The Effect of Noxa Serine-13 Phosphorylation on Hyperthermia-Induced Apoptosis

by

TREVOR MOREY

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Regulation of apoptosis is critical for cell survival during mild stress and for proper removal of damaged cells during severe stress including hyperthermia. Previous studies have shown that knockdown of the BH3-only protein Noxa prevents hyperthermia-induced Mcl-1 degradation and activation of apoptosis. Noxa is a pro-apoptotic BH3-only protein that is able to selectively bind to and disable anti-apoptotic Mcl-1. Phosphorylation of Noxa on serine-13 by the cyclin-dependent kinase CDK5 inhibits the apoptotic function of Noxa. In this study I investigated whether hyperthermia is able to induce apoptosis by preventing Noxa phosphorylation, due to reduced CDK5 activity, leading to activation of Noxa. I was able to demonstrate that both the phosphorylation status and solubility of CDK5 is reduced during hyperthermia. Furthermore, overexpression of a non-phosphorylatable Noxa (S13A) resulted in a significant decrease in cell viability and increase in caspase-3 activity compared to overexpression of wild-type Noxa at 37°C. However, I was unable to detect in vivo phosphorylation of Noxa serine-13 in lymphoid cells and therefore was unable to conclude whether or not hyperthermia affects the phosphorylation status of Noxa.
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List of Abbreviations

A1: Bcl-2-related protein A1

β-TrCP: Beta transducin repeat containing protein

B-CLL: B-cell chronic lymphoblastic leukemia

Bad: Bcl-2-associated death promoter

Bak: Bcl-2 homologous antagonist killer

Bax: Bcl-2-associated X protein

Bcl-2: B-cell lymphoma 2

Bcl-W: Bcl-2 like protein 2

Bcl-xL: B-cell lymphoma-extra large

BDNF: Brain-derived neurotropic factor

Bid: BH3 interacting-domain death agonist

Bik: Bcl-2-interacting killer

Bim: Bcl-2-like protein 11

BH: Bcl-2 homology

BH3: Bcl-2 homology domain 3

Bmf: Bcl-2-modifying factor

Bok: Bcl-2-related ovarian killer

c-Abl: c-Abelson

cAMP: Cyclic adenosine monophosphate

Caspase: Cysteine-aspartic protease

CDK: Cyclin-dependent kinase

CDK1: Cyclin-dependent kinase 1
CDK2: Cyclin-dependent kinase 2
CDK5: Cyclin-dependent kinase 5
DISC: Death-inducing signaling complex
Dox: Doxycycline
ER: Endoplasmic reticulum
ErbB: Epidermal growth factor receptor
ERK: Extracellular signal-regulated kinase
FoxO: Forkhead box protein O
FoxO1: Forkhead box protein O1
Fyn: Tyrosine-protein kinase Fyn
GFP: Green fluorescence protein
GSK-3β: Glycogen synthase kinase 3 beta
HA: Human influenza hemagglutinin
HEK: Human embryonic kidney cells
HIF-1α: Hypoxia-inducible factor 1, alpha subunit
Hrk: Harakiri, Bcl-2-interacting protein
HSP: Heat shock protein
HSP70: Inducible heat shock protein 70
IRF-1: Interferon regulatory factor 1
JNK: c-Jun N-terminal kinase
MAPK: Mitogen-activated protein kinase
Mcl-1: Myeloid cell leukemia sequence 1
MEF: Mouse embryonic fibroblasts
MOMP: Mitochondrial outer membrane permeabilization
MTD: Mitochondrial targeting domain
NGF: Nerve growth factor
NSV-1/2: Noxa splicing variant 1/2
OMM: Outer mitochondrial membrane
p38MAPK: p38 mitogen-activated protein kinase
PBS: Phosphate buffered saline
PI3K: Phosphoinositide 3-kinase
PMAIP1: PMA-induced protein 1
Puma: p35 upregulated modulator of apoptosis
PVDF: Polvinylidene fluoride
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
rtTA: Reverse tetracycline-controlled transactivator
tBid: Truncated Bid
TEV: Tobacco etch virus
TM: Transmembrane
TRE: Tetracycline response element
USP9X: Ubiquitin specific peptidase 9, X-linked
Wee1: Wee1-like protein kinase
WT: Wild-type
Introduction

1. Apoptosis

1.1. Introduction

Apoptosis is a regulated form of programmed cell death that has significant roles in development, tissue homeostasis, and in response to environmental stresses. Regulated cell death was first described as shrinkage necrosis, due to morphological observations, and later renamed apoptosis (Kerr et al., 1965; Kerr et al., 1972). During the first phase of apoptosis, termed the condensation phase, the cell separates from neighboring cells, the cytoplasm and nucleus become condensed, and nuclear DNA is fragmented (Kerr et al., 1972). The endoplasmic reticulum (ER), Golgi apparatus, and mitochondria become disrupted (Taylor et al., 2008), and regulated proteolysis occurs (Kihlmark et al., 2001; Yoo et al., 2008). The cell is then fragmented into apoptotic bodies (Kerr et al., 1972, Taylor et al., 2008). The second stage of apoptosis involves phagocytosis and destruction of apoptotic bodies (Kerr et al., 1972; Vaux & Strasser, 1996). Mutations in genes regulating apoptosis have been implicated in a number of human diseases including cancer (Liadis et al., 2005; Rohn et al., 2008; Hanahan & Weinberg, 2000).

1.2. Molecular mechanisms

In mammalian cells apoptosis is mediated through two distinct, but linked, pathways. The first, termed the intrinsic or stress-induced pathway, is activated in response to stimuli that originate from within the cell, such as oncogenic stress or protein damage (Riedl & Shi, 2004). The intrinsic pathway is activated by mitochondrial release of cytochrome c and formation of the apoptosome. (Jiang & Wang, 2000). The second pathway, termed the extrinsic pathway, is activated in response to binding of death ligands to cell-surface death receptors (Nagata, 1999;
Danial & Korsmeyer, 2004). Binding of death ligands promotes death receptor oligomerization and formation of the death-inducing signaling complex (DISC) (Peter & Krammer, 2003). These pathways are linked through DISC-mediated cleavage of the anti-apoptotic Bcl-2 family protein BID, forming pro-apoptotic tBID, which activates the intrinsic pathway (Li et al., 1998). Ultimately, activation of either pathway leads to the activation of caspases, which exist as inactive proteases in healthy cells.

Cysteine-aspartic proteases, also known as caspases, are proteases that contain a conserved cysteine residue within their catalytic domain and cleave target substrates after specific aspartic acid residues (Shiozaki & Shi, 2004). Caspases can be classified into two groups based on their mode of activation and role in apoptosis. The first group consists of initiator caspase-8, -9, and -10, which are synthesized as monomeric inactive zymogens (Li et al., 1997; Peter & Krammer, 2003; Shiozaki & Shi, 2004). Initiator caspases are activated by either DISC-mediated (caspase-8 and -10) or apoptosome-mediated (caspase-9) proximity-dimerization and autocatalysis (Shi, 2004; Jiang & Wang, 2000; Pop et al., 2006; Bao & Shi, 2007). Once activated the initiator caspases cleave and activate the second group of caspases, the effector caspases. In mammalian cells caspase-3, -6, and -7 are the crucial effector caspases, which are synthesized as inactive single-chain zymogen dimers that are activated by specific intra-chain cleavage by activated initiator caspases (Riedl & Shi, 2004; Shiozaki & Shi, 2004). Activated effector caspases then cleave specific substrates that results in the dismantling of the cell (Figure 1).
Figure 1: Regulation of apoptosis. Apoptosis can be activated by distinct, though connected, pathways. Shown on the left is activation of apoptosis by the stress pathway (intrinsic pathways) whereby stress signals cause activation of BH3-only proteins and inhibition of anti-apoptotic Bcl-2 family proteins, leading to activation of pro-apoptotic Bax and Bak. Bax and Bak form oligomeric pores in the mitochondrial outer membrane to allow for the release of cytochrome c. Released cytochrome c is able to bind to apaf-1 and the initiator caspase-9, forming the apoptosome. The apoptosome is then able to bind, cleave, and subsequently activate the effector caspase-3, -6, and -7, resulting in activation of apoptosis. Shown on the right is activation of apoptosis by the death receptor pathway (extrinsic pathway) whereby death receptor ligands bind to death receptors and cause oligomerization of caspase-8 and formation of the death-inducing signaling complex (DISC). Activated DISC is then able to bind, cleave, and subsequently activate the effector caspase-3, -6, and -7, resulting in activation of apoptosis. These pathways are linked through DISC-mediated cleavage of the Bcl-2 family protein BID into tBID, allowing for activation of Bax and Bak and cytochrome c release. Adapted from Adams and Cory, 2007.
Stress signals promote mitochondrial outer membrane permeabilization (MOMP), which in turn results in release of cytochrome c and activation of caspase-3 (Chipuk et al., 2006), which is necessary for the activation of the apoptosome (Li et al., 1997). Members from the Bcl-2 family of pro- and anti-apoptotic proteins are the main regulators of MOMP and stress-induced apoptosis.

