Radiation biology

1.1.1 Types and sources of radiation

There are many sources of background radiation present in our environment (Figure 1.1). Depending on the source, radiation can interact with matter in different ways. Radiation is the emission of energy in the form of subatomic particles or electromagnetic waves (Hall and Giaccia, 2018). When energy is absorbed by molecules, it can lead to excitation or ionization. The former is the result of non-ionizing radiation, where the transferred energy excites an electron into a higher energy orbit of a molecule; this type of radiation has insufficient energy to cause molecular damage. On the other hand, ionizing radiation (IR) carries large enough energy to expel electrons from a molecule, which can lead to radiolysis (breaking of chemical bonds) as well as the production of reactive free radicals (Hall and Giaccia, 2018).

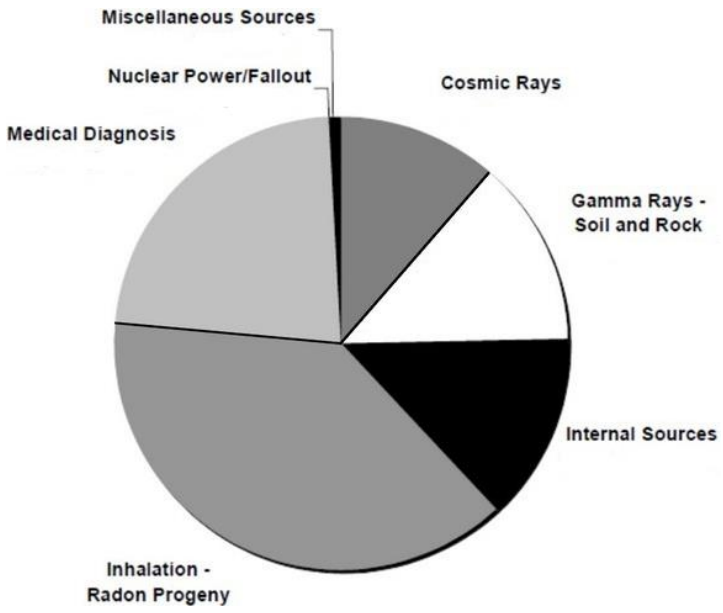


Figure 1.1 Distribution of background radiation in Canada (adapted from Canadian guidelines for the management of naturally occurring radioactive materials)

Electromagnetic radiation comes in the form of photons travelling in a wave-like pattern, delivered in a stream. Photons are mass-less and uncharged, and therefore can penetrate human tissue unimpeded. The consequent effect of radiation on molecules is determined by the energy deposited in tissues (Hall and Giaccia, 2018). Electromagnetic waves can be placed on a spectrum ranging from low energy, non-ionizing waves to high energy, ionizing waves (Figure 1.2). Photon energy can be determined by Planck's equation $E=hc/\lambda$, where h is Planck's constant, c is the speed of light and λ is the wavelength. Non-ionizing electromagnetic waves are characterized by long wavelengths and therefore have low photon energy. Examples of non-ionizing radiation includes microwaves and radio waves which emit radiofrequency radiation to confer a spin on molecules, and infrared rays that induce molecule vibration. Prolonged exposure can lead to tissue damage from the heat generated from this type of molecular excitation. Electromagnetic waves emitting from radioactive atoms (γ-rays), x-rays and ultraviolet light have shorter wavelengths and higher energy. The high photon energy ionizes atoms by expelling valence electrons, therefore altering the chemical composition and reactivity of molecules (Hall and Giaccia, 2018).

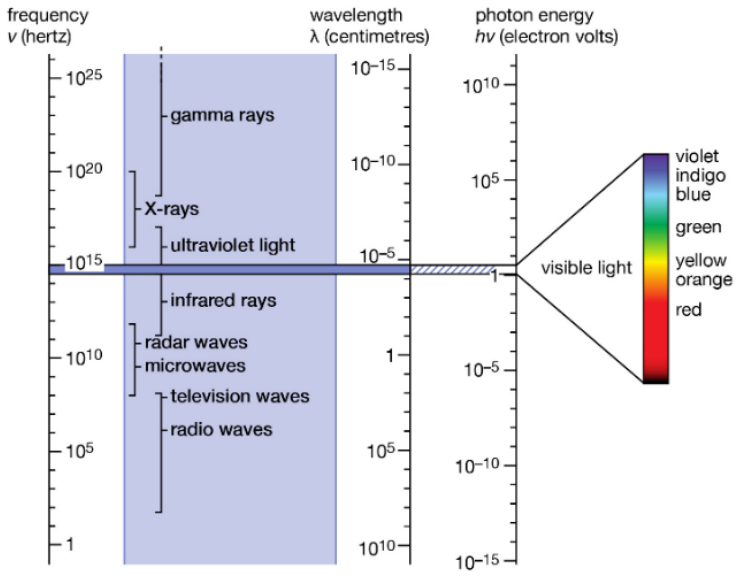


Figure 1.2 The electromagnetic spectrum (Encyclopædia Britannica)

Particulate radiation are fast-moving subatomic particles. When these collide with molecules, they are able to transfer large amounts of energy to the matter per unit distance traversed. Particulate radiation has a high linear energy transfer, therefore usually inducing more molecular damage than electromagnetic radiation. Charged particles forming natural IR can consist of electrons, protons, α-particles and heavy charged particles. Radioactive β-decay produces negatively charged electrons. Protons are positively charged particles 2000 times heavier than electrons, while neutrons have a heavier mass than protons but are uncharged. Both represent a fraction of background radiation from the sun. α-particles consist of two protons and two neutrons and are produced from radioactive α-decay. Decay of radium-226 for example gives rise to radon gas and α-particles. Radon itself is an active α-emitter and a major source of background radiation from the ground, causing damage to the lining of our lungs when inhaled (Hall and Giaccia, 2018). Heavy charged particles are nuclei of elements stripped of electrons, and these high energy particles are a source of cosmic radiation.

1.1.2 Consequences of ionizing radiation in a cell

Radiation can interact with all molecules within a cell, but damage to DNA has the most detrimental effect. DNA carries heritable information that determines the phenotype of a cell. Therefore damage to DNA can result in phenotypic changes that are passed onto progeny cells. DNA is a large molecule composed of two strands in a double helix structure. Each strand consists of a backbone of deoxyribose sugar linked by a phosphate group. Attached to the sugars are nucleic acids that make up the genetic code: adenine, thymine, cytosine, and guanine. The two DNA strands are held together by hydrogen bonds between nucleic acid pairs. Spontaneous DNA damage occurs from endogenous sources such as base misincorporation during DNA replication, or base modifications by reactive oxygen species produced from metabolism. Exogenous sources

of DNA damage mostly come from background radiation. Following irradiation, particulate or electromagnetic radiation with sufficient kinetic energy can directly ionize and damage DNA, causing DNA single strand or double strand breaks and nucleic acid damage. The majority of DNA damage induced by electromagnetic radiation is indirect, meaning that radiation interacts with molecules other than DNA, forming charged molecules called free radicals that can then go on to directly ionize DNA (Bolus, 2001). Free radicals are highly reactive due to their ionized state; the expelled electron leaves an unpaired orbital electron making molecules highly unstable and chemically reactive. The most abundant molecule in a cell is water. Ionization of H_2O forms a short-lived ion radical H_2O^+, which can further react with another H_2O molecule to form H_3O^+ and $OH^\bullet$. The free hydroxyl radical $OH^\bullet$ is reactive due to its unpaired valence electron and can diffuse to and damage DNA (Bolus, 2001). It is estimated that 1 Gray (Gy) of IR, i.e. the absorption of 1 joule of energy per kilogram, is sufficient to cause >1000 nucleic acid damages, 1000 single stranded breaks and 40 double stranded breaks (Costes et al., 2010).

The three main types of cell fate after DNA damage are temporary or permanent cell cycle arrest, cell cycle resumption after DNA repair, or cell death. Cell survival after DNA damage depends on the extent of damage and the activity of key players. Two of these, ATM and ATR are apical kinases of the DNA damage response: ATM is activated in the presence of double strand breaks while ATR is activated by RPA coated single-stranded DNA generated during replication fork uncoupling or double strand break repair. ATM and ATR phosphorylate many common substrates, but they specifically phosphorylate Chk2 and Chk1, respectively. Together, ATM and ATR are responsible for activating cell cycle checkpoints and DNA repair (Abraham, 2001). Cell cycle checkpoints prevent cells from propagating damaged DNA by pausing the cell cycle and allowing time for DNA repair. When sub-lethal levels of DNA damage have been repaired, checkpoints are

inactivated and the cell cycle resumes. Cells may also permanently exit the cell cycle and undergo senescence.

Damage to DNA can come in many forms: base modifications, base mismatch, single and double strand breaks and inter-strand crosslinks (Sancar et al., 2004). Both prokaryotic and eukaryotic cells have evolved dedicated repair mechanisms responsible for repairing different types of DNA damage (Figure 1.3). Base damage can be repaired by base excision repair or nucleotide excision repair. Base excision repair replaces single modified bases that have been alkylated, oxidized or deaminated. Bases chemically modified with adducts or crosslinked to neighbouring nucleotides will appear bulky and are repaired by excising and replacing multiple base pairs by the nucleotide excision repair pathway. The base excision repair pathway feeds into the single stranded break repair pathway which mends the nicked backbone. If left unrepaired, single strand DNA breaks can turn into double stranded breaks when cells undergo DNA replication. Single strand breaks formed on opposite strands in close proximity are double strand breaks. Since double strand breaks are rarely "clean-cut", the ends have to be processed before re-ligation (Sancar et al., 2004). There are two main pathways of double strand break repair: non-homologous end joining involves processing of the ends followed by direct ligation; loss of base pairs from processing makes this pathway error-prone. Homologous recombination on the other hand uses replicated DNA as a template to repair the broken strands in the presence of sister chromatids. Homologous recombination is therefore error-free (Sancar et al., 2004).

In circumstances where DNA damage extends beyond the point of repair, cells can die by apoptosis, a programmed cell death pathway driven by caspase-dependent cleavage of substrates. p53 activity is a key determinant of whether cells will survive or die as it plays a role in both cell cycle arrest and apoptosis. Cells may also die by necrosis or mitotic catastrophe.

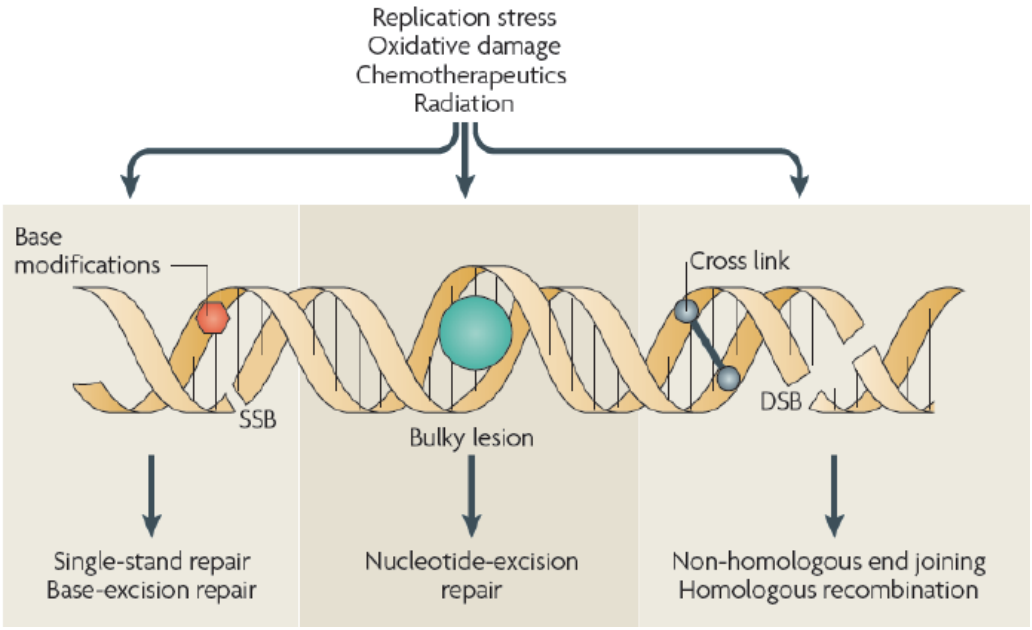


Figure 1.3 Types of DNA damage (Arjunan et al., 2015)

1.1.3 Use of radiation in cancer treatment

Treatment of cancer typically consists of surgical removal of the tumour bulk (where possible), combined with radiotherapy and/or chemotherapy. Radiation therapy is used to treat approximately 50% of cancer patients (Baskar et al., 2012). The rationale is to induce lethal levels of DNA damage to tumour cells and promote cell death. Recent improvements in radiotherapy efficiency can be attributed to advancements in imaging modalities, treatment planning and delivery. Since radiation damages normal and cancer cells indiscriminately, targeted therapy is crucial to specifically kill cancer cells while sparing damage to healthy cells. In order to achieve this, radiation beams can be situated to deliver the majority of the radiation dose to the tumour, thus sparing surrounding organs. In addition, doses of radiation are given in smaller fractions per treatment to allow for recovery of healthy cells by DNA repair.

1.2: Cellular plasticity

1.2.1 Stem cell plasticity

Cellular plasticity is a term used to describe the differentiation potential of a cell, which underlies the developmental hierarchy (Figure 1.4). Stem cells have high cellular plasticity and can self-renew and differentiate into multiple cell types. Maintenance of plasticity is dependent on both intrinsic and extrinsic factors, and cells with high plasticity can respond and adapt to these factors to change their phenotype accordingly (Lakshmipathy and Verfaillie, 2005). Transcription factors and epigenetic marks regulate plasticity by expressing genes required for self-renewal and by maintaining a 'naïve' state. The niche or microenvironment can have an impact on intrinsic properties of a cell as well. In humans, totipotent cells derived from the zygote represent cells with the highest cellular plasticity. Embryonic stem cells are pluripotent and derived from totipotent cells; they have the capacity to generate all cell lineages in the body. When cells differentiate during development, cell type specific proteins are expressed, thus limiting cell potency. Pluripotent cells that are primed for a certain cell lineage, or progenitor cells, are multipotent and have limited plasticity. Terminally differentiated cells are at the bottom of the hierarchy as they are fully committed to a specific cell type (Lakshmipathy and Verfaillie, 2005).

Cell differentiation during development has long been considered an irreversible process. However work by Yamanaka and others has shown that terminally differentiated cells can be reprogrammed into a stem-like state. Retroviral transduction of transcription factors Oct3/4, Sox2, KLF4 and c-Myc is sufficient to dedifferentiate human somatic cells into a pluripotent state, displaying functional and phenotypic similarities to embryonic stem cells (Takahashi et al., 2007). The discovery of induced pluripotency shows that plasticity can be established by stemness transcription factors aided by optimal growth medium *in vitro*. Following this finding, other groups

have shown that somatic cells can be reprogrammed into other cell lineages indirectly through a progenitor state, or directly via trans-differentiation. Together, research in cellular plasticity and reprogramming highlights the flexibility of cellular differentiation and dedifferentiation by manipulating transcription factor expression and the microenvironment.

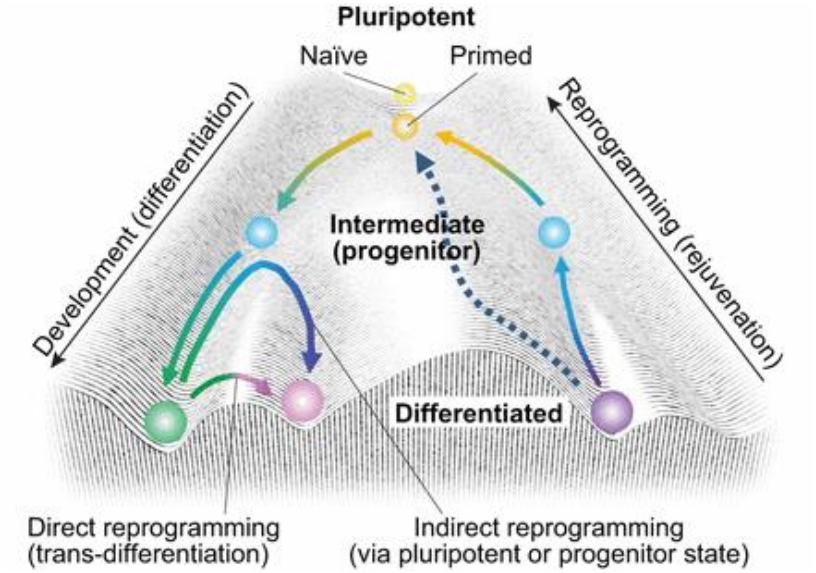


Figure 1.4 Waddington's epigenetic landscape of cell fate changes (Takahashi and Yamanaka, 2015)

1.2.2 Tumour heterogeneity

Tumour heterogeneity is a key contributor to therapy resistance. The emergence of heterogenous populations within a tumour can be explained by two hypotheses: the clonal evolution theory and the cancer stem cell theory (Rich, 2016). The clonal evolution theory posits that selective pressure within a tumour microenvironment leads to outgrowth of cells with genetic and epigenetic advantage. Since every cell is uniquely located in a niche, multiple subpopulations of cells with varying intrinsic phenotypes can stochastically form a tumour. The cancer stem cell (CSC) theory hypothesizes that only a biologically distinct subset of cells called cancer stem cells are

tumourigenic and contribute to long term tumour growth. In this model, a differentiation hierarchy exists where CSCs can divide asymmetrically to give rise to progenitor cells and the non-tumourigenic bulk of the tumour (Batlle and Clevers, 2017). Cancer stem cells have this high cellular plasticity characterized by their ability to self-renew and differentiate, much like normal tissue stem cells. The origin of CSCs may be the result of transformed tissue stem cells or progenitor cells, or dedifferentiation of neoplastic cells. CSCs are thought to be responsible for tumour initiation and metastasis and are often resistant to therapy, therefore, to prevent relapse the ideal treatment must target CSCs. The stochastic and CSC theories are not mutually exclusive, and it is likely that both are represented in human cancers, providing an explanation to therapy resistance and tumour relapse (Rich, 2016).

In vitro, CSCs can sometimes be identified by unique biomarkers and functional assays similar to the identifying criteria used for their normal tissue stem cell counterparts. For example, CSCs can be identified by expression of tissue specific stem cell markers. Low CD24 and high CD44 cell surface protein expression is used to identify breast CSCs, while CD133-positivity identifies glioma stem cells (Al-Hajj et al., 2003; Singh et al., 2003). Another marker, the aldehyde dehydrogenase ALDH1, is upregulated across multiple CSC types (Ginestier et al., 2007). Marker expression alone is not always accurate, and often requires co-expression of multiple markers to correctly identify CSC populations. Therefore assays that test functional phenotypes of CSCs often are more reliable. The side population assay is based on CSC ABC transporter expression. In CSCs, these pumps are able to efflux a variety of compounds. In the assay a lipophilic dye is used to stain the cells. Cells that lose dye retention due to efflux have been found to be stem-like (Golebiewska et al., 2011). *In vitro* sphere formation in suspension cultures tests self-renewal potential of cells. Yet the current gold standard assay for testing the stemness of cancer cells is the serial tumour

dilution assay, which determines the tumour-initiating potential of cells *in vivo* by xenografting different titres of cancer cells into mice (Kreso and Dick, 2014).

1.3: Radiation-induced plasticity

There is an existing paradox that cancer therapies can lead to adverse effects preventing complete tumour eradication. Preclinical and clinical studies over the past decades have documented that surgery, chemotherapy and radiotherapy all have the potential to implicate phenotypic and environmental changes that can prevent tumour eradication (Martin and Anderson, 2018). Radiation and some chemotherapies have been shown to promote plasticity in cancer cells and to induce metastasis. A literature review of radiation-induced plasticity will be discussed below.

1.3.1 Radiation-induced metastasis

Metastasis is the process of tumour cells disseminating from the primary tumour and establishing a secondary tumour at a distant site. Metastatic tumours are difficult to treat as they can be widely disseminated and sometimes remain undetected. Metastasis of cancer cells of epithelial origin require epithelial to mesenchymal transition (EMT), where cells first lose polarity and undergo phenotypic changes to increase mobility in order to escape into circulation. For metastatic tumours to form, cells must escape immune surveillance in circulation, exit and establish a new tumour at a distant site. These cells have considerable tumour initiating capacity as well as adaptability to various niches, which are characteristics closely tied to cellular plasticity.

Radiation therapy-induced metastasis has been observed in both preclinical and clinical studies for over 60 years (reviewed in Blyth et al., 2017). In 1949, Kaplan and Murphy published the first investigation of radiotherapy-induced metastasis in murine models. Despite the rarity of metastasis in untreated and adequately treated epidermoid carcinomas, clinical observations of under-

irradiated tumours disseminating to distant sites led to the speculation that radiotherapy has a role in metastasis. To test this hypothesis experimentally, the authors used a murine mammary carcinoma model. Subcutaneous tumours were locally irradiated with a single sublethal dose and surviving mice were examined for metastasis after 8 weeks. They found that irradiated mice had more frequent pulmonary metastases compared to unirradiated control tumours (Kaplan and Murphy, 1949). This work prompted further investigation on possible mechanisms of radiation-induced metastasis.

In 1991, von Essen proposed possible mechanisms of radiation-mediated enhancement of metastasis that remain relevant to this day. This includes release of tumour cells into circulation, bystander effects from non-tumour cells and directly induced changes in tumour cells (von Essen, 1991). The presence of circulating tumour cells (CTC) is an independent predictor of distant metastasis, however the significance of the timing of CTC presence (before, during or after irradiation) in establishing metastases is unclear (Janni et al., 2016). Furthermore, the extent of CTC release can vary between radiation modalities and treatment plans, illustrating the lack of knowledge in radiation-enhanced CTC release and the complexity of human tumour response (Blyth et al., 2017). Bystander effects of irradiated cells on non-irradiated cells may also promote malignant growth and metastasis. Because radiation can lead to systemic physiological changes, these effects can originate from many sources. Mixing lethally IR-damaged tumour cells with untreated tumour cells is found to enhance growth, suggesting an effect of radiation on cell-secreted factors (Seelig and Revesz, 1960). Irradiation alters the microenvironment of a cell as well; the tumour bed effect was coined when it was found that while pre-irradiated stroma reduces growth of implanted tumours, it can promote invasion and metastasis (Kuonen et al., 2012). Release of pro-metastatic factors such as TGF-β and IL1-β from normal tissue following

irradiation has been shown in mice (Biswas et al., 2007; Bouchard et al., 2017). Radiation can also lead to direct changes in tumour cell phenotype. For example, some irradiated cancer cells upregulate matrix metalloproteinase activity which aids in stromal invasion. However, the responses in various cell lines were not found to be always uniform or to correlate with cell motility and invasion (Fujita et al., 2015).

1.3.2 Radiation-induced stemness

Despite the effectiveness of cancer therapies in achieving tumour remission, there often will be some cells that escape killing due to heterogeneity in sensitivity to therapy. If the surviving cells have high plasticity, they may be capable of reconstituting a tumour or metastasize, leading to patient relapse. Using chemotherapy as an adjuvant to radiotherapy can aid in eradication of most cells, however cancer stem cells are frequently resistant to both treatments and their presence within a tumour at the time of treatment has been negatively correlated with patient outcome (Pallini et al., 2008; Ginestier et al., 2007). CSCs were found to exhibit characteristics that aid in radioresistance by dampening the effects of radiation, including activated DNA repair checkpoints and elevated free radical scavengers (Bao et al., 2006; Diehn et al., 2009). Radiation-induced enrichment of CSCs can be explained by selective pressure, but recent studies observe an increase in total CSC numbers that can't be explained by proliferation alone, suggesting the induction of stemness in non-stem cell like populations (Lagadec et al., 2012).

Maintenance of stemness in embryonic stem cells and induced-pluripotent stem cells is largely dependent on the expression of key pluripotency factors OCT4 and Sox2, and the transcriptional network driven by these regulators (Rizzino, 2013). With this knowledge, it seems possible that pluripotency factors are upregulated following radiation to induce stemness in non-stem cells. In hepatocellular carcinoma, radiation induces plasticity in non-tumourigenic cells. Using the side

population assay, a functional test of the ability of cells to efflux a dye based on the expression of drug transporters in CSCs, non-stem cells were sorted out and irradiated. Treated cells exhibited enhanced sphere formation, and increased expression of pluripotency factors OCT4 and Sox2 (Ghisolfi et al., 2012). In other tissues, treatment with sub-lethal doses of radiation not only leads to expression of stem cell markers, but also overexpression of an inhibitor of apoptosis, the protein survivin. Besides promoting survival following radiation by blocking apoptosis, survivin is also closely associated with many plasticity-maintaining pathways in neural stem cells (Dahan et al., 2014).

Although upregulation of pluripotency factors is commonly observed following radiation, the underlying mechanisms are unclear. OCT4 and Sox2 are expressed in embryonic stem cells. But as cells differentiate, Sox2 expression is limited to specific tissue stem cells and silenced in others, while OCT4 is ubiquitously inactivated and appears to have no role in adult stem cell maintenance (Dreissens and Blanpain, 2011; Lengner et al., 2007). In order to induce stemness, OCT4 and Sox2 levels must reach a specific threshold and stoichiometry as sub-optimal changes in protein levels can affect the efficiency of reprogramming (Rizzino, 2013).

It has previously been shown that transient polyploid cells formed following radiation upregulate pluripotency factor Nanog through increased transcriptional activity of OCT4 and Sox2. These proteins colocalize to PML nuclear bodies in p53 negative cells (Salmina et al., 2010). Approximately 5 days post-irradiation, polyploid cells undergo multi-polar reductive divisions and pluripotency factors can be passed on to daughter cells, thus increasing the fraction of stem-like cells within the population. Here the authors showed a pathway in which acquisition of stemness relies on formation of polyploid cells after radiation (Salmina et al., 2010). In this case, increased gene dosage in polyploid cells and accumulation of these factors at PML bodies may support their

interaction to drive pluripotent gene networks. Upregulation of pluripotency factors was not observed in all polyploid cells, and the majority of cells enter senescence via p16INKA4a activation or die by apoptosis after irradiation.

Lagadec and colleagues followed up on these observations by showing that radiation directly induces stem-like functional properties in previously non-tumourigenic breast cancer cells (Lagadec et al., 2012). Non-stem cells were sorted from the bulk population using three different stem cell parameters: high ALDH1 expression, surface markers $CD24^{low}/CD44^{high}$, or low proteasome activity, then treated with radiation and examined for changes in phenotype. In each case, non-stem cells acquired stem cell properties following irradiation in a dose-dependent manner. In addition, induced-stem cells were able to form mammospheres and seed tumours in mice at higher frequencies compared to their non-irradiated counterparts, illustrating self-renewal and tumourigenic potential reminiscent of cancer stem cells. Gene expression analysis revealed that induced stem cells expressed similar subset of genes as pre-existing CSCs, including stemness factors OCT4, Sox2, KLF4, and Nanog, presumably re-expressed in polyploid cells formed following radiation. Notch1 signalling and Nanog and Sox2 expression were also found to be required for establishing induced stemness. Pharmacological induction of polyploidy using the anti-mitotic drug noscapine was sufficient to mimic the phenotype of radiation-induced stem cells (Lagadec at al., 2012). Whether the source of stemness is the polyploid cell itself or its progeny was not specified by the authors.

