Characterization of the anti-apoptotic function of the lysine demethylase plant homeodomain finger protein 8 (PHF8)

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Graduate Program in Biochemistry
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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CHARACTERIZATION OF THE ANTI-APOPTOTIC FUNCTION OF THE LYSINE DEMETHYLASE PLANT HOMEODOMAIN FINGER PROTEIN 8 (PHF8)

(Thesis format: Monograph)

by

Kimberly Muranko

Graduate Program in Biochemistry

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
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Abstract

Apoptosis is an essential process in development and tissue maintenance. The tumor suppressor protein p53 initiates apoptosis through transactivation of pro-apoptotic genes when cellular stress is detected. This study identifies a regulatory role for the lysine demethylase, PHF8, in the p53-mediated apoptosis pathway. We initially suspected PHF8 of demethylating the adaptor protein Numb, however found this to be untrue. PHF8 has been found to have oncogenic properties including an anti-apoptotic effect, however how PHF8 negatively affects apoptosis has not been previously investigated. We found PHF8 inhibits translation of the pro-apoptotic genes TP53, BAX and CASP3. Chromatin immunoprecipitation revealed PHF8 binding to the TP53 and CASP3 gene promoters. H3K27 methylation at these promoters was significantly decreased with doxorubicin treatment in the presence of PHF8, and significantly increased when PHF8 was knocked down. Since methylated H3K27 is a repressive mark, we suspect other transcription factors or histone modifying proteins to be involved.

Keywords

Apoptosis, Numb, PHF8, p53, Caspase3, demethylation, epigenetics, demethylase, chemotherapy, cell death.
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<th>Description</th>
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<tbody>
<tr>
<td>Apaf-1</td>
<td>Apoptosis-protease activating factor 1</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia-telangiectasia, mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>ATM and Rad3-related</td>
</tr>
<tr>
<td>BAD</td>
<td>B-cell lymphoma (Bcl)-2-associated death promoter</td>
</tr>
<tr>
<td>BAX</td>
<td>Bcl-2-associated x protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>Caspase3</td>
<td>Cysteine-dependent aspartate-directed protease 3</td>
</tr>
<tr>
<td>Caspase9</td>
<td>Cysteine-dependent aspartate-directed protease 9</td>
</tr>
<tr>
<td>CDK1</td>
<td>Cyclin dependent kinase 1</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>Chk1</td>
<td>Checkpoint kinase 1</td>
</tr>
<tr>
<td>Chk2</td>
<td>Checkpoint kinase 2</td>
</tr>
<tr>
<td>CASP3</td>
<td>Caspase3 gene</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>DPF</td>
<td>Aspartic acid-proline-phenylalanine</td>
</tr>
</tbody>
</table>
E2F1  E2F transcription factor 1
EH    Esp15 homology
ESCC  Esophageal squamous cell carcinoma
FAS   First apoptosis signal
FBS   Fetal bovine serum
GAPDH Glyceraldehyde 3-phosphate dehydrogenase
H3    Histone H3
H4    Histone H4
HRP   Horseradish peroxidase
IP    Immunoprecipitation
JmjC  Jumonji C
JHDM  JmjC demethylase
K     Lysine
KDM   Lysine demethylase
KMT   Lysine methyltransferase
LSD1  Lysine specific demethylase 1
MDM2  Mouse double minute 2 homologue
mRNA  Messenger ribonucleic acid
NCBI  National Center for Biotechnology Information
NLS  Nuclear localization sequence/signal
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Noxa</td>
<td>Latin for damage; also known as Phorbol-12-myristate-13-acetate-induced protein 1</td>
</tr>
<tr>
<td>NPF</td>
<td>Asparagine-proline-phenylalanine</td>
</tr>
<tr>
<td>p21</td>
<td>Protein 21 kDa</td>
</tr>
<tr>
<td>p53</td>
<td>Protein 53 kDa</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>PHD</td>
<td>Plant homology domain</td>
</tr>
<tr>
<td>PHF8</td>
<td>Plant homeodomain finger protein 8</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PML</td>
<td>Promyelocytic leukemia</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>PolII</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>PRR</td>
<td>Proline rich region</td>
</tr>
<tr>
<td>PRRi</td>
<td>PRR region with sequence insertion</td>
</tr>
<tr>
<td>PRRo</td>
<td>PRR region without sequence insertion</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine binding</td>
</tr>
<tr>
<td>PTBi</td>
<td>PTB domain with sequence insertion</td>
</tr>
<tr>
<td>PTBo</td>
<td>PTB domain without sequence insertion</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------</td>
<td>------------</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 up-regulated modulator of apoptosis</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RARα</td>
<td>Retinoic acid receptor alpha</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Rpl13</td>
<td>60S ribosomal protein L13</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with tween</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor protein p53 gene</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SET1</td>
<td>Su(var)3-9, Enhancer of Zeste, Trithorax 1</td>
</tr>
<tr>
<td>SET7/9</td>
<td>Su(var)3-9, Enhancer of Zeste, Trithorax 7/9</td>
</tr>
<tr>
<td>SET8</td>
<td>Su(var)3-9, Enhancer of Zeste, Trithorax 8</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>XLMR</td>
<td>X-linked mental retardation</td>
</tr>
</tbody>
</table>
Chapter 1

1 Introduction

Post-translational modifications (PTM) play an important role in protein regulation. There are many different types of PTMs including phosphorylation, acetylation, glycosylation, as well as methylation an area of PMT research that is under rapid growth. The first histone methyltransferase, Su(var)3-9 human 1 (SUV39H1) was discovered in 2000 (Rea et al., 2000). Methylation of histone tail residues has become well characterized and is known to affect the regulation of gene expression by recruiting or inhibiting other regulatory proteins (Zhang and Reinberg, 2001). Methyltransferases have since been found to target either lysine or arginine histone tail residues (Zhang and Reinberg, 2001). Interestingly, until the discovery of the first demethylase, lysine specific demethylase 1 (LSD1), which removes methyl groups from histone H3 lysine 4, methylation was thought to be a static modification (Shi et al., 2004). Another relatively recent discovery was the methylation of non-histone proteins. The p53 protein was the first non-histone protein discovered to undergo post-translational methylation (Chuikov et al., 2004) and since this discovery, many non-histone methylation targets have been identified (Huang and Berger, 2008). Lysine methylation can act as either a positive or negative regulatory signal, existing in three states, mono-, di- and tri-methylation (Margueron et al., 2005). Each different methylation state on the same amino acid can elicit a different regulatory effect on the protein. Methylation has been found to play a role in regulating nearly all cellular processes, including cell proliferation, differentiation and apoptosis to name a few (Shi and Whetstine, 2006; Meek and Anderson, 2009). This study will focus on the role of methylation in regulating apoptosis.

1.1 Apoptosis

Cells death is an important process for the development and survival of multicellular organisms. During development, cells are required to undergo apoptosis in order for organ development and appendages to form (Horvitz, 1999; Renehan et al., 2001). By having a controlled manner of disposing of unwanted cells, an immune response is
avoided (Renehan et al., 2001). Apoptosis is also critical to the general maintenance and protection of tissues. Controlled cell death is the organism’s way of disposing cells that are no longer necessary or could become harmful and cause damage to the tissue (Renehan et al., 2001). For example when a cell acquires DNA damage that cannot be repaired, it is in the body’s best interest to prevent this cell from replicating and remove it, avoiding daughter cells which may have harmful mutations. Improper repair of DNA damage by avoiding cell cycle arrest and failure of cells to undergo apoptosis are hallmarks of cancer (Hanahan and Weinberg, 2000).

Apoptosis signalling occurs in response to a variety of different pathological and physiological stimuli. Depending on which stimuli the cell is responding to, different pathways and proteins will become active. The two main types of apoptotic pathways are the extrinsic pathway and the intrinsic pathway (Elmore, 2007). The extrinsic pathway involves an external ligand binding to a trans-membrane death receptor [i.e. Tumor necrosis factor receptor (TNFR), first apoptosis signal (FAS) receptor, etc.] (Locksley et al., 2001), whereas the intrinsic pathway results from an internal signal such as oncogene expression, excess DNA damage, hypoxia, toxins, or radiation (Elmore, 2007).

1.2 Tumor suppressor protein p53

1.2.1 Discovery of p53

The p53 protein was originally identified in 1979 as a protein found to bind the large ‘T’ antigen in simian virus 40 (SV40) transformed cells (Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979). It was identified as a 53kDa protein and therefore was named p53, representing a protein with a molecular weight of 53 kDa (Crawford, 1983). Since its discovery, p53 has been identified as a tumour suppressor protein in response to its cellular protective functions. In this regard, the p53 protein protects the cell in a number of ways such as regulating cell cycle check points (Kuerbitz et al., 1992), DNA damage response and repair, cell stress response and apoptosis induction (Meek, 2009), as well as regulating the inhibition of angiogenesis (Tokino and Nakamura, 2000). These functions are regulated by p53 through its role as a transcription factor. Therefore, p53 exerts its protective effects on cells by up-regulating the
transcription of genes required for these processes (Riley et al., 2008). The p53-induced gene expression includes the genes of proteins required for apoptosis such as pro-apoptotic proteins B-cell lymphoma (Bcl)-2-associated x protein (BAX) (Miyashita et al., 1994), Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA) (Oda et al., 2000) and p53 up-regulated modulator of apoptosis (PUMA) (Nakano and Vousden, 2001), as well as genes required for the cell cycle arrest and DNA damage response (Levine, 1997) such as the cell cycle cyclin dependent kinase (cdk) inhibitor p21 (El-Deiry, 1993). Although p53 functions largely as a transcription factor, there is also evidence that p53 directly regulates proteins during apoptosis such as BAX (Chipuk et al., 2004).

Critically, the p53 protein has been reported to be deregulated in at least 50% of cancers (Soussi and Berude, 2001). In these cases, the p53 gene itself is either mutated or there are mutations in one of the many proteins that regulate p53 function or stability. For this reason, p53 has been studied intensely over the years in hopes to treat these cancers by reversing the negative effect on p53 function. However, our understanding of p53 function and its regulation remains incomplete and should remain an important focus of study for the development of new therapies for personalized cancer treatment.

1.2.2 p53 mediated apoptosis

The p53 mediated apoptosis pathway is induced by a number of signals (Elmore, 2007). These include aberrant oncogene expression, excessive DNA damage and hypoxia (Elmore, 2007; Gotz and Montenarh, 1995). In this manner, p53 is able to activate both the intrinsic and extrinsic apoptosis pathways. The extrinsic apoptosis pathway can be activated by p53 transactivation of the FAS receptor. Whereas, the p53 mediated intrinsic apoptosis pathway signalling (Figure 1.1) involves activation of pro-apoptotic Bcl-2 family proteins such as BAX, facilitating the release of cytochrome C from the mitochondria. Since p53 plays such an important protective role, it is constantly being synthesized in cells, but is also being constantly degraded, stabilized only when required by the cell. This constant synthesis of p53 allows for a quick response to cellular stress.

Ataxia-telangiectasia, mutated (ATM) and ATM and Rad3-related (ATR) are two kinases which sense genotoxic stress (Yang et al., 2004). When DNA damage is present, these
two kinases become activated. They can either directly activate p53 by phosphorylating p53 themselves or indirectly by activating other proteins which can then act on p53 (Yang et al., 2004). Both ATM and ATR have been found to phosphorylate a number of residues on p53 including serine 15 (Ser15) which leads to p53 activation (Canman et al., 1998; Tibbetts et al., 1999). ATM has been found to phosphorylate Ser46 of p53 which has been suggested to lead to p53’s transactivation of proapoptotic genes (Saito et al., 2002).