1.3. Bcl-2 family members and interactions between Bcl-2 family members

Bcl-2 (B-cell lymphoma 2) family proteins are the main regulators of stress-induced apoptosis and are defined by containing at least one Bcl-2 homology domain (BH domain) (Cory & Adams, 2002). Bcl-2 family proteins are divided into three categories based on apoptotic function (Figure 2a). The first category consists of the pro-survival (anti-apoptotic) proteins Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and A1, whose function is to maintain mitochondrial, ER, and nuclear membrane integrity. All anti-apoptotic Bcl-2-like proteins contain multiple BH domains (Warr & Shore, 2008; Er et al., 2006; Youle & Strasser, 2008). Additionally, they contain (except A1) a C-terminal hydrophobic domain that assists in intracellular membrane targeting (Cory & Adams, 2002; Zamzami et al., 1998). In healthy and stressed cells Bcl-2 and Mcl-1 exists as integral membrane proteins (Janiak et al., 1994; Yang et al., 1995), while Bcl-w and Bcl-xL become membrane associated only once a cell has received stress signals (Wilson-Annan et al., 2003; Gonzalez-Garcia et al., 1994; Nijhawan et al., 2003). Deregulation of the anti-apoptotic Bcl-2-like proteins has been implicated in many autoimmune, degenerative (Merino & Bouillet, 2009), neurodegenerative (Shacka & Roth, 2005), and developmental disorders (Youle & Strasser, 2008), as well as in cancer (Kang & Reynold, 2009; Hanahan & Weinberg, 2000).
Figure 2

A

Pro-survival

Bcl2 family

Pro-apoptosis

Bax family

BH3-only family

B cl2, Bcl-XL, A1, Bcl-w, Mcl1

Bax, Bak, Bok

Bim, Bik, Bad, Bmf, Hrk, Noxa, Puma

B

Healthy

Dying

Displacement

Displacement

Inactivation and Degradation

Inactivation

Cell Death
Figure 2: Structure and function of the Bcl-2 family. (A) Structural comparison of Bcl-2 family proteins. Members of the Bcl-2 family can be divided into two categories. The first category contains pro-survival members and includes Bcl-2, Bcl-xL, Bcl-W, Mcl-1, and A1. These proteins contain multiple Bcl-2 homology (BH) domains (BH1-4), though certain members, such as Mcl-1, lack the N-terminal BH4 domain. Additionally, most pro-survival Bcl-2 family members, such as Mcl-1, contain a transmembrane domain (TM) that targets these members to specific intracellular compartments. The second category contains pro-apoptotic members, which can be further subdivided into the Bax family and the BH3-only family. The Bax family consists of Bax, Bak, and Box, all of which are responsible for forming oligomeric pores in the outer mitochondrial membrane, resulting in release of cytochrome c and activation of apoptosis. These proteins contain multiple BH domains (BH1-3) and contain a TM domain that targets them to the outer mitochondrial membrane. The BH3-only family consists of Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, and Puma. These proteins are responsible for activating Bax, Bak, and Bok upon receiving stress signals, though the exact mechanism is currently being investigated (Figure 3). All members of the BH3-only subfamily of Bcl-2 family proteins contain only a single BH domain (BH3), and most members, such as Noxa, also contain a TM domain responsible for targeting these proteins to specific intracellular compartments. Adapted from Cory and Adams, 2002. (B) Activation of Bak and Bax requires engagement of anti-apoptotic Bcl-2 family proteins with pro-apoptotic BH3-only proteins. In healthy cells Bak is inactivated by anti-apoptotic Bcl-2 family proteins, such as Mcl-1 and Bcl-xL. During stress BH3-only proteins, such as Noxa, are able to either bind to anti-apoptotic Bcl-2 family proteins, leading to release of Bak, or are able to directly bind and activate Bak, though the exact mechanism is still debatable (Figure 3). Once released/activated Bak then forms oligomeric pores in the outer mitochondrial membrane, leading to release of cytochrome c and activation of apoptosis. Adapted from Willis et al., 2005.

The second category of Bcl-2 family proteins contains the pro-apoptotic proteins Bax, Bak, and Bok, which all demonstrate sequence homology and 3D structural similarity and contain BH domains 1, 2, and 3 (Cory & Adams, 2002). In healthy cells Bax is found as an inactive monomer in the cytosol (Youle & Strasser, 2008; Chipuk et al., 2010), while Bak is constitutively targeted to the outer mitochondrial membrane (OMM) by its transmembrane (TM) domain, but is inactivated by binding to anti-apoptotic Mcl-1 or Bcl-xL (Willis et al., 2005; Youle & Strasser, 2008). Upon apoptotic stimulation Bax undergoes a conformational change, leading to exposure of its TM domain and translocation into the OMM (Youle & Strasser, 2008; Cartron et al., 2008). Bak, on the other hand, is released/activated by the interaction of pro-
apoptotic BH3-only proteins with Mcl-1 and Bcl-xL (Willis et al., 2005; Youle & Strasser, 2008). Bak and Bax then form homo-oligomeric pores in the OMM that cause MOMP, release of cytochrome c, and apoptosis (Figure 2b; Cory & Adams, 2002; Chipuk et al., 2010), though the exact mechanism for pore oligomerization and cytochrome c release remains controversial (Martinou & Green, 2001; Cory & Adams, 2002; Chipuk et al., 2010). Recently it was demonstrated that in healthy cells Bax is constantly retrotranslocated from the mitochondria to the cytosol in order to maintain mitochondrial integrity (Edlich et al., 2011). Retrotranslocation of Bax is dependent on anti-apoptotic Bcl-2 family proteins, such as Mcl-1, Bcl-2, and Bcl-xL, and inhibition of retrotranslocation results in Bax accumulation on the mitochondria and increases in both caspase 3/7 activity and cytochrome c release (Edlich et al., 2011).

The third category of Bcl-2 family proteins contains the pro-apoptotic BH3-only proteins Bid, Bad, Bim, Bik, Noxa, Puma, Hrk, and Bmf. These proteins all contain only one BH domain (BH3), while Bik also contains a TM and both Noxa and Puma contain an additional mitochondrial targeting domain (MTD) (Cory et al., 2003; Youle & Strasser, 2008; Lomonosova & Chinnadurai, 2009). BH3-only proteins are synthesized and activated by cellular stress through several mechanisms such as transcriptional upregulation, phosphorylation, and cleavage (Lomonosova & Chinnadurai, 2009; Chipuk et al., 2010). BH3-only proteins function to induce apoptosis by activating Bax and Bak, though the exact mechanism is still debatable and both direct and indirect models have been proposed (Figure 3). Overall, the indirect model proposes that death is the default pathway that must be constrained by the anti-apoptotic Bcl-2-like proteins, while the direct model purposes that life is the default pathway and that death requires direct activation of Bax and Bak by BH3-only proteins. Additionally, different BH3-only proteins have different apoptotic potential and binding specificity due to their respective BH3
**Figure 3**: Comparison of the indirect and direct model of Bax and Bak activation. (A) In the direct model (Letai *et al.*, 2002; Kuwana *et al.*, 2005) Bim and tBid act as “activators” by binding to Bak and Bax directly in order to induce pore formation. The remaining BH3-only proteins act as “sensitizers” and bind to the anti-apoptotic Bcl-2-like proteins, releasing bound Bim and tBid and allowing them to directly activate Bak and Bax. (B) In the indirect model the BH3-only proteins engage anti-apoptotic Bcl-2-like proteins, leading to the liberation of Bak and Bax, though do not bind directly to Bak and Bax (Willis *et al.*, 2005; Willis *et al.*, 2007). Additionally, some BH3-only proteins can only bind to specific anti-apoptotic Bcl-2 family proteins (selective) while others can bind to all anti-apoptotic Bcl-2 family proteins (promiscuous). This ability of BH3-only proteins to bind and neutralize certain, or all, anti-apoptotic proteins is based on their BH3 domains (Chen *et al.*, 2005). Adapted from Adams & Cory, 2007 (2).
domain (Chan et al., 2005). Currently the debate between the indirect and direct model, and of the role of the variable potential of BH3-only proteins in Bax/Bak activation, has yet to be concluded.

Upregulation of many of the anti-apoptotic Bcl-2 family proteins have been shown to play an important role in the development and maintenance of cancer cells (Hanahan & Weinberg, 2000). As such researchers have developed small-molecule BH3 mimetic anticancer drugs with the aim to disrupt the anti-apoptotic function of anti-apoptotic Bcl-2 family proteins. ABT-737 is a BH3 mimetic that is able to bind to the anti-apoptotic proteins Bcl-2, Bcl-xL, and Bcl-w, though is unable to bind to Mcl-1 (van Delft et al., 2006). As such cells expressing Mcl-1 demonstrate increased resistance to ABT-737 as compared to cells in which Mcl-1 was down-regulated (van Delft et al., 2006; Konopleva et al., 2006). Potential cancer treatments are currently being investigated by examining the synergistic effect of ABT-737 with other compounds that are able to decrease Mcl-1, such as sorafenib (Adams and Cory, 2007; Hikita et al., 2010). Another BH3 mimetic that is currently being investigated is S1 (Zhang et al., 2007), which has been shown to be able to disrupt both Bcl-2/Bax and Mcl-1/Bak complexes and induce apoptosis (Zhang et al., 2011). S1 can induce apoptosis in primary acute lymphoblastic leukemia cells regardless of Mcl-1 levels, and therefore S1 represents a novel class of compounds that are able to function solely as BH3 mimetics and as pan-Bcl-2 inhibitors (Zhang et al., 2011).
2. Mcl-1

2.1. Introduction

Mcl-1 is an anti-apoptotic protein that plays an essential role in stress-induced apoptosis. Mcl-1 was first identified in the differentiating myeloid cell line ML-1 (Kozopas et al., 1993), and has been mapped to chromosome 1q21. Mcl-1 is a 350 amino acid protein that belongs to the Bcl-2 family proteins and contains three BH domains (BH1, 2, 3) and lacks the N-terminal BH4 domain found in other Bcl-2 family proteins (Bingle et al., 2000; Youle & Strasser, 2008; Luke et al., 2010). Mcl-1 also contains a C-terminal TM domain that localizes Mcl-1 primarily to the outer mitochondrial membrane (Akgul et al., 2000; Bingle et al., 2000) and two PEST sequences that are commonly found in proteins with short half-lives (Kozopas et al., 1993; Akgul, 2009). Furthermore two residues within the Mcl-1 PEST sequence (Asp$^{127}$ and Asp$^{157}$) have been observed to be critical for caspase-3, -6, and -7 cleavage of Mcl-1 (Herrant et al., 2004; Weng et al., 2005). Alternative splicing of Mcl-1 produces two different Mcl-1 mRNAs that encode for Mcl-1$L$ and Mcl-1$S$ that either contain or lack exon 2 respectively (Bingle et al., 2000; Bae et al., 2000). Whereas Mcl-1$L$ contains 350 amino acid residues and the above features, Mcl-1$S$ contains 271 amino acid residues and only the BH3 domain and PEST sequences. Consequently, Mcl-1$S$ resembles BH3-only proteins and unlike Mcl-1$L$ (hereafter Mcl-1) has pro-apoptotic activity (Bingle et al., 2000; Bae et al., 2000).