The mechanism of radiation-induced stemness may be understood from examining signalling pathways activated by radiation that are also implicated in stem cell maintenance. These pathways may serve as potential therapeutic targets in the clinic. Notch signalling regulates cell fate, including the development of specific cell types from embryonic stem cells and the maintenance

of tissue-specific adult stem cells. Notch signalling is initiated by a notch ligand binding to a cell surface notch receptor, following which a cleavage event will release the intracellular domain of the notch receptor (NICD). This allows for the translocation of that domain to the nucleus and the transcriptional activation of target genes (Liu et al., 2010). Notch has been found to play an oncogenic role in many cancer types. However this role is dependent on tissue-specific epigenetic regulation and signal strength (Aster et al., 2017). The Notch receptor Notch-1 has been shown to be upregulated in breast cancer cell lines after radiation. Given its role in mammary epithelial stem cell self-renewal, Notch signalling may be a mechanism for radiation-induced stemness in breast cancer (Phillips et al., 2006). Subsequently, the expression of Notch receptors and ligands following radiation in breast cancer cells and whether inhibitors of Notch signalling could reduce cancer stem cell pool (Lagadec et al., 2013). *In vitro* studies with breast cancer cell lines expressing a reporter construct for CBF-1, a Notch target gene, show that NICD is transcriptionally active after a single dose of 3Gy radiation; *in vivo*, NICD is detectable in implanted tumours 4 hours post-irradiation. Expression of Notch receptors and ligands were upregulated after radiation, although the timing of upregulation differed between receptors and ligands and between doses. As mentioned, Notch signalling is dependent on cleavage of NICD from the surface bound receptors, which is carried out by the protease γ-secretase. In the absence of this protease, NICD is unable to translocate to the nucleus to activate Notch target genes. The authors found that inhibitors of γ-secretase ablate radiation-induced upregulation of Notch receptor 2 and Notch ligands DLL1, 3 and Jagged1. In addition, γ-secretase inhibition partially blocked radiation-induced formation of CSCs, seen by reduced sphere forming capacity, as well as sensitization to radiation (Lagadec et al., 2013). Despite this important insight into radiation-induced stemness, the use of γ-secretase inhibitors in other breast cancer models show opposite effects including increased proliferation

and radioprotection (Debeb et al., 2012). Radiation dose also changes Notch regulation: high dose radiation upregulates DLL3, an inhibitory notch ligand. The same effect was not observed after low dose fractions. Therefore, the use of Notch signalling inhibitors to block radiation-induced stemness would have to consider the timing of treatment, and cancer tissue type. Notch signalling is also dependent on cell-cell contact for activation. It likely is altered by changes to the microenvironment after radiation doses.

The non-receptor tyrosine kinase Src is involved in multiple cellular pathways including proliferation, motility and survival, and its activity is found to be upregulated in many tumours. Regulation of Src depends on the balance between upstream signalling from cell surface receptor tyrosine kinase and phosphatase activities (Sen and Johnson, 2011). The Src family kinases are expressed in self-renewing embryonic stem cells, and inhibition of their activity is important for the differentiation propensity of human pluripotent stem cells (Chetty et al., 2015). In non-small cell lung carcinoma, EGFR/Src/Akt signalling upregulates Sox2 expression in self-renewing, "side-population" cells (Singh et al., 2012). In breast cancer cells, radiation-induced Src activity is responsible for inducing an EMT phenotype; irradiation of breast cancer cells in the presence of Src inhibitor blocked radiation-induced migration and invasion, and expression of the EMT driver Slug (Kim et al., 2015). Src-dependent activation of downstream PI3K/AKT and p38 after radiation promotes migratory and invasive phenotypes and increases the population of $CD24^{low}/CD44^{high}$ stem-like cells. The radiation-induced stem cells showed increased resistance to follow-up treatments with chemotherapies and radiation treatment that was dependent on continuous Src activity. This study implicates the role of Src kinase in inducing EMT and stemness, however the mechanism of Src activation following radiation is unknown. The authors speculate

that radiation induces secretion of yet to be identified factors that activate upstream receptor tyrosine kinases.

Wnt is a secreted protein that binds to the extracellular domain of surface receptors, resulting in signal transduction leading to translocation of β-catenin to the nucleus as a transactivator. High Wnt activity is a marker for CSCs across multiple cancers, with both intrinsic and microenvironmental regulators of Wnt signalling contributing to elevated activity (de Sousa and Vermeulen, 2016). Wnt signalling has been implicated in conferring radioresistance to cancer cells by upregulating ROS scavengers and promoting CSC survival after IR (Zhao et al., 2018). In prostate cancer, Wnt directly upregulates ALDH1 expression in cells leading to radioresistance and stem-like properties (Cojoc et al., 2015). Wnt ligands are secreted by stromal cells, with one of the sources being tissue-associated macrophages that secrete Wnt via extracellular vesicles. In intestinal crypts, secretion of these extracellular vesicles is critical for regeneration of intestinal stem cells following radiation injury (Saha et al., 2016). The Wnt pathway also mediates radiation-induced metastasis in glioblastoma cells, possibly through crosstalk with radiation-induced EGFR signalling (Dong et al., 2015; Hu and Li, 2010). Although there is no direct link between radiation and Wnt activation, radiation-induced changes in the microenvironment and in other pathways have the potential to alter Wnt signalling and thereby to induce stemness.

The tumour microenvironment (TME) can determine the overall response of a tumour to radiation. The TME can consist of cancer-associated fibroblasts (CAFs), immune cells, blood and lymphatic vasculature, adipose cells and the extracellular matrix, all of which have the potential to contribute to tumourigenesis (Wang et al., 2017). Tumour-associated macrophages (TAMs) are recruited to tumours via cytokines where they can promote immunosuppression or anti-tumour inflammatory responses, depending on the macrophage polarization. Immune activation following radiation is

largely dependent on macrophage recruitment and activity, and recent studies find that TAMs are able to secrete pro-metastatic and stemness-promoting factors into the TME, implicating a role of TAMs in radiation-induced stemness (Chen et al., 2018; Shi and Shiao, 2018). Recently, CAFs were shown to promote stem-like properties in hepatocellular carcinomas through an IL-6/STATs/Notch signalling pathway (Xiong et al., 2018). Irradiated CAFs when co-cultured with non-irradiated pancreatic cancer cells enhance the invasive potential by altering their secretory phenotype (Ohuchida et al., 2004). The ECM is an "inter-cellular" mesh composed of various proteins, proteoglycans, and minerals that aid in cell-cell communication, adhesion and signal transduction. It serves as an anchor for cells, providing structural integrity as well as binding sites for growth factors. It establishes specialized niches for development and stem cell maintenance (Walker et al., 2018). In fast proliferating tumours the ECM has a dynamic relationship with surrounding cells. As tumours increase in size, mechanical strain placed on the basement membrane and collagen deposition has been shown to promote migration. In glioma cells, radiation-induced Src activity leads to matrix metalloproteinase-2 expression and changes in mechanical tension within the ECM promotes invasion (Park et al., 2006; Yoo et al., 2018).

1.4 Hypoxia-induced plasticity

Fast growing tumours typically have inadequate oxygenation due to poor vascular architecture and leaky vessels. In addition to the high metabolic activity and increased oxygen consumption of proliferative cells, inefficient oxygen delivery leads to areas of low to no oxygenation. In well vascularized peripheral tissues, the physiological oxygen level is approximately 5%, while hypoxic regions range from 0.3-4.2% oxygen (Mckeown, 2014). Cancer cells that can adapt to and tolerate hypoxic niches not only are chemoresistant due to impeded drug delivery from blood vessels but

are also radioresistant due to the "oxygen effect". The oxygen effect is named after observations that well oxygenated tumours are more sensitive to radiation compared to hypoxic tumours. The oxygen enhancement ratio (OER) calculates the ratio of a dose given at hypoxia to a dose given at atmospheric oxygen pressure, leading to the same biological effect (i.e. cell survival). However, the oxygen effect is only observed when molecular oxygen is present at the time of irradiation. The role oxygen plays in radiation-induced damage can be explained by the oxygen fixation hypothesis, which postulates that when hydroxyl radicals generated from ionized water molecules encounter macromolecules such as DNA, radicals are formed from these macromolecules. These radicals can interact with oxygen to form peroxyl radicals that are more damaging and difficult for the cell to chemically repair, thus resulting in more permanent or "fixed" DNA damage. In hypoxic cells, the DNA damage caused by free radicals is more readily repaired due to the lack of oxygen (Grimes and Partridge, 2015).

Human embryonic stem cells grown under hypoxia are more likely to maintain pluripotency and not spontaneously differentiate. Unsurprisingly, CSCs have been found to preferentially reside in hypoxic niches as well (Ezashi et al., 2005; Marie-Egytienne et al., 2013). The major signalling pathway of hypoxia involves the hypoxia-inducible (HIF) transcription factors, dimers composed of α and β subunits. While the α subunits are only stably expressed under hypoxia, the β subunits are constitutively expressed. HIF signalling allows for adaptive responses to TME changes in oxygen by activating survival pathways such as a metabolic shift, proliferation and angiogenesis. Two homologous HIFα proteins are expressed in cells: HIF1α is universally expressed whereas HIF2α expression is more tissue specific. Under normoxia, HIF1α activity may be downregulated via several mechanisms, most commonly through oxygen-dependent pathways regulating both protein stability and transactivation via post-translational modifications. Oxygen-independent

activation of HIF1α can occur through oncogenic mechanisms such as growth factor signalling, loss of VHL or binding to Hsp90 (Masoud and Li, 2015). HIF1α is responsible for expanding CD133-positive glioma stem cell populations, which is augmented by hypoxia-induced growth factor signalling (Soeda et al., 2009). Increased expression of stem cell markers OCT4, Sox2, Myc and Nanog under hypoxia was found to be dependent on HIF across 11 cancer cell lines. Furthermore hypoxia-associated gene expression signatures overlapped with human embryonic stem cells, indicating a role of HIF in implementing stem cell phenotypes under hypoxia (Mathieu et al., 2011). It was previously found that maintenance of neural progenitor cells under hypoxia is dependent on HIF1α-mediated Notch signalling, and more recently in ovarian cancer, a hypoxia/Notch-mediated activation of stem cell marker Sox2 was found to promote stem cell characteristics (Gustafsson et al., 2005; Seo et al., 2016). As previously discussed, Notch signalling is a major regulator of the CSC phenotype. Thus its interaction with HIF1α signalling postulates a mechanism for hypoxia-induced stemness.

HIF2α expression was first thought to be restricted to vascular endothelial cells during embryonic development. However HIF2α expression was later observed in adult tissues as well as in transformed cells and tumour-associated stromal cells. Although HIF1α and HIF2α exhibit overlapping patterns of gene expression in response to hypoxia, recent studies reveal non-overlapping functions of the two transcription factors (Hu et al., 2003). Specifically, HIF2α directly transcribes the OCT4 gene in embryonic stem cells and primordial germ cells to maintain an undifferentiated state. This activity contributes to teratoma tumourigenesis *in vivo*. This unique function of HIF2α may explain why HIF2α more effectively promotes tumour growth compared to HIF1α in renal cell carcinoma xenografts. However, discrepancies exist in HIF2α targets between cell types (Covello et al., 2006).

1.5 Sox2 background

1.5.3 Yamanaka factors

In 2007, Yamanaka and colleagues reported that retroviral transduction with the transcription factors OCT4, Sox2, KLF4 and c-Myc is sufficient to dedifferentiate human somatic cells into a pluripotent state resembling human embryonic stem cells (Takahashi et al., 2007). Induced-pluripotent stem cells (iPSCs) can be directed to differentiate into multiple cell types including neural, muscle and cardiac cells and display *in vivo* pluripotency via teratoma formation. Initially the efficiency of creating iPSCs was relatively low and required weeks to achieve, despite expression of Yamanaka factors across the population. It was thought that cell intrinsic epigenetic patterns and extrinsic interactions may play a role in determining the dynamics of direct reprogramming *in vitro*. Direct reprogramming appeared to be a mostly stochastic process that occurred continuously up to 18 weeks in some studies. This seemed to indicate that certain cells may be more susceptible to reprogramming at earlier stages or at later time points, but that all cells had the potential to give rise to iPSCs. However, this process was found to be amenable to acceleration by inhibiting the p53/p21 pathway or by overexpressing Lin28 or Nanog (Hanna et al., 2009). When epigenetic barriers are removed by knockdown of the histone methyltransferase DOT1L, OCT4 and Sox2 alone can directly reprogram human fibroblasts (Onder et al., 2012). Undoubtably OCT4 and Sox2 are key regulators in establishing pluripotency in iPSCs and in embryonic stem cells. Thus the expression of both have been used as markers for stemness in CSCs. Interestingly, follow-up studies revealed that while early reprogramming occurred stochastically, late stages were hierarchical and dependent on activation of endogenous Sox2 to drive gene expression and to activate the pluripotency circuitry (Buganim et al., 2012).

1.5.2 SOX family proteins

Sox2 is part of a family of 20 evolutionarily conserved SRY-related HMG box (Sox) proteins, originally identified based on sequence similarity to the high-mobility-group (HMG) domain of the mammalian testis-determining factor SRY. Sox proteins with more than 80% sequence similarity in their HMG domain are further clustered into sub-groups from A to H (Sarkar and Hochedlinger, 2013; Schepers et al., 2002). The HMG domain is the region that binds to DNA in the minor groove with sequence-specificity. From studies on SRY HMG domains, it is known that they not only have high binding affinity to target DNA motifs, they can also induce an 80° to 90° bend in the chromatin which is functionally critical as it aids in the assembly of nucleo-protein complexes (Koopman, 2010). Sox family members are transcription factors and proteins within the same subgroup typically display redundant biological functions due to their biochemical similarities. However Sox proteins between subgroups have evolved biologically distinct functions that were attained through HMG domain flanking sequences that affect gene targets, dimerization with other Sox proteins or co-factors, or unique post-translational modifications (Wegner, 2009). Sox family members all contain a DNA-binding HMG domain, but members of subgroups B1, C, F and E contain transactivation domains, and subgroup B2 members have repression domains. Sox2 is part of subgroup B1, characteristically having an HMG domain, two nuclear localization signals, one nuclear export signal, and a C-terminal transactivation domain.

1.5.3 Sox2 expression

Sox2 is an essential gene. Initial studies show that while Sox2 null blastocysts fail to survive following implantation, lasting maternal Sox2 is also required for pre-implantation development of the trophectoderm and derivation of embryonic and trophoblast stem cells (Avilion et al., 2003; Keramari et al., 2010). In embryonic stem cells, OCT4, Sox2 and Nanog are the central regulators

that specify pluripotency. Maintenance of stemness relies on a positive-feedback loop with OCT4, Sox2 and Nanog binding and targeting their own transcription as well as that of over 300 genes including Wnt and TGF-β pathways (Boyer et al., 2005). OCT4 and Sox2 can form a heterodimer and are able to bind together at Oct-Sox enhancers. Conditional knockout of Sox2 in mouse embryonic stem cells leads to trophectoderm differentiation, and while the lack of Sox2 does not affect Oct-Sox enhancer activity, suggesting a redundancy in Sox family protein function, Sox2 expression was found to be required to maintain OCT4 levels at an optimal level for pluripotency (Masui et al., 2007). In addition, maintenance of stemness is sensitive to Sox2 levels, as both over-expression or down-regulation of Sox2 leads to trophectoderm differentiation in human embryonic stem cells, suggesting that Sox2 is under tight regulatory control in these cells (Adachi et al., 2010). Studies in mouse and human embryonic stem cells indicate variations in stemness maintenance between organisms, but both stem cells rely on the activity of OCT4, Sox2 and Nanog. In human embryonic stem cells, Sox2 can be replaced by Sox3 to regulate self-renewal in conjunction with OCT4 and Nanog, and SOX2 knockdown does not affect levels of the other factors. Compared to the previous mouse models observed by Boyer and colleagues, where OCT4/Sox2/Nanog co-binding regulates a large subset of genes, the same factors appear to regulate stemness individually by repressing unique cell fates in human cells (Boyer et al., 2005; Wang et al., 2012). In adult tissue, Sox2-positvity marks rare unipotent and multipotent stem cells that have been identified to maintain homeostasis in various locations including reproductive organs, the gastrointestinal tract, lens, trachea and oral cavity (Figure 1.5). Importantly, lineage tracing studies show that Sox2-positive cells originate from early epithelial progenitors during fetal development and persist into adulthood, highlighting the role of Sox2 during all stages of development (Arnold et al., 2011). Pertaining to this, SOX2 mutations have been shown to cause anophthalmia-esophageal-genital

syndrome, which is characterized by poorly developed or no eyes, esophageal atresia, male urogenital abnormalities, hearing loss, delayed development of motor skills, and pituitary defects (Williamson et al., 2006). The significance of Sox2 dosage during development is illustrated in the spectrum of ocular-related diseases caused by various mutations in the SOX2 gene, mostly affecting the coding region leading to premature truncation mutants, or DNA-binding and transactivation domain mutants (Schneider et al., 2009).

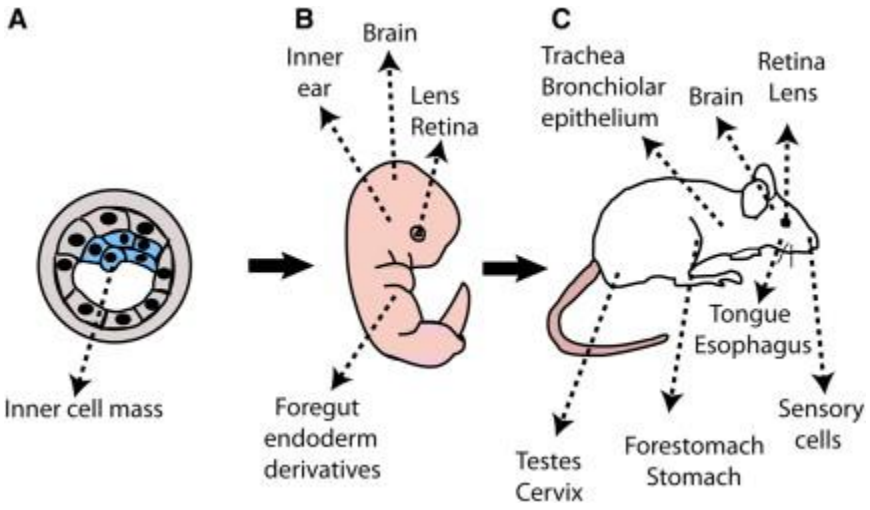


Figure 1.5 Sox2 expression throughout development (Driessens and Blanpain, 2011)

1.6 Sox2 cofactors and transcriptional targets

Sox2 has various sets of transcriptional targets depending on the tissue and available co-factors. In embryonic stem cells, proteomic studies show that Sox2 is integrated in large protein complexes consisting of transcription factors, chromatin modifying factors and co-factors involved in self-renewal (Ding et al., 2012). These large protein complexes, often with specific stoichiometry, likely act as "hubs" of transcriptional regulation and functionally influence each other. Sox2 co-

binds a large network of genes with OCT4 and Nanog, and the universal coactivator p300 has been found to associate with this complex (Boyer et al., 2005; Chen et al., 2008). Sox2 can form a heterodimer with OCT4 (also called POU5F1) via protein-protein interaction and cooperatively bind on enhancer elements or promoters with the respective consensus sequences for HMG/POU cassettes (Reményi et al., 2003). Sox2-OCT4 dimers are responsible for transcriptional activation of a large set of genes including growth factor FGF-4, stem factor Nanog, embryonic stem cell coactivator UTF1, and F-box protein Fbx15 (Ambrosetti et al., 1997; Kuroda et al., 2005; Nishimoto et al., 2001; Tokuzawa et al., 2003). A well studied example of cooperative Sox2-OCT4 binding is at the FGF-4 enhancer element, where the DNA binding domains function to promote protein-protein and protein-DNA interactions. Changes to the length of base pairs between HMG/POU cassettes abrogate enhancer activation, indicating the importance of spatial arrangement of recognition sites for transcriptional regulation. Despite the ability of Sox2-OCT1 dimers to bind to the same HMG/POU cassette, these dimers are unable to initiate transcription of FGF-4, as Sox2-OCT4 dimers are uniquely positioned for transactivation (Ambrosetti et al., 1997). Interestingly, the UTF1 regulatory element only binds Sox2-OCT4 while precluding Sox2-OCT1 complexes, despite only having a one base-pair difference to the FGF-4 regulatory element (Nishimoto et al., 1999). Sox2 and OCT4 also auto-regulate their own expression through a positive feedback loop (Okumura-Nakanishi et al., 2004; Chew et al., 2005). This interdependency of Sox2 and OCT4 generates a bi-stable system wherein embryonic stem cells either maintain self-renewal or differentiate, which may be the reason why changes in OCT4 or Sox2 dosage is sufficient to induce differentiation.

As cells undergo development, lineage-specific factors are expressed to aid in differentiation. In the early steps of lens development, Sox2 dimerizes with transcription factor Pax6 to initiate lens

placode formation by activating the lens-specific gene δ-crystallin (Kamachi et al., 2001). Pax6 is an essential gene, and like Sox2, heterozygous mutations have been shown to cause a spectrum of ocular and developmental disorders in humans (Hever et al., 2006). Sox2-Pax6 complexes bind DNA in a similar fashion to Sox2-OCT4 dimers, whereby complex binding to the consensus cassette on DNA is highly sensitive to base pair spacing between SOX and Pax6 recognition sites. In neural stem cells, Sox2 binds to Chd7, an ATP-dependent chromodomain-binding helicase that is responsible for chromatin remodelling. Sox2 and Chd7 are expressed at similar levels in the brain, olfactory bulbs, eyes and inner ears, and are found to co-regulate a subset of genes, including Notch ligand Jagged-1 and Gli2 and Gli3 of the Sonic hedgehog pathway (Engelen et al., 2011). These Sox2-Chd7 regulated genes are mutated in certain human diseases, and interestingly, the CHARGE syndrome associated with Chd7 mutation has many overlapping phenotypes with the Sox2-associated anophthalmia-esophageal-genital syndrome. In adult tissue, the Sox2-Chd7 complex is important in oligodendrocyte precursor cell activation following spinal cord injury to induce expression of genes that drive proliferation and promote tissue regeneration (Doi et al., 2017). Sox2 also cooperates with another POU transcription factor, BRN2, in neural progenitor cells. This heterodimer has functions different from Sox2-OCT4 complexes in embryonic stem cells (Lodato et al., 2013). Sox2-BRN2 binds a large group of distinct enhancers in neural progenitor cells, such as the intronic enhancer within the Nestin gene and the 3' enhancer SRR2 of the Sox2 gene (Tanaka et al., 2004; Miyagi et al., 2006).

Although Sox2 is usually a transcriptional activator, it has been found to associate with co-repressors of the groucho-related gene (Grg) family in neural stem cells. This interaction has been proposed to repress the expression of effectors involved in neuronal differentiation and help maintain stemness (Liu et al., 2014). In high Sox2-expressing radial glia cells, binding to low

affinity recognition sites on DNA and interaction with Tcf/Lef proteins mediates the recruitment of Gro/Tle corepressors and subsequent repression of Cyclin D1 transcription, which promotes apical symmetrical division and maintaining a dedifferentiated state (Hagey and Muhr, 2014).

1.7 Mechanisms of Sox2 regulation

Sox2 dosage has proven to be essential for maintenance of stemness, as both the increase and decrease in protein levels lead to differentiation of embryonic stem cells. In order to maintain activity at optimal levels, Sox2 is subject to multiple levels of regulation, from transcription over translation, to a myriad of post-translational modifications.

1.7.1 Transcriptional and post-transcriptional regulation

Sox2 transcription has been shown to be regulated through gene-proximal and distal enhancer elements. The first identified proximal enhancers are the Sox2 regulatory regions 1 and 2 (SRR1 and SRR2): SRR1 is located 5' of the transcriptional start site whereas SRR2 is downstream of the Sox2 gene. Chromatin immunoprecipitation studies show that OCT4 is bound to SRR1 in embryonic and neural stem cells (Catena et al., 2004). Similar to the FGF4 and UTF1 regulatory elements bound by Sox/OCT complexes, SRR2 contains HMG/POU consensus motifs. However, the SRR2 sequence is permissive to Sox2-OCT4 and Sox2-OCT6 complex binding, and its activity does not require the same cooperative binding observed with Sox2-OCT4 dimers at FGF4 enhancers (Tomioka et al., 2002). Interestingly, SRR1 and SRR2 element activity is restricted to embryonic and neural stem cells, suggesting that the majority of Sox2 transcription in stem cells is determined by enhancer activity that is dependent on an autoregulatory positive feedback loop and the presence of interacting POU factors (Tomioka et al., 2002; Miyagi et al., 2004). For example, in the developing brain Sox2 expression is restricted to neural stem and progenitor cells

in the telencephalon, which is conferred by specific POU factor expression and Sox2-POU interaction at SRR2 (Miyagi et al., 2006). One recent study identified three more downstream enhancers of the Sox2 gene: a proximal enhancer, SRR18, and two distal enhancers, SRR107 and SRR111, that are located more than 100k base-pairs downstream of the Sox2 transcriptional start site (Zhou et al., 2014). While deleting the proximal enhancers SRR1, SRR2 and SRR18 in embryonic stem cells had no negative effect on Sox2 transcription, loss of the distal enhancers led to a significant decrease in Sox2 transcripts, and distal enhancer activity is required for Sox2 transcription in embryonic stem cells. The distal enhancers SRR107 and SRR111 act as a "Sox2 control region" by forming a large chromatin loop to interact with the promoter region of the Sox2 gene, mediated by bound stemness factors and coactivators.

Sox2 enhancers have been reported to be bound by factors other than Sox2 and OCT4 as well. YAP1 of the Hippo pathway has been reported to activate Sox2 expression through the enhancer SRR1 by forming dimers with OCT4 in dedifferentiated lung cancer cells (Bora-Singhal et al., 2015a). YAP1-OCT4 dimers were elevated in the tumour compared to normal tissue and conferred stem-like properties such as drug efflux and self renewal that were dependent on this interaction. In adult neural stem cells originating from the subependymal zone, p21 associated with SRR2 to repress Sox2 expression, which is required for the maintenance of the stem cell population and the reduction of replicative stress (Marqués-Torrejón et al., 2013). Although there appear to be conflicting reports as to what transcription factors are bound at different enhancers, and which regulatory regions contribute the most to Sox2 transcription in different contexts, self-regulation of Sox2 transcription at enhancers is limited to the presence of the appropriate binding partners at corresponding enhancer elements.

In cancer cells, aberrant expression of transcription factors or dysregulated oncogenic signalling pathways may drive Sox2 expression that is otherwise limited to stem cells. Another SOX family member, Sox4, that is not known to be involved in stemness, regulates Sox2 expression through the 3' enhancer of Sox2. This regulation is controlled by TGF-β signalling in glioma-initiating stem-like cells (Ikushima et al., 2009). The transcription factor FOXM1 is a regulator of Sox2 expression in glioblastoma cells by directly binding the promoter region and upregulating transcription. FOXM1 is a well-known regulator of multiple cellular pathways including cell cycle, apoptosis and DNA damage repair. However in glioblastoma FOXM1 activity not only contributes to aberrant Wnt signalling, increased DNA damage repair, and therapy resistance, but also the acquisition of stemness through Sox2 induction (Lee et al., 2015). Similarly, Gli1 of the sonic hedgehog pathway binds the promoter region of Sox2 in non-small cell lung cancers and regulates Sox2 expression (Bora-Singhal et al., 2015b).

Transcription can also be regulated through epigenetic mechanisms in the forms of DNA methylation or histone modifications. Genome-wide sequencing of embryonic stem cells and neural progenitor cells reveal that the Sox2 gene is rich in histone markers associated with active promoter, H3K4me3, and active transcription, H3K36me3, that extend beyond the coding regions of the gene, which may regulate non-coding transcription or activity at proximal and distal enhancers (Mikkelsen et al., 2007). Interestingly, in embryonic fibroblasts the Sox2 promoter is instead marked with repressive mark, H3K27me3, indicating restriction of Sox2 expression as pluripotent cells differentiate. DNA methylation is another cellular mechanism to regulate transcription of Sox2. In glioblastoma, where Sox2 overexpression plays a role in CSC maintenance, Sox2 promoter analysis shows hypomethylation of CpG islands in patient samples and cell lines. This suggests that aberrant Sox2 expression in glioblastoma is in part due to loss of

promoter repression (Alonso et al., 2011). In contrast, the Sox2 promoter is hypermethylated in endometrial cancer and linked to a worse outcome, indicating that Sox2 downregulation may also be involved in carcinogenesis (Wong et al., 2010).

Post-transcriptional regulation of Sox2 is dependent on microRNAs (miRNAs) that have sequence specificity for target mRNAs to block translation or to induce degradation. Expression of miRNAs is important in maintaining Sox2 levels at an optimal level in stem cells. For example, miR-200c and miR-9* are found to target the 3' untranslated region of the Sox2 gene to inhibit Sox2 expression, and upregulation of both miRNAs is associated with differentiation of neural stem and progenitor cells. Additionally, Sox2 and E2F3 are involved in a negative feedback loop in regard to Sox2 regulation by activating the transcription of miR-200c (Peng et al., 2012). In glioma stem cells, ID4 supresses miR-9* expression, leading to upregulated Sox2 expression and self-renewal (Jeon et al., 2011). miRNAs that downregulate Sox2 expression and prevent induction of stemness in cancer cells are considered to be tumour suppressors, and negative regulators of tumour suppressive miRNAs may be potential therapeutic targets for cancer treatment.