Activation of p53 allows it to activate the expression of proapoptotic genes PUMA, BAX and Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA) (Nakano and Vousden, 2001; Miyashita et al., 1994; Oda et al., 2000). These proteins inhibit Bcl-2, an anti-apoptotic protein, while stimulating the release of cytochrome C from the mitochondria. BAX binds to anti-apoptotic Bcl-2 family members when the cell is not undergoing apoptosis, which inhibits its apoptotic function (Oltvai et al., 1993). PUMA is able to inhibit the anti-apoptotic Bcl-2 proteins, releasing BAX and allowing it to form dimers with other pro-apoptotic Bcl-2 family members (Kuwana et al., 2005; Follis et al., 2013). These BAX dimers can then permeabilize the outer mitochondrial membrane causing the release of cytochrome C (Gross et al., 1999). Cytochrome C is then able to bind to and activate apoptosis-protease activating factor 1 (Apaf-1) hepta-oligomer along with adenosine tri-phosphate (ATP), forming the apoptosome (Garrido et al., 2006). The apoptosome is a complex which cleaves pro-cysteine-dependent aspartate-directed protease-9 (pro-Caspase9) to activate it and causes subsequent cleavage and activation of downstream caspases (Acehan et al., 2002). This leads to fragmentation of the nuclear envelope, degradation of nuclear and cytoskeletal proteins, as well as the degradation of chromosomal DNA (Elmore, 2007). The apoptotic cells and fragments are engulfed by phagocytes through the recognition of a phosphatidylinerine on the cell surface (Wu et al., 2006). This phosphatidylinerine is normally found within the membrane bi-layer, however when cells undergo apoptosis it is flipped out to be recognized on the surface of the cell (Fadok et al., 1998).
Figure 1.1: p53 mediated apoptosis signaling pathway. DNA damage, aberrant oncogene expression and other stresses signal the activation of kinases (ATM, ATR) which activate p53 through phosphorylation as well as other proteins which inhibit mouse double minute 2 homologue (MDM2) polyubiquitination of p53. The activation and stabilization of p53 leads to transactivation of genes encoding proteins required for apoptosis such as BAX, PUMA and Noxa. P53 is also believed to directly activate BAX. Activation of PUMA, NOXA and BAX lead to the release of cytochrome C from the mitochondria. Cytochrome C then binds to Apaf-1 along with ATP activating Apaf-1 and forming the apoptosome. This complex is then able to cleave pro-caspase9 into its active form Caspase9. This is the initiation step of the Caspase signaling cascade which commits the cell to apoptosis. (Węsierska-Gądek and Schmidt V, 2007; http://www.cancer-therapy.org/CT/v5/B/HTML/25._We3Fsierska-Ga3Fde_and_Schmid,_203-212.html)
1.2.3 Post-translational modification of p53

As previously mentioned p53 is a crucial cell regulatory protein which is constantly being synthesized so that it is ready to respond rapidly to cellular stresses (Kubbutat et al., 1997). Therefore, p53 is highly regulated through various combinations of PTMs, with at least 50 different reported modifications including phosphorylation, methylation, acetylation, glycosylation, sumolyation, neddylation and poly-ribosylation (Figure 1.2) (Meek and Anderson, 2009). Many of these modifications have been found to be interdependent (Meek and Anderson, 2009).

When p53 function is not required the E3 ligase mouse double minute 2 homologue (MDM2) polyubiquitinates p53 (Haupt et al., 1997). This polyubiquitination targets p53 for proteasomal degradation (Haupt et al., 1997). When genotoxic stress is present MDM2 becomes inhibited through a number of mechanisms, including acetylation of p53 (Tang et al., 2008). Once MDM2 is inhibited p53 levels rise dramatically (Bates and Vousden, 1996).

The p53 protein is also inhibited by monomethylation on lysine 382 by the lysine methyltransferase Su(var)3-9, Enhancer of Zeste, Trithorax 8 (SET8) (Shi et al., 2007) and mono-methylation on lysine 370 by another lysine methyltransferase, lysine methyltransferase 3C (KMT3C) (Huang et al., 2006). Methylation at these sites inhibits p53’s transactivation activity. As mentioned above, p53 can be activated by phosphorylation of certain residues. The phosphorylation of p53 at Ser15 activates p53 activity and has been found to promote further phosphorylation at other sites enhancing p53 translational activity (Yang et al., 2004). Notably, p53 can also be activated by methylation at lysine 372 by Su(var)3-9, Enhancer of Zeste, Trithorax 7/9 (SET7/9) (Chuikov et al., 2007).
Figure 1.2: Identified post-translational modifications of human p53 and their suspected modifying proteins. The p53 protein undergoes a number of post-translational modifications, including phosphorylation, methylation, acetylation, ubiquitination and glycosylation as shown here. A number of modifying proteins regulate these modifications which lead to p53 activation, inhibition, stabilization or destabilization. (Meek and Anderson, 2009)
1.2.4 Stabilization of p53 by Numb

Numb was originally identified as a cell fate determinant in *Drosophila* embryos during the development of the Drosophila nervous system (Uemura et al. 1989). Ten years later, four human Numb isoforms were discovered (Verdi et al. 1999). Expression of Numb isoforms has been identified in almost all adult and embryo tissues, suggesting that Numb function is not specific to neuronal development.

Numb has been characterized as an adaptor protein with a wide range of functions including roles in differentiation (Zhong et al., 1997), cell polarity and adhesion (Wang et al., 2009), cell migration (Nishimura and Kaibuchi, 2007), endocytosis (Santolini et al., 2000) and ubiquitination and proteolytic degradation (McGill and McGlade, 2003). Numb interacts with its binding partners via two protein-protein binding domains, an amino terminal phosphotyrosine binding domain (PTB) (Bork and Margolis, 1995; Li et al., 1998; Li et al., 1997) and a carboxy-terminal proline rich region (PRR) with Src-homology 3(SH3) binding sites (Yan, 2010). Also at the C-terminus, there are Esp15 homology (EH) regions containing aspartic acid-proline-phenylalanine (DPF) and asparagine-proline-phenylalanine (NPF) motifs (Gulino et al., 2010). There have been 9 splice variants identified to date, with isoforms 1-4 the most well-known. Isoform 1 and 3 have predicted molecular weight of 72 and 71 kDa (Numb-PRR<sub>L</sub>) respectively and differ from isoform 2 and 4, with a molecular weight of 66 and 65 kDa (Numb-PRR<sub>S</sub>) respectively, by an insert of 48 amino acids within Numb’s proline rich region (PRR) (Yan, 2010). Isoform 1 and 2 also contain an 11 amino acid insert within the PTB domain which isoform 3 and 4 do not contain (Yan, 2010).

Numb can form binary complexes with MDM2 and p53 (Numb-MDM2 and Numb-p53) (Juven-Gershon et al., 1998; Yogosawa, 2008) as well as a tricomplex with both MDM2 and p53 (Numb-MDM2-p53) (Yogosawa, 2008). Numb binding to p53 stabilizes the
protein by inhibiting MDM2 polyubiquitination of p53, so that p53 is no longer targeted for degradation (Yogosawa, 2008). Critically, the Numb-p53 interaction has been shown to be regulated by Numb methylation (Dhami et al., 2013). In an unstressed cell, Numb has been shown to be methylated on lysine 158 (K158) and lysine 163 (K163) by the methyltransferase SET8 (Dhami et al., 2013). This methylation blocks Numb binding to p53 (Dhami et al., 2013). When the cell experiences a stress such as DNA damage, Numb will become demethylated allowing it to bind to and stabilize p53 (Dhami et al., 2013). Numb bound p53 is then able to activate target genes required for the induction of apoptosis (Dhami et al., 2013) (Figure 1.3).
Figure 1.3: The working model of Numb demethylation which leads to stabilization of p53. The lysine demethylase SET8 has been found to methylate Numb. However when the cell experiences a stress signal such as excessive DNA damage or aberrant oncogene expression, an unidentified lysine demethylase (KDM) demethylates Numb. This demethylation allows Numb binding to p53. The binding of Numb to p53 inhibits MDM2 polyubiquitination of p53 which leads to its proteasomal degradation. Stabilization of p53 by Numb binding leads to the up-regulation of pro-apoptotic genes transactivated by p53, which lead to the induction of apoptosis. (Dhami et al., 2013)
1.3 The lysine demethylase PHF8

1.3.1 The identification of PHF8

Plant homeodomain finger containing protein 8 (PHF8) is a lysine demethylase (KDM). The PHF8 gene is located in the Xp11.21 chromosome (Laumonnier et al., 2005). It was first identified in 2005 for its association with X linked mental retardation (XLMR) and cleft lip/ cleft palate (Laumonnier et al., 2005). A mutation in phenylalanine 279 of PHF8 to a serine (F279) is a common mutation seen in patients with XLMR (Koivisto et al., 2010). This F279S mutation is located within the catalytic domain of PHF8 and therefore causes the enzyme to have defective demethylase activity. Truncation mutations have also been identified in XLMR patients (Laumonnier et al., 2005; Abidi et al., 2007). Since PHF8 is largely a nuclear protein, it is thought that these truncating mutations delete putative nuclear localization sequences (NLS) therefore inhibiting PHF8 from entering the nucleus and acting on its target genes (Abidi et al., 2007).

Although PHF8 is a ubiquitously expressed protein, murine PHF8 expression levels were found to be high in both the embryonic and adult brain of mice (Laumonnier et al., 2005). PHF8 has also been shown to be involved in neuronal differentiation (Qui et al., 2010). Exogenous overexpression of PHF8 in the mouse embryonic cell line P19 drove cells towards neuronal differentiation (Qui et al., 2010). At least one of the ways PHF8 drives neuronal differentiation is through the retinoic acid receptor alpha (RARα). PHF8 has been found to activate RARα expression as well as act as a co-activator of RARα through direct interaction with the receptor (Qui et al., 2010).

1.3.2 Characterization of PHF8

The PHF8 protein is a lysine demethylase that belongs to the largest family of demethylases, the Jumonji C (JmjC) family also known as JmjC histone demethylases (JHDM) (Loenarz et al., 2009; Yue et al., 2010). Unlike other demethylases, JmjC domain containing proteins are able to remove all three histone methylation states, mono-, di- and tri-methylation (Klose et al. 2006). JHDMs catalyze lysine demethylation in an oxidative reaction and they require cofactors α- ketoglutarate and
iron [Fe (II)] for the demethylation reaction as demonstrated in Figure 1.4A (Klose et al. 2006; Tsukada et al., 2006). Within the JHDM family, PHF8 belongs to the lysine demethylase 7 (KDM7) subfamily containing three members: KDM7A/JHDM1D, KDM7B/PHF8, and KDM7C/PHF2 (Fortschegger and Shiekhattar, 2011). All KDM7 family members seem to target mono- and dimethylated histone H3 lysine 9 (H3K9me1/2), dimethylated histone H3 lysine 27 (H3K27me2) and monomethylated histone H4 lysine 20 (H4K20me1) (Fortschegger and Shiekhattar, 2011).

Within the JHDM family, PHF8 belongs to the lysine demethylase 7 (KDM7) subfamily containing three members: KDM7A/JHDM1D, KDM7B/PHF8, and KDM7C/PHF2 (Fortschegger and Shiekhattar, 2011). All KDM7 family members seem to target mono- and dimethylated histone H3 lysine 9 (H3K9me1/2), dimethylated histone H3 lysine 27 (H3K27me2) and monomethylated histone H4 lysine 20 (H4K20me1) (Fortschegger and Shiekhattar, 2011).

The PHF8 protein exists in at least 4 different isoforms created by alternate splicing. Isoform 1 is the largest, containing 1060 amino acids and weighing approximately 118kDa (Kikuno et al., 1999). Isoform 2 of PHF8 is missing amino acids 1-36 and therefore contains 1024 amino acids (Bechtel et al., 2007). Isoform 3 is missing amino acids 478-578 and isoform 4 is missing both amino acids 1-36 and 478-578 but also has an additional 24 amino acids added to the C terminal of the protein (Ota et al., 2004). PHF8 contains a PHD zinc finger domain at amino acids 7-53 in isoform 2 (Laumonnier et al., 2005) or 43-89 in isoform 1(Kikuno et al., 1999). The JmjC domain is located between amino acids 195-294 in isoform 2 (Laumonnier et al., 2005) and 231-330 in isoform 1 (Kikuno et al., 1999). Both of these domains are highly conserved among PHF8 homologues in other species. PHF8 is a highly conserved protein though evolution with orthologues present in yeast (Laumonnier et al., 2005).