2.2. Transcriptional, translational, and post-translational regulation

Regulation of Mcl-1 transcription is controlled by constitutively active and/or extracellular signal-activated transcription factors. Growth factor stimulation of Mcl-1 transcription has been observed in cholangiocarcinoma cells upon interleukin-6 treatment by
STAT3 activation (Isomoto et al., 2005), and upon interleukin 3 treatment by the PI3K/Akt signaling pathway (Wang et al., 1999) and a p38MAPK-dependent pathway (Wang et al., 2003). Human papillomavirus (HPV) infection-induced expression of interleukin 17 leads to increased transcription of Mcl-1, which is dependent on PI3K signaling (Chang et al., 2010). Protection from tert-butyl hydroperoxide-induced hypoxia has been observed in HepG2 cells due to hypoxia-induced HIF-1α-dependent Mcl-1 transcription (Piret et al., 2005). Staurosporine down-regulates Mcl-1 transcription in a caspase-dependent manner in Jurkat cells (Iglesias-Serret et al., 2003). Lastly, co-culturing of chronic lymphoblastic leukemia (CLL) cells with vascular endothelial cells results in enhanced CLL cell viability in vitro and increased Mcl-1 gene transcription due to increased Rel A activation, whereas inhibition of Rel A prevents transcription of Mcl-1 (Buggins et al., 2010).

Mcl-1 has been shown to be regulated at the translational level by micro-RNAs and RNA-binding proteins. Micro-RNA 29b (Mir-29b) binds the 3’-untranslated region (UTR) of Mcl-1 mRNA and prevents its translation (Mott et al., 2007). Interestingly, mir-29b was found to be either highly expressed or down-regulated in non-malignant immortalized or malignant cholangiocytes, respectively (Mott et al., 2007). In HCUG2 cells, the RNA-binding protein CUG-binding protein 2 binds to the 3’-UTR of Mcl-1 mRNA and both stabilizes Mcl-1 mRNA and prevents Mcl-1 translation (Subramaniam et al., 2008).

Multiple modes of post-translational modification have been identified to regulate Mcl-1 turnover and function. Proteasome inhibition leads to Mcl-1 accumulation (Fernandez et al., 2005; Qin et al., 2006; Nijhawan et al., 2003; Cuconati et al., 2003), and it was observed that Mcl-1 is ubiquitinated and targeted for proteasomal degradation by the BH3-containing E3 ligase Mule/ARF-BP1/LASU1 in both healthy and stressed cells (Zhong et al., 2005). Occupation of
the BH groove of Mcl-1 by other BH3-only proteins can prevent the interaction between Mcl-1 and Mule resulting in increased levels of Mcl-1 (Warr et al., 2005). Mcl-1 is also ubiquitinated by the E3 ligase β-TrCP, though interaction between Mcl-1 and β-TrCP requires Mcl-1 phosphorylation by GSK-3β (Ding et al., 2007). The deubiquitinase USP9X has been shown to stabilize Mcl-1 by removing the Lys-48-linked polyubiquitin chains that mark Mcl-1 for proteasomal degradation (Schwickart et al., 2010; Opferman & Green, 2010). In addition to ubiquitin-dependent degradation of Mcl-1, evidence has been gathered that demonstrates ubiquitin-independent degradation of Mcl-1. Stewart et al. (2010) observed that removal of the lysine residues required for ubiquitination of Mcl-1 (Mcl-1KR) had no effect on the rate of degradation in both healthy and stressed cells as compared to wild-type Mcl-1. Additionally, inhibition of ubiquitin activating enzyme E1 failed to prevent degradation of Mcl-1KR, and as well the 20S proteasome was able to degrade in vitro translated Mcl-1 in a cell free system (Stewart et al., 2010). Association of Mcl-1 with different BH3-only proteins also regulates Mcl-1 turnover. Overexpression of Noxa leads to the formation of Noxa/Mcl-1 complexes and subsequent Mcl-1 degradation via a proteasome-dependent mechanism (Willis et al., 2005; Czabotar et al., 2007), whereas interaction of Bim and Puma with Mcl-1 leads to stabilization of Mcl-1 (Czabotar et al., 2007; Mei et al., 2005). Lastly, the BH3-like ligand Bim s2A can bind Mcl-1 and promote apoptosis in cells when Bcl-xL is absent or neutralized, though is unable to induce Mcl-1 degradation and conversely stabilizes Mcl-1 upon binding (Lee et al., 2008).

As discussed above, phosphorylation has been observed to regulate Mcl-1 turnover and function. ERK-mediated phosphorylation of Mcl-1 at residues Thr163 and Thr92 is required for the interaction between Mcl-1 and Pin1 resulting in stabilization of Mcl-1 (Domina et al., 2004; Ding et al., 2008). GSK-3β-mediated phosphorylation of Mcl-1 at residues Ser155 and Ser159 is
required for β-TrCP-mediated ubiquitination and degradation of Mcl-1 (Maurer et al., 2006; Ding et al., 2007). Stress-activated JNK has also been implicated in the regulation of many Bcl-2-family proteins, and upon overexpression of activated JNK, apoptosis is rapidly induced (Lei et al., 2002). Residues Ser\textsuperscript{121} and Thr\textsuperscript{163} of Mcl-1 are phosphorylated by JNK in response to oxidative stress leading to Mcl-1 inactivation (Inoshita et al., 2002). Interestingly, priming of Mcl-1 by JNK-mediated phosphorylation of Thr\textsuperscript{163} is required for phosphorylation of Ser\textsuperscript{159} by GSK-3β, and phosphorylation of both Thr\textsuperscript{163} and Ser\textsuperscript{159} is required for UV-stimulated Mcl-1 degradation (Morel et al., 2009). JNK1 has been shown to phosphorylate Ser\textsuperscript{64} of Mcl-1 leading to enhanced anti-apoptotic activity and increased interaction of Mcl-1 with Bim, Noxa, and Bak (Kobayashi et al., 2007). These observations implicate Mcl-1 as a protein for signal integration between pro-apoptotic (JNK) and pro-survival (AKT) protein kinase signalling pathways (Morel et al., 2009). Additionally, CDK1-cyclin B1 phosphorylates Mcl-1 on Thr\textsuperscript{92}, and depletion of CDK-cyclin complexes results in inhibited phosphorylation of Mcl-1 at both Thr\textsuperscript{92} and Ser\textsuperscript{64} (Harley et al., 2010). Phosphorylation of Thr\textsuperscript{92} leads to proteasomal-dependent degradation of Mcl-1 during mitotic arrest, demonstrating an intrinsic link between progression through mitosis and regulation of apoptosis (Harley et al., 2010).

### 2.3. Function, relevance, and hyperthermia

Mcl-1 promotes cell survival by binding and neutralizing pro-apoptotic Bak and Bax, subsequently suppressing MOMP and cytochrome c release (Willis et al., 2005; Chipuk et al., 2010). Mcl-1 also interacts with the pro-apoptotic BH3-only proteins Bim, Puma, Bmf, Bik, Hrk, tBid, and Noxa (Chen et al., 2005). In healthy cells it has been suggested that Mcl-1 sequesters Bak at the OMM preventing the activation and oligomerization of Bak. When a cell receives
apoptotic signals, BH3-only proteins, such as Noxa, selectively bind to Mcl-1 and either disrupt Mcl-1/Bak complexes leading to release and oligomerization of Bak (indirect model; Willis et al., 2005; Willis et al., 2007), or disrupt Mcl-1/direct activator (Bim, tBid) complexes allowing for direct activation of Bak (Letai et al., 2002; Kim et al., 2006).

Mcl-1 is an essential anti-apoptotic protein involved in development, homeostasis and response to apoptotic stress, and as such deregulation has been observed in various diseases. Mcl-1 is a critical and specific regulator of homeostasis and development of early hematopoietic progenitors, and depletion of Mcl-1 mRNA in mice results in ablation of bone marrow (Opferman et al., 2005). Overexpression of Mcl-1 has been shown in a number of human hematopoietic and lymphoid cancers, as well as in many solid tumors (Akgul, 2009). Additionally, Mcl-1 is a key factor in resistance to cancer therapies in various cancer types (Akgul, 2009). Overexpression of Mcl-1 provides hematopoietic cells resistance to various cytotoxic agents, such as cyclophosphamide, disrupts lymphopoiesis, and promotes malignant transformation of both hematopoietic progenitor and stem cells (Campbell, KJ, et al., 2010). Knock-out of Mcl-1 in ontogenetically primitive human pluripotent stem cells results in decreased stem cell self-renewal but does not affect survival of hematopoietic stem/progenitor cells (Campbell, CJ, et al., 2010). Degradation of Mcl-1 upon apoptotic stimuli, such as ultraviolet irradiation (UV), adenovirus-mediated DNA damage, growth factor withdrawal, exposure to hyperthermia and bortezomib treatment is essential for initiation of apoptosis (Nijhawan et al., 2003; Cuconati et al., 2003; Maurer et al., 2006; Qin et al., 2006; Stankiewicz et al., 2009). It has been observed that decreasing Mcl-1 protein levels by anti-sense oligonucleotide treatment induces apoptosis in multiple myeloma cells (Zhang et al., 2002). Conversely, Mcl-1 depletion by siRNA or by interaction with BH3-only proteins failed to induce
apoptosis, suggesting that additional Bcl-2-like anti-apoptotic proteins regulate apoptosis in various cell types (Nijhawan et al., 2003; Willis et al., 2005).

In the Mosser lab (University of Guelph) it has been shown that hyperthermia leads to reduced levels of Mcl-1 by a combination of reduced synthesis, increased ubiquitination by Mule, and caspase cleavage (Stankiewicz et al., 2009). Additionally, overexpression of Hsp70, which prevents heat-induced Bax activation (Stankiewicz et al., 2005), stabilizes Mcl-1 in hyperthermia-exposed cells due to reduced Mule binding, possibly by inhibiting Noxa/Mcl-1 complexes (Stankiewicz et al., 2009). Interestingly, depletion of Noxa mRNA by shRNA prevents hyperthermia-induced Mcl-1 depletion, Bax activation and oligomerization, and provided resistance to hyperthermia-induced apoptosis (Stankiewicz et al., 2009). In order to elucidate the anti-apoptotic role of Mcl-1 in hyperthermia further investigation into the interaction between Mcl-1 and Noxa is required.
3. Noxa

3.1. Discovery

Noxa was first identified in 1990 by Hijikata et al. as a cDNA clone during a screen in adult T-cell leukemia cells for gene products involved in tumorigenesis. When peripheral blood mononuclear, human embryonic lung, and Jurkat T acute lymphoblastic leukemia cells were treated with the tumor promoter mitogen phorbol-12-myristate-13-acetate (PMA) Hijikata et al. observed a rapid induction of a novel transcript, which he named ATL-derived PMA-responsive gene. It was later renamed PMA-induced protein 1 (PMAIP1) under the HUGO system (Ploner et al., 2009). In a separate study a cDNA was identified in X-ray-irradiated wild-type and IRF-1/p53 double deficient mouse embryonic fibroblasts (MEF) (Oda et al., 2000). This cDNA was termed Noxa (Latin for damage), and a human homolog of Noxa was identified in Saos2 cells (Oda et al., 2000). Though both PMAIP1 and Noxa are both acceptable names for this protein, many current studies, including my own, utilize Noxa for simplicity.