1.7.2 Translational and post-translational regulation

Translational regulation of Sox2 has been reported to be mediated by the eukaryotic translation initiation factor 4E (eIF4E), which is a 5' mRNA cap binding protein that directs ribosomes to mRNA molecules in order to initiate translation. eIF4E upregulation is observed in many cancers where it is proposed to increase the translation of malignancy-related mRNAs. Interestingly, eIF4E is found to translationally activate Sox2 in glioma-initiating cells and irradiated pancreatic cancer cells (Ge et al., 2010; Mamane et al., 2004; Yu et al., 2016). IR-mediated activation of caspase-3 leads to a signalling pathway involving PKCδ, p38 kinase and MNK1 kinase that phosphorylates and activates eIF4E in pancreatic cancer, resulting in increased Sox2 translation and proliferation.

The mechanism of Sox2 selective upregulation by eIF4E is suggested to be through the 5' untranslated region of the Sox2 mRNA, as regions with high GC content that are highly structured have higher a affinity for eIF4E. Translation initiation at GC-rich Sox2 mRNA is therefore more likely to be targeted when eIF4E is upregulated (Mamane et al., 2004).

Post-translational modifications (PTMs) are enzymatic, covalent modifications of a protein that can regulate its activity, localization or interaction with other molecules. PTMs are an effective way of rapidly modulating proteins and can occur at any time point following translation. The Sox2 protein is subject to many types of PTMs including phosphorylation, acetylation, methylation, SUMOylation, ubiquitylation and glycosylation (Figure 1.6). The implications of the different modifications will be discussed here.

Phosphorylation is the addition of a phosphate group on an amino acid and introduces a negative charge. Sox2 phosphorylation at multiple sites have been proposed to be involved in stem cell maintenance. Akt1 kinase-mediated phosphorylation at T118 enhances the stability and transcriptional activity of Sox2 and this PTM is involved in the self-renewal capacity of human embryonic stem cells (Fang et al., 2014; Jeong et al., 2010). Also other Sox2 phosphorylation sites have been implicated in stemness; for example, Aurora B kinase-mediated phosphorylation at S220 and S251 increases the proportion of stem-like cancer cells (Qi et al., 2016). The cyclin dependent kinase CDK2 phosphorylates Sox2 at S39 and S253, which is required for induced-pluripotency associated reprogramming, but is dispensable for embryonic stem cell maintenance (Ouyang et al., 2015). Similarly, while CDK2 phosphorylation at S39 maintains self-renewal in neural stem cells, loss of the phosphorylation site through the cleavage of Sox2 induces neuronal differentiation. Interestingly, the truncated form of Sox2 is still able to bind to neuron-specific

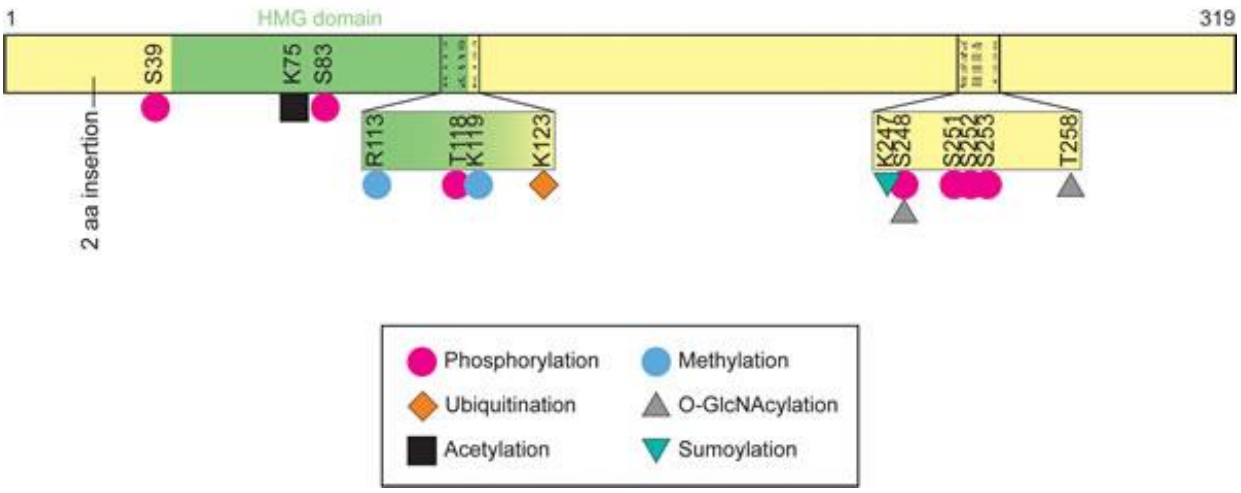


Figure 1.6 Post-translational modifications of mouse Sox2. 2 amino acid insertion refers to human Sox2 sequence (Wuebben and Rizzino, 2017).

genes through its HMG domain and effectively drive differentiation (Lim et al., 2017). Taken together, Sox2 phosphorylation could be a marker for stem-like cells in cancer as well. Indeed, in breast cancer, Sox2 phosphorylation at T116 marks cancer cells with high tumourigenicity and stem-like properties (Gupta et al., 2018). In lung cancer, PRKCI is co-amplified with SOX2 on chromosome 3q26, and PRKCI phosphorylates Sox2 at T118 leading to increased Sox2 binding to the HHAT promoter. HHAT encodes the Hedgehog acyl transferase that is a rate limiting enzyme in the hedgehog signalling pathway, thus creating a cell-autonomous signalling axis that drives tumourigenesis and stemness (Justilien et al., 2014).

Phosphorylation can also act as an intermediate PTM to recruit other modulators such as SUMO-ligases. SUMOylation is the addition of small ubiquitin-like modifiers (SUMO) molecules, that regulate protein structure or subcellular localization. For example, Sox2 phosphorylation at three consecutive sites, S249, S250 and S251 occurs within a phosphorylation dependent SUMOylation motif identified also in other SOX family proteins (Van Hoof et al., 2009). The subsequent

SUMOylation at an upstream residue K247 interferes with DNA binding of Sox2 with target genes, such as FGF4 and Nanog, through conformational changes in protein structure that affect the HMG domain or its interaction with OCT4 (Tsuruzoe et al., 2006; Wu et al., 2012).

Acetylation involves the transfer of an acetyl group from the acetyl co-enzyme A to a target protein. Sox2 is acetylated by p300/CBP on K75 located within the nuclear export signal in the HMG domain. This promotes Sox2 association with the nuclear export machinery and its subsequent cytoplasmic localization (Baltus et al., 2009). This PTM was found to be induced during embryonic stem cell differentiation, and when cellular acetylation is increased, Sox2 is targeted for ubiquitin-mediated degradation, although the link between acetylation and ubiquitylation is unclear. Follow-up studies show that the lysine deacetylase SIRT1 binds Sox2 to remove the K75 acetyl modification, leading to nuclear retention and stabilization of Sox2. This in turn is important for maintenance of stemness in bone marrow-derived mesenchymal stem cells (Yoon et al., 2014).

Methyltransferases add methyl groups onto proteins. Sox2 protein stability is regulated by a methylation-phosphorylation switch in embryonic stem cells, whereby methylation targets Sox2 for proteasomal degradation and phosphorylation at an adjacent site blocks methylation. The lysine methyltransferase Set7 mono-methylates Sox2 at K117. K117 methylation inhibits Sox2 transcriptional activity and recruits the E3 ubiquitin ligase WWP2 that binds Sox2 through its HECT domain, leading to the subsequent Sox2 ubiquitylation and proteasomal degradation (Fang et al., 2014). This pathway is inhibited when T118 is phosphorylated by Akt1, as previously discussed, resulting in Sox2 stabilization and embryonic stem cell maintenance. Sox2 is also monomethylated at K42 by Set7 and targeted for proteasomal degradation through L3MBTL3 binding-mediated recruitment of E3 ubiquitin ligase CRL4DCAF5 (Zhang et al., 2019). In contrast, Sox2 can be protected from proteolysis via demethylase LSD1 activity or by PHF20L1 binding to

methylation sites to prevent the interaction with E3 ligases – this is true for both methylation sites (Zhang et al., 2018). Sox2 transcriptional activity can be regulated by methylation as well. The protein arginine methyltransferase CARM1 di-methylates Sox2 at R113 to enhance Sox2-mediated transactivation and increases self-association of Sox2 proteins, with no observed impact on protein stability or localization (Zhao et al., 2011).

Ubiquitylation is a common PTM and can be the transfer of a single or a chain of ubiquitin moieties. K48-mediated poly-ubiquitylation of proteins via ubiquitin ligases targets them to the 26S proteasome for proteolysis. Ubiquitylation of a protein takes part in multiple steps involving three enzymes that regulate activation (E1), conjugation (E2) and ligation (E3) of ubiquitin onto the substrate. As mentioned above, Sox2 PTMs can recruit ubiquitin ligases leading to protein degradation. In neural progenitor cells, several enzymes determine Sox2 ubiquitylation status; a balance between $CUL4A^{DET1\text{-}COP1}$ ubiquitin ligase and OTUD7B de-ubiquitylase activity directly govern differentiation and Sox2 stability (Cui et al., 2018). In embryonic stem cells, the ubiquitin conjugating E2 enzyme, Ube2s forms K11 linked poly-ubiquitin chains directly on Sox2 at K123, marking it for proteolysis and neuroectoderm differentiation (Wang et al., 2016).

O-linked-N-acetylglucosamine (O-GlcNAc) is a molecule added to proteins and a form protein glycosylation. Sox2 O-GlcNAcylation is mediated by O-GlcNAc transferase, OGT, and is associated with the pluripotent state of embryonic stem cells. Three residues in Sox2 are proposed to be modulated, S248, T258 and S259, the latter two being within the transactivation domain (Myers et al., 2011). Interestingly, while T258A and S259A double mutants lead to reduced Sox2 transcriptional activity and reprogramming efficiency, S248A, T258A and S259A triple mutants had no such effect (Jang et al., 2012). This may be due S248 being a site for both phosphorylation and O-GlcNAcylation, resulting in a competition between OGT and kinase activity; if either S248

modifications already reduce Sox2 activity, a S248A mutant that increases Sox2 activity may cancel out effects of T258A and S259A mutants in a triple mutant setting (Jang et al., 2012). Indeed, follow up studies show that mutation of S248A that prevents O-GlcNAcylation promotes reprogramming efficiency of somatic cells, suggesting that O-GlcNAcylation of Sox2 at S248 is inhibitory of pluripotency (Myers et al., 2016).

1.8 Sox2 in cancer

Aberrant Sox2 expression has been detected in many types of cancers and is associated with stem-like properties of CSCs. Due to the tissue-specific activities of Sox2, either increased or decreased expression can lead to different prognoses between cancer types.

Sox2 has a large role in neural stem cell maintenance and differentiation. Unsurprisingly, increased Sox2 is detected in gliomas as well. Examination of patient samples and cell lines show that high Sox2 levels correlate with high grade, undifferentiated glioblastomas and are associated with poor prognosis (Annovazzi et al., 2011). An increase in Sox2 transcription in glioma has been reported to be due to Sox2 promoter hypomethylation, with overexpression of both Sox2 mRNA and protein detectable in glioblastoma samples (Alonso et al., 2011; Schmitz et al., 2007). Sox2 contributes to the glioma stem cell phenotype, including tumourigenicity and drug resistance. ID4 suppression of the Sox2-targeting miRNA, miR-9*, leads to de-repression of Sox2. High Sox2 levels lead to increased self-renewal and expression of ABC transporters that mediate drug resistance (Jeon et al., 2011). Sox2 expression promotes proliferation and tumourigenicity of glioma stem cells. However Sox2 dosage appears to remain important for stem cell maintenance also in glioma, as both silencing and overexpression of Sox2 can lead to loss of proliferative and stem-like properties (Cox et al., 2012; Gangemi et al., 2009).

Sox2 expression is observed in the various subtypes of breast carcinomas, although one study finds that it is more frequently expressed in basal-like breast cancers (Rodriguez-Pinilla et al., 2007). Together with β-catenin, Sox2 regulates Cyclin D1 expression to drive cell cycle progression and proliferation in breast cancer cells (Chen et al., 2008). Sox2 also promotes breast CSC formation and stem cell properties. Studies in cell lines show that Sox2 mediates mammosphere and *in vivo* tumour formation independent of OCT4 or Nanog, and elevated expression is dependent on an activated upstream enhancer SRR1 (Leis et al., 2011). In estrogen receptor-positive breast cancer cells estrogen reduces the stem cell pool, however expression of Sox2 in CSCs downregulates estrogen receptor expression. Sox2 also mediates estrogen receptor inhibitor (tamoxifen) resistance by activating Wnt signalling and overcoming the growth inhibitory effects, which leads to a worse prognosis for Sox2 high staining tumours (Piva et al., 2014; Simões et al., 2011).

In osteosarcoma, Sox2 is also overexpressed and maintains CSC self-renewal. Yet contrary to observations in breast cancer, Sox2 inhibits Wnt signalling in osteosarcoma to sustain an undifferentiated state (Basu-Roy et al., 2012). Follow-up studies show that Sox2 further promotes CSC properties and proliferation by transcriptional repression of Hippo pathway effectors, resulting in active YAP signaling. This pathway activation by Sox2 is found in glioblastoma as well (Basu-Roy et al., 2015). The essential role of Sox2 and YAP in osteosarcoma growth is further highlighted by Sox2 deletion resulting in the lack of tumour formation in mouse osteosarcoma models. This may indicate a reliance on Sox2 activity in bone cancers (Maurizi et al., 2018).

Sox2 is overexpressed and implicated in CSC maintenance and a worse prognosis in a number of other cancer types as well, including lung, gastric, colorectal, esophageal, melanoma, ovarian, pancreatic, and prostate cancer (Wuebben and Rizzino, 2017). Interestingly, amplification of Sox2 is associated with a better prognosis in squamous cell lung carcinoma. Sox2 amplification was

found to be unique to squamous cell lung carcinoma compared to lung adenocarcinomas, with higher levels of Sox2 correlating with lower tumour grade and increased overall survival (Wilbertz et al., 2011). Meanwhile, Sox2 overexpression in small cell and non-small cell lung carcinoma promotes CSC maintenance and oncogenic signalling (Karachaliou et al., 2013). In general, Sox2 is linked to oncogenic pathways that promote invasive and CSC phenotypes.

1.9 Hypothesis and Objectives

Given the roles of Sox2 in cellular plasticity and cancer progression and the observation that radiation treatment can lead to increased cancer cell stemness, we wondered if IR regulated Sox2. We therefore hypothesize that IR regulates Sox2 in cancer cells, and, if so, aimed to elucidate the subsequent effect on cell fate.

There are three main approaches to this study: the first section focuses on Sox2 regulation after DNA damage at the protein level. We also investigated the regulation of Sox2 transcriptional activity using Sox2 reporter cell lines. Finally, we attempted to identify Sox2 interacting partners after IR using a proximity-dependent labelling method.

MATERIALS AND METHODS

Cell culture

All cell lines were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with, 10% fetal bovine serum (FBS), and 100U/mL penicillin and 100µg/mL streptomycin (Gibco), incubated at 37°C and 5% CO_2, with the exception of J1 mouse embryonic stem cells (ATCC #SCRC-1010), which were grown in DMEM F12 supplemented with 15% FBS, 100U/mL penicillin and 100µg/mL streptomycin, 0.1mM β-mercaptoethanol, and 1000U/mL mouse leukemia inhibitory factor. U-2 OS cells were from ATCC (#HTB-96). MCF7 cells (ATCC #HTB-22) were from the lab of Dr. Razmik Mirzayans (Department of Oncology, University of Alberta), denoted **MCF7-RM,** and of Dr. Lynne Postovit (Department of Oncology, University of Alberta), denoted **MCF7-LP**. HEK293 FlpIn TREx cells (Invitrogen) and HeLa FlpIn TREx cells were grown in DMEM, 10% FBS and 1% P/S with 10 µg/mL blasticidin-HCl; stable HEK 293 and HeLa FlpIn TREx Sox2-BirA-FLAG and FLAG-BirA-Sox2 cells were maintained with selective pressure with 50µg/mL hygromycin B (Invitrogen) and 10µg/mL blasticidin-HCl (Gibco). MCF7 RR and RU cells (received from Dr. Raymond Lai, Department of Laboratory Medicine and Pathology, University of Alberta) were maintained in media supplemented with 1µg/mL puromycin (Tocris Bioscience). Cells were passaged with 0.5% Trypsin-EDTA diluted in PBS (Gibco). For hypoxia treatments, cells were cultured in the Heracell VIOS tri-gas incubator (Fisher) set to 1% oxygen. Cells were seeded then grown in 1% oxygen overnight prior to irradiation. For transport of cells between the incubator or irradiator, plates were sealed with parafilm then placed in an airtight container.

Expression vectors

Plasmids obtained or cloned are listed in the following table.

Table 2.1

Construct	Origin
pcDNA5 FRT/TO $BirA_{R118G}$-FLAG	Dr. Anne Claude Gingras, Department of molecular genetics, University of Toronto
pcDNA5 FRT/TO FLAG-$BirA_{R118G}$	Dr. Anne Claude Gingras, Department of molecular genetics, University of Toronto
pcDNA5 Sox2-$BirA_{R118G}$-FLAG	Cloned
pcDNA5 FLAG-$BirA_{R118G}$-Sox2	Cloned
pOG44	Purchased (Invitrogen)
pIRES FLAG-HAneo	Robert Roeder lab, Rockefeller University
pIRES FLAG-HAneo-Sox2	Cloned
pIRES FLAG-HAneo 24-317 Sox2	Cloned
pIRES FLAG-HAneo 41-317 Sox2	Cloned
pIRES FLAG-HAneo 1-109 Sox2	Cloned
pIRES FLAG-HAneo 1-180 Sox2	Cloned
pIRES FLAG-HAneo 1-250 Sox2	Cloned

Cell transfection and drug treatments

Cells were transfected with DNA using Lipofectamine 2000 according to manufacturer's protocol (Thermo Fisher). Various drugs were used at the following concentrations unless otherwise indicated: 0.5μM doxorubicin, 10μM gemcitabine, 5μM cisplatin, 10μM KU55933 ATM inhibitor (ATMi), 1μM AZD6738 ATR inhibitor (ATRi), 100nM PD0332991 CDK4/6 inhibitor, 50μg/mL cycloheximide (CHX), and 10μM MG132. ATM and ATR inhibitors were added 15 minutes prior to irradiation. CDK4/6 inhibitor was added overnight, after cells were seeded. shRNAs for ubiquitin ligase knockdowns were obtained from the University of Alberta RNAi core facility.

Cell irradiation

The gamma cell irradiator Shepherd Mark I irradiator with radioactive source Cesium-137 was used to treat cells. Plates were placed on a rotating platform to ensure even distribution of radiation. Dose rates ranged between 0.85 and 0.76 Gy per minute in the span of four years.

Lysate collection and immunoblotting

For lysate collection, cells seeded uniformly in 60mm plates were washed with cold phosphate-buffered saline (PBS) then lysed with TGN buffer (150mM NaCl, 5mM NaF, 1% Tween-20, 0.5% NP-40, and 50mM Tris-HCl pH 7.5) supplemented with 1mM phenylmethylsulfonyl fluoride (PMSF), or lysed with 1x Laemmli buffer (12mM Tris-HCl pH 6.8, 0.4% SDS, 2% glycerol, 1% β-mercaptoethanol, and 0.0025% bromophenol blue) followed by sonication for 20 cycles of 30 second intervals (Diagenode bioruptor) at the indicated time points. Lysates collected with TGN buffer were centrifuged at 16000 x g at 4°C, and the supernatant was mixed with 5x Laemmli buffer. All lysates were heated at 95°C on a heat block for 5 minutes before gel electrophoresis.

Equal volumes of protein lysates were loaded onto 10% SDS-polyacrylamide gels and separated by electrophoresis at 150V, using running buffer consisting of 2.5mM Tris, 192mM glycine and 0.1% SDS. Proteins were wet transferred to a nitrocellulose membrane in transfer buffer (25mM Tris, 192mM glycine and 10% methanol) for 2 hours at 90mA, then membranes were blocked in 5% non-fat dry milk (NFDM) for at least 1 hour at room temperature. Primary antibodies were diluted in 5% NFDM with 0.01% sodium azide then incubated with the membrane on a shaker overnight at 4°C. Primary antibodies were used at the following concentrations: Sox2 (Cell Signaling Technology #3579, 1:4000), Sox2 (Thermo Fisher 20G5, 1:1000), Chk1 (Cell Signaling

Technology #2360, 1:5000), Chk2 (Cell Signaling Technology #6334, 1:5000), phospho-Chk1 Ser345 (Cell Signaling Technology #2348, 1:2000), phospho-Chk2 Thr68 #2661, 1:2000), Cyclin A2 (Cell Signaling Technology #9870, 1:4000), Lamin A/C (Santa Cruz sc-7292, 1:1000), Geminin (Santa Cruz sc-13015, 1:1000), HA (Santa Cruz sc-57592, 1:5000), β-Actin (Cell Signaling Technology #4970 1:5000), α-Tubulin (Cell Signaling Technology #2144, 1:5000).

The following day, membranes are washed 3x for 5 minutes with 1x TBS (tris-buffered saline) with 0.05% tween-20 (TBS-T) then incubated with secondary antibody diluted in 5% NFDM for 2 hours at room temperature. Secondary antibodies were used at the following concentrations: for chemiluminescence anti-mouse IgG HRP and anti-rabbit IgG HRP (Cell signaling technology #7076, 7074 1:5000); for fluorescence IRDye 680RD anti-rabbit and IRDye 800CW anti-mouse (Li-cor) were used at 1:10k. Membranes were incubated with Amersham ECL prime (GE life sciences) before exposing Fuji medical x-ray films (Fujifilm) in a dark room, which were developed with the Kodak X-OMAT 2000A processor for HRP probes. Fluorescent antibody tagged membranes were visualized using the Odyssey Fc imaging system (Li-cor).

Statistical analysis

All statistical analysis was performed using GraphPad Prism 7 software. Experiments performed at least 3 times were analyzed and P values were calculated using one-way ANOVA. Means of treated samples were compared to that of controls. Significance is denoted on graphs: *=$P<0.05$, **=$P<0.01$, ***=$P<0.001$, ****=$P<0.0001$. Where the analysis was non-significant, no asterisks are shown.

Quantitative PCR

Cells were trypsinized and total RNA was isolated using the Illustra RNAspin mini kit (GE life sciences). 5µg RNA was reverse transcribed into cDNA using SuperScript IV reverse transcriptase (Thermo Fisher). cDNAs were amplified using Sox2 specific primers and detected using the 7900HT fast real time PCR system (Thermo Fisher) with PowerUp SYBR green master mix (Thermo Fisher). Relative changes in Sox2 mRNA were calculated using the ΔΔct method and normalized to GAPDH. Primers used and annealing temperatures are listed in the table below.

Table 2.2

Primer	Sequence (5' to 3')	Annealing temperature
SOX2-forward	GGGAAATGGGAGGGGTGCAAAAGAGG	61°C
SOX2-reverse	ACACCAATCCCATCCACACTCACGCAA	61°C
GAPDH-forward	GAAGGTGAAGGTCGGAGTC	53°C
GAPDH-reverse	GAAGATGGTGATGGGATTTC	53°C

Cellular fractionation

Cells were washed with PBS then lysed with cold hypotonic buffer (20mM HEPES pH 7.9, 1mM EDTA, 1mM EGTA, 1mM DTT, 0.2% NP-40, 2mM PMSF) and spun down at 16000 x g for 20 seconds. The supernatant was collected as the cytosolic fraction. The pellet was then resuspended in high salt buffer (hypotonic buffer, 420mM NaCl, 20% glycerol, 2mM PMSF) and rotated at 4°C for 30 minutes before spinning down at 16000 x g for 20 minutes. This supernatant was collected as the nuclear fraction. The new pellet was resuspended in 0.2N HCl and rotated at 4°C for 1.5 hours before spinning down at 16000 x g for 10 minutes. This supernatant was collected as the

chromatin fraction. Each fraction was mixed with Laemmli buffer and heated before immunoblotting.

Flow cytometry

Cells were trypsinized, spun down, and resuspended in cold PBS. Single cell populations were gated and analyzed for GFP expression using the FACSCanto II system (BD Biosciences). Parameters for background GFP were set using wild-type MCF7.

BioID and silver stains

HEK293 and HeLa FlpIn TREx cells with inducible expression of Sox2-BirA*-FLAG fusion protein were grown on 15cm plates and induced with 0.5µg/mL tetracycline for 7 to 9 hours. Tetracycline was then removed, and media replaced with 50µM biotin overnight for labelling. Two separate protocols were used for biotin pulldown experiments. The first one uses the cellular fractionation protocol to isolate cytosolic, nuclear and chromatin fractions (see protocol above). With the isolation of each fraction, protein extracts were incubated with 5µL of magnetic streptavidin beads (New England Biolabs) and rotated overnight. The second protocol was received from Dr. François Michel Boisvert (Department of Anatomy and Cell Biology, University of Sherbrooke). Cells were scraped from the plates and lysed in RIPA buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 0.1% SDS, 1% NP40) supplemented with protease inhibitor cocktail (Sigma Aldrich), 1mM PMSF, 1mM DTT, 0.4% sodium deoxycholate, 1mM NaF, and 2mM Na_2VO_4. Samples were sonicated at an amplitude of 30 at 3 cycles for 10 seconds, then rotated for an hour with benzonase (1µL per mL lysis buffer). SDS was added to a

final concentration of 0.4%, then placed on a rotator for another 15 minutes. The chromatin was spun down at 20000g for 20 minutes, and the supernatant was removed and added to 5µL of magnetic streptavidin beads which were rotated for 3 hours. Magnetic streptavidin beads were washed with RIPA buffer and 10% SDS twice for 5 minutes before use. Samples were washed with BioID wash buffer (2% SDS, 50mM Tris-HCl pH 7.5) then with RIPA buffer. To elute proteins from the beads, beads were boiled in 1x Laemmli buffer with 50µM biotin.

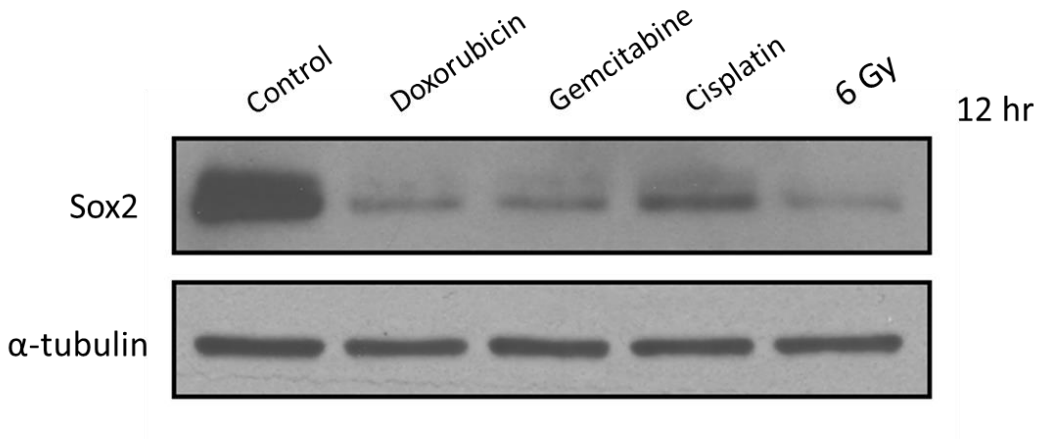
For silver stains, 12µL of protein extract was loaded onto a 10% SDS gel. Silver stains of SDS-polyacrylamide gels were done using the proteosilver silver stain kit (Sigma Aldrich).