The PHD domain of PHF8 is believed to recognize and bind to tri-methylated histone H3 lysine 4 (H3K4me3) (Figure 1.4B)(Feng et al., 2010). This binding localizes PHF8 to its target methylated lysines, giving it substrate specificity (Feng et al., 2010). The targeting of PHF8 to H3K4me3 suggests that it is involved in promoting transcription as H3K4me3 is a suggested marker for gene activation. Methylated H3K27, H4K20 and H3K9 are all found to repress transcription (Vakoc et al., 2006). Since PHF8 removes these methylation marks it can be said that it is involved in transcriptional activation. Previous studies have also showed a direct interaction of PHF8 with the C-terminal domain of RNA polymerase II (PolII) (Fortschegger et al., 2010). Both PHF8 and PolIII have also been found to bind to Su(var)3-9, Enhancer of
Zeste, Trithorax 1 (SET1) (Liu et al., 2010; Ng et al., 2003). SET1 methylates H3K4 (Roguev et al., 2001), therefore providing PHF8 with its binding site.

Additionally, PHF8 has been found to be a regulator of the cell cycle, specifically in the promotion of the G2/M transition (Lim et al., 2013). Lim et al., (2013) found the levels of PHF8 to be highest at both the G2 phase and the beginning of M phase. This study found that when PHF8 is knocked down, it results in delayed G2/M phase transition and therefore impairs mitosis (Lim et al., 2013). Sun et al., (2013) discovered that when they knocked down PHF8 there was a decrease in cell proliferation, supporting PHF8’s involvement in promoting cell cycle progression. Phosphorylation of PHF8 by cyclin dependent kinase 1 (CDK1) has been found to occur on serine 33 and serine 84 causing the dissociation of PHF8 from its bound chromatin (Liu et al., 2010). This phosphorylation and subsequent dissociation occurs towards the end of the G2 phase allowing accumulation of H4K20me1 and progression into mitosis (Liu et al., 2010). During interphase PHF8 is recruited to the chromatin again by its interaction with H3K4me3 (Liu et al., 2010).
Figure 1.4: The mechanism of PHF8 catalyzed demethylation reaction. A. The lysine demethylase PHF8 requires iron Fe (II) binding and α-ketogluterate as a cofactor for demethylation activity. The methyl group becomes oxidized forming an unstable hydroxymethyl (CH$_2$OH). This hydroxymethyl group becomes released as formaldehyde (CH$_3$O), (Klose et al., 2006). B. A ribbon model of PHF8 demonstrates binding to a H3K4me3K9me2 peptide (green stick structure). The H3K4me3 is interacting with the PHD domain (cream), while the H3K9me2 is interacting with the JmjC domain (indigo) along with the Fe (II) molecule (red sphere) and α-ketogluterate (yellow stick structure) (Krishnan et al., 2011).
1.3.3 Oncogenic properties of PHF8

The PHF8 protein has recently been identified as an oncogenic protein (Bjorkman et al., 2012). Elevated PHF8 expression has been reported in many prostate cancer patient samples (Bjorkman et al., 2012). PHF8 has also been found to play a role in the pathogenesis of esophageal squamous cell carcinoma (ESCC) (Sun et al., 2013). PHF8’s oncogenic effects are a result of its ability to promote cell migration, cell cycle progression/cell proliferation and inhibit apoptosis (Bjorkman et al., 2012; Sun et al., 2013).

PHF8 promotes cell motility by regulating cytoskeletal remodeling as well as up-regulating the expression of genes involved in cell migration such as fibronectin, integrins and proteins involved in the integrin pathway (Bjorkman et al., 2012; Asensio-Juan et al., 2012). When PHF8 is silenced in prostate cancer cells there is a significant decrease in cell motility (Bjorkman et al., 2012). The opposite is seen when PHF8 is overexpressed, cells become more motile (Bjorkman et al., 2012). Sun et al., (2013) also found that PHF8 knockdown ESCC cells were more motile and invasive then their PHF8 expressing counterparts.

As an example of its oncogenic roles, PHF8 has also been found to promote cancer cell proliferation. Knockdown of PHF8 significantly decreased cell proliferation in prostate cancer cells (Bjorkman et al., 2012). One of the ways PHF8 is suggested to affect cell cycle progression is through the demethylation of H4K20 (Liu et al., 2010). Methylated H4K20 is a repressive mark found on many of the oncogenic transcription factor E2F, target genes (Abbas et al., 2010), and PHF8 binds to E2F1 (Liu et al., 2010). PHF8 has also been found to promote cell proliferation in esophageal squamous carcinoma cells (Sun et al., 2013). Sun et al., (2013) reported that when they knocked down PHF8 they saw significant inhibition of cell proliferation. They also found that both anchorage- dependent and independent growth of ESCC cells were decreased when PHF8 was knocked down (Sun et al., 2013). This same study also reported that when prostate cancer cells were injected into nude mice they formed larger tumours than those injected with prostate cancer.
cells with PHF8 knocked down by shRNA (Sun et al., 2013). This suggests that PHF8 may be a potential target for cancer therapeutics.

Finally, an effect of PHF8 on apoptosis has also been identified. Bjorkman et al., 2012 report a weak yet significant effect on apoptosis in cells with PHF8 knocked down. Knockdown of PHF8 in ESCC cells was also found to significantly increase apoptosis (Sun et al., 2013). Although PHF8 has been found to negatively affect apoptosis, there has been no research done to investigate the mechanism behind its anti-apoptotic effect.

1.4 The purpose of this thesis

It is known that Numb is methylated by SET8 at sites K158 and K163; however the protein responsible for demethylating Numb remains unknown. The purpose of this thesis was to determine which lysine demethylase is demethylating Numb. We identified PHF8 as a possible candidate for the Numb demethylase since it targets methylated H3K27 and H4K20 which share sequence similarities to Numb K159 and K163 (Dhami et al., 2013). Previous research has indicated PHF8 as an oncogene; therefore it would be of interest to further investigate any possible roles for the lysine demethylase in regulating p53 mediated apoptosis.

We hypothesize that PHF8 demethylation activity plays a regulatory role in the intrinsic p53-mediated apoptosis pathway.

Aim 1: Identify and characterize candidate Numb demethylases

To address this aim we selected possible demethylases by identifying demethylases known to target lysines on histone tails with similar surrounding sequences to Numb K158 and K163. The chemotherapeutic drug, doxorubicin, was used to induce apoptosis and the levels of mRNA of these demethylases were observed during apoptosis to see if there were any changes compared to non-apoptotic/untreated cells. SET8 levels have been found to decrease when cells undergo apoptosis, so we
suspected that the potential Numb demethylase levels would increase when apoptosis is induced.

Aim 2: Observe the effect of PHF8 on apoptosis

To address this second aim, flow cytometry analysis was done to observe the effect of PHF8 on apoptosis. PHF8 was either overexpressed or knocked down and the levels of apoptosis were observed. Quantitative real time-PCR (qRT-PCR) was used to observe any effect PHF8 has on genes involved in apoptosis. The mRNA levels of the apoptotic proteins were observed in cells with PHF8 overexpressed and in cells with PHF8 knocked down. Chromatin immunoprecipitation (ChIP) was also done to determine if PHF8 is directly regulating the promoters of the observed apoptotic genes it seemed to have an effect on. ChIP analysis was also done with H3K27me3 immunoprecipitated from cells overexpressing PHF8 and cells with PHF8 knocked down, to determine if PHF8 was regulating the genes through the methylation status of H3K27.
Chapter 2

2 Materials and Methods

2.1 Solutions and buffers

All solutions and buffers used are listed here in table 2.1.

Table 2.1: Solutions and buffers

<table>
<thead>
<tr>
<th>Solution</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>1×TAE buffer (pH8.5)</td>
<td>Tris base 40 mM, EDTA 1 mM, Glacial acetic acid adjusting pH to 8.5</td>
</tr>
<tr>
<td>1× TBST</td>
<td>Tris base 20 mM, NaCl 137 mM, Tween20 0.05% (v/v)</td>
</tr>
<tr>
<td>1× PBS buffer</td>
<td>KH$_2$PO$_4$ 1 mM, Na$_2$HPO$_4$ 10 mM, KCl 2.7 mM, NaCl 137 mM</td>
</tr>
<tr>
<td>5× SDS-PAGE (Laemmli) Sample buffer</td>
<td>Tris base (pH 6.8) 312 mM, SDS 10%, α-mercaptoethanol 25%, Bromophenol Blue 0.05%, Glycerol 50%</td>
</tr>
<tr>
<td>Western blot transfer buffer</td>
<td>Methanol 20%, 24 mM Tris base, 194 mM Glycine</td>
</tr>
<tr>
<td>1x MOPS buffer</td>
<td>50 mM MOPS, 50 mM Tris base, 0.1% SDS, 1 mM EDTA</td>
</tr>
<tr>
<td>ChIP SDS lysis buffer</td>
<td>1% SDS, 10 mM EDTA, 50 mM Tris (pH 8.1)</td>
</tr>
<tr>
<td>ChIP dilution buffer</td>
<td>0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCL (pH 8.1), 167 mM NaCl</td>
</tr>
<tr>
<td>LiCl wash</td>
<td>0.25 M LiCl, 1% IGEPL-CA630, 1% deoxycholic acid (sodium salt), 1mM EDTA, 1 mM Tris (pH8.1)</td>
</tr>
<tr>
<td>High salt wash</td>
<td>0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCL (pH8.1), 500 mM NaCl</td>
</tr>
<tr>
<td>Low salt wash</td>
<td>0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCL (pH8.1)</td>
</tr>
<tr>
<td>TE pH 8.0</td>
<td>10 mM Tris-HCl, 1 mM EDTA (pH adjusted with HCl)</td>
</tr>
</tbody>
</table>
2.2 Cell culture, transfection and siRNA knock down

2.2.1 Cell culture

HeLa cells, a cervical cancer cell line and U2OS cells, an osteosarcoma cell line, were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (FBS) and 2 mg/ml glutamine at 37 °C with 5% CO₂.

2.2.2 Cell transfection

Cells were subcultured the day prior to the transfection to be 50-80% confluent in a 100 mm tissue culture dish. 8 µg of DNA was mixed with 440 µl warm Opti-MEM (Life Technologies) and incubated at room temperature for 5 minutes. 60 µl (1 mg/ml) warm Polyethylenimine (PEI) was then added to the diluted DNA and mixed immediately for 15 seconds. The solution was left for 20 minutes at room temperature prior to adding it to the cells. Before the addition of DNA to the cells, cells were washed three times with warm 1 x phosphate buffered saline (PBS) and cells were left in 4.5 mL antibiotic free media. 24 hours after the addition of the DNA solution to the cells, cells were washed three times with warm 1 x PBS and 10 mL of fresh media was added to the plate. Cells were then grown for another 18-24 hours prior to use.

2.2.3 SiRNA transfection

Cells were subcultured the day prior to the transfection to be 40-70% confluent. 10 µl of 100 µM siRNA (Sigma-Aldrich) was diluted in 990 µl serum free media and incubated at room temperature for 5 minutes. 20 µl DharmaFECT (ThermoFisher Scientific, Catalogue #T-2001-01) was diluted in 980 µl serum free media and also incubated at room temperature for 5 minutes. The two were then mixed together and incubated at room temperature for 20 minutes. Cells were washed three times with 1 x PBS then replenished with 8 ml of antibiotic free media. The siRNA mixture was then added to the cells and incubated at standard conditions for 48 hours.
2.2.4 Doxorubicin treatment

Cells were treated with 1 µM doxorubicin hydrochloride (Sigma-Aldrich, Catalogue # D1515-10MG) dissolved in ddH₂O for 18 hours at 37°C. Doxorubicin was added directly to cell culture media. For a 100 mm dish with 10 ml of media, 2 µl of 5 mM doxorubicin stock was used.