3.2. General features and transcript variants

Human Noxa encodes a 54-amino acid protein that contains a single Bcl-2 homology 3 (BH3) domain (Oda et al., 2000) and a C-terminal MTD (Seo et al., 2003) that are both conserved between multiple mammalian species (Figure 4). The core gene structure of human Noxa contains three exons and two introns (Wang & Sun, 2008). To date, three splice variants of human Noxa have been identified. Transcript 1, which includes exon 1 and 3, encodes for the 54 amino acid Noxa where both the BH3 domain and MTD are encoded within exon 3.
<table>
<thead>
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ΦΣXΦ2XXΣ’D2Φ3Γ

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<td>Fish</td>
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**Figure 4**: Sequence comparison of Noxa protein from various species. Mouse and rat BH3 domain A is shown in orange while BH3 domain B is shown in red with red residues showing homology to human Noxa. BH3 domain consensus sequence is shown above the BH3 domain B, where Φ is a hydrophobic residue (Φ2 is a conserved leucine residue), Σ and Σ’ are small residues, X is any residue, D is a conserved aspartate residue, Z is normally an acidic residue, and Γ is a hydrophilic residue (Day et al., 2008). The mitochondrial targeting domain (MTD) is shown in green (Seo et al. 2003).
Transcripts 2 and 3, named Noxa-splicing variants 1 and 2, respectively (NSV-1/2), both contain exons 1 and 3, although NSV-I contains a portion of exon 2 (2a), and NSV-2 contains the entire exon 2 (Wang & Sun, 2008). Sequence analysis of NSV-I and NSV-2 predict the synthesis of 136 amino acid and 70 amino acid proteins respectively (Wang & Sun, 2008). Both NSV-1/2 lack BH3 domains, due to differences in the reading frame of NSV-1/2 as compared to Noxa, and have extremely short protein half-lives, as they are undetectable without treatment with the proteasome inhibitor MG132 (Wang & Sun, 2008). The in vivo function of these Noxa splice variants is still to be determined.

3.3. Regulation of Noxa expression and post-translational modification

Early observations indicated that Noxa transcription was primarily induced by p53. In wild-type and IRF-1-deficient MEFs, X-ray irradiation caused a rapid induction of Noxa mRNA, whereas no Noxa induction was observed in p53<sup>−/−</sup> MEFs (Oda <i>et al.</i>, 2000). Analysis of the Noxa promoter region revealed a bona fide p53-response element 195 bp upstream of the transcription start site (Oda <i>et al.</i>, 2000, Seo <i>et al.</i> 2003). Additional studies have investigated p53-dependent transcription of Noxa either by in situ hybridization in p53<sup>−/−</sup> mice or treatment of multiple cell lines with various chemical compounds (Fei <i>et al.</i>, 2002; Saha <i>et al.</i>, 2010; Ghavami <i>et al.</i>, 2011; Raats <i>et al.</i>, 2011). The p53 independent regulation of Noxa by various stimuli has also been observed. Noxa induction was observed when multiple p53<sup>−/−</sup> melanoma cell lines, along with PC-3 prostate cells and Saos-2 osteosarcoma cells (both p53 null cell lines) were treated with the γ-secretase inhibitor GSI (Qin <i>et al.</i>, 2004). Hypoxia-induced HIF-1α has been shown to induce Noxa mRNA and protein expression in H719 and Saos-2 cells independent of p53 by binding to a hypoxia response element (HRE) at -1275 bp within the Noxa promoter (Kim <i>et al</i>.,
Overexpression of adenovirus E1A protein in the neuroblastoma cell line SH-SY5Y (non-functional p53) and SaOS-2 cells results in activation of p73 and induction of Noxa mRNA (Flinterman et al., 2005). H$_2$O$_2$-induced activating transcription factor 4 is able to induce Noxa mRNA expression in Jurkat cells by binding to a cAMP response element binding site within the Noxa promoter (Aikawa et al., 2010). A FoxO-binding site has been identified within the Noxa promoter by treatment of Jurkat cells with α-tocopheryl succinate, resulting in activation of FoxO1 and FoxO1-mediated transcription of Noxa (Valis et al., 2011). Additional studies have been completed that have investigated p53-independent regulation of Noxa transcription (Alves et al., 2006; Lau et al., 2008; Martin et al., 2009; Santidrián et al., 2010; Iglesias-Serret et al., 2010; Sheridan et al., 2010; Jin et al., 2010; Jin et al., 2011).

In addition to transcriptional regulation of Noxa, proteasomal degradation has been implicated in the control of Noxa protein stability. Noxa has a short half-life (Wang & Sun, 2008), although it does not contain any PEST or known E3-ligase binding domains. KLF6-SV1 was observed to bind to Noxa and lead to its HDM2-mediated proteasomal degradation upon KLF6-SV1 overexpression in SKOV3 cells (DiFeo et al., 2009). Additionally, proteasome inhibition in SKOV3 cells by MG132 causes an increase in both KLF6-SV1 and Noxa (DiFeo et al., 2009). Treatment of MM.1S cells with the novel proteasome inhibitor MLN2238 results in increased expression of both p53 and Noxa (Chauhan et al., 2011). Treatment with the proteasome inhibitor bortezomib (PS-441, Velcade) results in Noxa mRNA and protein induction in both p53 wild-type and p53-null melanoma cells, but not in normal melanocytes (Fernandez et al., 2005). This effect of bortezomib was also observed in vivo and in multiple other cell lines (Fernandez et al., 2005; Perez-Galan et al., 2006; Fribley et al., 2006). A cellular myelocytomatosis viral oncogene (c-MYC) binding site within the Noxa promoter has been
identified, and siRNA knockdown of c-MYC reduced bortezomib-induced Noxa mRNA expression in multiple melanoma cell lines, MDA-MB-231 cells, and HeLa cells (Nikiforov et al., 2007). Treatment of LX-2 cells (human hepatic stellate cells) with MG132 resulted in increased expression of both Noxa mRNA and Noxa protein, and the MG132-induced apoptosis in LX-2 cells was shown to require Noxa (Sosa Seda et al., 2010). Recently, siRNA knockdown of the E3 ligase SAG was found to lead to an increase in Noxa, whereas overexpression of SAG leads to a decrease in Noxa (Jia et al., 2010). However, further studies into the interaction between SAG and Noxa are needed as it has not been identified if SAG interacts directly with Noxa or if Noxa is ubiquitinated by SAG.

Post-translational modifications, such as ubiquitination and phosphorylation, have been shown to have essential roles in the function and degradation of many different proteins. Baou et al. demonstrated that ubiquitinated Noxa can be detected in both untreated and MG132-treated HEK293T cells when transfected to overexpress HA-tagged or untagged Noxa, leading to proteasome-dependent degradation of Noxa (Baou et al., 2010). To date a specific E3 ligase responsible for the ubiquitination of Noxa has not been identified. It has been demonstrated that Noxa is phosphorylated on Ser\textsuperscript{13} by the atypical cyclin-dependent kinase CDK5 in the presence of glucose, and that phosphorylation of Ser\textsuperscript{13} prevents the pro-apoptotic function of Noxa (Lowman et al., 2010). Furthermore, immunoprecipitation of Mcl-1 revealed that Mcl-1 is unable to associate with phosphorylated Noxa, although analysis of phospho-Noxa/Mcl-1 interaction was measured using a phospho-Ser\textsuperscript{13}-Noxa mouse monoclonal antibody that was shown to detect phospho-Noxa generated from \textit{in vitro} kinase assays or \textit{in vivo} only when Noxa was ectopically overexpressed (Lowman et al., 2010). No data has been published to date on
whether phosphorylation of Noxa on Ser\textsuperscript{13} has an effect on Noxa degradation or whether endogenous Noxa is phosphorylated \textit{in vivo} by CDK5.

Heat-shock is another cellular stress that has effects on apoptosis. A 2.95-fold up-regulation of \textit{Noxa} mRNA expression was observed in one study that investigated the effects of heat-shock on IMC-3 cells (Narita \textit{et al.}, 2002). In a separate study, it was observed that heat shock causes Noxa protein levels to initially drop, but then to return to higher-than-basal levels in PErTA cells, a human acute lymphoblastic T cell line derived from the PEER cell line that expresses rtTA, (Stankiewicz \textit{et al.}, 2009). Additionally, depletion of Noxa by shRNA prevented heat-induced apoptosis in PErTA cells (Stankiewicz \textit{et al.}, 2009). The mechanism of heat-induced Noxa expression is currently unknown.

3.4. \textbf{Subcellular localization and association with Bcl-2-like proteins}

Assessment by immunostaining has shown that overexpressed mouse Noxa preferentially localizes to the mitochondria, and that mutations within either the BH3 domain or MTD prevent mitochondrial localization (Oda \textit{et al.}, 2000; Seo \textit{et al.}, 2003). Furthermore, Noxa constructs that are missing either the BH3 domain or MTD fail to induce apoptosis (Seo \textit{et al.}, 2003). This suggests that both the BH3 domain and MTD are required for Noxa-induced apoptosis due to the proximity of the BH3 domain and MTD mutations in either domain may change the overall conformation of Noxa and impair Mcl-1 binding (Ploner \textit{et al.}, 2009). One study has also observed Noxa localization in the ER in melanoma cells where Noxa overexpression leads to accumulation of intracellular Ca\textsuperscript{2+} (Hassan \textit{et al.}, 2008). The mechanism behind Noxa-induced Ca\textsuperscript{2+} accumulation is still unknown.
Noxa contains only a single BH3 domain, placing it in the growing category of pro-apoptotic BH3-only proteins. Studies have shown that overexpression of Noxa is able to significantly induce apoptosis in various cell lines (Oda et al., 2000; Seo et al., 2003; Fribley et al., 2006), as well as correlate with MOMP, reactive oxygen species accumulation (ROS), and cytochrome c release (Hassan et al., 2008; Seo et al., 2003). Additionally, Noxa<sup>−/−</sup> mice show a severe osteoporotic phenotype due to increased survival of short-lived osteoclasts (Idrus et al., 2011). Conversely, other studies have shown that Noxa overexpression has little to no apoptotic potential (Chen et al., 2005; Weber et al., 2009; Willis et al., 2005; Hagenbuchner et al., 2010), and that Noxa knockdown has no developmental effect in mice (Shibue et al., 2003; Villunger et al., 2003). Additionally, mature neutrophils, differentiated from established neutrophil progenitor cells from Noxa-deficient mice, have a slight resistance to spontaneous apoptosis (Kirschnek et al., 2011). This result was also observed in cultured primary neutrophils from Noxa-deficient mice, though substantially reduced apoptosis was observed in cultured primary neutrophils and mature neutrophils both established from Bim/Noxa-deficient mice (Kirschnek et al., 2011).