CHAPTER 3: REGULATION OF SOX2 BY IONIZING RADIATION

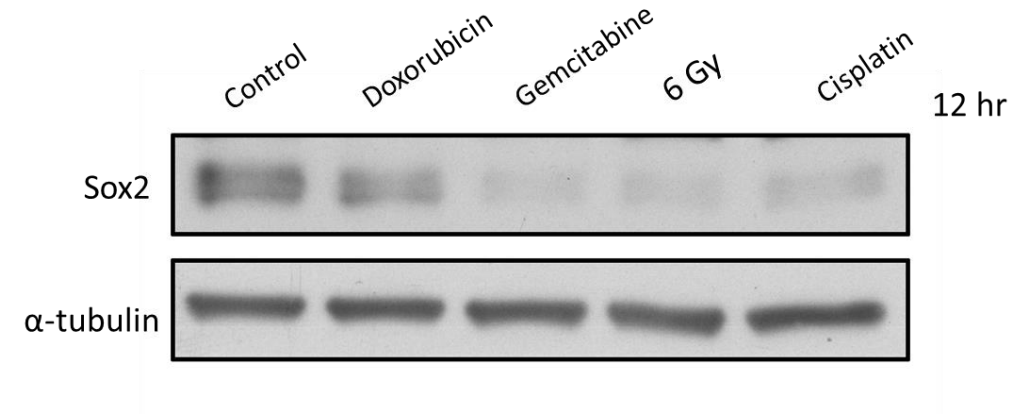
3.1 Regulation of Sox2 protein

3.1.1 Sox2 protein is down-regulated after DNA damaging treatments

To assess the effect of DNA damage on Sox2 protein expression, we used human cell lines that expressed high levels of Sox2, namely the breast adenocarcinoma cell line MCF7 and the osteosarcoma cell line U-2 OS. A variety of DNA damaging agents, including the DNA topoisomerase II inhibitor doxorubicin, the nucleoside analogue gemcitabine, the DNA crosslinker cisplatin, and ionizing radiation were used to treat cells. Sox2 protein levels were examined by immunoblot of lysates obtained 12 hours later. Using two different antibodies against Sox2, biological replicates show significant decrease in Sox2 protein level 12 hours post DNA damage treatments in U-2 OS cells (Figure 3.1.1a). Although this observation was consistent, the kinetics of down-regulation differed between replicates; with IR and gemcitabine there was at least a 50% down-regulation in Sox2 protein 12 hours after. In MCF7-RM cells, Sox2 down-regulation was also observed with the same treatments, however this was not significant and with more variability between replicates (Figure 3.1.1b). In addition, we performed similar treatments of J1 mouse embryonic stem cells and examined Sox2 protein expression 4 and 8 hours post-treatment (3.1.1c). A preliminary experiment shows that Sox2 protein appears to decrease at 8 hour time points with gemcitabine and IR, suggesting that DNA damaged induced Sox2 down-regulation may be a conserved mechanism between cell types.


Control
Doxorubicin
Gemcitabine
Cisplatin
6 Gy
12 hr
Sox2
α-tubulin

(#20G5)


Control
Doxorubicin
Gemcitabine
6 Gy
Cisplatin
12 hr
Sox2
α-tubulin

(#3579)

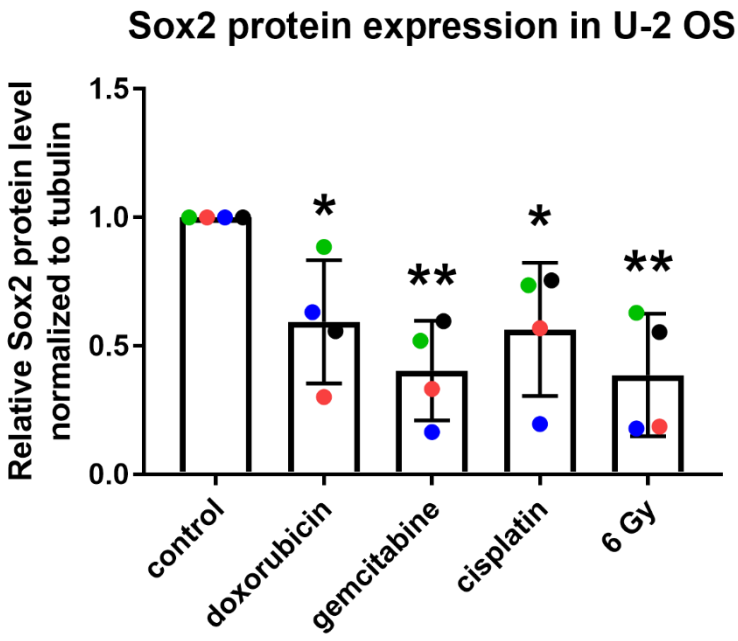

Sox2 protein expression in U-2 OS
Relative Sox2 protein level normalized to tubulin
1.5
1.0
0.5
0.0
*
**
*
**
control
doxorubicin
gemcitabine
cisplatin
6 Gy

Figure 3.1.1a. Sox2 protein down-regulation 12 hours after DNA damaging treatments in U-2 OS cells. Representative immunoblots of biological replicates; cells were treated with 0.5μM doxorubicin, 10μM gemcitabine, 5μM cisplatin, or 6Gy IR. Top blotted using Thermo Fisher Sox2 antibody (20G5) and bottom using Cell Signaling Technology Sox2 antibody (#3579). Cells lysed with TGN buffer; imaged on film. Quantification of four separate replicates, Sox2 protein levels were normalized to tubulin. Same coloured points correspond to the same replicate (n=4).

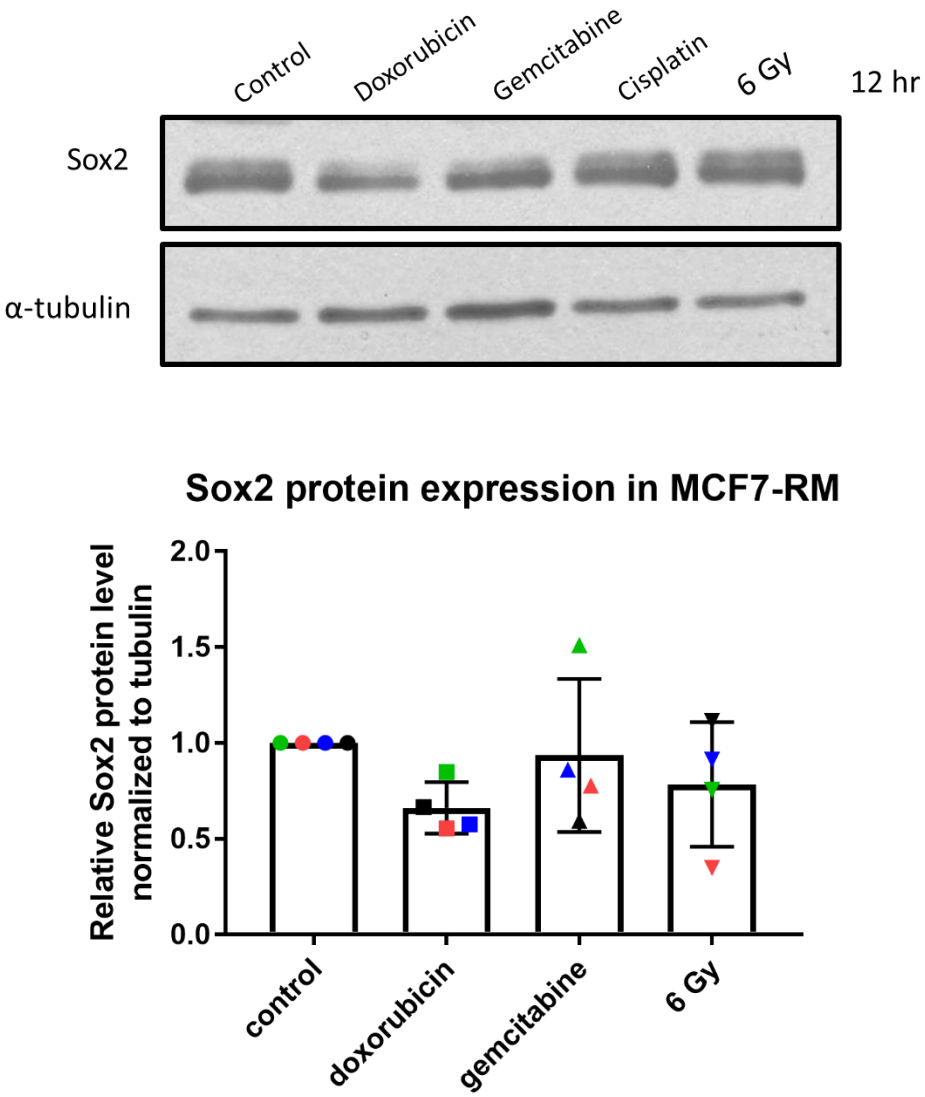


Figure 3.1.1b. Sox2 protein down-regulation 12 hours after DNA damaging treatments in MCF-RM cells. Representative immunoblots of biological replicates. Cells were treated with 0.5µM doxorubicin, 10µM gemcitabine, 5µM cisplatin, or 6Gy IR. Immunoblots of TGN lysates were probed using Thermo Fisher Sox2 antibody (20G5) and imaged on film. Same coloured points correspond to the same replicate (n=4).

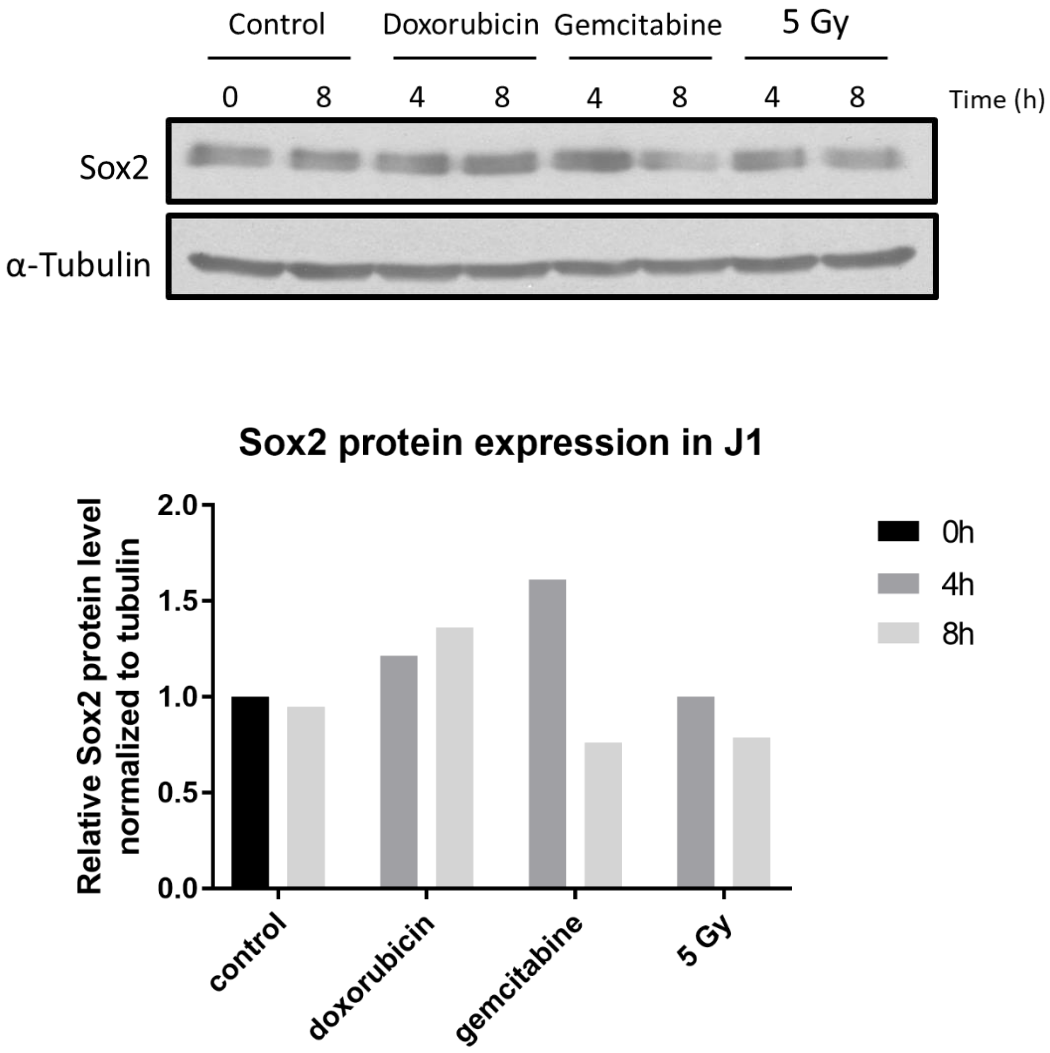


Figure 3.1.1c. Sox2 protein expression 4 and 8 hours after DNA damaging treatments in J1 mouse embryonic stem cells.; Cells were treated with 0.5μM doxorubicin, 10μM gemcitabine, or 5Gy IR. Immunoblots of TGN lysates were probed using Thermo Fisher Sox2 antibody (20G5) and imaged on film (n=1).

Next we performed time course studies to observe Sox2 protein levels over time. U-2 OS cells were irradiated at 3Gy and 6Gy. Lysates were collected at 6, 12, and 24 hours after IR. With 3Gy treatment, Sox2 protein decreases significantly over time, up to 24 hours (Figure 3.1.1d, top graph). To address the variability seen with Sox2 protein down-regulation, and to rule out potential inconsistencies with the cell lysis process, cells were lysed with either TGN lysis buffer or Laemmli buffer. TGN buffer contains a milder detergent NP-40, which may lead to incomplete lysis of subcellular membranes and explain inconsistent results, compared to Laemmli buffer which contains a stronger detergent, SDS. U-2 OS cells were treated with 6Gy, then lysates were collected at 6, 12, and 24 hours post- IR with either TGN buffer or Laemmli buffer. Sox2 down-regulation was observed in U-2 OS cells up to 24 hours post-IR with both lysis buffers (Figure 3.1.1d, middle graph).

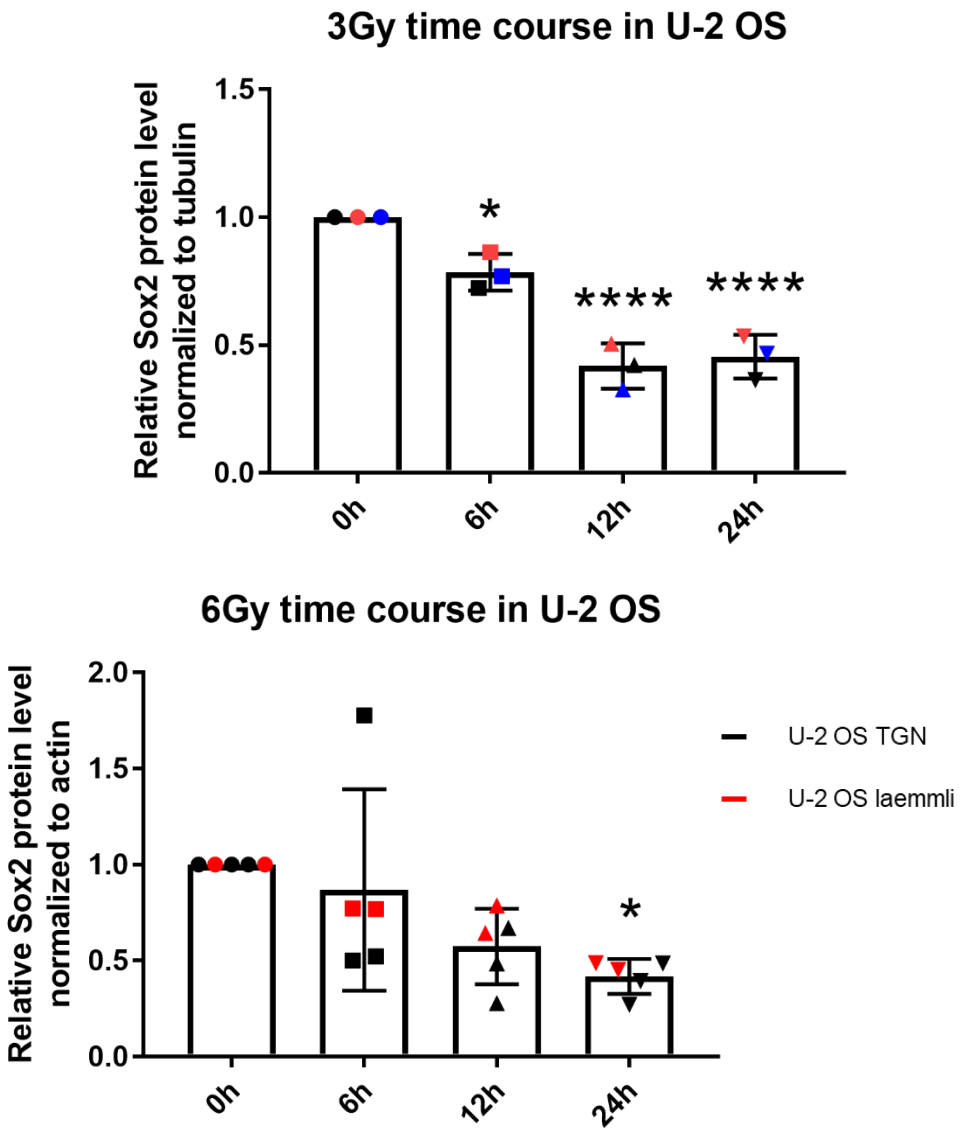


Figure 3.1.1d. Time course evaluation of Sox2 protein expression after 3Gy and 6 Gy in U-2 OS cells. **Top graph**: Cells were treated with 3Gy and lysates were collected with TGN lysis buffer at the indicated time points. Same coloured points correspond to the same replicate (n=3). **Bottom graph**: Cells were treated with 6Gy IR then lysates were collected at the indicated time points with either TGN buffer (black points) or Laemmli buffer (red points). Representative immunoblot of TGN lysed cells shown, Sox2 detected using Cell signaling technology Sox2 antibody (#3579).

With regards to MCF7, we had received this cell line from two different sources and noticed differences in cell morphology. Neither of the cell lines had been verified in our hands so we decided to test both cell lines for Sox2 protein regulation after IR and see if they would behave differently. MCF7-RM and MCF7-LP cells were treated with 6Gy and lysates were collected at 6, 12, and 24 hour time points with either TGN or Laemmli buffer (Figure 3.1.1e, top graph). In MCF7-LP cells, Sox2 protein extraction was similar between the two lysis buffers. Interestingly, Sox2 down-regulation was not seen with IR treatment in MCF-LP cells, compared to previous observation in MCF-RM cells. On the other hand, lysis buffer had an impact on Sox2 protein extraction from MCF7-RM cells; while TGN lysis shows Sox2 down-regulation over time, Laemmli lysis shows Sox2 upregulation with 6Gy treatment (Figure 3.1.1e, top graph). Further experiments conducted using MCF7-RM with 3Gy treatment showed that the two lysis buffers were not equally extracting Sox2 protein from cells, especially at 24 hour time points (Figure 3.1.1e, bottom graph).

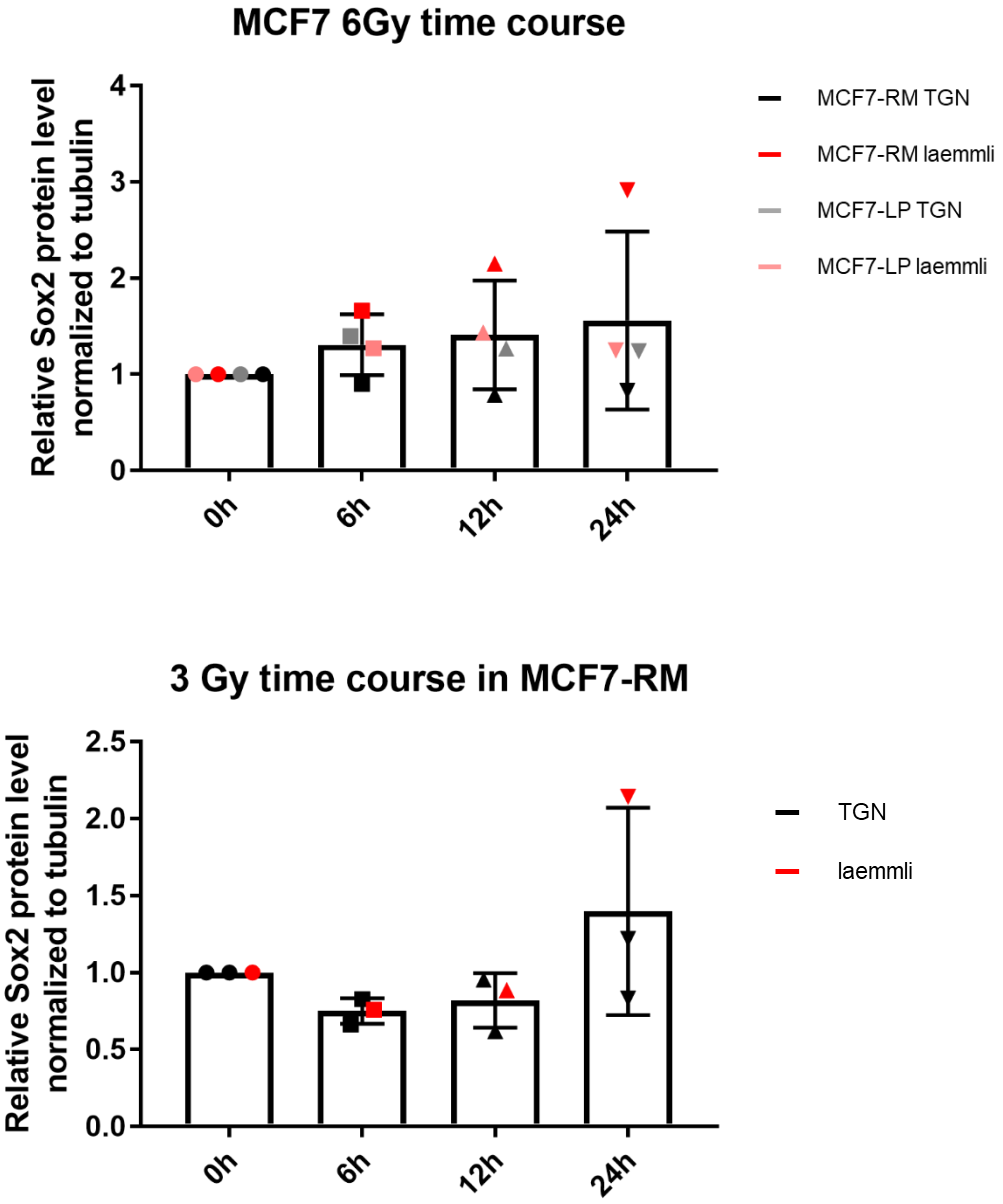
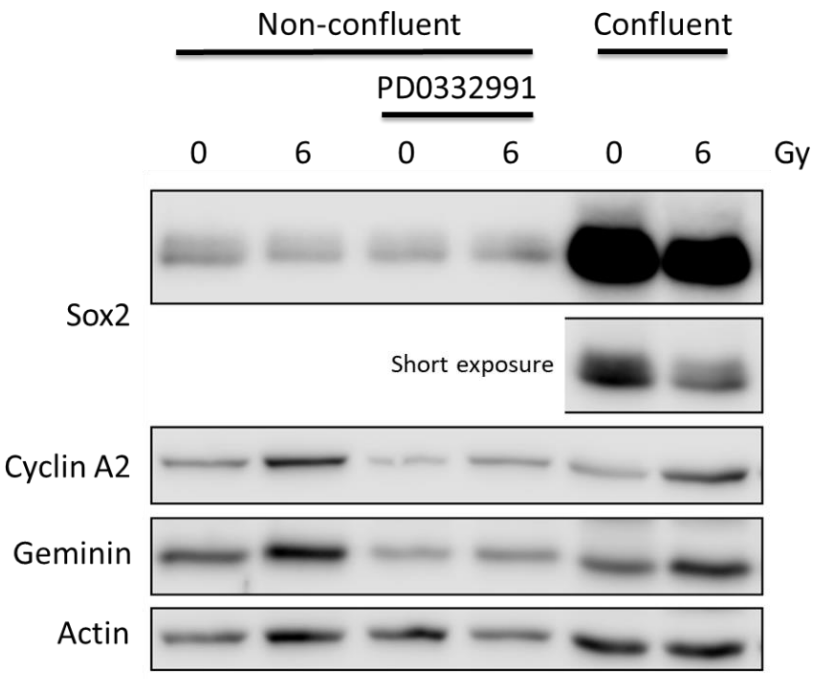
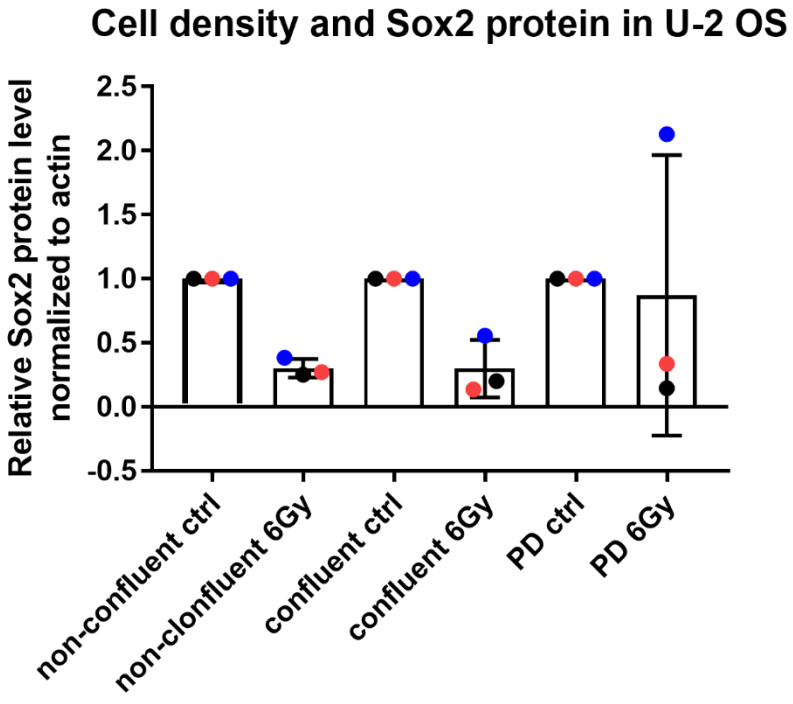
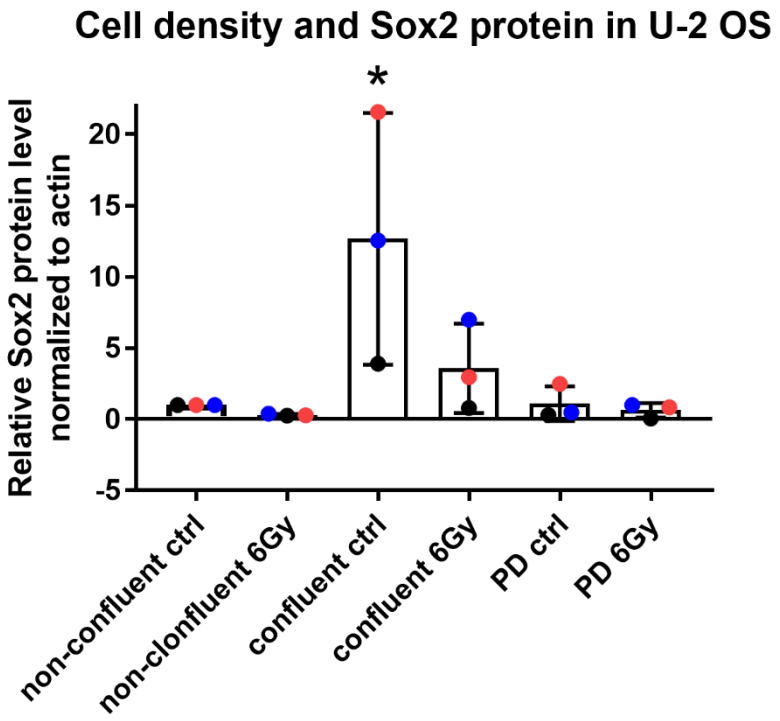


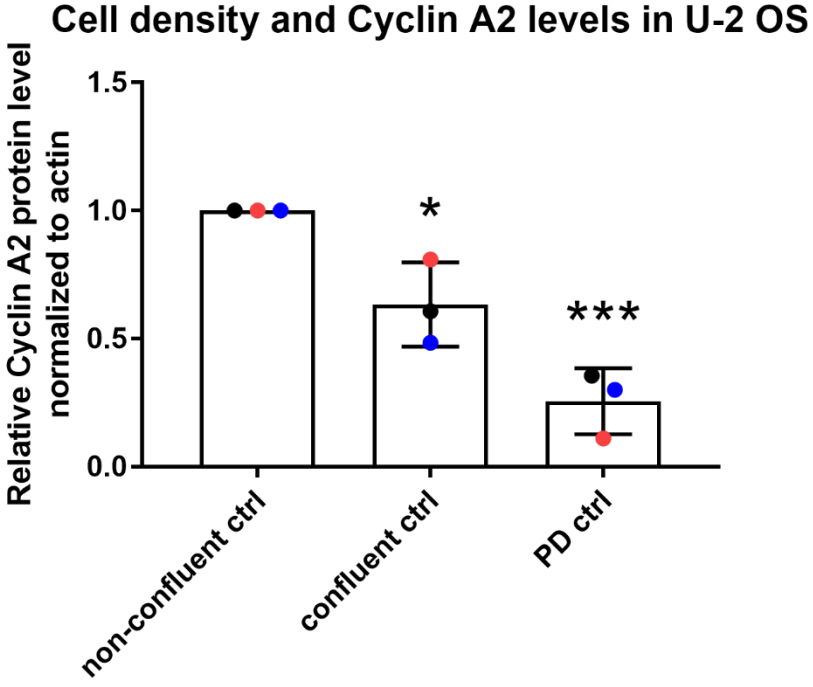
Figure 3.1.1e. Time course evaluation of Sox2 protein expression after IR in MCF7 cell lines. **Top graph**: MCF7-RM and MCF7-LP cells were treated with 6Gy and lysates were collected with TGN or laemmli lysis buffer at the indicated time points. **Bottom graph**: MCF7-RM cells were treated with 3Gy IR then lysates were collected at the indicated time points with either TGN or laemmli lysis buffer. Sox2 levels were normalized to actin or tubulin.