2.3 Quantitative real-time polymerase chain reaction (qRT-PCR)

2.3.1 TRIzol RNA isolation

Cells were grown in 6-well dishes, 2 wells per condition. Cells were transfected on day one, media changed and doxorubicin was added on day 2 for an 18 hour treatment. Media was aspirated and 600 µl of TRIzol (Life Technologies, Catalogue#15596-026) was added per well. Cells were scraped off the dish in TRIzol and collected in micro-centrifuge tubes and the mixture was pipetted up and down a few times. Cells were left at room temperature for 5 minutes prior to the addition of 120 µl chloroform. Tubes were shaken vigorously for 15 seconds and left at room temperature for 3 minutes. Samples were then centrifuged for 15 minutes at 4°C at 12 000 x g. The upper aqueous phase was transferred to a new centrifuge tube and 300 µl isopropanol was added and left at room temperature for 10 minutes to precipitate the RNA. The precipitated RNA was the pelleted by centrifugation 12 000 x g for 10 minutes at 4°C. The RNA pellet was then washed with 1 mL 75% ethanol and centrifuged again for 5 minutes 12 000 x g at 4°C. The ethanol was aspirated away and the pellet was left to air dry for 5 to 10 minutes. The RNA was re-suspended in DEPC treated water (RNase-free) by pipetting up and down several times and incubating 15 minutes in a 55°C water bath. RNA concentration was calculated by measuring absorbance A₂₆₀ and using the following formula

\[ A_{260} \times \text{dilution} \times 40 = \frac{\mu g\text{RNA}}{mL} \]

Absorbance at A₂₈₀ was also measured and the ratio A₂₆₀/A₂₈₀ was calculated to ensure a ratio >1.8. Samples were either stored at -80°C or used for cDNA synthesis for qPCR.
2.3.2 cDNA synthesis

Isolated RNA was reverse transcribed since cDNA is more stable than RNA. BioRad’s iScript™ Reverse Transcription Supermix for RT-qPCR was used (Bio-Rad, Hercules, CA, Catalogue # 170-8840). 500 ng-1 µg of RNA was used depending on the concentration of each sample. For each set of experiments the RNA concentration was adjusted so all samples were at the same concentration. 2 µl of RNA solution was added to 4 µl 5x iScript reverse transcription supermix (Bio-Rad, Hercules, CA, Catalogue # 170-8840) and 14 µl of water for a final volume of 20 µl for each sample. The reaction mixtures were prepared in PCR tubes. In a PCR thermocycler (iCycler- Biorad, Hercules, CA) the following protocol was used for cDNA synthesis: 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C and a final hold at 4°C. A no-RT control was also done to exclude genomic DNA contamination.

2.3.3 qRT-PCR procedure

iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, Catalogue # 170-8884) was used for RT-qPCR reactions performed in the Bio-Rad CFX connect Real-Time system (Bio-Rad, Hercules, CA). iQ™ SYBR® Green Supermix contains iTaq DNA polymerase, dNTPs, MgCl₂, SYBR® Green I dye, fluorescein as well as enhancers and stabilizers. Each reaction was a final volume of 10 µl consisting of 4 µl cDNA and 0.2 µl forward primer, 0.2 µl reverse primer, 0.6 µl nuclease free H2O, 5 µl SYBR Green supermix. The forward and reverse primers, nuclease-free H₂O and SYBR Green were combined into a master mix and 6 µl of mater mix was added to the cDNA. The reactions were carried out with the following parameters:

Cycle 1: Denaturation: 95°C 3 minutes

Cycle 2 (40-50 x): Denaturation: 95°C 10 seconds

Annealing/extension: 60°C 30 seconds

Finally samples were heated from 65°C to 95°C in 0.5°C increments every 5 seconds to create a melt curve. Further analysis such as normalization, graphs and statistics were done using Microsoft Excel.
2.3.4 qRT-PCR primers

Primers used in this study for qPCR analysis were designed using the National Center for Biotechnology Information’s (NCBI) Primer-BLAST search and ordered from Sigma Aldrich. The sequences for the primers used can be found in Table 2.2.

Table 2.2: List of qRT-PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>SETD8</td>
<td>GGGGAAACCATTAGCCCGGAA</td>
<td>TGGTCCTCGGACTTCATGGC</td>
</tr>
<tr>
<td>KDM6B</td>
<td>TGAAGAACGTCAGTCCATTG</td>
<td>TCCCGCTGTACCTGACAGT</td>
</tr>
<tr>
<td>KDM6A</td>
<td>ATGGAAACGTCGCTACCTG</td>
<td>ATTAGGACCTGCGGAATGTG</td>
</tr>
<tr>
<td>PHF8</td>
<td>TAGCACAGGGCACAAGGTTTC</td>
<td>CGATAGGCTGGGCTCTTTC</td>
</tr>
<tr>
<td>TP53</td>
<td>GAGGTTGGCCTGACTGTACC</td>
<td>TCGTCCCAGTAGATACCAC</td>
</tr>
<tr>
<td>BAX</td>
<td>TTTGCTTCAGGGTTTTCATCC</td>
<td>CAGTTGAAGTTGCGTCAGA</td>
</tr>
<tr>
<td>CASP3</td>
<td>TGAGCGGATTGAGAAGAGTT</td>
<td>TGAACCAGGAGCCATCATTT</td>
</tr>
<tr>
<td>PUMA</td>
<td>GCCAGATTGTAGACAGAGG</td>
<td>CAGGCACCTATTTGGGCTC</td>
</tr>
<tr>
<td>Rpl13</td>
<td>GCCCTACGACAAGAAAAGCG</td>
<td>TACTTCCAGCAAACCTCGT</td>
</tr>
<tr>
<td>GAPDH (ChIP)</td>
<td>CAATTTCCCATCTCATGT</td>
<td>TAGTACCGGGCCTACTTT</td>
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<tr>
<td></td>
<td>Nucleotides region 467-482</td>
<td>Nucleotides region 654-673</td>
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<tr>
<td>BAX (ChIP)</td>
<td>TTTGCTTCAGGGTTTTCATCC</td>
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<td>Nucleotides region 144-163</td>
<td>Nucleotides region 370-389</td>
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<td>TP53 (ChIP)</td>
<td>CAGCACATGACGGAGGTGT</td>
<td>TCATCCAAATACTCCACACGC</td>
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<td></td>
<td>Nucleotides region 562-581</td>
<td>Nucleotides region 666-686</td>
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</table>
2.4 Transformation of *Escherichia coli* (*E. coli*) and DNA plasmid amplification

500 ng – 1 μg of plasmid DNA (Table 2.3) was added to a microcentrifuge tube on ice. *E. coli* DH5α cells were thawed on ice. 30 μl of the competent cells were added to the DNA and mixed by gently pipetting up and down several times. The cells and DNA were incubated on ice for 15 minutes. The bacteria/plasmid mixture was then heat shocked at 42°C for 60 seconds, followed by incubation for 2 minutes on ice. 70 μl of antibiotic free LB media was added to the bacteria and plasmid mixture and was incubated at 37°C on a shaker for one hour.

DH5α cells were then plated on agar plates with the appropriate antibiotic resistance. pFLAG-CMV constructs confer ampicillin resistance, therefore 50 μg/ml ampicillin agar plates were used. Plates of bacteria were left to grow overnight at 37°C. The next day colonies were picked and grown in 3 ml LB media with the appropriate antibiotic. Cell cultures were left to grow overnight at 37°C on a shaker.

Plasmid DNA was purified using a miniprep DNA plasmid purification kit (Sigma-Aldrich, St. Louis, MO, USA).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Protein Description</th>
<th>Host</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFLAG-CMV2</td>
<td>Empty vector with FLAG tag</td>
<td><em>E. coli</em> DH5α</td>
<td>Li lab</td>
</tr>
<tr>
<td>pFLAG-hPHF8-CMV2</td>
<td>Human PHF8 isoform 2</td>
<td><em>E. coli</em> DH5α</td>
<td>Li lab (Courtney Voss); The ORFeome Collaboration (hPHF8 insert)</td>
</tr>
</tbody>
</table>

**Table 2.3: List of plasmids**
2.5 Cell extract preparation, immunoprecipitation and western blot

2.5.1 Cell lysis

Cells were placed on ice and washed three times with ice cold 1 x PBS. 500 µl-1 mL lysis buffer (Table 2.1) supplemented with 1 mM sodium orthovanadate, 2 µg/ml aprotinin, 10 µM leupeptin, 1 µM E64, 1 µM bestatin, 1 µM pepstatin A and 1 mM phenylmethanesulfonyl fluoride (PMSF) was added to the 100 mm dish of cells and left on ice for 5 minutes. Cells were then scraped into a 1.5 ml Eppendorf tube using a rubber policeman on ice. The cell lysates were rotated for 15 minutes at 4°C prior to centrifugation at max speed (16000 x g) for 20 minutes at 4°C. The supernatant was removed and used for subsequent experiments.

2.5.2 Measurement of protein concentration

Protein concentration was measured using Bradford dye assay (Bio-Rad protein assay). The dye was diluted in a 1 part dye to 4 parts ddH$_2$O ratio. 1-2 µl of sample was added to 800µl of diluted dye in a plastic cuvette. The absorbance was measured in a GENESYSSTM spectrophotometer (Spectronic Unicam, Rochester, NY, USA) at a wavelength of 595 nm. Protein concentration of the sample was determined using a bovine serum albumin (BSA) standard curve.

2.5.3 Measurement of DNA and RNA concentration

5 µl of DNA or RNA sample was added to 495 µl ddH$_2$O in a glass cuvette. 500 µl ddH$_2$O was used as a blank. Both DNA and RNA samples were measure at 260 nm absorbance. DNA concentration was calculated using the formula: $A_{260} \times \text{dilution} \times 50 = \frac{\mu g \text{DNA}}{mL}$.

RNA concentration was calculated using the formula: $A_{260} \times \text{dilution} \times 40 = \frac{\mu g \text{RNA}}{mL}$.

Absorbance was also read at 280 nm to get the 260 nm/280 nm ratio.
2.5.4 Immunoprecipitation (IP)

To pre-clear the cell lysate 3 µg of anti-rabbit IgG was added to 1 mg total protein cell lysate. 40 µl of a 1:1 ratio mixture of protein A agarose beads (made in lab) to lysis buffer was added to the cell lysate with anti-IgG and incubated for one hour at 4°C on a shaker. Samples were centrifuged at 4°C 3000 x g for 5 minutes and the supernatant was transferred to a new 1.5 ml Eppendorf tube.

3 µg of antibody for IP per 1 mg of total protein was then added to the pre-cleared lysate and incubated at 4°C on a shaker for 4 hours or overnight. 40 µl of protein A bead slurry in lysis buffer was added to each sample and incubated another hour at 4°C on a shaker. Samples were then centrifuged for 5 minutes 3000 x g at 4°C. Supernatant was removed and beads with the antigen and protein attached were washed three to five times with cell lysis buffer. After the last wash, supernatant was removed and 2 x SDS Laemmli sample buffer was added to the beads to elute the protein.

2.5.5 Western blot (WB)

20-40 µg of protein sample was loaded onto a 8-12 % Bis-Tris gel. Gels were run in 1 x 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (Table 2.1) at 110 V for approximately one to two hours. The separated proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane solubilized in methanol. A semi-dry blot transferring system (BioRad, Hercules, CA) was used to transfer the gel at 21 V for 30 minutes. The gel and PVDF membrane were placed between two filter papers soaked in 1 x transfer buffer (Table 2.1).