One study observed that Noxa selectively binds to the pro-survival proteins Mcl-1 and A1, and that overexpression of Noxa alone has weak apoptotic potential in MEFs (Chen et al., 2005) (Figure 5). The specificity of Noxa towards Mcl-1 and A1 is dependent on key amino acid residues within the Noxa BH3 domain. Mutations in the Noxa BH3 domain (m3) allows Noxa to bind to Bcl-xL with a 100-fold increased affinity versus wild-type Noxa and is a more potent inducer of apoptosis (Chen et al., 2005), while other mutations within the BH3 domain rendered Noxa inactive (Oda et al., 2000; Seo et al., 2003). Additionally, Bim<sup>s</sup> chimeras containing the Noxa BH3 domain showed reduced apoptotic ability, as compared to wild-type Bim<sup>s</sup>, which has a high apoptotic potential. Bim<sup>s</sup>-Noxa BH3 chimeras were also restricted to binding Mcl-1,
Figure 5: Noxa interacts with selective anti-apoptotic Bcl-2 family proteins. (A) Binding specificity of different BH3-only proteins. Promiscuous members (Bim, Puma, tBid) are able to bind to all anti-apoptotic Bcl-2 family proteins, while selective members (Bad, Noxa) are able to bind only certain anti-apoptotic Bcl-2 family proteins (Chen et al., 2005). (B) Viability assays in fibroblasts demonstrated that certain BH3-only proteins (BimEL, Puma) are potent killers when overexpressed, while others (Bad, Noxa) are weak killers. Conversely, a Noxa construct that contains the Bad BH3 domain instead of the Noxa BH3 domain proved to be a potent killer (Chen et al., 2005). Adapted from Adams & Cory, 2007 (2).
further demonstrating how the Noxa BH3 domain controls binding specificity and apoptotic potential of Noxa (Chen et al., 2005). Interaction of Noxa with Mcl-1 has also been observed in melanoma cells treated with bortezomib (Qin et al., 2006), and in MDN and Jurkat cells where endogenous Noxa/Mcl-1 complexes were detected (Gomez-Bougie et al., 2007; Alves et al., 2006). A recent study observed that Noxa is able to bind to Mcl-1 and Bcl-xL in NB15-BclcL neuroblastoma cells demonstrating that Noxa can bind to different Bcl-2-like proteins in different cell types (Hagenbuchner et al., 2010). Additionally, treatment of HeLa cells with either UV, thapsigargin (ER stress inducer), or MG132 results in generation of Mcl-1-free Noxa which is then able to bind to Bcl-xL and induce apoptosis (Lopez et al., 2010; Zhang et al., 2010). Another study has demonstrated that Noxa is able to bind to Bcl-2, though this interaction can only be observed in Jurkat cells when they are treated with either bortezomib or MG132. Conversely, it was also demonstrated that interaction between Bcl-2 and Noxa can be observed in vivo in RL cells (diffuse large B cell lymphoma) that constitutively overexpressed Bcl-2 and in HCT116 cells that were treated with the topoisomerase I poison camptothecin, which causes upregulation of Noxa levels (Smith et al., 2011).

Due to the specificity of Noxa for both Mcl-1 and A1 (Chen et al., 2005), the cellular levels of Mcl-1 and A1 control sensitivity to Noxa-induced apoptosis. Overexpression of Noxa in MEFs leads to Mcl-1 degradation without significant induction of apoptosis (Willis et al., 2005). Consistent with the idea that Noxa needs to be complemented by Bad, which targets Bcl-xL, Bcl-2, and Bcl-w, in order to induce apoptosis (Chen et al., 2005), overexpression of both Noxa and Bad was shown to induce apoptosis in MEFs (Willis et al., 2005). Overexpression of Noxa in Bcl-xL+/− MEFs induced Bak-dependent apoptosis, demonstrating that Mcl-1 and Bcl-xL constrain Bak and that Noxa specifically engages Mcl-1 in order to promote Bak-dependent
apoptosis (Willis et al., 2005). Overexpression of Noxa has also been shown to disrupt Mcl-1/Bak complexes in multiple myeloma and B-cell lymphomas and in Jurkat cells (Morales et al., 2008; Inoue et al., 2007). Noxa has also been shown to disrupt Mcl-1/Bim complexes in bortezomib-treated MDN cells (Gomez-Bougie et al., 2007).

Another feature of Noxa is interaction between Noxa and Mcl-1 promotes proteasome degradation of Mcl-1. Willis et al. (2005) demonstrated that overexpression of Noxa leads to proteasome-dependent Mcl-1 degradation. Furthermore they showed that this Noxa-induced Mcl-1 degradation requires association of Noxa with Mcl-1 (Willis et al., 2005). Noxa-induced Mcl-1 degradation has also been observed when Noxa was overexpressed in U266 myeloma cells (Morales et al., 2008) and MEFs (Czabotar et al., 2007). Mcl-1 basal levels appear to be modulated by the HECT- and BH3-domain containing Mule/ARF-BP1 E3 ligase (Zhong et al., 2005). Recently it has been demonstrated that overexpression of Noxa causes decreased Mcl-1/USP9X interaction and, conversely, increased Mcl-1/Mule interaction, overall leading to increased Mcl-1 ubiquitination and degradation (Gomez-Bougie et al., 2011). Structural analysis of recombinant Noxa/Mcl-1 complexes has demonstrated that a C-terminal portion of the Noxa BH3 domain (FRQKLL) is required for Mcl-1/Noxa degradation (Czabotar et al., 2007). Overall these studies have established a pro-apoptotic role for the BH3-only protein Noxa through its interaction with Mcl-1.
4. Cdk5

4.1. Discovery

The proline-directed serine/threonine cyclin-dependent kinase 5 (CDK5) is an atypical member of the well-studied family of cyclin-dependent kinases (CDKs) (Dhariwala and Rajadhyaksha, 2008). Multiple separate groups first identified CDK5 in 1992. Hellmich et al. first identified CDK5 as neuronal cdc2-like kinase (nclk) as it is able to phosphorylate the lysine-serine-proline (KSP) motif of neurofilaments in vitro and shares 58% and 61% amino acid sequence homology to mouse CDK1 and human CDK2 (Hellmich et al., 1992). CDK5 was also reported as tau protein kinase II (TPKII) due to its association with and ability to phosphorylate tau (Ishiguro et al., 1992). Lew et al. reported the same kinase as brain proline-directed protein kinase due to its functional similarity to cdc2 in the bovine brain (Lew et al., 1992). Lastly, in 1993 Kobayashi et al. identified that the 30 kDa protein subunit of TPKII was the active enzyme and termed it CDK5. CDK5 has been mapped to 7q36 within the human genome. Translation of the 987 bp CDK5 transcript yields a 33 kDa protein that phosphorylates target proteins on serine and threonine residues within a S/TPXK/R motif, where X is any amino acid and P is a required proline residue at position +1 (Dhariwala and Rajadhyaksha, 2008; Shetty et al., 1993). CDK5 appears to have no intrinsic cellular distribution, but instead tends to co-localize with its substrates and activators (Ko et al., 2001; Paglini and Cáceres, 2001; Lee et al., 1996). Being a member of the CDK family, CDK5 shares structural features and characteristics with other CDKs, though its activation pattern is strikingly different (Mapelli and Musacchino, 2003; Terricone et al., 2001).
4.2. Activators of CDK5

Unlike other CDKs that require the binding of cyclins in order for their activation, CDK5 requires the binding of p35, p39, or p25 (a proteolytic fragment of p35) for activation. p35 (NCK5a, neuronal CDK5 activator) was first discovered due to its association and activation of CDK5 (Lew et al., 1994; Tsai et al., 1994), whereas p39 (NCK5ai, neuronal CDK5 activator isoform) was first identified as a 39 kDa isoform of p35 that shared 57% amino acid homology with p35 (Tang et al., 1995). p25 was first discovered as a truncated form of p35 that was found in the neurons of Alzheimer patients (Patrick et al., 1999), and subsequent studies identified that cleavage of p35 into p25 was calpain- and dephosphorylation-dependent (Kusakawa et al., 2000; Kamei et al., 2007). Lastly, p29, a similarly-cleaved product of p39, has also been identified and is known to play a role in the deregulation of CDK5 (Patzke et al., 2003). p35, p39, and p25 show limited amino acid sequence homology to cell-cycle cyclins, though, they are able to interact with CDK5 by folding into a tertiary structure containing a CDK5-binding domain that is similar to the CDK-binding domains of other cyclins (Mapelli and Musacchino, 2003; Terricone et al., 2001; Morgan, 1995; Brown et al., 1995, Tang et al., 1997). Studies regarding the age and regional distribution of p35 and p39 in embryonic and postnatal rat brains have demonstrated that the expression pattern of p35 and CDK5 is the inverse of p39, suggesting that they might have a developmental stage- and region-specific function (Wu et al., 2000). Interestingly, the subcellular localization of CDK5 is dependent on the activator to which it is bound. Specifically, CDK5 remains primarily cytosolic when not bound to any activators, whereas interaction with either p35 or p39 results in localization of CDK5 primarily to the plasma membrane and perinuclear region, due to myristoylation of p35 and p39, though CDK5/p35 complexes can also be seen in the cytosol (Asada et al., 2008). It has been observed that p39 can mask p35
deficiency by compensating for some, but not all, function of p35 in p35\(^{-/-}\) mice (Paglini and Cáceres, 2001; Hallows \textit{et al}., 2003), and that p39\(^{-/-}\) mice show no detectable abnormalities (Ko \textit{et al}., 2001). However, p35/39\(^{-/-}\) double knockout mice show a more severely affected phenotype, as compared to p35\(^{-/-}\) mice, that is identical to CDK5\(^{-/-}\) mice (Ko \textit{et al}., 2001). Due to lack of CDK5 activity and the severe phenotypic abnormalities in p35/39\(^{-/-}\) mice, p35 and p39 are thought to be necessary and sufficient for both normal CDK5 activity and proper neurodevelopment (Ko \textit{et al}., 2001; Dhariwala and Rajadhyaksha, 2008; Hisanaga and Endo, 2010). Studies have also found that cyclin D and E are able to bind to CDK5, though these associations do not activate CDK5 kinase activity (Xiong \textit{et al}., 1992; Miyajima \textit{et al}., 1995; Lalioti \textit{et al}., 2010). Lastly, CDK5 has been reported to bind and be activated by cyclin I, which is predominantly expressed in terminally differentiated cells, such as podocytes and neurons (Brinkkoetter \textit{et al}., 2009). Binding of cyclin I to CDK5 has been proposed to have an antiapoptotic function that is specific to terminally differentiated cells, possibly due to activation of the MEK/ERK pathway and increased expression of Bcl-2 and Bcl-x\(_L\) (Brinkkoetter \textit{et al}., 2009; Brinkkoetter \textit{et al}., 2010).