We noted that Sox2 protein levels also fluctuated between control time points, perhaps pointing to the sensitivity of Sox2 protein to cell cycle. To test this, U-2 OS cells were seeded onto plates at low or high densities such that at the time of irradiation, plates of cells were either non-confluent, or fully confluent. Cells were also treated with the CDK4/6 inhibitor, PD0332991, which halts the cell cycle in G1. Sox2 down-regulation was observed in all growth conditions 24 hours post-IR (Figure 3.1.1f). Interestingly, Sox2 protein levels in confluent control plates were at least 4 times higher compared to non-confluent control plates, even when normalized to actin (Figure 3.1.1f). We also probed for cell cycle regulators with low expression in G1 phase, including cyclin A2 and geminin, to confirm G1 arrest with PD0332991 treatment by comparing levels to cells that are cycling in non-confluent controls (Figure 3.1.1f). Further experiments were mostly conducted using U-2 OS cells, due to reproducible Sox2 protein down- regulation after IR, regardless of the lysis buffer used. A preliminary experiment where U-2 OS cells were treated with 6Gy for 12 hours before fractionating cell lysates into cytoplasmic, nuclear and chromatin fractions, shows that Sox2 down-regulation post-IR occurs in all three fractions, which may explain why lysis buffer strength did not affect our observations of Sox2 down-regulation (Figure 3.1.1g).


Non-confluent
Confluent
PD0332991
0
6
0
6
0
6
Gy
Sox2
Short exposure
Cyclin A2
Geminin
Actin


Cell density and Sox2 protein in U-2 OS
Relative Sox2 protein level normalized to actin
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
non-confluent ctrl
non-clonfluent 6Gy
confluent ctrl
confluent 6Gy
PD ctrl
PD 6Gy


Cell density and Sox2 protein in U-2 OS
Relative Sox2 protein level normalized to actin
20
15
10
5
0
-5
*
non-confluent ctrl
non-clonfluent 6Gy
confluent ctrl
confluent 6Gy
PD ctrl
PD 6Gy


Cell density and Cyclin A2 levels in U-2 OS
Relative Cyclin A2 protein level normalized to actin
1.5
1.0
0.5
0.0
*
***
non-confluent ctrl
confluent ctrl
PD ctrl

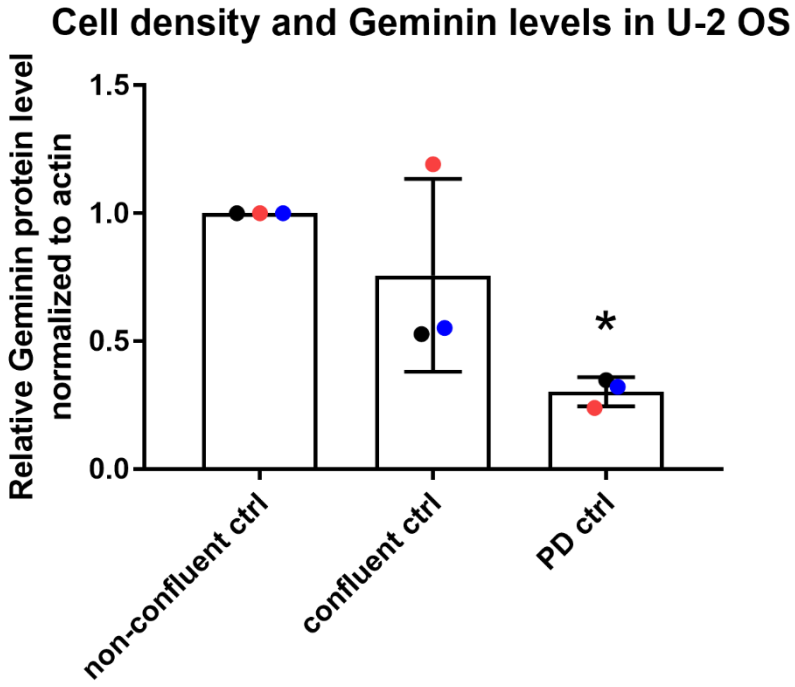


Figure 3.1.1f. The effect of cell density on Sox2 down-regulation after IR in U-2 OS. U-2 OS cells were seeded at low density or high density and treated with 6Gy. For G1 arrest, cells were treated with 100nM PD0332991 at the time of seeding for 24 hours. Lysates were collected with Laemmli buffer 24 hours post-IR and probed for Sox2, cyclin A2 and geminin. Representative immunoblot; detected with fluorescent probes on the Odyssey Fc imager as shown. Protein levels were normalized to actin. Sox2 quantification data is shown in two graphs; the **top graph** depicts Sox2 protein relative to Sox2 levels of the respective growth conditions, while the **bottom graph** depicts Sox2 levels relative to non-confluent control. Same coloured points correspond to each replicate. The final two graphs show quantification of cyclin A2 and geminin in the three conditions (n=3).

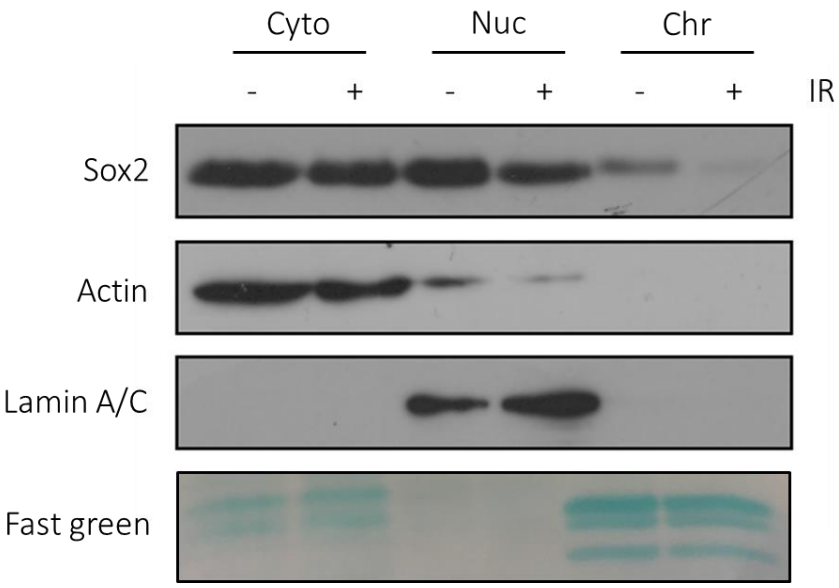


Figure 3.1.1g. Cell fractionation of U-2 OS cells treated with 6Gy for 12h. U-2 OS cells were treated with 6Gy for 12 hours. Then the indicated fractions were isolated, as described in the methods section. Actin, lamin A/C and fast green protein stain for histones were used as controls for cytoplasmic, nuclear and chromatin fractions, respectively.

In summary, Sox2 protein is down-regulated following DNA damage in cancer cell lines. In U-2 OS, significant decrease in Sox2 levels can be observed from 6 hours up to 24 hours post-IR. We also uncovered a cell density sensitivity of Sox2 protein level, where high cell density alone leads to increased Sox2 protein. Despite this, IR-mediated Sox2 down-regulation can be observed at both low and high cell densities and did not appear to be cell cycle dependent.

3.1.2 Sox2 down-regulation is dependent on ATM signalling

In order to understand the signalling pathway leading to Sox2 down-regulation post-IR, we investigated DNA damage signalling apical kinases, ATM and ATR. In a preliminary experiment, U-2 OS cells were treated with inhibitors of ATM and ATR, combined with gemcitabine or 6Gy, and Sox2 protein levels were quantified after 12 hours (Figure 3.1.2a). ATM inhibition appeared to rescue the effect of IR-mediated Sox2 down-regulation, whereas ATR inhibition did not. Follow up experiments were performed to confirm the role of ATM in Sox2 regulation, while it was unclear whether ATR inhibition had an effect due to large variations between replicates (Figure 3.1.2b). ATM and ATR specific targets Chk2 and Chk1, respectively, were probed for phosphorylation status to determine if the inhibitors effectively blocked kinase activity (Figure 3.1.2b, bottom two graphs). Sox2 down-regulation following IR is partially restored by ATM inhibition, suggesting that ATM activation may be upstream of this regulation. ATM activation triggers a signalling cascade of many effectors that could potentially interact with Sox2, so it would be difficult to pinpoint and validate potential regulators of Sox2 through known ATM substrates.

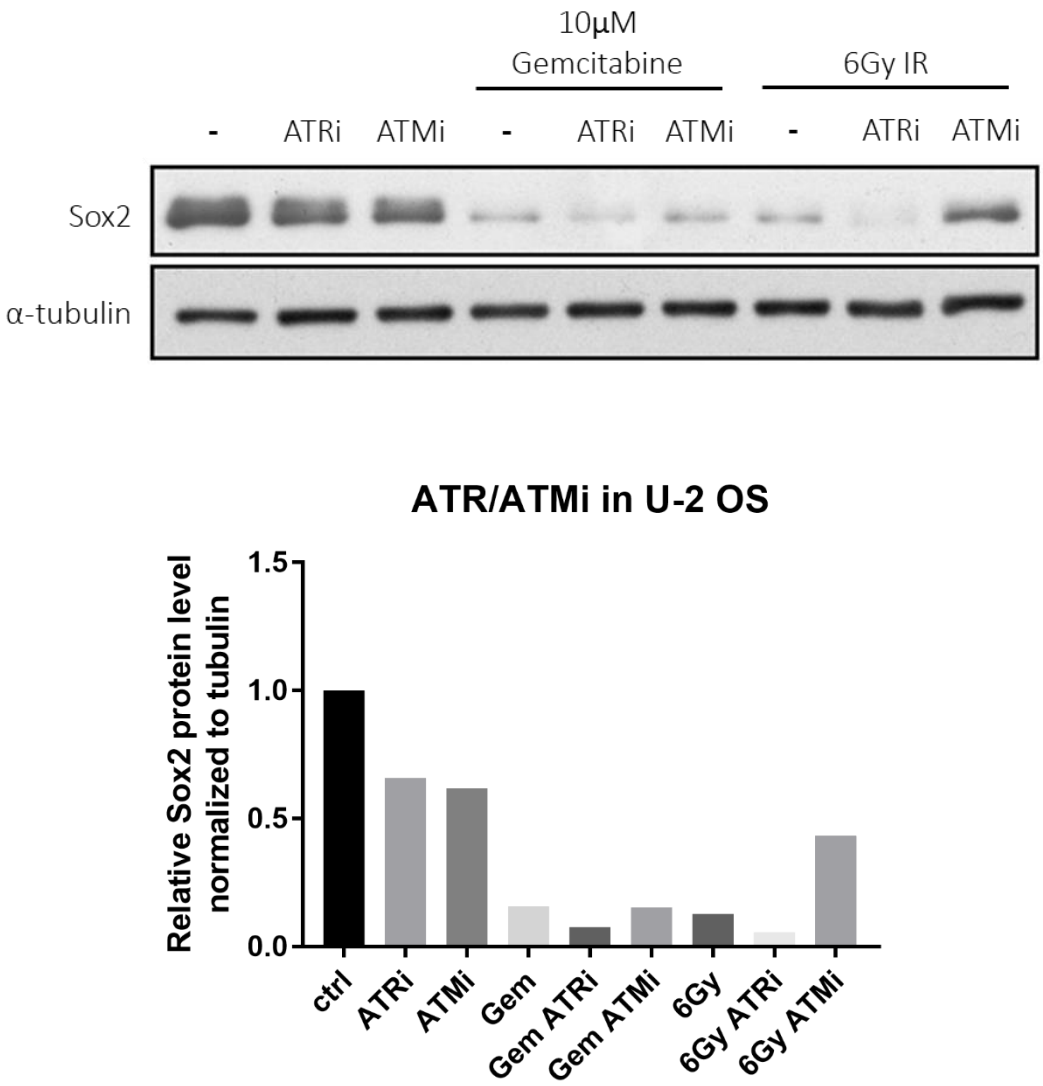
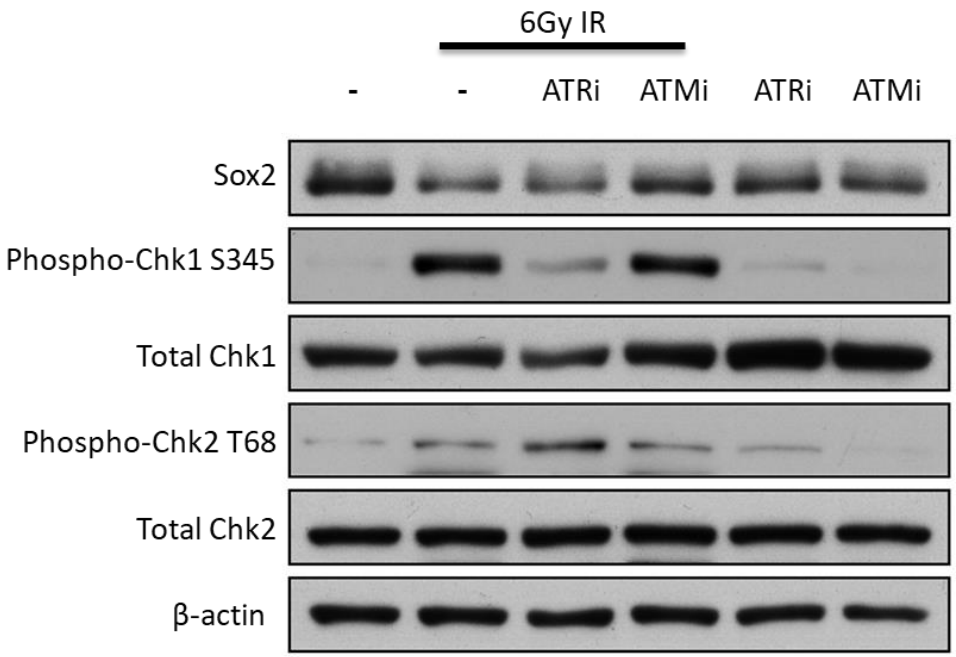
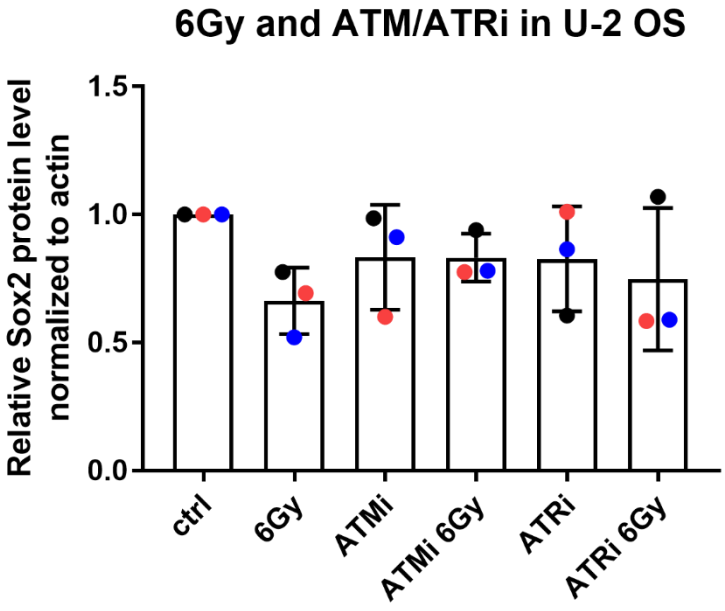


Figure 3.1.2a. ATR and ATM inhibition in gemcitabine or 6Gy treated U-2 OS cells. U-2 OS cells were treated with 10μM ATM inhibitor KU-55933 or 1μM ATR inhibitor AZD6738, added 15 minutes before irradiation or addition of 10μM gemcitabine (Gem). Lysates were then collected 12 hours later for immunoblot analysis.

3.1.3 Sox2 down-regulation is post-transcriptional

As mentioned in the introduction, Sox2 is subject to regulation at multiple levels. However in light of the short time frame in which we observe IR-mediated protein down-regulation, we hypothesized that a post-transcriptional regulatory mechanism control Sox2 expression after IR. We repeated the 3Gy time course experiment in U-2 OS cells but collected total mRNA to quantify Sox2 mRNA levels. Unlike the rapid drop in Sox2 protein levels after IR, no significant changes in *SOX2* mRNA levels were observed (Figure 3.1.3a). There was a large variation in *SOX2* mRNA levels at 12 and 24 hour time points, which may be related to our previous observation that plate density alone increases Sox2 protein levels. It could be possible that transcriptional autoregulation of Sox2 is competing at later time points post-IR with density-induced Sox2 changes. However, our results were too variable at the 12 and 24 hours time points to confirm up or down-regulation of Sox2 transcription.


6Gy IR
-
-
ATRi
ATMi
ATRi
ATMi
Sox2
Phospho-Chk1 S345
Total Chk1
Phospho-Chk2 T68
Total Chk2
β-actin


6Gy and ATM/ATRi in U-2 OS
Relative Sox2 protein level normalized to actin
1.5
1.0
0.5
0.0
ctrl
6Gy
ATMi
ATMi 6Gy
ATRi
ATRi 6Gy

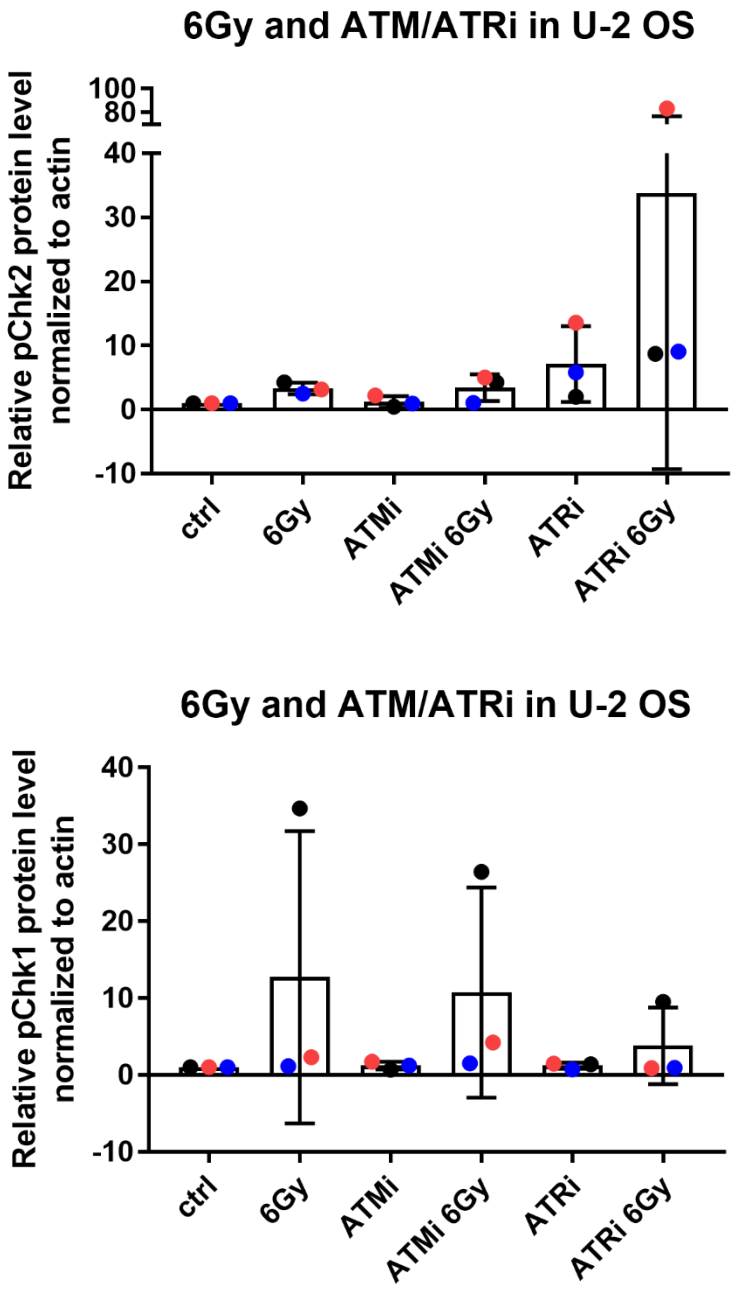


Figure 3.1.2b. ATM and ATR inhibition in 6Gy treated U-2 OS cells. U-2 OS cells were treated with 10μM ATM inhibitor KU-55933 or 1μM ATR inhibitor AZD6738, 15 minutes before irradiation. Lysates were then collected 12 hours later for immunoblot analysis Representative immunoblot shown. **Top graph**: quantification of Sox2 protein. **Bottom graphs**: quantification of pChk2 T68 (left) and pChk1 S345 (right). (n=3)

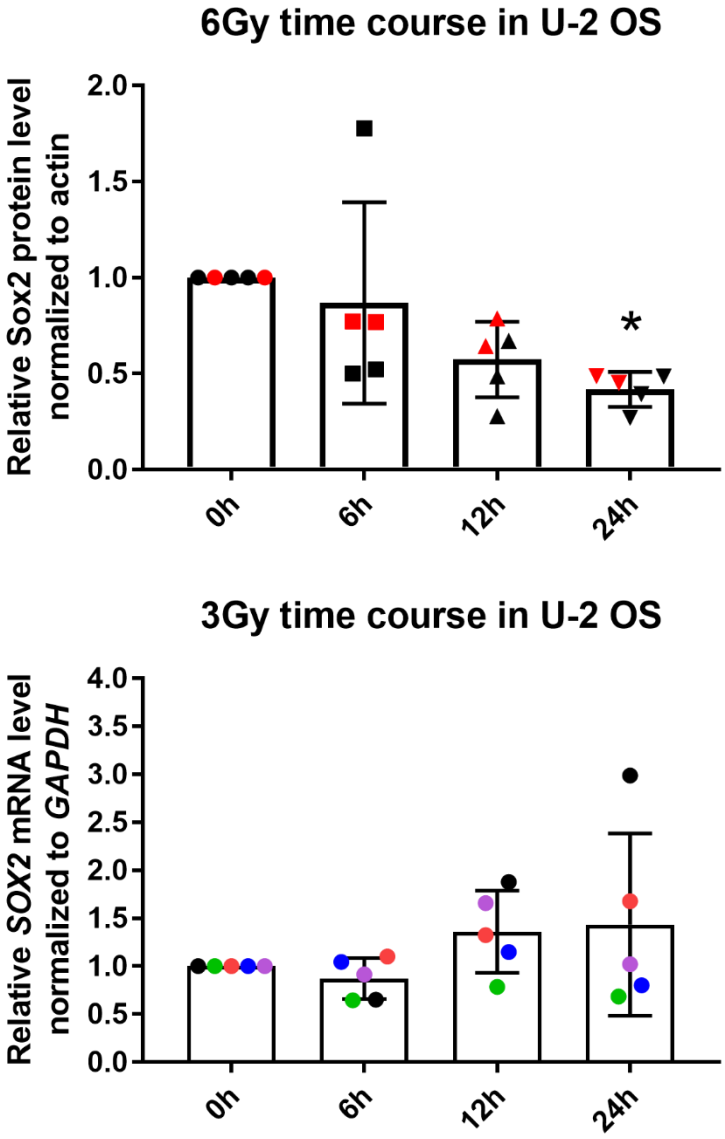


Figure 3.1.3a. Sox2 protein and mRNA levels in 3Gy treated U-2 OS cells. **Top graph**: taken from figure 3.1.1c, Sox2 protein quantification at indicated time points post-IR. **Bottom graph**: quantitative PCR of Sox2 mRNA in U-2 OS cells post-IR, total mRNA was isolated at indicated time points and reverse transcribed into cDNA. Sox2 mRNA was amplified and normalized to GAPDH mRNA. (Immunoblot n=3, qPCR n=5)

Since we observe Sox2 protein down-regulation at early (6 hour) time points when no significant changes in *SOX2* mRNA level is observed (and variations are small), it is likely that post-transcriptional mechanisms are responsible for the drop in Sox2 levels. To further test post-transcriptional Sox2 down-regulation, we used an exogenous Sox2 expressing cell line, in which Sox2 expression is tetracycline-inducible under a non-mammalian promoter. This avoids confounding effects by the autoregulatory Sox2 loop. Both exogenous and endogenous Sox2 levels are down-regulated 12 hours after treatment with the DNA damaging drug gemcitabine, importantly, treatment with the proteasome inhibitor, MG132, led to accumulation of Sox2 either alone or when combined with gemcitabine (Figure 3.1.3b). Although exogenous Sox2 transcription is under a viral promoter, gemcitabine treatment resulted in down-regulation, suggesting that DNA damage-mediated Sox2 down-regulation is promoter-independent and therefore a post-transcriptional event. Accumulation of Sox2 with MG132 treatment implicates that Sox2 is subject to proteosomal degradation after gemcitabine. When MG132 was combined with gemcitabine, Sox2 levels remained high – suggesting that DNA damage-mediated Sox2 down-regulation is via the ubiquitin-proteasomal pathway.

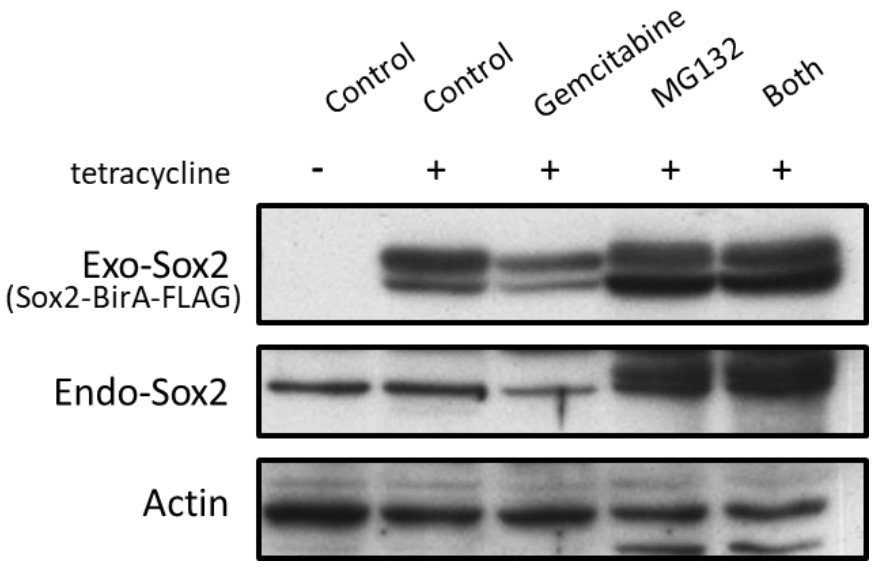


Figure 3.1.3b. Regulation of exogenous Sox2 after DNA damage. A HeLa cell line stably expressing a tetracycline-inducible Sox2-BirA-FLAG fusion protein was incubated with 2µg mL tetracycline for 10 hours and then treated with 10µM gemcitabine, 10µM MG132 or a combination of both. Lysates were collected 12 hours later for immunoblot analysis. Exogenous Sox2 (exo-Sox2) and endogenous Sox2 (endo-Sox2) were distinguishable by molecular weights of ~70kDa and ~35kDa, respectively.