Following the transfer, PVDF membranes were blocked in either 5 % skim milk in 1 x Tris-buffered saline with Tween (TBST) or 3% bovine serum albumin (BSA) in 1 x TBST for one hour at room temperature on a shaker. The blocking buffer was removed and the membrane was incubated with primary antibody diluted in blocking buffer for one hour on a shaker at room temperature or overnight at 4°C on a shaker. The dilution of primary antibody used was in accordance to the recommended dilution given by the manufacturer (Table 2.3).
After the primary antibody was removed, the membrane was rinsed three times with 1 x TBST then washed 3 x 5 minutes with 1 x TBST. Next the membrane was incubated in secondary antibody conjugated with horseradish peroxidase (HRP) diluted in a ratio of 1:5000 in 5% skim milk in 1 x TBST for one hour. The membrane was then rinsed three times and washed 3x 15 minutes with 1 x TBST. An enhanced chemiluminescence (ECL) detection solution (GE Healthcare Life Sciences) was added to the membrane and incubated for 5 minutes. Excess ECL solution was removed and signals were exposed onto X-ray film over a range of 15 seconds to 15 minutes. Film was then developed using a Kodak M35A X-OMAT processor.

**Table 2.4: List of antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Source</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Numb (H-70)</strong></td>
<td>rabbit</td>
<td>Santa Cruz Biotech</td>
<td>1:1000 dilution in 5% milk in 1xTBST</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Catalogue number: sc-25668)</td>
<td></td>
</tr>
<tr>
<td><strong>PHF8 (ab36068)</strong></td>
<td>rabbit</td>
<td>Abcam (Catalogue number:ab36068)</td>
<td>1:1000 dilution in 5% milk in 1xTBST</td>
</tr>
<tr>
<td><strong>p53(DO-1)</strong></td>
<td>mouse</td>
<td>Santa Cruz Biotech</td>
<td>1:1000 dilution in 5% milk in 1xTBST</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Catalogue number: sc-126)</td>
<td></td>
</tr>
<tr>
<td><strong>β-tubulin (H-235)</strong></td>
<td>rabbit</td>
<td>Santa Cruz Biotech</td>
<td>1:1000 dilution in 5% milk in 1xTBST</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Catalogue number: sc-9104)</td>
<td></td>
</tr>
<tr>
<td><strong>FLAG (M2)</strong></td>
<td>mouse</td>
<td>Sigma Aldrich</td>
<td>1:1000 dilution in 5% milk in 1xTBST</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Catalogue number: F3165)</td>
<td></td>
</tr>
<tr>
<td><strong>Pan-lysine mono- and di- methylation</strong></td>
<td>rabbit</td>
<td>Abcam (Catalogue number: ab23366)</td>
<td>1:1000 dilution in 3% BSA in 1xTBST</td>
</tr>
</tbody>
</table>
2.6 Flow cytometry analysis

Cells were plated on day 0, transfected with pFLAG-PHF8-CMV or pFLAG-CMV empty vector on day 1, media changed and treated with doxorubicin or not on day 2. On day 3 cells were rinsed with 1 x PBS and trypsinized to remove the cells from the dish. The cells were collected and centrifuged at 1000 x g for 5 minutes and the supernatant removed. An Annexin V-FITC apoptosis detection kit (BioVision incorporated, Cat. # K101-100) was used to detect apoptotic cells according to the manufacturer's protocol. Cells were re-suspended in binding buffer at a concentration of approximately 5x10⁵ cells/ 500 µl. Cells were then passed through a cell strainer to prevent large clumps of cells from clogging the machine. 5 µl of each Annexin V-FITC and propidium iodide (PI) were added to each experimental sample and incubated at room temperature for 10 minutes before being placed on ice. Control tubes were also set up containing no dye, PI alone Annexin V-FITC alone, and both PI and Annexin V-FITC. An unstained non-doxorubicin treated sample was also used to observe the effect of doxorubicin on the emission spectra.

Cells were analyzed on the LSRII flow cytometer (BD Bioscience) using FACS Diva software (BD Bioscience) in Robarts Research Facility. AnnexinV FITC samples were excited by the 488 nm blue laser and detected by the 530/30 detector. For PI stained cells the yellow-green 561 nm laser was used to excite samples and the 610/20 detector was used to detect the fluorochromes. Data analysis was done using FlowJo software (FlowJo Enterprise) and Microsoft Excel.
2.7 Chromatin immunoprecipitation (ChIP) assay

Cells were plated on day 0, transfected with pFLAG-PHF8-CMV or pFLAG-CMV empty vector on day 1, media changed and treated with doxorubicin or vehicle ddH₂O on day 2. On day 3, 270 µl of 37% formaldehyde was added to each 100 mm dish of cells containing 10 ml of media. Cells were incubated with the formaldehyde on a shaker at room temperature for 10 minutes to allow cross-linking of chromatin bound proteins to the nucleotides. 687.5 µl of 2 M glycine was added to the media to quench the cross-linking. The medium mixture was them removed and cells were washed with cold 1 x PBS containing protease inhibitors. 1 ml of cold 1 x PBS plus protease inhibitors was added to the cells and scraped into microcentrifuge tubes. Cells were centrifuged at 3000 x g at 4°C for 5 minutes. Supernatant was removed and the cell pellet was re-suspended in 200 µl ChIP lysis buffer (Table 2.1). Cells were incubated at room temperature for 5 minutes prior to being put on ice. 1.2 ml ChIP dilution buffer (Table 2.1) was added to each sample. Next the lysed cells were sonicated (60 Sonic Dismembrator, Fisher Scientific) for 5 x 15 second on 45 seconds off pulses on number 5. 40 µl of a mixture of 0.2 mg/ml sheared salmon sperm DNA (ssDNA) in ChIP dilution buffer and protein G sepharose beads at a 1:1 ratio was added to the lysate and incubated at 4°C on a nutator to pre-clear the sample. Samples were centrifuged for 2 minutes at 3000 x g 4°C and supernatant transferred to new tubes. 100 µl of supernatant was saved from each sample for ChIP input DNA for normalization. 3 µg of antibody was added to the samples and incubated overnight on a nutator at 4°C. 40 µl of Protein G sepharose/ ssDNA bead slurry was added to each sample and incubated at 4°C for 1 hour on a nutator. Samples were centrifuged 4 minutes at 3000 x g at 4°C. Supernatant was removed and beads were washed with 1 ml low salt buffer (Table 2.1) for 30 minutes, then high salt buffer (Table 2.1) for 15 minutes, then LiCl buffer (Table 2.1) for 10 minutes. Each of these washes was at 4°C on a nutator with centrifugation at 3000 x g at 4°C between to remove each wash. The final two washes were in TE buffer (pH 8.0) (Table 2.1) at room temperature for 5 minutes each. The sample was eluted off the beads using 100 µl 1% SDS and 0.1 M NaHCO₃ in ddH₂O. Samples were incubated on a shaker at room temperature for 4 hours then centrifuged for 1 minute at 3000 x g and supernatant kept. Elutant was incubated at 65°C overnight to de-crosslink DNA along with the ChIP input samples.
DNA was purified from the sample using QIAEX II Gel Extraction kit (Qiagen, Venlo, Limburg, Netherlands, Catalogue #20021) following the manufacturer’s protocol to extract DNA from solutions. DNA fragments were analyzed using qRT-PCR.
Chapter 3

3 Results

3.1 Prediction of possible Numb demethylases

The original goal of this study was to identify the lysine demethylases responsible for demethylating Numb. Numb has been shown to be methylated by the lysine methyltransferase SET8 on lysine residues K158 and K163 (Dhami et al., 2013). The first step in identifying SET8 as the possible lysine methyltransferase targeting Numb was to look at the peptide sequence surrounding the two methylation sites (Dhami et al., 2013). Dhami et al., 2013 preformed a sequence alignment and found that the sequence was closely related to histone H3 lysine 27 (H3K27) and histone H4 lysine 20 (H4K20), as well as the p53 lysine 382 (p53K382) methylation site (Dhami et al., 2013). The latter two are known targets of SET8 (Nishioka et al., 2002; Shi et al., 2007). Table 3.1 shows the protein sequences surrounding the lysines of interest targeted for methylation.

Since H3K27 and H4K20 were found to have similar surrounding sequences to those of the Numb K158 and K163 residues (Dhami et al., 2013), we narrowed down the possible lysine demethylase by identifying demethylases known to target H3K27 and H4K20. We decided to investigate lysine demethylase 6B (KDM6B) and lysine demethylase 6A (KDM6A) which both demethylate H3K27 (Lan et al., 2007; Hong et al., 2007). In addition to the KDM6 demethylases, plant homeodomain (PHD) finger containing protein 8 (PHF8) which is known to demethylate monomethylated H4K20 (Lui et al., 2010) and dimethylated H3K27 (Loenarz et al., 2010) was also investigated.

3.2 PHF8 expression increases during doxorubicin induced apoptosis

In order to test which of the suspected lysine demethylases may be demethylating Numb we first looked at which of those demethylases are elevated during apoptosis. The Numb methyltransferase, SET8, has previously been found to be down regulated in cells undergoing apoptosis (Dhami et al., 2013), therefore we suspected that the possible Numb demethylase would be up-regulated when apoptosis is induced. Quantitative real
Table 3.1: Sequence comparison of Numb lysine methylation sites to histones and p53 methylation sites. H3K27 and H4K20 were previously identified by sequence alignment to have similar sequence similarity of residues surrounding the methylated lysine as Numb K158 and K163. The methylated lysine K382 of p53 was also identified to have a similar sequence to both Numb methylation sites, H3K27 and H4K20. The alignment gave a score to each of the lysine residues based on sequence similarity of the surrounding amino acids compared to those of Numb (K158). (Dhami et al., 2013)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence containing lysine methylation site</th>
<th>Alignment Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>hNumb</td>
<td>143-HAVGCAFAACLERKQKREKECGVTATFDAS-174</td>
<td>N/A</td>
</tr>
<tr>
<td>H4K20</td>
<td>1-GRGKGGKGLGKGGAKHRKVLRDNIQGITYLEPS-34</td>
<td>2.5</td>
</tr>
<tr>
<td>p53K382</td>
<td>363-HSSHLKSKQGQSSTHRKLMKTEGPDS-393</td>
<td>1.5</td>
</tr>
<tr>
<td>H3K27</td>
<td>10-TGGKAPRQLTAKAARKSAPATGKVKKPH-40</td>
<td>1.5</td>
</tr>
</tbody>
</table>
time polymerase chain reaction (qRT-PCR) was used to identify elevated lysine demethylases. U2OS cells were either untreated or treated with 1.0µM doxorubicin for 18 hours to induce apoptosis. Cells were lysed and the total mRNA was extracted. PUMA, a protein involved in p53 mediated apoptosis was also selected as a positive control since its expression is known to increase when treated with doxorubicin (Dhami et al., 2013). SET8 was observed as a negative control since it has been found to decrease with doxorubicin treatment (Dhami et al., 2013).

As shown in Figure 3.1, the levels of KDM6A remained relatively constant with the 18 hour doxorubicin treatment compared to the untreated cells. The level of KDM6B mRNA however, decreased significantly with doxorubicin treatment. The only lysine demethylase tested whose levels increased significantly was PHF8. As expected, PUMA levels increased with doxorubicin treatment, well SET8 levels of mRNA decreased.

To further confirm the observed increase in PHF8 expression during apoptosis, we repeated the experiment in HeLa cells (Figure 3.2). Once again the levels of PHF8 were significantly elevated with 18 hours of doxorubicin treatment compared to untreated cells. SET8 was used again as a negative control, which was significantly decreased in both cell lines.