\textbf{4.3. Regulation of CDK5 activity}

Treatment of PC12 cells (rat pheochromocytoma) with nerve growth factor (NGF) results in the strong induction of p35 expression that is ERK-dependent and mediated by early growth response protein 1 (Egr1; Harada et al., 2001). In support, Egr1−/− mice have reduced p35/p25 protein levels and CDK5 activity in brain tissues (Li et al., 2007). Chang et al. identified that both p35 and p39 are targets for the transcription factor heat shock factor protein-2 (HSF2) by observing reduced p35/p39 protein levels in the telencephalon of Hsf2−/− mice during cortical development (Chang et al., 2006). p35 and p39 have overlapping, but distinctive, expression profiles in the developing mouse brain. Expression of p35 can be observed in the cerebral cortex as early as embryonic day 15, whereas p39 expression is not present until postpartum day 0 (P0). Furthermore, expression of p35 decreases while expression of p39 is increased after P0 in the cerebral cortex, while expression of both p35 and p39 is highest during embryonic development and decreases after birth in the cerebellum, brain stem, and spinal cord (Takahashi et al., 2003).

In the adult rat, expression of p35 mRNA is predominantly greater in the brain as compared to the spinal cord, whereas expression of p39 mRNA is the opposite of p35 (Zheng et al., 1998).

Protein levels of p35 and p39 are also regulated by protein degradation. Studies have shown that p35 has a half-life of ~30 min and is ubiquitinated in COS-7 cells (monkey kidney fibroblast) and mouse primary cortical neurons (Patrick et al., 1998; Endo et al., 2009). Furthermore, phosphorylation of p35 at Thr138 by activated CDK5 results in proteasome-dependent degradation of p35, resulting in a negative feedback loop that inhibits CDK5 activity (Patrick et al., 1998; Kamei et al., 2007; Lalioti et al., 2010). Conversely, phosphorylation of p35 residues Ser59, Ser65, and Ser124 by protein kinase Cδ results in stabilization of p35 (Zhao et al., 2009). Lastly, prevention of protein phosphatase 1 and 2A activity by treatment of COS-7 cells with okadaic acid results in maintained phosphorylation of Thr138 and prevention of
calpain-dependent cleavage of p35 into p25 (Kamei et al., 2007). One study investigated the relative rates of degradation of p35 as compared to p39 and observed that p39 both cleaved and degraded at a slower rate than p35 (Minegishi et al., 2010). The variance in degradation and cleavage rates were attributed to the N-terminal p10 regions of p35 and p39 that, as a result of membrane association predominantly controlled by myristoylation, regulate the cleavage and degradation of p35 and p39 respectively (Minegishi et al., 2010). Lastly, the cleaved products of p35/p39, p25/p29 respectively, are protected from degradation as they lack the N-terminal p10 region. This leads to aberrant subcellular localization and interaction of p25/p29 with CDK5 results in deregulation of CDK5 target specificity and promotes neurodegeneration (Liebl et al., 2011).

In addition to levels of p25/p35/p39 protein, CDK5 activity is also regulated by direct phosphorylation of CDK5, though the effect CDK5 phosphorylation on CDK5 activity is dramatically different from that of other CDKs. Commonly, CDK activity is inhibited by phosphorylation of both Thr\textsuperscript{14} and Tyr\textsuperscript{15} by the dual specific kinases Wee1 and myelin transcription factor 1, while phosphorylation of CDK1/2 on residue Thr\textsuperscript{160/161} by CDK-activating kinase (CAK) is required for activation of CDK1/2 activity (Liebl et al., 2011; Hisanaga and Endo, 2010; Lalioti et al., 2010). Conversely, CDK5 does not require phosphorylation of Ser\textsuperscript{159} (equivalent to Thr\textsuperscript{160/161} in CDK1/2) as CDK5, prepared from E. coli, becomes activated upon binding with p35 and addition of CAK does not result in phosphorylation or enhanced kinase activity of CDK5 (Qi et al., 1995, Poon et al., 1997). Analysis of the crystal structure of CDK5/p25 complexes demonstrates that binding of p25 forces the activation loop of CDK5 to adopt an open conformation that is typical of other activated proline-directed kinases, such as CDK1/2, once phosphorylated (Tarricone et al., 2001; Lalioti et al., 2010; Hisanaga and Endo,
In addition, CDK5 is not phosphorylated at Tyr^{15} by Wee1, although phosphorylation of Thr^{14} by CDK T14 kinase, purified from bovine thymus, results in inactivation of CDK5 (Poon et al., 1997; Matsuura and Wang, 1996). In opposition to the inhibitory role of Tyr^{15} phosphorylation for CDK1/2, phosphorylation of Tyr^{15} results in increased CDK5 enzymatic activity and is mediated by non-receptor Src family tyrosine kinases, such as c-Abelson (c-Abl) and Fyn, and the receptor-type tyrosine kinases ephrin type-A receptor 2/4 (Zukerberg et al., 2000; Sasaki et al., 2002; Cheng et al., 2003; Fu et al., 2007). Phosphorylation of Tyr^{15} by c-Abl is mediated by CDK5 and Abl enzyme substrate (Cables), which facilitate interaction between CDK5 and c-Abl and lead to enhanced Tyr^{15} phosphorylation of CDK5 (Zukerberg et al., 2000). Phosphorylation of Tyr^{15} by Fyn is mediated by association of Plex-A2 with both active Fyn and CDK5, resulting in CDK5 phosphorylation and increased kinase activity that is dependent upon Fyn activity (Sasaki et al., 2002). Molecular dynamic simulations have predicted that phosphorylation of Tyr^{15} allows for ATP to adopt a more favorable conformation within CDK5, allowing for enhanced substrate phosphorylation (Zhang et al., 2007). It is currently unknown whether phosphorylation of Tyr^{15} plays a direct role in the binding of CDK5/p35, therefore having control over CDK5 activity, or if Tyr^{15} phosphorylation only affects substrate specificity and/or the rate of kinase activity (Hisanaga and Endo, 2010).

CDK5 activity can also be inhibited by various pharmacological compounds. The earliest, and currently the most popular, inhibitors of CDK5 are the purine-based compounds olomoucine and roscovitine (Veselý et al., 1994; Meijer et al., 1997). Though, due to the interaction of these purine-based compounds with the conserved ATP-binding pocket present in all members of the CDK family both olomoucine and roscovitine are not selective inhibitors of CDK5 (Liebl et al., 2011). Various targets for both compounds have been identified, where
olomoucine and roscovitine has been shown to inhibit CDK1, CDK2, CDK5, Erk1, and Erk2, while in addition roscovitine is able to also inhibit pyridoxal kinase (Veselý et al., 1994; Meijer et al., 1997; Knockaert et al., 2002; Bach et al., 2005; Galimberti et al., 2010). These compounds are still cited as “specific” inhibitors of CDK5 though information regarding the semi-specific binding properties of olomoucine and roscovitine have been previously published, and subsequently companies, such as Boehringer Ingelheim and Pfizer, are currently developing more specific CDK5 inhibitors (Liebl et al., 2011).

4.4. Function of CDK5 in neuronal development and function

CDK5 is an essential player in central nervous system development, function, and disease. It’s function is indispensable for proper neuronal migration and differentiation, axonal elongation, and synaptic function. Also, association of CDK5 with p25 leads to altered regulation of CDK5 that promotes neuronal apoptosis and development of neurological diseases such as Alzheimer's and Parkinson's disease (Dhariwala and Rajadhyaksha, 2008; Liebl et al., 2011; Smith and Tsai, 2002; Lalioti et al., 2010). Studies of CDK5−/− and p35/p39−/− mice have revealed that these mice die during the perinatal period of development due to widespread disruption of neuronal migration in the cerebral cortex, hippocampus, and cerebellum, resulting in a lack of cortical laminar structure and cerebellar foliation (Ohshima et al., 1996; Hirasawa et al., 2004; Ko et al., 2001; Gilmore et al., 1998; Smith and Tsai, 2002; Liebl et al., 2011). The requirement for CDK5 in development and survival of p35-expressing neurons was demonstrated by rescue of neuronal development by re-expression of CDK5, under the control of the p35 promoter, in CDK5−/− mice (Tanaka et al., 2001). Studies investigating the molecular basis for the pathological effect of CDK5 knockout have found that caspase-3 activity is increased in the brain cortex of
CDK5<sup>+/−</sup> mice as compared to wild-type mice (Li et al., 2002). In addition to CDK5<sup>+/−</sup> mice, p35<sup>+/−</sup> mice have severe defects in cortical lamination, due to lack of CDK5/p35 kinase activity and improper neuronal migration, and suffer from seizures and sporadic adult lethality (Chae et al., 1997; Smith and Tsai, 2002). Though CDK5<sup>+/−</sup> and p35<sup>+/−</sup> mice do share similar phenotypic abnormalities, it should be noted that these two phenotypes are not identical as p39 can maintain limited function of CDK5 in p35<sup>+/−</sup> mice (Paglini and Cáceres, 2001; Hallows et al., 2003).