Sox2 has previously been reported to be ubiquitylated by the E3 ligase WWP2 as well as regulated by the E2 ligase UBE2S in embryonic stem cells (Fang et al., 2014; Wang et al., 2016). In a preliminary experiment, we performed a transient knock down of either ubiquitin ligase and probed for Sox2 protein in MCF7-RM cells (Figure 3.1.3c). Stabilization of Sox2 protein was seen when WWP2 is knocked down with short hairpin RNA against WWP2, suggesting that under normal conditions the WWP2 E3 ligase targets Sox2 for turn over. An immunoblot is needed to conform that shWwp2 #2 knocks down WWP2.

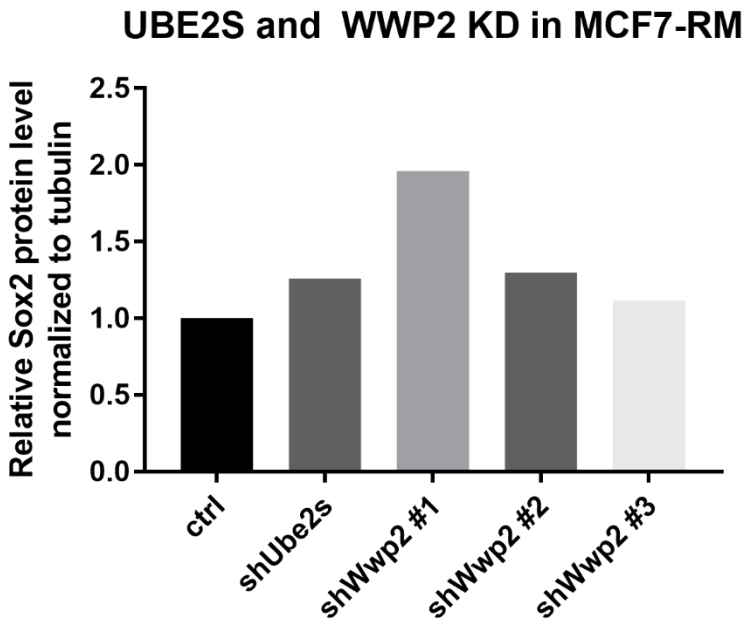
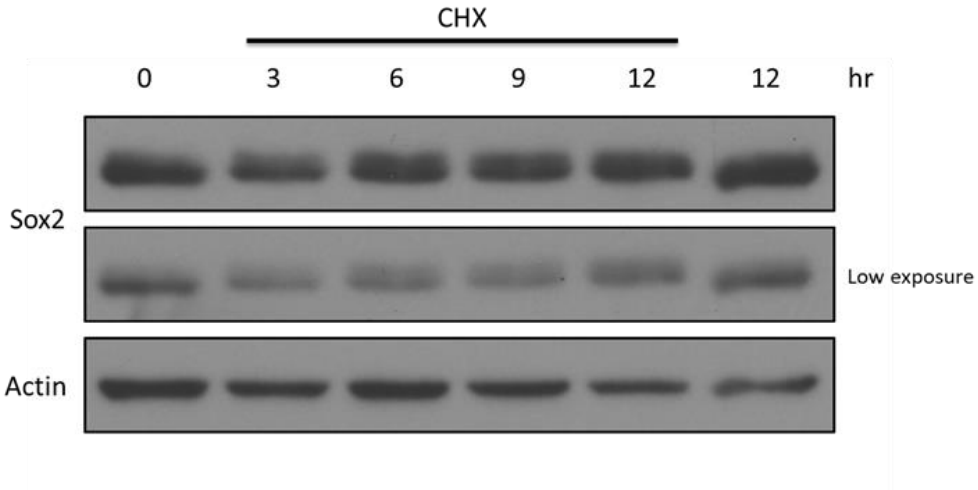


Figure 3.1.3c. Transient knock down of UBE2S and WWP2 in MCF7-RM cells. MCF7 cells were transfected with shRNAs against UBE2S and WWP2, then lysates were collected 24 hours after. Sox2 protein levels were compared to untransfected control and normalized to tubulin. (n=1)

Sox2 levels in stem cells are relatively stable in order to maintain a threshold level for stemness. For example, either an overexpression or down-regulation of Sox2 leads to trophectoderm differentiation in human embryonic stem cells (Adachi et al., 2010). To test Sox2 protein stability in U-2 OS cells, we treated cells with the protein synthesis inhibitor cycloheximide (CHX) and collected lysates every 3 hours over 12 hours (Figure 3.1.3d). Sox2 protein levels appear to be stable over this time period in two replicates, however there were varying levels in 12 hour controls, and one replicate showed Sox2 protein decreasing over time (Figure 3.1.3d, red points).



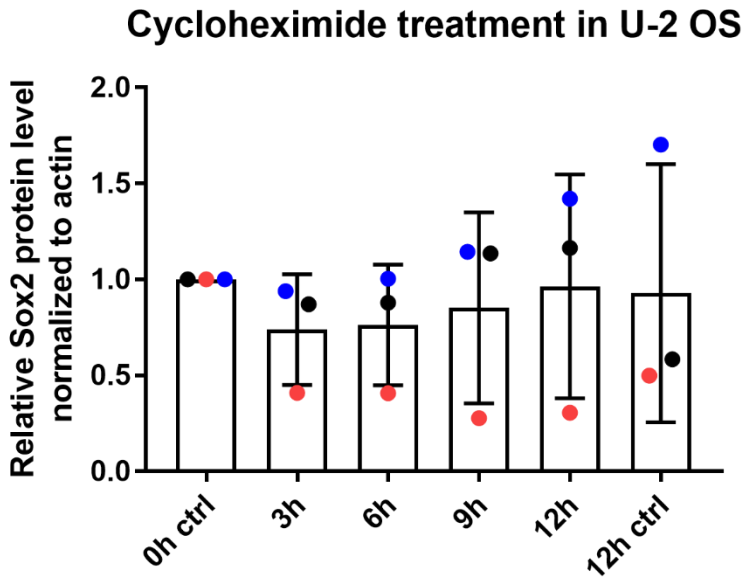


Figure 3.1.3d. Sox2 protein turnover in U-2 OS cells. U-2 OS cells were treated with 50µg/mL cycloheximide (CHX) then lysates were collected at the indicated time points. Quantification of Sox2 protein normalized to actin. Same coloured points correspond to each replicate (n=3).

We repeated this experiment, except that we treated U-2 OS cells with CHX, 3Gy, ATM inhibition, or combinations of the three; four replicates were performed, and lysates were collected at 8 and 12 hour time points (Figure 3.1.3e). If IR inhibits Sox2 translation, we would expect to see enhanced Sox2 down-regulation when IR is combined with CHX, compared to either treatment alone. Given that Sox2 levels decreased after CHX alone, it is difficult to deem the conclusion that Sox2 turnover is accelerated by IR with statistical significance.

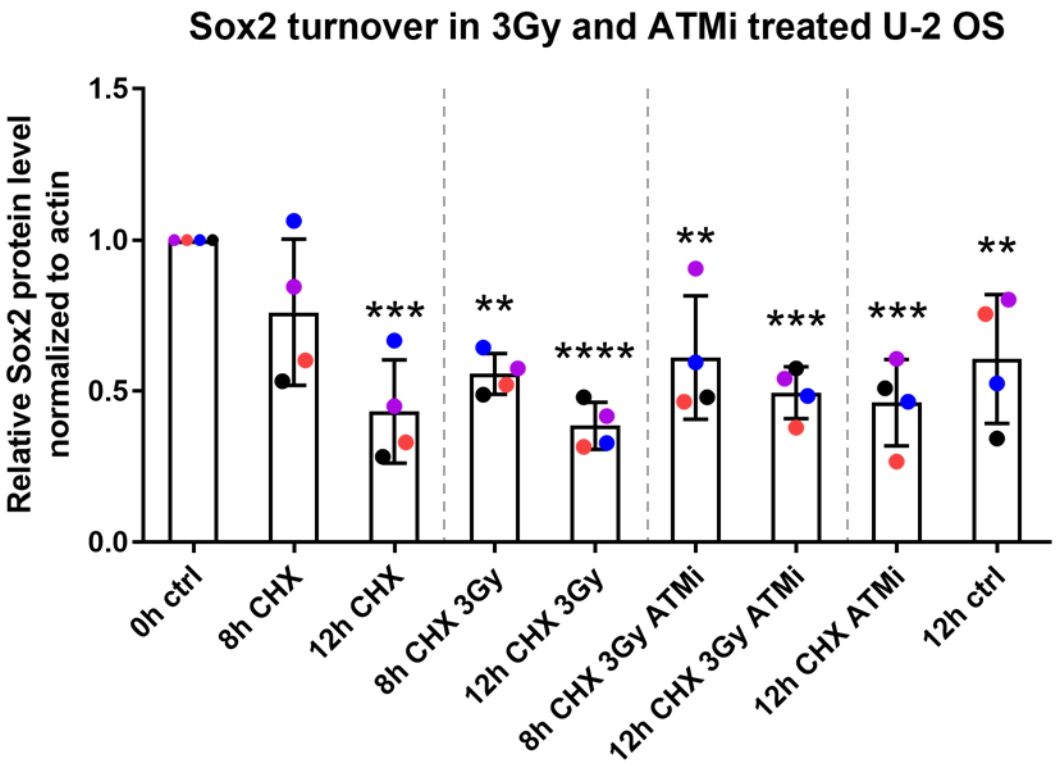


Figure 3.1.3e. Sox2 turnover in 6Gy and ATMi treated U-2 OS. U-2 OS cells were treated with 10μM ATM inhibitor KU55933 15 minutes before 6Gy or 50μg/mL cycloheximide (CHX) treatment. Lysates were collected at 8 and 12 hours and Sox2 levels were quantified and normalized to actin levels. Same colour points correspond to same replicates (n=4).

Despite several proteomic studies investigating ATM and ATR targets, Sox2 has not previously been identified as a phosphorylation target of ATM or ATR (Matsuoka et al., 2007). Although Sox2 has three ATM/ATR consensus target sequences (SQ motif), phosphorylation has not been reported at these residues. It is therefore likely that ATM indirectly (through intermediate factors) targets Sox2 for degradation. E3 ligases often recognize ubiquitylation targets through another PTM, for example WWP2 only binds to methylated Sox2 for targeted ubiquitylation. We propose that following IR, ATM activity triggers down stream pathways that either post translationally modify Sox2 for targeted degradation, or activate ubiquitin ligases that target Sox2, or both (Figure 3.1.3f).

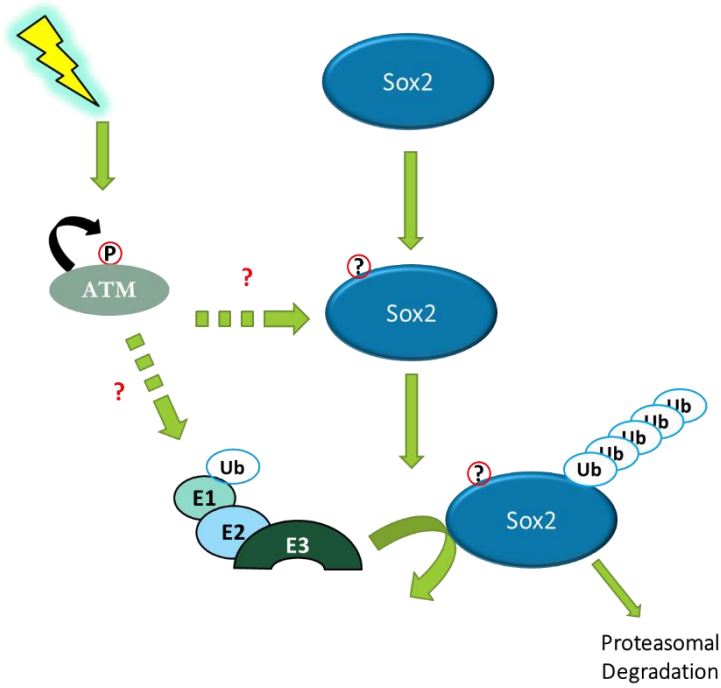


Figure 3.1.3f. Proposed model of Sox2 proteasomal degradation following IR. After IR, ATM is activated and initiates downstream signalling cascades. This may lead to post-translational modification of Sox2 that makes it vulnerable to ubiquitin ligase targeting and subsequent degradation. ATM signalling may also lead to ubiquitin ligase activation that then targets Sox2 for degradation.

3.1.4 Elucidation of a Sox2 degron

Specific PTM sites on Sox2 have been identified to be integral to ubiquitin ligase recruitment and targeted degradation. In order to understand the mechanism of IR-mediated Sox2 degradation, we created expression vectors for various Sox2 truncation mutants and expressed them in U-2 OS cells. A schematic of the truncation mutants is illustrated below (Figure 3.1.4a). We first treated truncation mutant expressing cells with cycloheximide to assess protein stability. Only exogenously expressed Sox2 was probed for immunoblot analysis using its HA tag. While most of the mutants and wild type Sox2 were subject to protein turnover, the mutant with a truncated carboxy-terminus remained stable (Figure 3.1.4b).

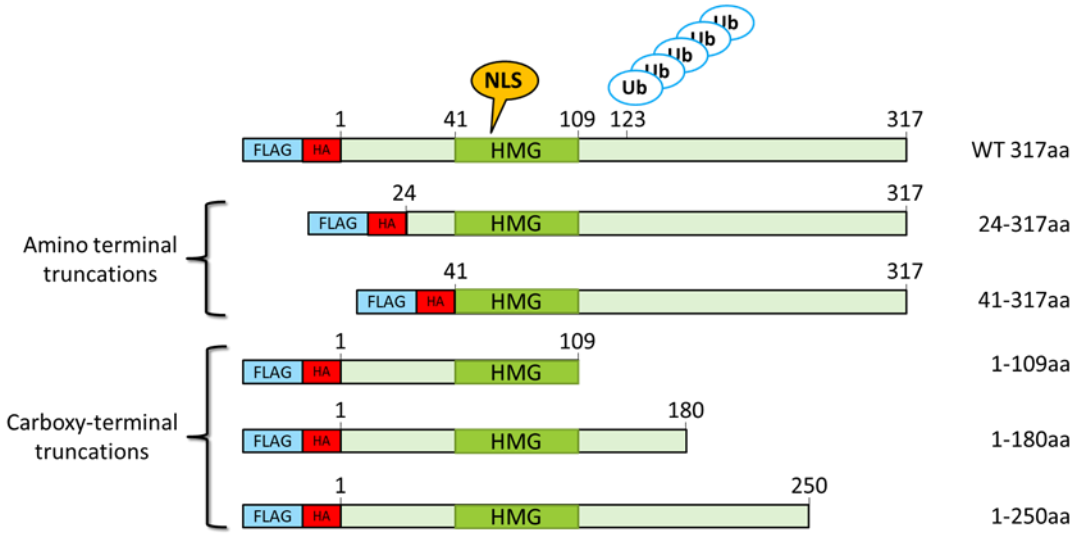
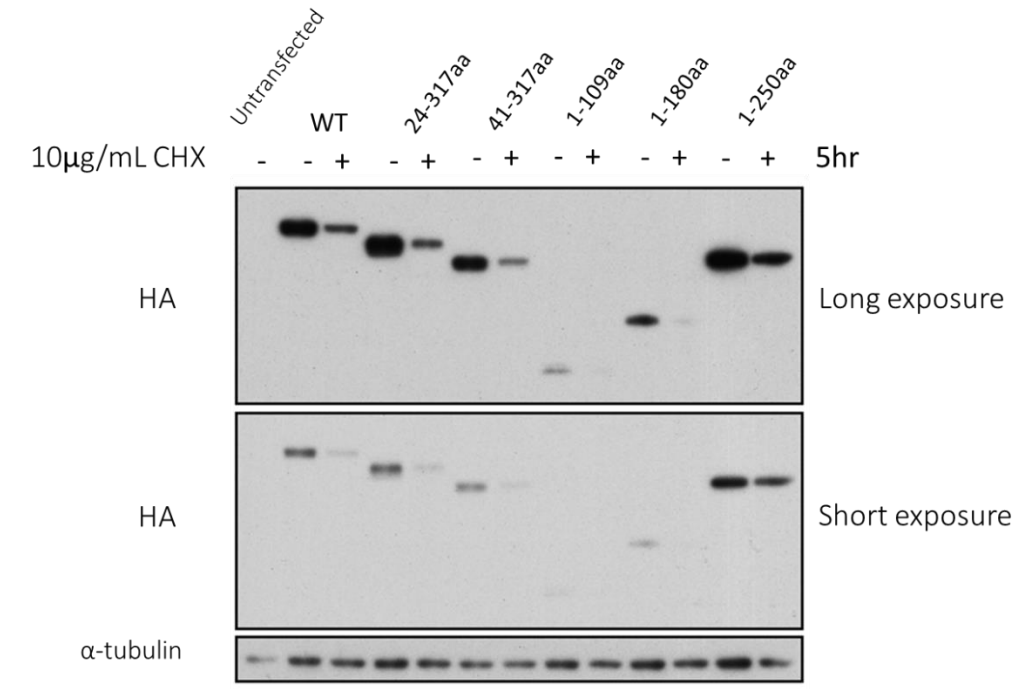


Figure 3.1.4a. Schematic of Sox2 truncation mutants. Sox2 mutants were sub-cloned into a plasmid containing a FLAG-HA tag. The HMG domain was left intact to avoid changes in nuclear localization and DNA binding. K123 denotes the currently known ubiquitylation site on Sox2.



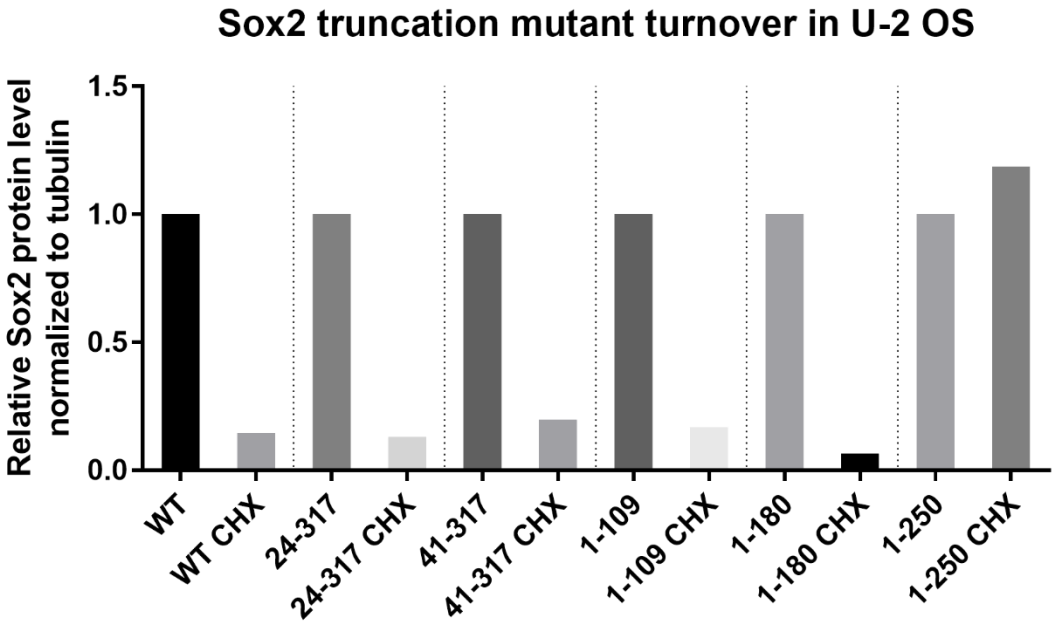
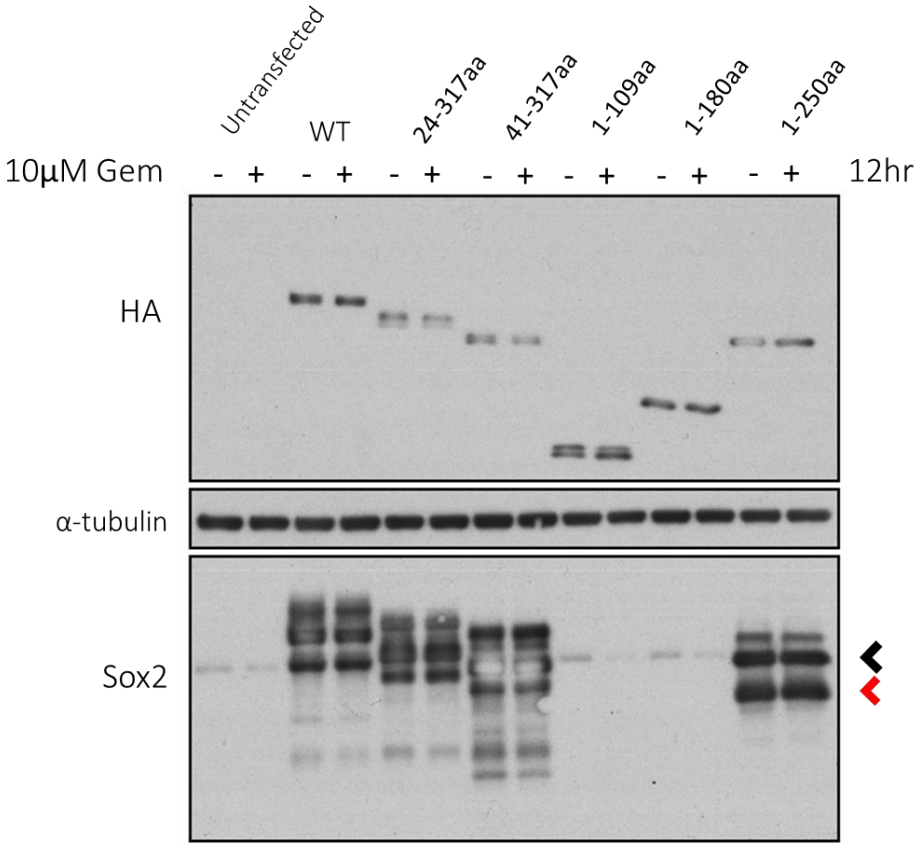


Figure 3.1.4b. Sox2 truncation mutant turnover in U-2 OS cells. Cells were transfected with Sox2 expression constructs for 24 hours then treated with 10µg/mL CHX for 5 hours. Lysates were collected for immunoblot analysis. Exogenous Sox2 probed using anti-HA antibody and normalized to tubulin. (Wild type = WT).


Untransfected
WT
24-317aa
41-317aa
1-109aa
1-180aa
1-250aa
10μM Gem
- + - + - + - + - + - + - +
12hr
HA
α-tubulin
Sox2

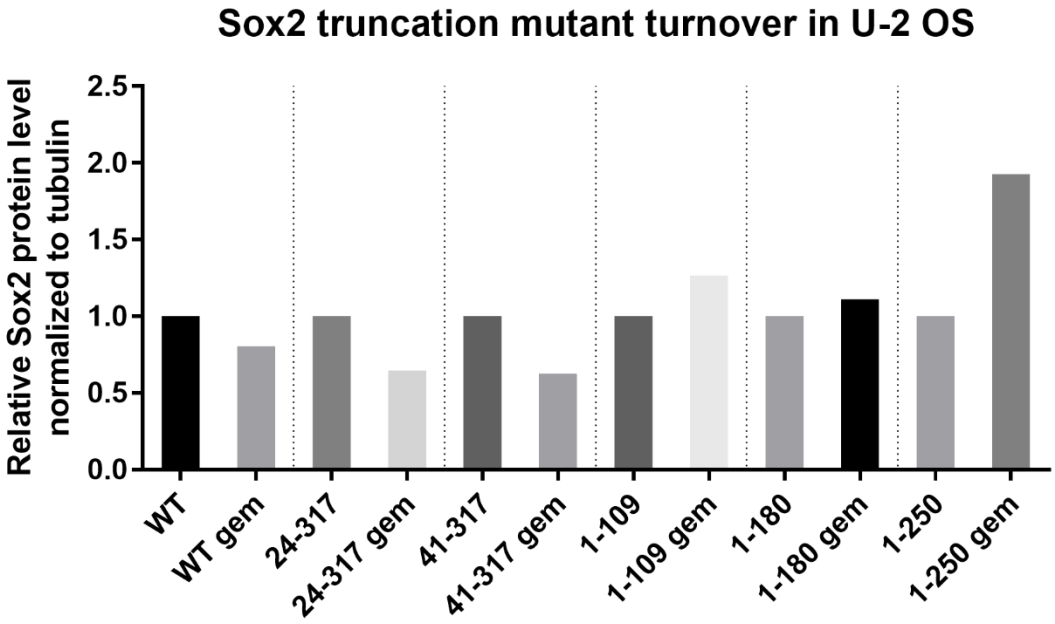

Sox2 truncation mutant turnover in U-2 OS
Relative Sox2 protein level normalized to tubulin
2.5
2.0
1.5
1.0
0.5
0.0
WT
WT gem
24-317
24-317 gem
41-317
41-317 gem
1-109
1-109 gem
1-180
1-180 gem
1-250
1-250 gem

Figure 3.1.4c. Sox2 truncation mutant turnover with gemcitabine treatment U-2 OS cells. Cells were transfected with Sox2 expression constructs for 24 hours then treated with 10μM gemcitabine (gem) for 12 hours. Lysates were collected for immunoblot analysis. Exogenous Sox2 probed using anti-HA antibody and normalized to tubulin, shown is the quantification of exogenous Sox2. Black arrowhead points to endogenous Sox2, red arrowhead points to1-250 Sox2 mutant in last two lanes (Wild type = WT).

Next, the same truncation mutants were expressed, except that cells were treated with gemcitabine for 12 hours. Exogenous Sox2 levels decreased slightly in WT and the amino-terminus mutants with gemcitabine treatment, not in the mutants lacking the carboxy-terminus (Figure 3.1.4c). The 1-109 truncated Sox2 is the only mutant that does not contain the reported K123 ubiquitylation site, and we did not observe accumulation of Sox2 when treated with CHX or gemcitabine over the other mutants. It is unclear why the 1-250 Sox2 mutant displayed an increase in protein levels after CHX and gemcitabine, however it is likely independent of the ubiquitin-proteasomal pathway. In the CHX treated cells, the increase may be due to loss of an unreported degron, or an error in immunoblot development underestimating loading control (Figure 3.1.4b, tubulin last lane). However, both experiments were only performed once and truncation mutants were overexpressed, which may mask changes in protein levels.

3.2 Sox2-dependent transcription

3.2.1 Sox2 responsive and non-responsive cell lines

To assay Sox2-dependent transcription following DNA damage, we used a reporter cell line for Sox2 transcriptional activity (kindly provided by Dr. Raymond Lai). Stable MCF7 cells expressing the reporter construct pGreenFire1-Sox2SRR2-mCMV-EF1-Puro had been generated by lentiviral transduction (Figure 3.2a). This reporter construct carries the SRR2 Sox2 enhancer element that drives CMV promoter expression of GFP, which acts as a readout for Sox2 transcriptional activity. When Lai and colleagues generated the cell line, they noted that the bulk population of cells can be divided into two distinct groups: Sox2 active vs Sox2 inactive subsets, despite similar expression of Sox2 protein in both sub-populations (Wu et al., 2012). Sox2 active cells comprised only a small percentage of the total MCF7 population. Sox2 active cells can be isolated based on their GFP expression, whereas Sox2 inactive cells do not express GFP (Figure 3.2a). The two populations were sorted out using fluorescence activated cell sorting (FACS) and cultured separately as MCF7 Sox2 inactive (inact) and Sox2 active (act) cells. Because the Sox2 active population tended to shift and lose GFP expression over time, Sox2 inactive and active cells were re-sorted every two months.