Although the mRNA levels of a gene may be elevated, it does not always result in elevated protein levels. Therefore our next step was to observe the protein expression level of PHF8 with doxorubicin treatment. To test this, cells lysates of both HeLa and U2OS cells treated with doxorubicin for 6 hours, 18 hours or left untreated, were analyzed by western blot (Figure 3.3). Both cell lines showed an increase in PHF8 expression level at 18 hours compared to untreated cells (Figure 3.3, top panels). Numb and p53 expression levels were also observed. Both Numb and p53 expression levels also increased in both cell lines with doxorubicin treatment (Figure 3.3, middle panels).
Figure 3.1: Transcript expression levels of lysine demethylases with doxorubicin treatment. Total mRNA was extracted using TRIzol, from U2OS cells treated with 1.0μM doxorubicin for 18 hours. The mRNA was reverse transcribed into cDNA. Primers targeting lysine demethylases were used to analyze levels of demethylase mRNA. SET8 was used as a negative control and PUMA was used as a positive control. Data presented is the mean ± SEM of three biological repeats. *- indicates significant difference from the corresponding control (p<0.05).
Figure 3.2: Transcript expression of PHF8 increased with doxorubicin treatment in both HeLa cells and U2OS cells. Total mRNA was extracted using TRIzol, from U2OS cells treated with 1.0 μM doxorubicin for 18 hours or from untreated cells. The mRNA was reverse transcribed into cDNA. Primers targeting PHF8 and SET8 were used to analyze levels of mRNA using qPCR. Values were normalized to Rpl13. Data presented is the mean ± SEM of three biological repeats. *- indicates significant difference from the corresponding control (p<0.05).
Figure 3.3: PHF8 protein expression level was up-regulated with doxorubicin treatment for 18h. Both HeLa cells and U2OS cells were either left untreated or treated with 1.0µM doxorubicin for 6 hours or 18 hours. Cells were lysed and samples were separated using an 8% Bis-Tris gel. Western blot analysis was done using anti-PHF8 (upper panel), anti-Numb (second panel), anti-p53 (third panel) and anti-β-tubulin (bottom panel). β-tubulin was observed as a loading control to ensure equal levels of protein were added.
3.3 PHF8 does not demethylate Numb

The significant increase in PHF8 mRNA (Figure 3.1 and Figure 3.2) and protein levels during apoptosis (Figure 3.3) led us to believe it could be the demethylase responsible for demethylating Numb. To further test this prediction we set out to observe whether or not PHF8 has an effect on Numb methylation. To do this we overexpressed pFLAG-PHF8-CMV or pFLAG-CMV empty vector in HeLa cells and immunoprecipitated endogenous Numb. Cells were either treated with 1.0µM doxorubicin or left untreated. Numb immunoprecipitated samples along with cell lysates were observed using SDS-PAGE and western blot analysis. As seen in Figure 3.4 A, PHF8 appears to have no effect on Numb methylation status.

3.4 PHF8 has an anti-apoptotic function

Since PHF8 expression increases with doxorubicin induced apoptosis, we predicted that PHF8 would promote apoptosis. To test this hypothesis we used flow cytometry analysis. We first observed cells with PHF8 knocked down using siRNA specific to PHF8. As a control we also observed cells transfected with a scrambled control siRNA. Cells were treated with 1.0 µM doxorubicin for 18 hours or left untreated. Cells were analyzed using Annexin V FITC to observe cells in early apoptosis and propidium iodide (PI) was used to analyze cells in late apoptosis. A significantly higher percentage of cells with PHF8 knocked down were undergoing apoptosis when treated with doxorubicin compared to cells containing the scrambled control siRNA (Figure 3.5). Even untreated PHF8 knockdown cells showed slightly more apoptosis than control cells, however this was not significant.

To further confirm that PHF8 promotes cell survival, HeLa cells were transfected with FLAG tagged PHF8 to observe how PHF8 overexpression affected apoptosis. Cells were also transfected with the empty vector pFLAG-CMV as a control. Again cells were stained with Annexin V FITC and PI and analyzed using flow cytometry to detect apoptotic cells. As expected, cells overexpressing PHF8 were significantly less apoptotic compared to cells transfected with empty vector pFLAG-CMV (Figure 3.6). There was
Figure 3.4: PHF8 does not demethylate Numb. HeLa cells were transfected with FLAG-PHF8 or pFLAG-CMV and treated with 1.0µM doxorubicin for 18 hours or left untreated. Cells were lysed and endogenous Numb was immunoprecipitated with anti-Numb antibody. The immunoprecipitate (A) and cell lysate (B) were run on 8% Bis-Tris SDS-PAGE and analyzed by western blot. Immunoprecipitate blots were blotted with anti-Numb and a pan-methylation antibody (A). Lysate blots were blotted with anti-FLAG, anti-PHF8, anti-Numb, anti-p53 and anti-β-tubulin (B).
Figure 3.5: Cells transfected with siRNA against PHF8 undergo more apoptosis when treated with doxorubicin. HeLa cells were transfected with either siRNA against PHF8 or a scrambled control siRNA. Cells were treated with 1.0μM doxorubicin for 18 hours or left untreated. Annexin V FITC and propidium iodide were used to select for the apoptotic cells within the population. Apoptotic cells were identified using flow cytometry analysis. Data presented is the mean ±SEM of five biological repeats. *-indicates significant difference from the corresponding control (p<0.025).
Figure 3.6: Cells overexpressing PHF8 undergo similar apoptosis as control cells when treated with doxorubicin. HeLa cells were transfected with either FLAG-PHF8 or pFLAG-CMV. Cells were treated with 1.0μM doxorubicin for 18 hours or left untreated. Annexin V FITC and PI were used to select for the apoptotic cells within the population. Apoptotic cells were identified using flow cytometry analysis. Data presented is the mean ±SEM of six biological repeats. *- indicates significant difference from the corresponding control (p<0.025).
no significant difference in apoptosis between untreated PHF8 overexpressing cells compared to untreated control cells.

3.5 PHF8 expression down regulates apoptotic genes

To further investigate the anti-apoptotic effect of PHF8, qRT-PCR was used to observe the effect of PHF8 activity on the expression of apoptotic genes that are involved in p53-mediated apoptosis. HeLa cells with PHF8 knocked down using siRNA or transfected with a scrambled control siRNA, were treated with 1.0 µM doxorubicin for 18 hours or left untreated prior to RNA extraction. The observed mRNA expression levels were normalized to the mRNA levels of the ribosomal protein Rpl13. As shown in figure 3.7 A, PHF8 mRNA levels were significantly reduced in knockdown cells confirming that PHF8 was successfully knocked down in these cells. The mRNA expression levels of p53 (TP53), Caspase3 (CASP3) and BAX (BAX) were all significantly up-regulated in doxorubicin treated PHF8 knockdown cells (Figure 3.7 B, C and D). Total p53 mRNA levels were significantly up-regulated in both doxorubicin treated and untreated cells when PHF8 was knocked down (Figure 3.7 B). Interestingly, no significant effect was seen on the mRNA levels of PUMA in either treated or untreated cells (Figure 3.7 E).

The same qRT-PCR experiments were repeated in cells overexpressing PHF8. HeLa cells were transfected with pFLAG-PHF8-CMV or pFLAG-CMV empty vector. The transfected cells were treated with 1.0 µM doxorubicin or left untreated prior to mRNA extraction. HeLa cells transfected with FLAG-PHF8 showed significantly higher levels of PHF8 mRNA when induced by doxorubicin compared to cells transfected with pFLAG-CMV (Figure 3.8 A). In cells overexpressing PHF8, the mRNA expression levels of p53, Caspase3 and BAX decreased significantly compared to cells transfected with the empty control vector, when cells were treated with doxorubicin (Figure 3.8 B, C and D).
**Figure 3.7: Transcript expression of apoptosis genes when PHF8 is knocked down.**

HeLa cells were transfected with siRNA against PHF8 (siPHF8) or scrambled siRNA (siCtrl). Total mRNA was extracted using TRIzol from the siRNA transfected cells treated with 1.0μM doxorubicin for 18 hours or from untreated cells. The mRNA was reverse transcribed into cDNA. Primers targeting the apoptotic genes *PHF8* (A), *TP53* (B), *CASP3* (C), *BAX* (D) and *PUMA* (E) were used to analyze levels of mRNA with qRT-PCR. Observed mRNA values were normalized to Rpl13. Data presented is the mean ±SEM of five biological repeats. *- indicates significant difference from the corresponding control (p<0.025). **- indicates significant difference from the corresponding control (p<0.1).
Figure 3.8: Transcript expression of apoptosis genes with PHF8 overexpressed. Total mRNA was extracted using TRIzol, from HeLa cells treated with 1.0μM doxorubicin for 18 hours or from untreated cells. The mRNA was reverse transcribed into cDNA and primers targeting the apoptotic genes PHF8 (A), TP53 (B), CASP3 (C) and BAX (D) were used to analyze levels of mRNA with qPCR. The observed mRNA values were normalized to Rpl13 mRNA. Data presented is the mean ±SEM of five biological repeats. *- indicates significant difference from the corresponding control (p<0.05). **- indicates significant difference from the corresponding control (p<0.1).
3.6 PHF8 overexpression reduces p53 protein level

Since p53 is such an important protein, we found it interesting that PHF8 expression appeared to regulate the mRNA expression level of p53. To further confirm the down regulation of p53 expression by PHF8, SDS-PAGE and western blot analysis were used to observe the effect of PHF8 on p53 protein expression. pFLAG-PHF8-CMV or pFLAG-CMV plasmids were transfected into HeLa cells and the effects of PHF8 overexpression on p53 were observed. The protein expression level of p53 was decreased in cells overexpressing PHF8 with or without doxorubicin treatment compared to cells transfected with empty vector pFLAG-CMV (Figure 3.9 C). The levels of PHF8 and FLAG were also observed using anti-PHF8 and anti-FLAG antibodies respectively (Figure 3.9 A, B). To ensure equal loading, the levels of the housekeeping protein β-tubulin was also observed (Figure 3.9 D).

3.7 PHF8 binds to the promoters of the p53 and Caspase3 genes

Since we saw an effect of PHF8 overexpression and knockdown on the mRNA expression levels of p53, Caspase3 and BAX, we wondered in PHF8 was directly regulating their transcription. To investigate this chromatin immunoprecipitation (ChIP) was done to observe PHF8 binding to the promoters of the TP53, CASP3 and BAX genes. HeLa cells were transfected with FLAG-PHF8 or pFLAG-CMV and treated with or without doxorubicin. Antibodies against either FLAG or RNA polymerase II (PolII) were used to immunoprecipitate PHF8 and PolII with their bound DNA. PolII was used as a positive control since it should be found at all active promoters. PolII was found to bind the promoter of GAPDH (Figure 3.10 E), whereas PHF8 only bound slightly and not significantly to the GAPDH promoter compared to the empty vector and IgG controls (Figure 3.10 A). Both PHF8 and PolII bound the TP53 promoter significantly compared to the pFLAG-CMV and IgG controls (Figure 3.10 B and E), however there was no significant difference between doxorubicin treated and untreated cells (Figure 3.10 B). PHF8 bound the TP53 promoter significantly more than that of GAPDH (Figure 3.10 A and B). PHF8 did not bind to the BAX promoter to the same extent as TP53 or CASP3 (3.10 D).
**Figure 3.9: PHF8 overexpression reduces p53 protein levels.** pFLAG-PHF8-CMV or pFLAG-CMV was transiently transfected into HeLa cells. Cells were either untreated or treated with 1.0μM doxorubicin for 18 hours. The cells were lysed and whole cell lysates were run on 8% Bis-Tris SDS-PAGE and analyzed by western blot. Blots were analyzed using anti-FLAG (A), anti-PHF8 (B), anti-p53 (C) and anti-βtubulin (D).
Figure 3.10: PHF8 binds the promoter of p53 and Caspase3 but not BAX. Cells over expressing PHF8 or an empty vector control (CMV) were fixed with formaldehyde to crosslink protein to their bound DNA. PHF8, PolII (E) and IgG were immunoprecipitated along with their bound DNA fragments. DNA was purified and GAPDH (A), TP53 (B), CASP3 (C) and BAX (D) genes were amplified using qRT-PCR. Values were normalized to input DNA. Data presented is the mean ±SEM of four biological repeats. *- indicates significant difference from the corresponding empty vector (CMV) and/or no doxorubicin control (p<0.05). **- indicates significant difference from the corresponding empty vector (CMV) control (p<0.1).
Since PHF8 appeared to be directly regulating p53 and Caspase3 transcription we then wanted to determine whether or not this regulation was through PHF8 demethylation of methylated H3K27. HeLa cells were transfected with siRNA to knock down PHF8 or scrambled control siRNA. The cells were treated with doxorubicin or left untreated. Anti-H3K27me3 was used to immunoprecipitated methylated H3K27 from the samples and qRT-PCR analysis was used to analyze the methylation status of H3K27 at the promoters of TP53, CASP3 and BAX. As shown in Figure 3-11 B, cells with PHF8 knocked down and treated with doxorubicin had a significantly higher level of methylated H3K27 on the TP53 promoter than the control siRNA cells. The untreated cells with PHF8 knocked down also showed significantly more methylated H3K27 than untreated control cells (Figure 3.11 B). Doxorubicin treated PHF8 knock down cells had more H3K27 methylation of the CASP3 gene compared to cells transfected with scrambled siRNA (Figure 3.11 C). GAPDH H3K27 methylation levels were not significantly different between PHF8 knockdown cells and the control cells, with the exception of the significantly lower levels of H3K27 methylation in untreated control siRNA cells (Figure 3.11 A).