CDK5 has also been demonstrated to be involved in axonal elongation. Studies have demonstrated that there exists a high degree of temporal correlation between CDK5 activation, p35 expression, and formation of axonal tracts in the developing brain (Paglini et al., 2001; Smith and Tsai, 2002; Dhariwala and Rajadhyaksha, 2008). CDK5 and p35 are both present at the leading edge of axonal growth cones in developing neurons where CDK5 is co-distributed with actin filaments but not with microtubules (Nikolic et al., 1996). siRNA-mediated knockdown of p35 in cultured neurons results in decreased laminin response and inhibition of axonal elongation, which can be rescued by co-expression of p35 but not CDK5 (Paglini et al., 1998; Nikolic et al., 1996). At the molecular level inhibition or silencing of CDK5 in PC12 cells suppresses phosphorylation of protein phosphatase 1 (PP1) and prevents NGF-induced neurite outgrowth, whereas overexpression of wild-type PP1 promotes NGF-induced differentiation of PC12 cells (Li et al., 2007). Additional studies have also shown that CDK5 may play a role in neurite branching, which is vital for proper neuronal patterning (Carter et al., 2003; Carter et al., 2008; Lalioti et al., 2010).

CDK5 has been shown to have various roles in synapse formation, maintenance, and synaptic communication. A role for CDK5 in the formation of dendritic spines was identified due to inhibition of BDNF-induced dendritic growth when rat primary hippocampal neurons lack
CDK5 activity (Cheung et al., 2007). Conversely, CDK5 regulates retraction of dendritic spines by phosphorylating ephexin1 upon being itself phosphorylated on Tyr\textsuperscript{15} by activated EphA4, leading to activation of the small Rho GTPase RhoA and retraction of dendritic spines (Fu et al., 2007). CDK5 has also been identified to regulate neuron secretion at the synapse by phosphorylating key mediators, such as Synapsin1, Munc18, and Amphipysin, resulting in functional changes in the activity of these key mediators (Dhariwala and Rajadhyaksha, 2008, Smith and Tsai, 2002; Lalioti et al., 2010). CDK5 and p35 are abundant in embryonic muscle and at the neuromuscular junctions in adulthood (Fu et al., 2001), and additional studies have shown that CDK5, p35, and p39 are present in synaptic membranes (Fu et al., 2001; Niethammer et al., 2000; Humbert et al., 2000). CDK5 is able to phosphorylate the membrane receptor ErbB, resulting in ErbB endocytosis and increased acetylcholine receptor transcription in nearby synaptic sites (Fu et al., 2001). CDK5 has also been demonstrated to play a role in the activity of dopamine-and cAMP-regulated neuronal phosphoprotein 32, which controls dopamine signalling in specific neurons in adult mice (Bibb et al., 1999; Smith and Tsai, 2002). Lastly, in addition to the roles for CDK5 previously discussed, CDK5 function has also been implicated in many other cellular processes such as cell cycle control and gene regulation, cell survival and apoptosis, membrane dynamics, focal adhesion formation, intracellular trafficking, and glucose metabolism, overall demonstrating the varied and essential roles of CDK5 in proper cell function (Dhariwala and Rajadhyaksha, 2008, Smith and Tsai, 2002; Lalioti et al., 2010; Hisanaga and Endo, 2010).
4.5. Function of CDK5 in non-neuronal cells

CDK5 has also been identified to play important roles in processes such as, cell death and proliferation, angiogenesis, migration of epithelial and cancer cells, inflammation, myogenesis, glucose metabolism, and insulin secretion in non-neuronal cells (Contreras-Vallejos et al., 2012; Liebl et al., 2011). These studies have investigated CDK5 function in cells such as cells of hematopoietic lineage, HEK293, COS7, MEF, HCT116, HeLa, adipocytes, pancreatic β cells, and many other non-neuronal cell types (Contreras-Vallejos et al., 2012; Liebl et al., 2011).

CDK5 has been demonstrated to be present in both podocytes and pancreatic β cells (Brinkkoetter et al., 2010; Ubeda et al., 2004; Lilja et al., 2004). Additionally, both p35 and p39 have been found to be expressed in pancreatic β cells (Ubeda et al., 2004; Lilja et al., 2004).

Ubeda et al. (2004) observed that elevated extracellular glucose concentration results in increased expression of p35 and a correlative increase in CDK5 kinase activity. CDK5/p35 is able to stimulate the insulin promoter in response to elevated glucose levels as inhibition of CDK5 prevents stimulation of the insulin promoter (Ubeda et al., 2004). Additionally, suppression of CDK5 and p39 results in inhibition of \( \text{Ca}^{2+} \)-induced insulin exocytosis in pancreatic β cells (Lilja et al., 2004).

The function of CDK5 has also been studied in cells of hematopoietic lineage and has been implicated in processes such as response to inflammation, proliferation, and apoptosis (Studzinski and Harrison, 2003; Liebl et al., 2011). Overexpression of a dominant-negative CDK5 in IPC-81 rat leukemia cells results in inhibition of cAMP-induced caspase-3 activation and apoptosis (Sandal et al., 2002). Additionally, treatment of IPC-81 cells with cAMP results in moderately increased expression of CDK5 that correlates with cAMP-induced apoptosis (Sandal et al., 2002). Treatment of various B-CLL cell lines (B cell chronic lymphocytic leukemia),
which are deficient in p53-dependent apoptosis, with roscovitine results in p53-independent apoptosis within 24 hours of treatment due to decreased expression of genes involved in transcription, translation, survival, and DNA repair (Alvi et al., 2005). One anti-apoptotic protein that has been identified to be down-regulated in roscovitine-treated MM cells is Mcl-1, whereby rapid down-regulation of Mcl-1 transcription and translation is independent of caspase cleavage (Raje et al., 2005). The mechanism for Mcl-1 transcriptional down-regulation was later shown to be caused by roscovitine-induced dephosphorylation of RNA polymerase II (MacCullum et al., 2005). Phosphorylation of RNA polymerase II is required for its function, and subsequently roscovitine-induced dephosphorylation of RNA polymerase II results in inhibited transcription, leading to decreased Mcl-1 levels that were shown to be sufficient for induction of apoptosis in MM cells (MacCullum et al., 2005). In addition to down-regulation of Mcl-1 mRNA it has been shown that treatment of B-CLL cells with roscovitine induces rapid proteasomal degradation of Mcl-1 and apoptosis (Hallaert et al., 2007). In addition Mcl-1 remains associated with Noxa when B-CLL cells are treated with roscovitine, and RNAi-induced reduction of Noxa protein levels confers resistance to roscovitine-induced apoptosis in B-CLL cells (Hallaert et al., 2007). Though Noxa has been shown to be phosphorylated by CDK5, it has yet to be determined if roscovitine-induced apoptosis is due to inhibition of Noxa phosphorylation via inhibition of CDK5 (Lowman et al., 2010).
Hypothesis

Regulation of apoptosis is critical for cell survival during stress and for proper removal of aged and damaged cells. Deregulation of apoptosis has been found to be essential for many diseases, including cancer and many neurodegenerative diseases. Post-translational modifications, such as phosphorylation, have been shown to regulate the function of various apoptotic regulatory proteins, such as members of the Bcl-2 family of pro- and anti-apoptotic proteins. Hyperthermia is one type of stress that is able to induce apoptosis through activation of the intrinsic pathway and results in changes in phosphorylation and activity of members of the Bcl-2 family. My primary interest during this study was to investigate how members of the Bcl-2 family regulate hyperthermia-induced apoptosis. Published results have demonstrated that the pro-apoptotic BH3-only protein Noxa is required for hyperthermia-induced Mcl-1 degradation and apoptosis (Stankiewicz et al., 2009). Also, it has been demonstrated that Noxa is phosphorylated on serine-13 by the cyclin-dependent kinase CDK5, resulting in prevention of the apoptotic function of Noxa (Lowman et al., 2010). I hypothesize that exposure to hyperthermia results in decreased Noxa phosphorylation, due to reduced CDK5 activity, leading to activation of Noxa, Mcl-1 degradation, and apoptosis (Figure 6). I plan to investigate this hypothesis by first exposing cells to hyperthermia and observing the effect of hyperthermia on CDK5 phosphorylation and solubility. I next plan to determine if Noxa is phosphorylated on serine-13 in lymphoid cells and to investigate the effect of hyperthermia on serine-13 phosphorylation. These experiments will be performed through transient transfection of FLAG-tagged Noxa followed by western blotting with a phospho-S*PXK antibody and with a phospho-specific fluorescent stain. Lastly, I will determine the mechanistic role of Noxa serine-13 phosphorylation on hyperthermia-induced apoptosis by examining the effect of Noxa
Figure 6: Hypothetical model for the role of Noxa serine-13 phosphorylation on hyperthermia-induced apoptosis. Under standard growing conditions Noxa is phosphorylated on serine-13 by CDK5, suppressing the pro-apoptotic function of Noxa (Lowman et al., 2011). I propose that Noxa becomes dephosphorylated during hyperthermia, due to reduced activity of CDK5, resulting in activation of Noxa. Once activated Noxa is able to interact with the anti-apoptotic Bcl-2 family protein Mcl-1, leading to their mutual degradation and activation of apoptosis.

serine-13 phosphorylation on cell viability, caspase-3 activation, and on Noxa-induced Mcl-1 degradation during normal growth conditions and following exposure to hyperthermia. These experiments will be performed by alamar blue assay, caspase-3 assay, and by western blotting, respectively, in cells exposed to hyperthermia. Overall this study seeks to establish a role for Noxa serine-13 phosphorylation on hyperthermia-induced apoptosis, and may aid in the hunt for additional targets with potential therapeutic relevance.
Materials and Methods