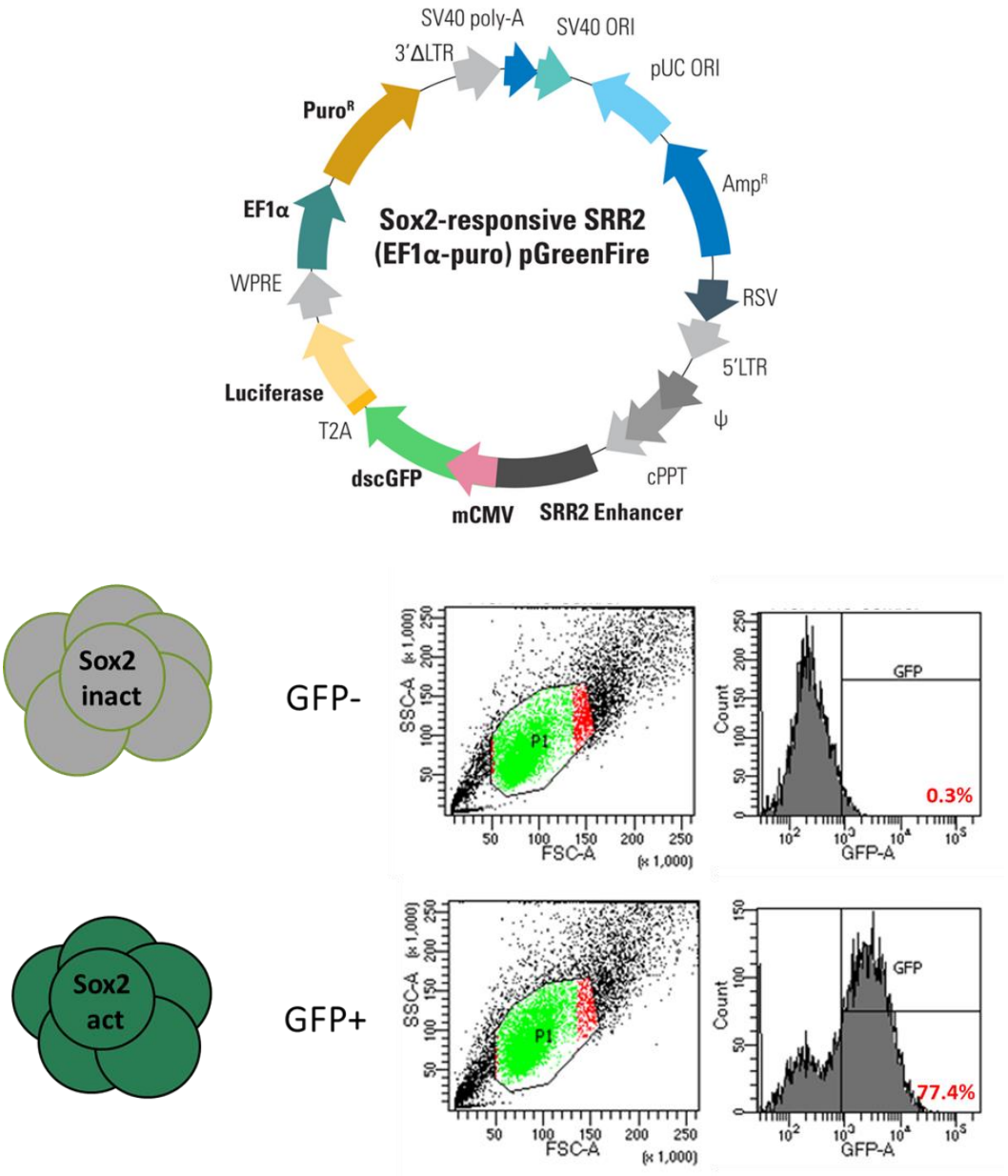
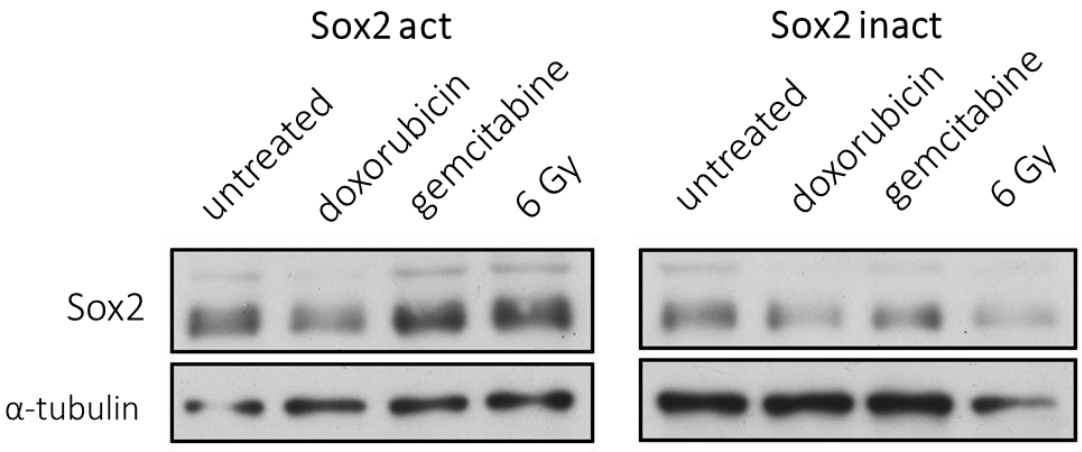


Figure 3.2a. Schematic of Sox2 reporter cell lines. **Top:** pGreenFire1-Sox2SRR2-mCMV-EF1-Puro construct from SBI system biosciences. SRR2 enhancer drives CMV promoter expression of destabilized CopGFP (dscGFP) and luciferase. EF1α drives the puromycin resistance gene. **Bottom:** Sox2 inactive (inact) cells are GFP negative and Sox2 active (act) cells are GFP positive. Flow cytometry analysis of the two cell lines were performed for GFP expression. Scatter plot of side scatter vs forward scatter area shows gating of single cell populations. Histograms show percentage of cells with GFP expression above threshold.

3.2.2 Sox2-dependent transcription after IR and hypoxia

We were interested in how Sox2 protein levels in active and inactive cells respond to DNA damage. A preliminary experiment with these reporter cell lines showed that Sox2 protein decreased with DNA damaging agent treatment after 12 hours (Figure 3.2b). To understand how DNA damage impacts Sox2 transcriptional activity and if a decrease in Sox2 protein correlates to its activity, we analyzed GFP expression of single cell populations with flow cytometry. Sox2 inactive and active cells treated with various DNA damaging agents were collected for flow analysis after 24 hours.

In Sox2 inactive cells, an increase in Sox2 active cells was observed in two out of four replicates when treated with 6Gy after 24 hours, indicating transcriptional activation of Sox2 in previously Sox2 inactive cells (Figure 3.2c, top graph). When Sox2 active cells were treated with the same drugs, GFP levels remained stable, despite treatment with CHX which inhibits the translation of new GFP protein (Figure 3.2c, bottom graph). This indicated that the GFP protein was stable for 24 hour, even in the presence of CHX, and thus using the Sox2 *active* cell line to assess changes in Sox2-dependent transcription through GFP was not possible for our treatment time frames.



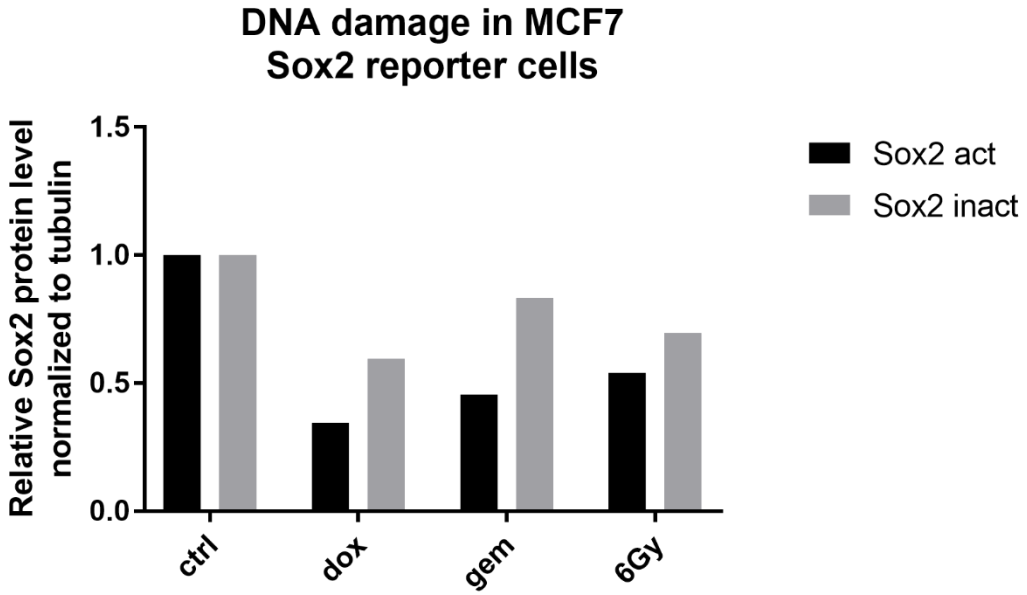


Figure 3.2b. DNA damaging treatments in MCF7 Sox2 reporter cell lines. MCF7 Sox2 act and inact cells were treated with the indicated drugs for 12 hours and lysates were collected for immunoblot analysis of Sox2. Sox2 levels were normalized to tubulin, n=1.

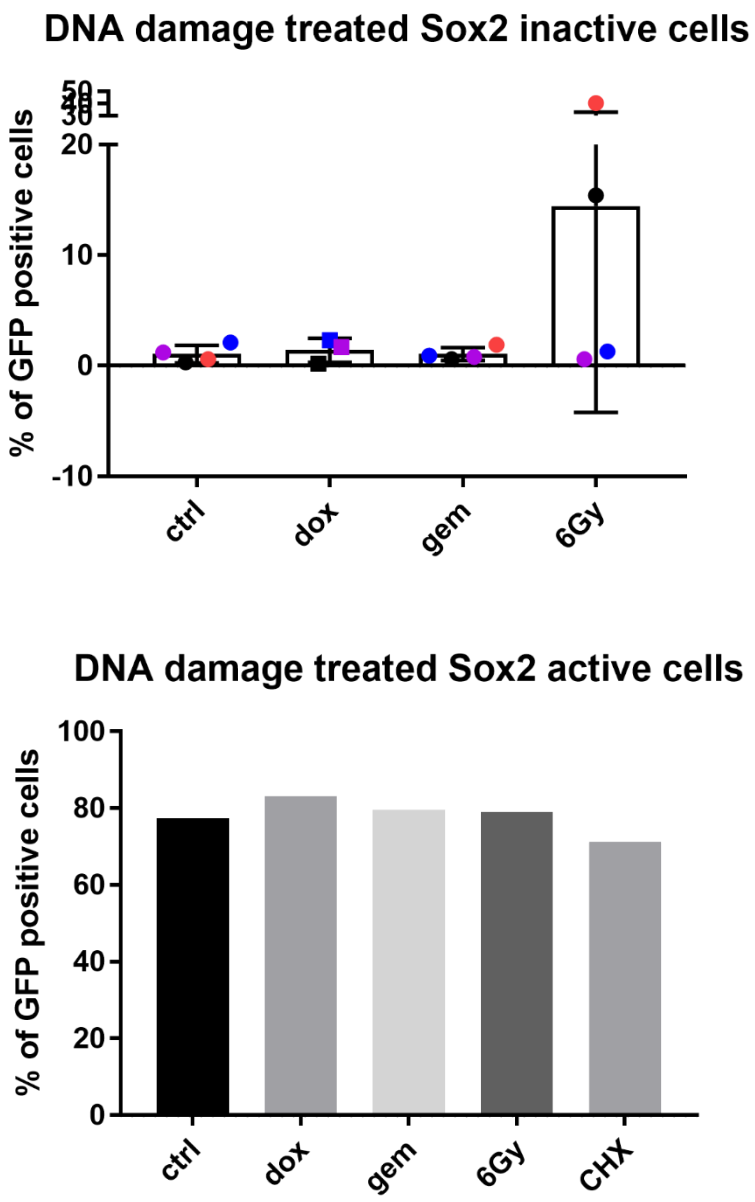


Figure 3.2c. Analysis of Sox2-dependent transcription following DNA damaging treatments in MCF7 Sox2 active and inactive cells. **Top**; Sox2 inactive cells were treated with IR or the indicated drugs for 24 hours and collected for flow analysis of GFP expression. Same coloured points correspond to the same replicate n=4, dox n=3. **Bottom;** Sox2 active cells treated with the indicated drugs or IR for 24 hours then collected for flow analysis, n=1. GFP expression was analyzed from gated single cells and the % of GFP-positive cells was determined in this population.

Hypoxia has been reported to induce expression of Sox2 in MCF7 cells within 48 hours and up to 72 hours (Park et al., 2016). We tested whether this was the case in our MCF7 Sox2 reporter cells. Sox2 active and inactive cells were incubated in 1% oxygen overnight then treated with or without 6Gy IR. Cells were placed back under hypoxia and lysates were collected at 24 and 48 hour post-IR (Figure 3.2d). Combined hypoxia and 6Gy led to decreases in Sox2 protein in both cell lines at 24 hours. At 48 hours, Sox2 levels were approximately half of control, but there was almost no difference between hypoxia alone and combined treatments. To understand how combined hypoxia and IR impacts Sox2-dependent transcription, we treated Sox2 inactive cells with either 2Gy or 6Gy under normoxic or hypoxic conditions, then collected cells for flow analysis after 72 hours. Under normoxia, we observed a dose-dependent induction of Sox2-dependent transcription, seen with the increase of GFP-positive cells (Figure 3.2e). Hypoxia alone increased the percentage of Sox2 active cells, and combination with IR showed further increase in GFP positive cells, suggesting a possible additive effect of hypoxia and IR in increasing Sox2 activity.

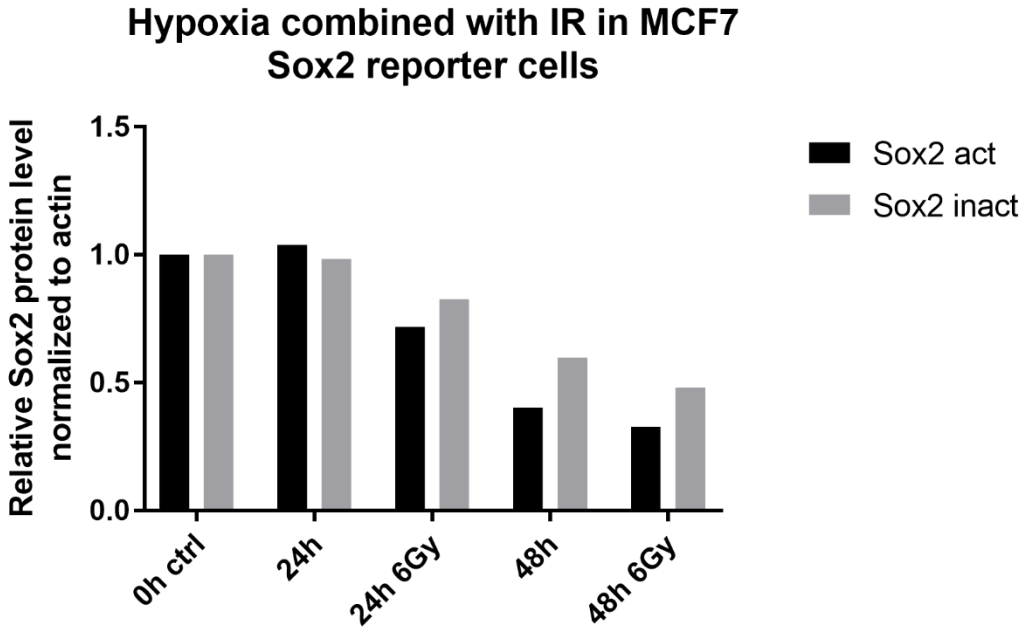


Figure 3.2d. Combined hypoxia and IR treatment in MCF7 Sox2 reporter cell lines. MCF7 Sox2 active (act) and inactive (inact) cells were grown in 1% oxygen overnight then treated with or without 6Gy. 0 hour control cells were grown in normoxia. Lysates were collected at 0, 24 and 48 hours post-IR for immunoblot analysis. Quantification of Sox2 protein levels in Sox2 active and inactive cells shown, normalized to actin (n=1).

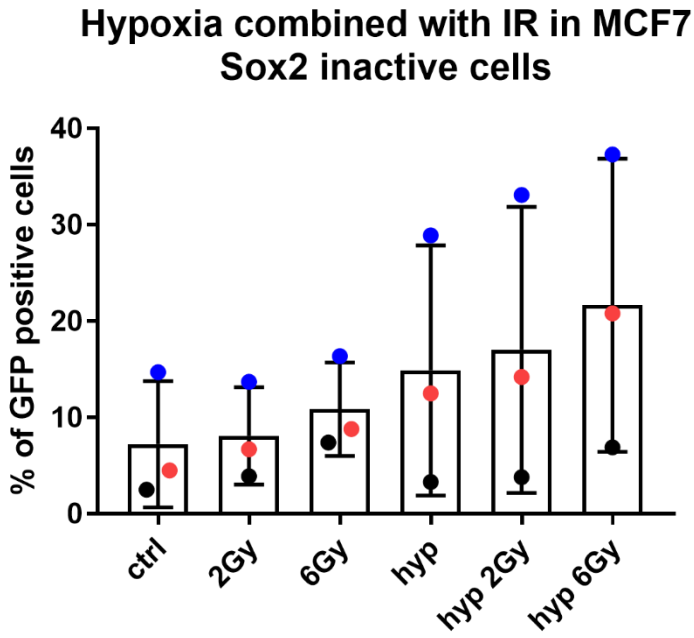


Figure 3.2e. Combined hypoxia and IR treatment in Sox2 inactive MCF7 cells. Sox2 inactive cells were incubated under normoxia (ctrl, 21% oxygen tension) or under hypoxia (hyp) at 1% oxygen tension overnight. The following day cells were treated with 2Gy or 6Gy and placed back under normoxia or hypoxia. 72 hours post-IR cells were collected for flow analysis of GFP expression (n=3).

Sox2 protein in MCF7 cells has been reported to increase following radiation treatment (Lagadec et al., 2012) and under hypoxia (Park et al., 2016). Our data shows that Sox2 protein decreases with IR and DNA damaging drugs in both Sox2 active and inactive MCF7 cells. Hypoxia alone did not affect Sox2 levels compared to normoxic control at 24 hours, however we observed a decrease in Sox2 protein at the 48 hour time point. When cells were grown under hypoxia then irradiated, Sox2 protein was down-regulated relative to cells grown under hypoxia alone. Comparing the immunoblot results to the flow cytometry data of Sox2 activity, we see that IR and hypoxia, either alone or combined, lead to an increase in Sox2 transcriptional activity. Hypoxia-induced Sox2 transcriptional activity has previously been shown using the same Sox2 reporter

system using a triple-negative breast cancer cell line (Soley-Abyaneh et al., 2018). Together this suggests that in MCF7, hypoxia and IR mediated Sox2 protein down-regulation corresponds to an increase in Sox2 transcriptional activity.

3.3 Identification of Sox2 interacting partners

3.3.1 BioID overview

To gain a further understanding of Sox2 regulation following IR ang hypoxia, we sought to identify interacting partners of Sox2 using a proximity-dependent biotin-labelling technique called BioID. BioID utilizes a mutant *E. coli* biotin ligase, $BirA^{R118G}$ (BirA*) (Roux et al., 2013). In the presence of its substrate, biotin, wild-type BirA converts biotin and ATP to form activated biotin (biotinyl-5'-AMP). Activated biotin is bound to the BirA active site until recognition of specific lysine residues on the BCCP subunits of acetyl-CoA carboxylase. Following which activated biotin is covalently linked to the substrate site. Choi-Rhee et al. identified a BirA mutant that increases the labelling efficiency while decreasing substrate lysine specificity. BirA* with the R118G mutation was found to have lower affinity to activated biotin, leading to premature release and indiscriminate biotinylation of lysine residues (Choi-Rhee et al., 2004). In BioID, BirA* fused to a protein of interest acts as a tagging enzyme for any interacting or close by proteins, allowing for either strongly interacting, transiently interacting, or adjacent proteins to be labelled with biotin. The covalent nature of biotinylation permits harsh lysis and washing conditions during purification with streptavidin, which improves protein solubilization and reduces non-specific binding to the column. Protein eluted from the streptavidin pull-down can be sent for subsequent protein identification by mass spectrometry. Alternatively, the protein of interest, which is also tagged with a FLAG peptide, can be used for identification of more stable interacting partners by co-immunoprecipitation. This would be a complementary technique to BioID (Figure 3.3a).

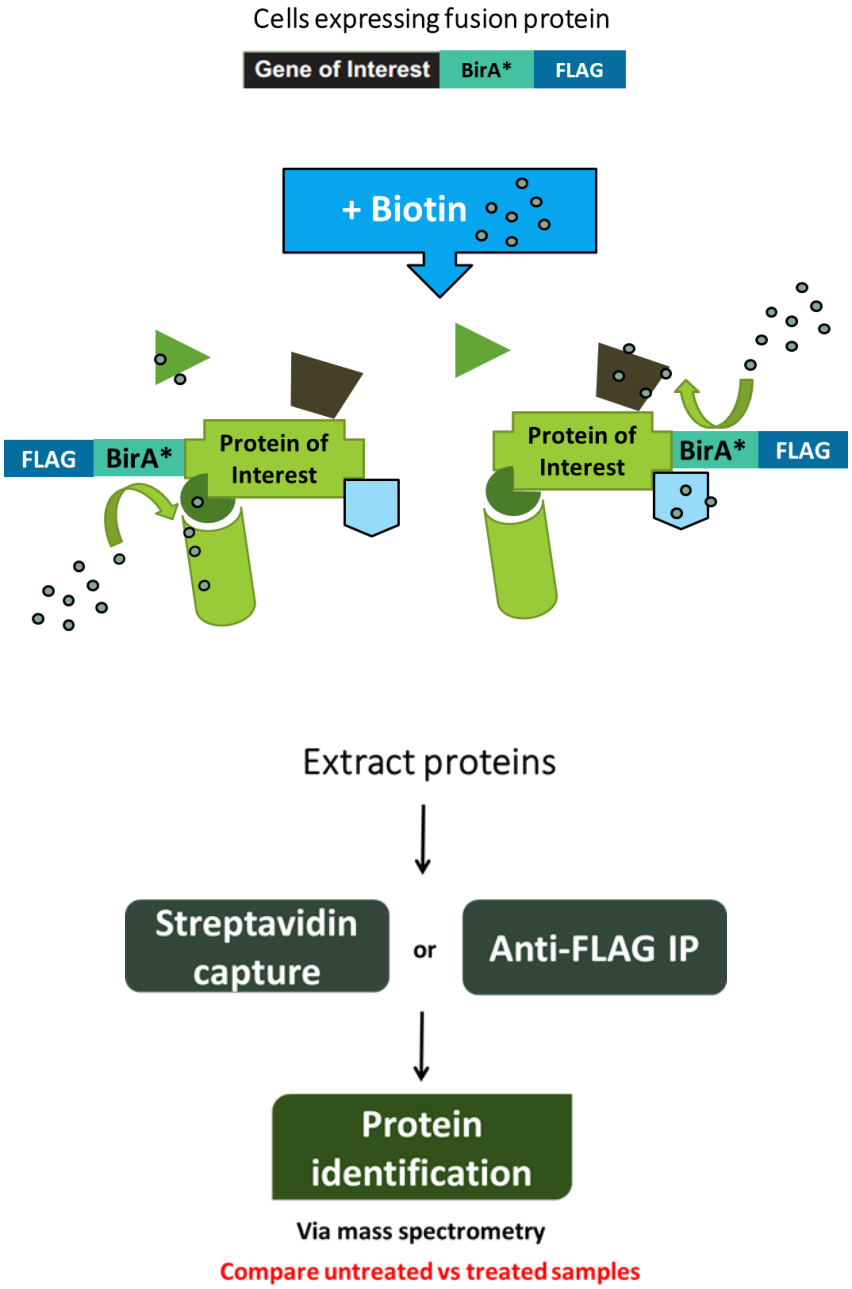
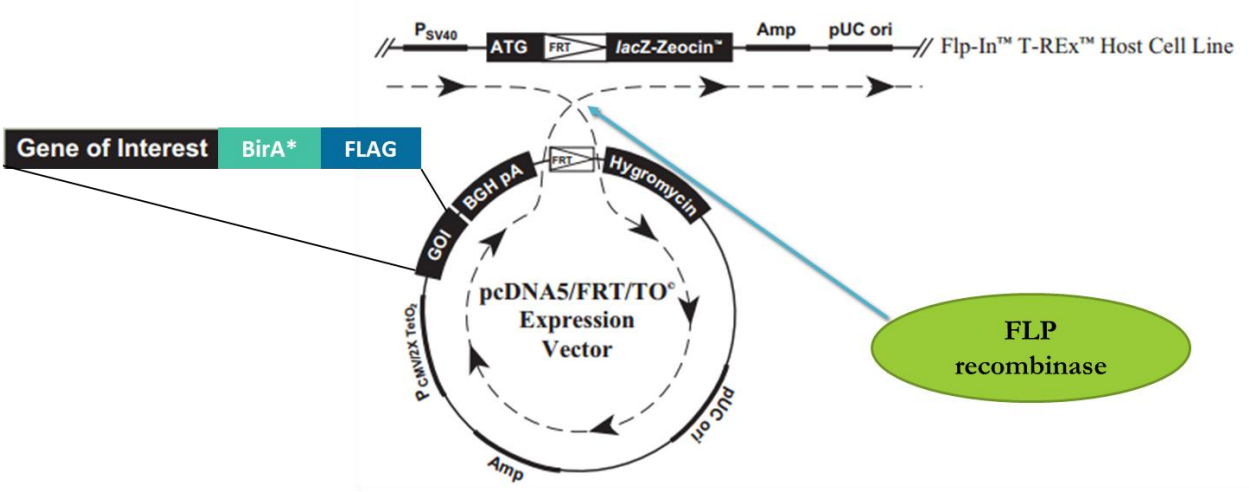


Figure 3.3a. Schematic of BioID. In the presence of biotin, cells expressing BirA* fused to its amino- or carboxyl- terminal will create a "cloud" of active biotin that covalently modifies lysine residues of surrounding proteins. Protein extracts can be subject to streptavidin capture or FLAG immunoprecipitation to identify both transiently and strongly interacting partners of the protein of interest by mass spectrometry in control vs treated samples.

3.3.2 Generation of tetracycline-inducible Sox2 expressing cells

We generated stable cell lines expressing Sox2 fused to BirA* using the Flp-In TREx system (Invitrogen). This system allows for the integration of a tetracycline-inducible gene at a specific genomic location of host cell lines. Host cell lines express two plasmids: pFRT/*lac*Zeo contains a single FRT site and a Zeocin resistance gene that is genomically integrated, while pcDNA6/TR has a constitutively expressed Tet repressor gene and blasticidin resistance. Once the host cell line is established, the expression vector (pcDNA5/FRT/TO) and the FLP recombinase (pOG44) are co-transfected into the cells. The genomic and expression vector FRT sites are recognized by the FLP recombinase, which in turn catalyzes a homologous recombination event (Figure 3.3b, top). Successful genomic integration of the expression vector disrupts zeocin resistance gene expression resulting in zeocin sensitivity. The tetracycline response element within the expression vector is composed of two TetO operator sequences ($TetO_2$) and a CMV promoter. In the absence of tetracycline, constitutive expression of the tet repressor, tetR, blocks transcription of the fusion protein by binding to TetO as a homodimer. When tetracycline is added, it binds to TetR, causing a conformation change and releasing it from the operator. This allows for transcription of the fusion protein driven by the CMV promoter (Figure 3.3b, bottom).

We generated two stable cell lines with BirA* fused to either the amino- or carboxyl- terminal of Sox2 and tested for inducibility of fusion protein expression (figure 3.3c). Both Sox2 and BirA* have a molecular weight of ~35kDa, so the fusion protein was detected at ~70kDa.