The same ChIP experiment was repeated with PHF8 overexpressed in HeLa cells transiently transfected with pFLAG-PHF8-CMV or transfected with the empty vector pFLAG-CMV. The cells were either treated with doxorubicin or left untreated and prepared for ChIP the same as above. Again anti-H3K27me3 was used to immunoprecipitate methylated H3K27. Cells with PHF8 overexpressed and treated with doxorubicin had a significantly lower level of methylated H3K27 on the TP53 promoter than untreated PHF8 overexpressing cells (Figure 3.11 B). CASP3 also displayed significantly lower levels of methylated H3K27 when cells were overexpressing PHF8 and treated with doxorubicin compared to the untreated PHF8 cells and vector control cells (Figure 3.11 C). Significantly higher levels of GAPDH H3K27 methylation was observed in control cells compared to PHF8 overexpressing cells (Figure 3.11 A). H3K27 methylation of the GAPDH gene was also significantly lower in cells treated with doxorubicin than the same cells untreated (Figure 3.11 A).
Figure 3.11: H3K27 methylation of GAPDH, p53 and Caspase3 genes. Cells over expressing PHF8 and PHF8 knockdown cells were fixed with formaldehyde to crosslink protein to their bound DNA. Anti-H3K27me3 was used to immunoprecipitate DNA fragments bound to methylated H3K27. DNA was purified and GAPDH (A), TP53 (B) and CASP3 (C) promoter DNA was amplified using qRT-PCR. Values were normalized to input DNA. Data presented is the mean ±SEM of four biological repeats. *- indicates significant difference from the corresponding SiRNA, empty vector (CMV) or no doxorubicin control (p<0.05). **- indicates significant difference from the corresponding empty vector (CMV) or no doxorubicin control (p<0.1).
Chapter 4

4 Discussion

The lysine demethylase PHF8 is a relatively recently discovered protein (Laumonnier et al., 2005). It was originally believed that methylation was a static modification until the identification of the first demethylase LSD1, which led to the realization that it is in fact a dynamic process (Shi et al., 2004). PHF8 is a lysine demethylase known to target H3K9me/me2, H3K27me2 (Yokoyama et al., 2010) and H4K20me (Liu et al., 2010). The majority of studies done on PHF8 have been on characterizing the role of PHF8 in X-linked mental retardation as well as its structure. More recently PHF8 has been identified as an oncogene (Björkman et al., 2012). The PHF8 protein has been found to have involvement in cell migration, proliferation and cycle progression (Asensio-Juan et al., 2012; Björkman et al., 2012; Sun et al., 2013; Liu et al., 2010). These functions of PHF8 have proven to play a role in the progression and pathogenicity of both prostate cancer and esophageal squamous cell carcinoma (Björkman et al., 2012; Sun et al., 2013). The anti-apoptotic effect of PHF8 has also been previously identified to add to its oncogenic function (Björkman et al., 2012; Sun et al., 2013), however until now no one had investigated the mechanism of how PHF8 affects apoptosis in any detail.

4.1 PHF8 is up-regulated during apoptosis but does not demethylate Numb

PHF8, among other demethylases, was identified as a possible Numb demethylase due to its ability to demethylate the histone lysine residues H3K27me/me2 (Yokoyama et al., 2010) and H4K20me (Liu et al., 2010). These two methylated histone residues share similar surrounding sequences to those of the methylated lysines of Numb (K158 and K163) (Dhami et al., 2013) (Table 3.1). Quantitative RT-PCR and western blot analysis revealed elevated PHF8 levels when apoptosis was induced by doxorubicin in both HeLa and U2OS cells (Figures 3.2 and 3.3), which was not seen for any other tested demethylase (Figure 3.1). Since other pro-apoptotic genes have been found to be elevated
during apoptosis (i.e. PUMA) (Dhami et al., 2013), we initially believed this meant that PHF8 has a pro-apoptotic function during apoptosis. We suspected that the pro-apoptotic function of PHF8 might have been in demethylating Numb so that it is able to bind to and stabilize p53. The mRNA levels of SET8, the methyltransferase responsible for methylating Numb, decreased when apoptosis was induced by doxorubicin as shown previously (Dhami et al., 2013) (Figure 3.2). Since the methyltransferase expression decreases with doxorubicin treatment, we suspected that the Numb demethylase expression would increase with doxorubicin treatment, leading us to believe that PHF8 could be a possible Numb demethylase. Unfortunately, our western blot analysis of Numb immunoprecipitated from PHF8 overexpressing cells with and without doxorubicin treatment suggested that PHF8 was not involved in Numb demethylation (Figure 3.4). Even though PHF8 does not demethylate Numb and has not yet been identified to act on any non-histone proteins, it is still possible that PHF8 may target non-histone proteins.

Histone modifying proteins have been known to carry out many diverse functions. They can act on a number of different genes by modifying the histone tail residues at the promoters of their target genes (Shi and Whetstine, 2007). They also sometimes act on non-histone proteins, such as SET8 methylating both Numb (Dhami et al., 2013) and p53 (Shi et al., 2007). The expression level of a given histone modifying enzyme does not necessarily change during a certain cellular process that it is or is not involved in. Therefore it is possible that a lysine demethylase whose mRNA expression level does not change significantly during apoptosis, such as KDM6A (Figure 3.1), may be responsible for Numb demethylation or possibly play some other role in apoptosis. Further investigation into which demethylase is responsible for demethylating Numb is still required. Identification of the Numb demethylase could potentially lead to the identification of the demethylase responsible for demethylating p53 K382, which shares sequence similarity and the same methyltransferase as Numb K158 and K163 (Dhami et al., 2013).

When observing PHF8 protein levels with doxorubicin treatment, we did not see an increase in PHF8 levels at the 6 hour time point (Figure 3.3). In U2OS cells there
appeared to be a decrease in PHF8 expression (Figure 3.3). Doxorubicin induces apoptosis through intercalation of the chemical into the DNA, causing excessive DNA damage (Yang et al., 2014). At the 6 hour time point it is possible that the cells are going through the DNA damage response and have not yet entered apoptosis. Therefore since we do not see an increase in PHF8 until the 18 hour time point (Figure 3.3), it is unlikely that PHF8 plays a role in the DNA damage response.

4.2 PHF8 promotes cell survival

Further study into PHF8’s effect on apoptosis suggested an anti-apoptotic effect, contrary to our originally hypothesised that PHF8 was a pro-apoptotic protein. Flow cytometry analysis of PHF8 knockdown (Figure 3.5) and PHF8 overexpressing cells (Figure 3.6) demonstrated an anti-apoptotic effect on cells. PHF8 knockdown has previously been found to cause an increase in apoptosis (Björkman et al., 2012; Sun et al., 2013), however there was no further investigation into the mechanism behind this anti-apoptotic effect.

We decided to explore how PHF8 is negatively affecting p53-mediated apoptotic signaling in cells treated with doxorubicin. We found that PHF8 acts, at least in part, by down regulating genes required for apoptosis. Our qPCR analysis showed that PHF8 down-regulates p53, Caspase3 and BAX (Figures 3.7 and 3.8). BAX is a target gene of p53 (Miyashita et al., 1994), so it is likely that the effect seen on BAX expression is due to the down regulation of p53 by PHF8. PUMA is also an identified p53 target gene for p53-mediated apoptosis (Nakano and Vousden, 2001). We expected PHF8 to have a negative effect on PUMA mRNA expression since p53 is down regulated by PHF8; however we found that PUMA mRNA expression was not significantly affected by PHF8 (Figure 3.7). It has been suggested that PUMA up-regulation can occur in the absence of p53 (Luo et al., 2005; Dhami et al., 2013), therefore it is possible that we do not see an effect of PHF8 because PUMA is being regulated by a p53 independent mechanism. It would be interesting to observe the effects of PHF8 on other pro-apoptotic genes including other p53 target genes, such as Noxa another apoptotic protein. It would also be
of interest to observe the possible effects of PHF8 on anti-apoptotic genes. Since PHF8 is thought to promote transcription (Feng et al., 2010; Liu et al., 2010), it is possible PHF8 could be promoting transcription of anti-apoptotic genes to suppress apoptosis.

The negative effect of PHF8 on p53 expression was also observed at the protein level (Figure 3.9). As observed with qPCR, western blot analysis revealed that both the untreated and doxorubicin treated cell lysates experienced a decrease in p53 protein expression (Figure 3.9). The protein levels of the other apoptotic proteins were not analyzed due to limitations of reagent availability. The protein levels should be analyzed in the future to confirm the effect seen by qPCR. We would expect to see a decrease in protein expression of BAX and Caspase3 as well, at least when the cells are treated with doxorubicin. Since the expression levels of p53 was decreased at both the mRNA (Figure 3.8) and protein level (Figure 3.9) when PHF8 is overexpressed, we can conclude that PHF8 is affecting p53 at a transcriptional level, rather than post-translationally.

Histone modifying proteins typically bind to the gene promoters of the genes they are regulating. Chromatin immunoprecipitation revealed that PHF8 directly binds to the promoter of TP53 and CASP3, but less significantly to that of BAX with PHF8 binding less than 1% of the total DNA (Figure 3.10). This further confirms that PHF8 is directly regulating the transcription of TP53 and CASP3. As mentioned above, the negative effect seen on BAX mRNA level is likely a result of the inhibition of p53 transcription by PHF8 (Figures 3.7 and 3.8). However PHF8 has been shown to bind to PolIII (Fortschegger et al., 2010), therefore it is possible that the localization seen at the promoters of TP53, CASP3 and GAPDH could be due solely to this binding.

Since PHF8 demethylates H3K27me2 (Yokoyama et al., 2010), we decided to investigate if PHF8 is regulating the transcription of the affected apoptosis genes through demethylation of H3K27me2. A significant increase in H3K27 methylation was seen at both the TP53 and CASP3 promoters, when PHF8 was knocked down (Figure 3.11). Interestingly, when PHF8 is overexpressed H3K27 methylation at the TP53 and CASP3 promoters is significantly increased when untreated and significantly decreased when treated with doxorubicin, compare to empty vector control cells (Figure 3.11 B and C).
These findings suggest that PHF8 is regulating the promoters of *TP53* and *CASP3* through demethylation of methylated H3K27. Therefore, although PHF8 may be binding to PolII helping localize it to the genes of interest, PHF8 is also directly regulating these genes.