Modified from Life Technologies Flp-In T-REx core kit user guide (2012)

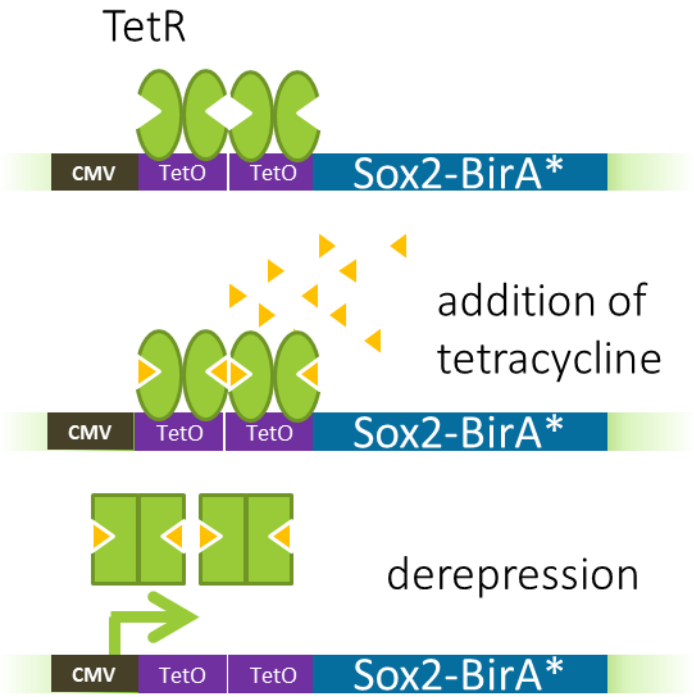


Figure 3.3b. Schematic of stable cell line generation and fusion protein expression. **Top**, fusion protein expression vector is integrated into genomic FRT sites through FLP recombinase activity. Stable integration leads to zeocin sensitivity and hygromycin resistance. **Bottom**, constitutive tet repressor expression blocks fusion protein expression in the absence of tetracycline. Addition of tetracycline derepresses the promoter, allowing for tetracycline-controlled transcriptional activation.

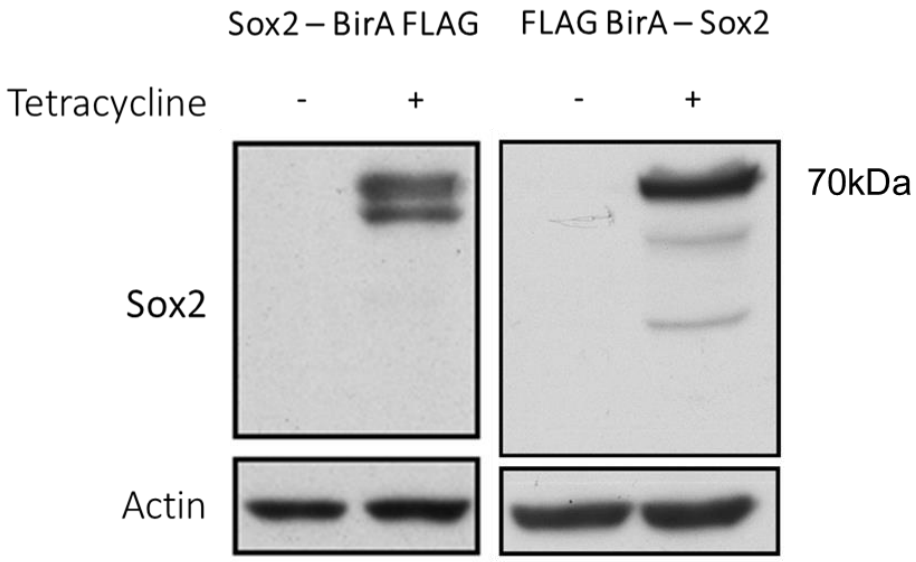


Figure 3.3c. Inducible expression of Sox2-BirA* fusion proteins. Stable HeLa cell lines expressing BirA* fusion proteins were induced with 2µg/mL tetracycline overnight and lysates were collected the following day for immunoblot analysis of Sox2. Sox2 and BirA* are both ~35kDa, the fusion protein is ~70kDa.

3.3.3 Experimental procedure and results

We performed biotin pull down with HEK293 Sox2-BirA* expressing cells treated with gemcitabine and ran the eluted protein on a gel for silver staining (Figure 3.3d). Considering that Sox2 is directly fused to BirA*, we expected the fusion protein to be highly enriched in the eluted fraction. However Sox2-BirA* (~70kDa) was not identifiable from the tetracycline-induced samples (lanes 2 and 3), and there was no distinct band at this molecular weight that was lacking from the non- induced sample in lane 1. In addition, there were many background bands present in all three samples, making it difficult to identify potentially unique interacting proteins in gemcitabine treated cells.

We later received the HeLa FlpIn TREx cell line and generated stable Sox2- BirA* expressing cells in place of the HEK293 cells. We chose to use the HeLa cell line instead of HEK293 to more closely represent the Sox2 interactome in the context of cancer. We generated HeLa cell lines expressing tetracycline-inducible Sox2 fused with BirA* both at amino- and carboxy- terminal ends (Figure 3.3c). We grew the Sox2-BirA* expressing cells under normoxia or hypoxia (1% oxygen) and performed a biotin pull down using two different protocols. In the first experiment, cells were induced to express Sox2-BirA* with tetracycline for 5 hours then placed under hypoxia for 4 hours. The medium was replaced with biotin for labelling overnight. Then cells were collected for cellular fractionation. Protein eluted from each fraction was loaded and ran on a gel for silver staining (Figure 3.3e). As the "bait" protein, we were expecting Sox2- BirA* to be enriched in all fractions, but this was not the case in the silver stain. There were still many precipitated background proteins, making it difficult to identify bands unique to the hypoxic fractions, especially in the chromatin fraction. In the cytosolic fraction, less biotinylated proteins

were pulled down from the hypoxic cells, however it is unclear if this is due to differences in protein input.

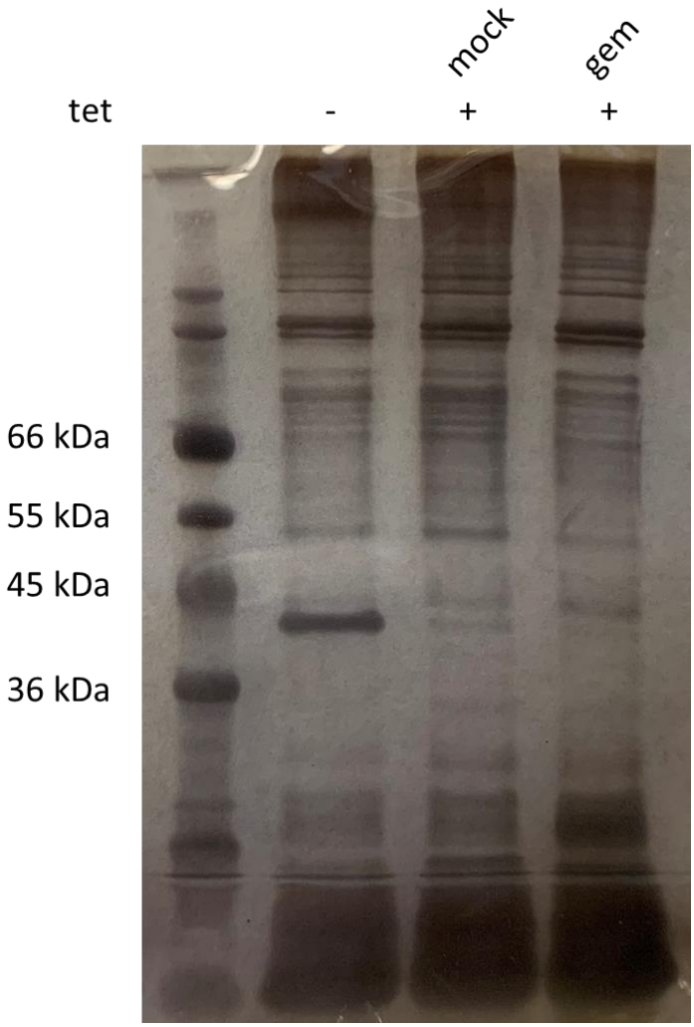


Figure 3.3d. Gemcitabine treatment and biotin pulldown of HEK293 Sox2-BirA* cells. HEK293 FlpIn TREx cells with stable tetracycline-inducible Sox2-BirA*-FLAG fusion protein expression were grown in 15cm plates and induced with 2µg/mL tetracycline (tet) for 7 hours. Media was then replaced with 50µM biotin and cells were either treated with DMSO (mock) or 10µM gemcitabine overnight. Nuclear soluble extracts were prepared and incubated with streptavidin beads for biotin pull down, then 8µl of protein samples were loaded on an SDS gel for silver stain.

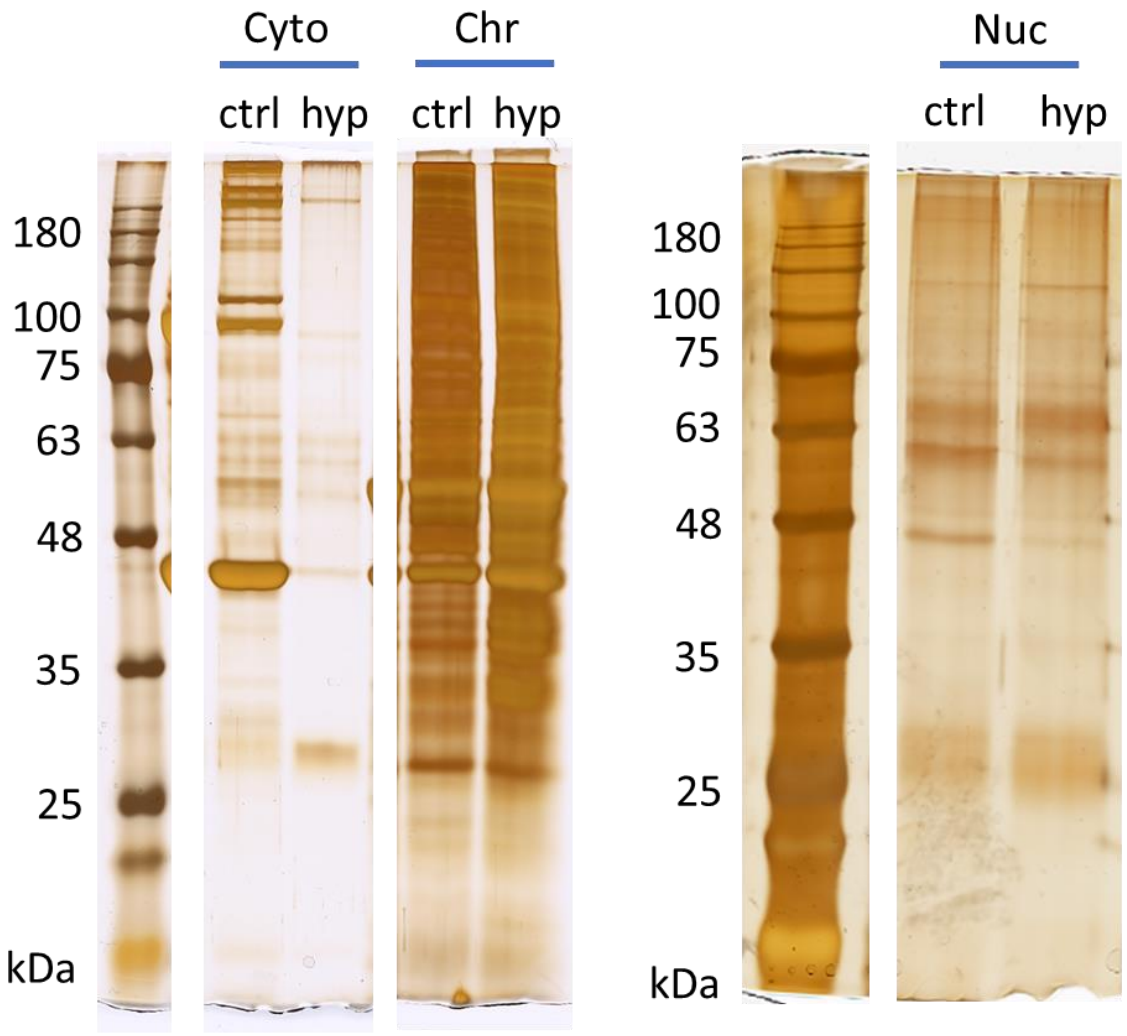


Figure 3.3e. Biotin pull down of HeLa Sox2-BirA* cells under normoxia and hypoxia. HeLa Sox2-BirA* cells were induced with 0.5µg/mL tetracycline for 5 hours then incubated under normoxia (ctrl) or 1% oxygen (hypoxia, hyp) for 4 hours. Media was then replaced with 50µM biotin for 14 hours before cellular fractionation. 12µL of protein eluted from each fraction was loaded on a 10% SDS gel for silver staining. Cytosolic (cyto) and chromatin bound (chr) fractions were loaded on one gel and the nuclear soluble fraction (nuc) was loaded on a separate gel because of differences in silver stain developing time. Lanes omitted in between were from unrelated experiments.

We repeated this hypoxia treatment experiment using a whole cell lysis protocol received from Dr. Boisvert (University of Sherbrooke), however the silver stain did not yield any bands (not shown). This protocol uses RIPA lysis buffer combined with benzonase and sonication to extract chromatin bound proteins, so it is unlikely that the lysis was incomplete. Because there were no bands detected in the silver stain and cells were labelled with biotin overnight, it is possible that proteins were loss during some washing steps.

CHAPTER 4: DISCUSSION

4.1 Discussion and Future Directions

According to the 2017 Canadian cancer statistics report, nearly half of all Canadians will be diagnosed with cancer in their lifetime (Canadian Cancer Society, 2017). Despite current advancements to improve cancer treatment, long term survival is often hindered by incomplete tumour eradication, leading to local recurrence and/or secondary tumours at distant sites. Recent efforts aim to target the tumour-initiating CSCs to achieve local tumour control, however emerging studies of cancer therapy-induced cellular plasticity point to potentially unexpected outcomes of therapy. Radiotherapy is commonly utilized in cancer treatment by targeting high doses of IR to a tumour and inducing lethal damage. IR has been found to induce stemness in non-stem like cancer cells, resulting in dedifferentiated cancer cells expressing pluripotency factors and with increased self-renewal and tumourigenic capacity. Expression of the pluripotency factor Sox2 is associated with CSC maintenance, as well as radiation induced stemness. Our interest in radiation induced stemness led to the hypothesis that radiation regulates Sox2 expression and activity, which may alter cancer cell fate. In the present study, we examined Sox2 regulation by IR in osteosarcoma and breast cancer cell lines. In osteosarcoma, Sox2 is a potent driver of tumourigenesis and CSC phenotypes (Basu-Roy et al., 2012; Maurizi et al., 2018). In breast cancer, Sox2 activates oncogenic pathways that promote proliferation, and its expression is associated with CSC self-renewal (Chen et al., 2008; Piva et al., 2014). Radiation-induced stemness has been demonstrated in the breast cancer cell line MCF7, and Sox2 expression can be induced in previously non-stem like cancer cells (Lagadec et al., 2012).

4.1.1 Sox2 protein down-regulation following IR

To determine if Sox2 plays a role in linking radiation and induction of stemness in cancer cells, we examined how Sox2 protein was regulated following IR in osteosarcoma and breast cancer cell

lines. Contrary to previous reports of Sox2 induction in radiation-induced osteosarcoma CSCs (Basu-Roy et al., 2012), we unexpectedly observed a decrease in Sox2 protein expression. In U-2 OS cells, Sox2 protein down-regulation was consistently observed when cells were treated with DNA damaging agents, however the kinetics of down-regulation varied between replicates. While the differences in Sox2 down-regulation between DNA damaging agents can be explained by the varying mechanisms of DNA damage induction, the degree of Sox2 down-regulation observed between IR treated replicates varied as well. This was not due to inconsistencies in cell lysis because comparisons of mild and harsh lysis conditions yielded similar results, and a preliminary experiment showed that Sox2 down-regulation occurs in the cytosolic, nuclear and chromatin fractions following IR. However it may be due to the effect of cell density on baseline levels of Sox2 we observed, where cells at higher densities have increased Sox2 protein compared to cells at lower densities. This was evident in our time course studies where untreated cells at later time points displayed an increase in Sox2 protein compared to early time points. We were unable to find studies referring to cell density mediated changes in Sox2 levels. While Sox2 is known to interact with signalling pathways regulated by cell adhesion (Wnt pathway) and organ size (Hippo pathway), these pathway interactions are not dependent on cell density and are only observed in the CSC populations (Basu-Roy et al., 2012, 2015). Regardless, we observed Sox2 down-regulation following IR at both low and high cell densities, suggesting that this regulation occurs independent of the cell cycle. Knowing the sensitivity of Sox2 protein to cell density, moving forward it will be important to maintain consistent seeding densities between experiments.

With regards to MCF7 cells, Sox2 down-regulation following IR was not as consistent or to the same extent as in U-2 OS cells. In addition, our results were confounded by the observation that MCF7-RM and MCF7-LP cells possessed different morphologies as well as proliferation rates.

Unfortunately, we have not genotyped either MCF7 cell lines. However there is the possibility that tissue-specific factors are involved in regulating Sox2 protein after IR.

Quantitative PCR of *SOX2* transcripts in U-2 OS cells after IR showed no significant changes in *SOX2* expression at early time points (6 hours), indicating that Sox2 protein down-regulation after IR is post-transcriptional, at least after 12 hours, the end point of most of our experiments. At later time points (24 hours) there is a large variation between replicates. It is possible early Sox2 protein down-regulation triggers an autoregulatory positive feedback loop, resulting in increased transcriptional activation of *SOX2* at later time points. Sox2 transcribed from a viral promoter (see Figure 3.1.3b) is also subject to IR-mediated down-regulation. This is a strong indicator that Sox2 down-regulation is post-transcriptional. We suspected that Sox2 down-regulation was via the ubiquitin-proteasome pathway due to the observed rapid decrease in protein levels after IR. While transient knockdown of Sox2 regulating ubiquitin ligase WWP2 showed increased protein stability, ubiquitylation assays will be required to determine if Sox2 is indeed ubiquitylated after IR. A recent study identified Set7-mediated methylation of Sox2 as a prerequisite for WWP2 recruitment and subsequent proteolysis. Sox2 degradation via this pathway can be rescued by LSD1 demethylase activity (Fang et al., 2014; Zhang et al., 2018). It will be interesting to test how LSD1 or Set7 inhibition affects Sox2 degradation following IR.

Inhibition of the DNA damage response kinases ATM and ATR revealed that ATM signalling is upstream of Sox2. While it is unlikely that ATM phosphorylates Sox2 directly, the ATM signalling cascade may mark Sox2 for E3 ligase recognition, indirectly activate ubiquitin ligase complexes to target Sox2, or both. Furthermore, the E2 ubiquitin ligase Ube2S has been shown to be activated in an ATM-dependent manner at DNA damage sites where it conjugates K11-linked ubiquitin for H2AX modification (Paul and Wang, 2017). Ube2S also ubiquitylates Sox2 at the currently only

known ubiquitylation site on Sox2, K123 (Wang et al., 2016). More recently, WWP2 was shown to promote non-homologous end joining repair in response to double stranded DNA breaks, raising the possibility that WWP2 is activated by DNA damage (Caron et al., 2019). The lysine methylase Set7 that methylates Sox2, which then is recognized by WWP2, is also implicated in the DNA damage response (Lezina et al., 2015). Considering the large subset of ATM substrates following DNA damage, this approach to identifying upstream regulators of Sox2 is not efficient. Our next approach therefore would be to validate already known Sox2 regulating ubiquitin ligases by with immunoprecipitation assays to verify DNA damage-induced interaction.

To this end, we constructed Sox2 truncation mutants to help pinpoint the Sox2 degron for IR-mediated degradation. However these mutants were highly overexpressed compared to endogenous Sox2 levels, which may overload the proteasome machinery and mask changes in protein stability at endogenous levels of expression. Interestingly, our preliminary experiment shows that the carboxyl-terminal truncation mutant with a 67 amino acid deletion increased in stability when cells were treated with CHX or gemcitabine. There are no reported ubiquitylation sites within this deletion, though it may be involved in mediating E3 ubiquitin ligase binding. There are also two sites of O-GlcNAcylation within the deletion, however the role of this PTM has only been reported to increase Sox2 transcriptional activity and reprogramming efficiency (Myers et al., 2016). It remains unclear how loss of this segment leads to Sox2 stabilization after gemcitabine or CHX treatment. Future experiments will focus on further dissecting the residues involved in IR-mediated down-regulation, as well as site directed mutagenesis of known sites of Sox2 regulation.

The sensitive nature of Sox2 protein to cell density made it difficult to interpret time course studies. When Sox2 turnover experiments were performed with cycloheximide treatment, most variation

in Sox2 levels between replicates occurred at later (12 hour) time points. Usually when cells are seeded for time course studies, we would compensate for growth by seeding less cells for later time points to avoid reaching high cell density and increasing Sox2 protein. In the experiments with combination treatments of CHX, IR or ATMi, 12 hour control plates ended up with overall lower Sox2 levels compared to the 0 hour control. We were then unable to use the 12 hour control data points as a reference for 12 hour treated data points, as we can't be certain that decrease in Sox2 is due to the treatment or the seeding density. The purpose of this experiment was to compare Sox2 half-life under normal conditions and IR-treatment. If IR-mediated Sox2 down-regulation is dependent on translational regulation, combined IR and CHX treatment would have an additive effect on Sox2 protein down-regulation.

Knowing that cell density can affect Sox2 levels, cells prepared for time course experiments should be seeded with more consistency, so that plates collected at each time point contain roughly the same number of cells. Plates may be seeded at low densities to avoid growth into higher densities at the experimental end point. However it is hard to predict how cellular proliferation rates will respond to drug treatments over a time course experiment. Since Sox2 down-regulation after IR is observed at both low and high densities, cells may also be treated at high densities, which closer represents *in vivo* cell-cell interactions and signalling.

4.1.2 Sox2-dependent transcription following IR and hypoxia

The Sox2 reporter cell line was used to assess Sox2-dependent transcription following IR, as changes in Sox2 protein level may not correlate to changes in Sox2 transcriptional activity. Immunoblot analysis of Sox2 inactive and active cells- the activity being measured by GFP

expression from a Sox2-dependent promoter - showed similar trends of Sox2 protein down-regulation when treated with various DNA damaging agents. Yet treatment with 6 Gy led to an induction of Sox2 activity in Sox2 inactive cells, while Sox2 protein levels decreased at the same 24 hour time point. This indicates that Sox2 protein down-regulation in MCF7 Sox2 reporter cells, shows an inverse correlation to Sox2 transcriptional activity.

This Sox2 reporter system utilizes an enhancer element SRR2 to measure Sox2-dependent transcription, with GFP as a readout for Sox2 activity at SRR2. We were only able to use the Sox2 inactive cells to look for induction of activity, as the GFP in Sox2 active cells was too stable to assess potential changes in GFP-expression in GFP-positive populations. Hypoxia had been shown to induce Sox2 protein expression in breast cancer cells (Park et al., 2016). We therefore investigated how combining IR and hypoxia affects Sox2 protein levels and activity. We were unable to observe the previously reported increase in Sox2 protein at 48 hours, even when grown under the same conditions of 1% oxygen with the same MCF7 cell line (Park et al., 2016). The difference in response to hypoxia between MCF7 cells may be explained by a recent report detailing clonal and genetic variation in drug responses between strains of MCF7 cells (Ben-David et al., 2018).

An IR dose-dependent induction of Sox2 activity was observed in previously inactive cells, and when IR was combined with culture under hypoxic conditions, there was a further increase in Sox2 activity. Our observation that hypoxia alone increased Sox2 activity was in line with a recent study using the same reporter system in triple-negative breast cancer cells. They found that hypoxic induction of Sox2 in Sox2-inactive cells was mediated by STAT3 binding to the SRR2. These hypoxia induced-Sox2 active cells displayed stem-like properties (marker expression,

tumourigenicity, drug resistance) similar to pre-existing Sox2 active cells (Soleymani-Abyaneh et al., 2018).

The SRR2 enhancer element was initially reported to be only permissive to Sox2-OCT4 or Sox2-OCT6 complex binding in embryonic stem cells (Tomioka et al., 2002). Emerging studies show that the SRR2 element may also be bound by other factors such as p21 and p27, which can lead to repression of Sox2 transcription during differentiation (Li et al., 2012; Marques-Torrejon et al., 2013). This potentially compromises the reporter system we used for isolating Sox2 active and inactive cells, because the SRR2 element is not exclusive to Sox2 binding and thus could lead to the isolation of false Sox2 inactive or active cells. Despite these limitations, this reporter system is useful for verifying transcriptional regulators of Sox2. Yet in addition, in order to assess Sox2 transcriptional activity, we may need to look at the mRNA levels of endogenous – and potentially tissue-specific - Sox2 transcriptional targets such as YAP, Nf2, and WWC1 (Basu-Roy et al., 2015; Seo et al., 2013).

4.1.3 Identification of novel Sox2 interacting partners after IR

BioID offers an alternative approach to affinity purification, which often fails to identify weak or transient protein interactions. Covalent modification of biotin on proteins makes the process of protein purification permissive to harsher lysis and washing conditions. To date, BioID has been used to unveil interacting partners of kinases, membrane bound proteins and chromatin-associated proteins (May and Roux, 2019). One disadvantage to BioID is the necessary labelling time of 15-18 hours, which is unsuitable for examining dynamic protein interactions induced by the DNA damage response. Our purpose for using BioID was to identify potential regulators of Sox2 that

may be involved in IR-mediated Sox2 down-regulation – most of this regulation is observed between 6 and 12 hours. After biotin labelling following IR, Sox2 may no longer be bound to the same proteins by the time for lysate collection as during the initial response. In addition to timing, the large amount of background biotinylated proteins that are precipitated by streptavidin make it difficult to identify interacting partners that are unique to a treatment. Our biggest concern with the BioID experiments performed was the inability to identify Sox2-BirA* from the streptavidin pull down, which should theoretically be the most frequently biotinylated target in the cell. It's possible that our tetracycline-induction times at 4 hours were inadequate, however our silver stains show that there are indeed proteins pulled down from the streptavidin beads (Figure 6e). Non-specific binding of proteins to beads was also reduced by stringent washing steps with buffers containing SDS. We suspect that overexpression of our fusion protein may be leading to degradation products with intact BirA*, explaining biotinylation in the absence of Sox2-BirA*. This might be circumvented by titrating fusion protein expression by adjusting tetracycline concentration and incubation time. However this requires scaling up of our starting material.

Recently, alternative labelling methods have been developed that offer shorter incubation times. For example, the modified soybean ascorbate peroxidase, APEX2, only takes 1 minute for biotin labelling neighbouring proteins (Hung et al., 2014). However, this requires the presence of hydrogen peroxide which can lead to oxidative stress and damage DNA. TurboID is a more promiscuous biotin ligase than BirA*, with a total of 15 mutations relative to wild-type BirA. Biotin labelling for 10 minutes with TurboID was shown to yield similar amounts of biotinylated products as labelling with BirA* (BioID) for 18 hours (Branon et al., 2018). For future experiments, TurboID may prove to be the better method for proximity-dependent labelling to identify Sox2 regulators following IR.

4.2 Conclusions

Sox2 is a master regulator of cell fate during development and for the maintenance of stem cells. Unsurprisingly, aberrant Sox2 expression is associated with oncogenic pathways and invasive phenotypes of a variety of cancer types. Recent studies show that radiation is able to induce CSC phenotypes in non-stem like cancer cells. Not only is Sox2 elevated in these induced CSCs, its role in CSC maintenance has been described in multiple cancer types. Here we reveal a radiation-mediated regulation of Sox2 in cancer cell lines. Our observations indicate that down-regulation of Sox2 protein levels after post-IR can occur concomitantly to an induction of Sox2 transcriptional activity. It remains unclear whether short-term changes to Sox2 protein stability and transcriptional activity have long-term implications on cancer cell fate. However, elucidation of Sox2 regulators and mechanistic insights of Sox2 regulation after IR may provide answers to this question. Our preliminary results point to a role of ATM in Sox2 regulation, an apical kinase for which recently bioavailable inhibitors have been developed and are entering the clinic. Furthermore, if Sox2 regulation by IR plays a pivotal role in CSC induction, Sox2 may serve as predictive biomarker for cancer therapy outcome.

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