Methylated H3K27 has been described as a transcriptionally repressive mark (Vakoc et al., 2006). Therefore the results shown in this study appear to conflict with previously published literature. In the qPCR analysis, PHF8 appeared to have a suppressive effect on p53 and Caspase3 expression (Figures 3.7 and 3.8). However, the ChIP experiments reveal that PHF8 is demethylating H3K27me2, therefore removing a transcriptionally repressive mark (Figure 3.11). The ChIP data would suggest a transcriptionally activating activity of PHF8 whereas the qPCR data suggest a repressive function. There is likely more going on at these promoters that has yet to be identified, including a wide number of other histone tail modifications. PHF8 is also responsible for demethylating H3K9me/me2 (Yokoyama et al., 2010) and H4K20me (Liu et al., 2010). Therefore these ChIP experiments need to be repeated to observe the effect of PHF8 on these histone modifications at the *TP53* and *CASP3* promoters as well. The results may help us to better understand the mechanism of PHF8 transcriptional inhibition. It is possible that the demethylation of H3K27me2 is required for the recruitment of a repressive transcription factor. Another possibility could be that PHF8, although demethylating H3K27me2 may also be demethylating a transcriptionally activating transcription factor or histone modifying protein, thereby inhibiting the transcriptionally activating function. PHF8 has been found to interact with the transcription factors E2F transcription factor 1 (E2F1) and host cell factor C1 (HCF-1) as well as the methyltransferase SET1 (Liu et al., 2010), all of whose activity PHF8 could be affecting.

The transcription factor E2F1 has been found to activate p53 transcription in HeLa cells (Massip et al., 2013). Since PHF8 has been found to bind E2F1 (Liu et al., 2010), it is possible that E2F1 and PHF8 are interacting at the p53 promoter. These two previously published findings support our results that identify PHF8 binding to the promoter of p53 and regulation of p53 transcription. E2F1 is also known to up-regulate pro-apoptotic genes in response to genotoxic stress inducing apoptosis (Wu et al., 2009). Methylation
of lysing 158 of E2F1 by SET9 can increase apoptosis, whereas demethylation of this site by LSD1 decreases apoptosis (Xie et al., 2011). PHF8 may be involved in a similar mechanism to down regulate p53 and Caspase3 expression.

4.3 PHF8 regulation of p53

The p53 protein is known to be largely regulated at the post-translational level, since p53 is continuously being synthesized (Kubbutat et al., 1997). A lot less is known about p53’s transcriptional regulation. Recently a number of proteins have been found to activate p53 transcription during DNA damage response and apoptosis. For example HoxA5 and Pitx1 have both been found to up-regulate transcription of the p53 gene to promote apoptosis (Raman et al., 2000; Liu and Lobie, 2007), whereas the proto-oncogene BCL6 is an example of a protein that inhibits p53 transcription (Phan and Ia-Favera, 2004). It is possible that PHF8 may be affecting p53 transcription by regulating one of these transcription factors through demethylation, either activating BCL6 or inhibiting HoxA5 or Pitx1.

We found that PHF8 is regulating the transcription of p53 (Figures 3.7 and 3.8); however it may also be directly affecting the p53 protein as well. The p53 protein is known to be methylated on a number of lysine residues, many of which the methyltransferases and demethylases responsible are unknown (Meek and Anderson, 2009). Lysine residue 382 of p53 has been found to have sequence similarities to H4K20 (Dhami et al., 2013), a target histone tail lysine of PHF8 (Liu et al., 2010). The methyltransferase SET8 is known to methylate p53K382 (Shi et al., 2007), however the demethylase responsible for its demethylation is unknown. It is possible that PHF8 could be demethylating p53K382.

Since PHF8 is known to promote cell cycle progression it is possible that PHF8 is inhibiting p53 during the cell cycle as well. The effect may be seen more in cancer cells which have elevated levels of PHF8, since we did not see much effect of PHF8 without doxorubicin treatment. The known role of PHF8 during the cell cycle is promoting the G2-M transition (Lim et al., 2013) and the G1-S transition (Liu et al., 2010), whereas p53
halts the cell cycle at the G1 to S phase transition when DNA damage is present (Kuerbitz et al., 1992). It is possible that PHF8 may be down regulating p53 when p53 is not required for cell cycle regulation.

4.4 PHF8 regulation of Caspase3

The lysine demethylase PHF8 plays a role in neuronal differentiation (Qiu et al., 2010). Mutations in the PHF8 gene lead to X-linked mental retardation (XLMR) and cleft lip pallet (Laumonnier et al., 2005). Recently Caspase3 has also been found to be involved in neurogenesis (Tzeng et al., 2013). Since PHF8 regulates Caspase3 expression during p53-mediated apoptosis, it is possible that PHF8 may have an effect on the CASP3 gene in neuronal cells during neurogenesis. If PHF8 is somehow regulating Caspase3, this may be one way that inactivating mutations of PHF8 leads to XLMR. Further studies are needed to investigate this possibility.

4.5 PHF8 as an oncogene

The down regulation of p53 by PHF8 supports the previously identified oncogenic effects of PHF8. The p53 protein plays a very important tumor suppressor role for cells and tissues. It blocks cell cycle progression when the cell is experiencing some sort of stress or there is DNA damage detected that needs repairing (Kuerbitz et al., 1992; Read and Strachan, 1999). Inhibition of the cell cycle by p53 stops the mutation in the DNA from being replicated and passed on to daughter cells which could potentially lead to cancer (Read and Strachan, 1999). The p53 protein is also involved in the DNA damage response and DNA repair itself (Meek, 2009). Finally when the DNA damage is too great to be repaired p53 protects the tissue again by signaling for the cell to undergo apoptosis (Meek, 2009). We have found that PHF8 inhibits p53 from inducing apoptosis (Figures 3.6 and 3.8) and although not yet determined, it is possible that PHF8 may be inhibiting the transcription of p53 during the cell cycle as well. The suppressive effect that PHF8 has on p53 could lead to cancer if not properly regulated.
It is well known that cancer is the additive result of a number of mutations that lead to the hallmarks of cancer (Hanahan and Weinberg, 2000). Therefore it would be unfair to say that PHF8 mutations can cause cancer but it could have an oncogenic effect. It is also well known that p53 is a highly regulated protein that is acted on by a large number of proteins (Meek and Anderson, 2009). Therefore the effect PHF8 has as a single protein might not be that significant in vivo. However, since PHF8 is regulating the transcription of p53 directly rather than post-translationally (Figures 3.7, 3.8 and 3.10) like the majority of other p53 regulating proteins (Meek and Anderson, 2009), PHF8 could have a more significant effect on p53. PHF8 also down regulates Caspase3 expression (Figures 3.7 and 3.8) which is an important protein for apoptosis. Therefore the significant down-regulation of apoptosis by PHF8 (Figures 3.5 and 3.6) does suggest that it can cause one of the hallmarks of cancer; evasion of apoptotic signaling (Hanahan and Weinberg, 2000).

There is a lot that remains to be learned about PHF8’s oncogenic functions. So far we have only observed the effects PHF8 has on the cell, such as cell cycle progression/ cell proliferation, cell migration and apoptosis (Bjorkman et al., 2012; Sun et al., 2013). A lot of work is still required to determine the underlying mechanisms causing these oncogenic effects. Once we can better understand this protein, we can attempt to develop better therapies to target PHF8 in cancer patients with PHF8 overexpression, for custom designed treatment programs.

4.6 Therapeutic potential of PHF8

Cancer treatments typically involve the use of radiation therapy and/or chemotherapy. The main goal of these treatments is to kill the cancer cells; unfortunately these treatments are not always successful. Sometimes cancer cells have the ability to evade apoptosis, which can lead to failed treatments and relapse. Due to its oncogenic anti-apoptotic effects, PHF8 would make a good target for cancer therapy. Used in combination with other treatments, a drug designed to inhibit PHF8 could potentially be used to target the cells that are evading apoptosis, making them more susceptible to cell
death. There has already been work done to create small peptide inhibitors to block the function of lysine methyltransferases and lysine demethylases (Rose et al., 2010). Both the PHD domain and the JmjC domain could be targeted on PHF8, however one study found that mutating the PHD domain did not inhibit its demethylase activity (Yue et al., 2010). Therefore, targeting the catalytic JmjC domain would be the best target to inhibit PHF8 activity.

PHF8 and other JmjC containing demethylases require both iron Fe(II) and α-ketoglutarate as co-factors for the catalysis of the demethylation reaction. These demethylases can therefore be inhibited by metal chelators such as desferoxamine (Barman-Aksözen et al., 2013). α-ketoglutarate mimics have also proven to inhibit JmjC domain containing demethylases, such as N-oxalylglycine (Hamanda et al., 2009) and pyridine-2,4-dicarboxylic acid (Mackeen et al., 2010; Loenarz et al., 2010). Unfortunately, chemicals which target co-factor binding to these demethylases are not going to be specific to inhibit a specific JmjC demethylase. Small molecule inhibitors have also been designed to inhibit JmjC demethylases. Luo et al., (2011) has identified a small molecule which mimics a methylated lysine residue connected to an α-ketoglutarate mimic that is able to bind to and inhibit the JmjC containing demethylase KDM6A. Another small molecule, 2-4(4-methylphenyl)-1,2-benzisothiazol-3(2H)-one, another metal ion chealator, has been identified to inhibit the JmjC demethylase Jumonji AT-rich interactive domain 1B (JARID1B)(Sayegh et al., 2013). Given the observed oncogenic activity of PHF8 a small molecule designed to specifically inhibit PHF8 would prove beneficial.

4.7 Future directions

This study has only started to give us insight into PHF8’s anti-apoptotic effects. There are many questions that remain to be answered. First, the still unanswered question of which lysine demethylase is demethylating Numb during the induction of apoptosis. Further investigation into the other suspected demethylases could lead to an answer to this question. Next, the biggest question that arises from the findings of this study is how
PHF8 is negatively regulating the transcription of p53 and Caspase3. What proteins are binding along with PHF8 at the promoters of these genes? How might PHF8 be affecting the activity of these proteins? Or how might these proteins affect PHF8 activity? PHF8 could also be regulating a number of other proteins involved in apoptosis. Does PHF8 bind to the promoters of other pro-apoptotic genes negatively regulating their transcription? Is PHF8 up-regulating any anti-apoptotic proteins during apoptosis? As mentioned previously, there is also the possibility that PHF8 could be regulating p53 or Caspase3 transcription during other cellular processes, such as whether or not there is any effect of PHF8 on p53 during the cell cycle or if PHF8 affects Caspase3 during neurogenesis. Future research to answer these questions will give us a better understanding of PHF8’s pro-apoptotic activities as well as further insight into the overall oncogenic effect of PHF8.

4.8 Conclusions

In summary, PHF8 has a negative regulatory effect on p53-mediated apoptosis, even though its expression levels increase during apoptosis. We found that PHF8 does not demethylate Numb as we had initially suspected, which would have been a pro-apoptotic effect. Instead, PHF8’s anti-apoptotic effect is at least in part due to its down-regulation of the pro-apoptotic genes TP53, BAX and CASP3. PHF8 directly binds to the promoters of TP53 and CASP3, demethylating H3K27me2. The negative effect on BAX transcription was likely the result of an indirect effect of p53 down-regulation, since BAX is transactivated by p53. However, it is unlikely that the inhibition of TP53 and CASP3 transcription by PHF8 is simply the result of PHF8 affecting H3K27 methylation. H3K27 demethylation is typically thought to have an activating effect on transcription. Therefore we suspect other proteins could be involved in the observed transcriptional inhibition and that PHF8 possibly could be regulating them. The question of how PHF8 is affecting the methylation status of H3K9me/me2 and H4K20me also requires further investigation.
References


Appendices

Appendix A. Raw PHF8 knockdown flow cytometry data of three biological replicates. The upper sets of histograms represent the apoptotic cells from samples treated with doxorubicin. The lower sets of histograms are the untreated samples. PHF8 knockdown cells are represented by the red line while control siRNA transfected cells are represented in blue. Western blot analysis demonstrates sufficient PHF8 knock down (bottom panel).
Appendix B: Raw PHF8 over expression flow cytometry data of three biological replicates. The upper sets of histograms represent the apoptotic cells from samples treated with doxorubicin. The lower sets of histograms are the untreated samples. PHF8 overexpressing cells are represented by the red line while pFLAG-CMV transfected cells are represented in blue. Western blot analysis demonstrates sufficient PHF8 over expression (bottom panel).
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