Cell lines and generation of stable cell lines

PErTA cells were derived from the human acute T-cell lymphoblastic leukemia PEER cell line by stably transfecting PEER cells to express the reverse tetracycline-controlled transactivator (rtTA; Gossen et al., 1995), allowing for tetracycline-induced expression of a protein of interest that is under the control of a tetracycline response element (TRE; Mosser et al., 2000; Figure 7). PErTA cells were stably transfected with the pTR5-DC/GFPQ*TK/hygro expression plasmid containing the cDNA sequence for wild-type Noxa (Noxa-WT), or a mutant form of Noxa containing either a serine-13 to alanine (S13A) or serine-13 to glutamic acid (S13E) mutation. Stable cell lines were generated by electroporating 10 x 10^7 PErTA cells in 400 μl RPMI-1640 (Thermo Scientific) with 10 μg of linearized plasmid DNA. Electroporation was accomplished with a BTX T820 electroporator using a single pulse of 200 V for 50 ms in a 0.4 cm gap cuvette (Genetronics). Stably transfected cells were selected for 2 weeks in bulk culture with 200 μg/ml hygromycin (Sigma-Aldrich). Transfected cells were then diluted to ~1 cell/100 μl and seeded into 96-well plates at 100 μl per well. Media was changed every 2-3 days with RPMI-1640 supplemented with 200 μg/ml hygromycin and wells that demonstrated growth were collected and cultured up to 1.0 x 10^7 cells in 10 ml. All PErTA/Noxa clones were subsequently characterized by measuring GFP expression by flow cytometry and Noxa expression by western blotting after 24h incubation with 1 μg/ml of doxycycline. PErTA70 cells were derived from PErTA cells that were stably transfected to co-express HSP70 and GFP when induced with doxycycline (Mosser et al., 2000). 293rtTA cells were derived from HEK293T cells that were stably transfected to express rtTA.
Figure 7: Tet-ON system of inducible ectopic expression. Cells stably transfected to overexpress the reverse tetracycline transcriptional activator (rtTA) are transfected with expression vectors that contain a gene of interest (GI) under the control of a tetracycline response element (TRE). (A) Without the addition of doxycycline rtTA is unable to bind to the TRE, and transcription of the GI is suppressed. (B) Doxycycline is able to bind to rtTA, resulting in a conformational change that allows rtTA to bind to the TRE and subsequently activate transcription of the GI.
Cell line maintenance

PErTA, PErTA70, and 293rtTA cells were grown at 37°C and 5% CO₂ in a water-jacketed incubator (Forma Scientific, Series II). PErTA and PErTA70 cells were grown in RPMI-1640 media supplemented with 10% fetal bovine serum (Invitrogen), 5% L-glutamine (Thermo Scientific), and 5% PSA (Thermo Scientific). 293rtTA cells were grown in DMEM media (Thermo Scientific) supplemented with 10% fetal bovine serum, 5% L-glutamine, and 5% PSA. PErTA and 293rtTA cells were maintained in media supplemented with 200 μg/ml G418 (Thermo Scientific). PErTA70 and PErTA/Noxa cells were maintained in media supplemented with 200 μg/ml of both G418 and hygromycin. PErTA, PErTA70, and PErTA/Noxa cells were maintained at a concentration between 0.1 x 10⁶ cells/ml and 1.0 x 10⁶ cells/ml. 293rtTA cells were maintained at between 10% and 100% confluence in 10 cm dishes.

Plasmids and plasmid construction

The pTR5-DC/GFPQ*TK/hygro plasmids containing Noxa-WT, Noxa-S13A, or Noxa-S13E were constructed by Alyssa Cho in the Mosser lab (University of Guelph) from the pTR5-DC/GFPQ*TK/hygro plasmid (Mosser et al., 2000). This plasmid contains a tetracycline regulated dicistronic expression cassette containing an internal ribosome entry site. This allows for tetracycline-induced expression of a dicistronic mRNA encoding for both GFP and either Noxa WT, Noxa-S13A, or Noxa-S13E. Co-expression of GFP assists with identification and selection of stably transfected cells with tetracycline-induced expression. The pINX-C-FF-ZZ-B plasmid was obtained from Dr. Ray Lu (University of Guelph) and was described by Tsai & Carstens (2006). The pINX-C-FF-ZZ-B plasmid allows for expression of a protein of interest that is fused to a TAP-tag containing two FLAG domains (FF) and two protein-A IgG binding
domains (ZZ). A TEV cleavage site separates the FF and ZZ domains, allowing for separation of the FF and ZZ domains when incubated with TEV protease. pINX-C-FF-ZZ-B was used to construct plasmids with constitutive expression of Noxa-WT or Noxa-S13A tagged to the FF-ZZ tag at the C-terminal of Noxa by PCR amplification of Noxa-WT or Noxa-S13A from the pTR5-DC/GFPQ*TK/Noxa-WT/hygro or pTR5-DC/GFPQ*TK/Noxa-S13A/hygro plasmids, respectively, and ligation of PCR products into the pINX-C-FF-ZZ-B plasmid. PCR products were generated with the following primers: Noxa-WT forward – TCGAATTCTACCATGCGT GGGAAGGCGCGCAAGAACGCTCAACCGGAGC; Noxa-S13A forward – TCGAATTCT ACCATGCCTGGGAAGGCGCGCAAGAACGCTCAACCGGAGC; Reverse – GTAATCGGCG GCCGCGGATGCCTTCTGAGCAGAAGAG. PCR amplification was completed under the following conditions: 1 cycle of 98°C for 30 seconds, 35 cycles of 98°C for 10 seconds and 72°C for 30 seconds, and 1 cycle of 72°C for 10 minutes using Phusion High-fidelity DNA polymerase (Finnzymes).

The pBI-EYFP-N1(ΔBsrG1)*tk/hygro plasmid was created by Stefani Radicioni in the Mosser lab (University of Guelph) by removing the BsrG1 restriction site from the pBI-EYFP-N1 plasmid obtained from Clonetech. The pBI-EYFP-N1(ΔBsrG1)*tk/hygro plasmid allows for tetracycline-induced expression of two separate mRNAs that encode for either a protein of interest or YFP. pBI-EYFP-N1(ΔBsrG1)*tk/hygro plasmids containing either Noxa-WT-FF-ZZ or Noxa-S13A-FF-ZZ where generated by PCR amplification of both Noxa-WT-FF-ZZ and Noxa-S13A-FF-ZZ from pINX-C-Noxa-WT-FF-ZZ-B and pINX-C-Noxa-S13A FF-ZZ-B plasmids respectively and ligation of PCR products into pBI-EYFP-N1(ΔBsrG1)*tk/hygro. PCR products were generated with the following primers: Forward - ATATATTCTAGCCACCATGCGCTGGGAAG
AAGG; Reverse – GCTTGCTCTAGATTAATTCGCTCTACTTTTCG. PCR amplification was accomplished as previously described.

pBI-EYFP-N1(ΔBsrG1)*tk/hygro plasmids were also generated containing Noxa-WT that was tagged with a single FLAG epitope at the N-terminal of Noxa. Noxa-nFLAG was generated by PCR amplification of Noxa-WT from pTR5-DC/GFPQ*TK/Noxa-WT/hygro and ligation of PCR products into pBI-EYFP-N1(ΔBsrG1)*tk/hygro. N-terminal FLAG domain was added to Noxa-WT during PCR amplification with the following primers: Forward – ATATAT CAGCTGGCCACCAGGACTACAAGGACGACGATGACAAAGCCTGGGAAGAAGGCG CGCAAG; Reverse - CTGCACGCTAGCTCAGGTTCCTGAGAAGAGTTTGGAATATC AGATTC. PCR amplification was performed as previously described.

**Western blotting**

Cell pellets were lysed in either Laemmli lysis buffer (2% SDS, 10% glycerol, 50 mM Tris; pH 6.8) or Triton X-100 lysis buffer (10 mM HEPES pH 7.4, 100 mM NaCl, 5 mM MgCl2, 1 mM EGTA, 1% Triton X-100). All lysis buffers were supplemented with a in-house prepared inhibitor cocktail (protease inhibitors: 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin; phosphatase inhibitors: 10 mM sodium fluoride, 1 mM sodium vanadate, 20 mM sodium phosphate, 3 mM β-glycerolphosphate, 5 mM sodium pyrophosphate; deubiquitinase inhibitor: 10 mM N-ethylmaleimide; proteasome inhibitor: 50 μM MG132). Cells lysed with Laemmli buffer were resuspended in buffer and sonicated for ~ 30 seconds and the lysate was centrifuged at 20,800 x g for 10 minutes at 4°C. Cells lysed with Triton X-100 buffer were resuspended in buffer by gentle pipetting and incubated at 4°C on a rocker for 30 minutes. Lysates were centrifuged at 20,800 x g for 10 minutes at 4°C and soluble lysate was transferred
to a separate 1.5 ml microcentrifuge tube. Protein concentrations of both Laemmli and Triton-soluble lysates were determined using the BCA Protein Assay Kit (Thermo Scientific). Samples were prepared for loading by diluting lysates with 5x Laemmli loading buffer (10% SDS, 50% glycerol, 0.25 M Tris; pH 6.8, 25% β-mercaptoethanol) and denaturing samples at 75°C for 10 minutes. Pellets obtained from Triton X-100 lysis were resuspended in an equal volume of 1x Laemmli loading buffer (2% SDS, 10% glycerol, 50 mM Tris; pH 6.8, 10% β-mercaptoethanol) as compared to Triton X-100 soluble lysates after addition of 5x Laemmli loading buffer.

Pelleted samples were subsequently sonicated for ~30 seconds and denatured at 95°C for 10 minutes. Samples were loaded onto lab-cast 12-15% SDS-PAGE gels, along with 3 μl of Pageruler prestained protein ladder (Fermentas), and electrophoresed at 200V. SDS-PAGE gels were transferred onto 0.22 μm polyvinylidene fluoride (PVDF) membranes (Millipore) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) at 300 mA for 60 minutes with constant heat exchange. PVDF membranes were blocked in blocking solution (5% w/v skim milk powder, 1x TBS-T (50 mM Tris; pH 7.4, 150 mM NaCl, 0.1% v/v Tween20)) for 60 minutes at room temperature on a rocker. Blocked membranes were incubated overnight at room temperature in various primary antibodies: Noxa (Enzo Life Sciences; ALX-804-408), Mcl-1 (Santa Cruz; SC-819), FLAG-M2 (Sigma-Aldrich; F3165), CDK5 (Cell Signalling; #2506), Phospho-CDK5 (Cell Applications; CG1085), Phospho-MAPK/CDK substrates (PXS*P or S*PXR/K; Cell Signalling; #2325), HSP70 (Stressgen Biotechnologies; SPA-810), Caspase-3 (Enzo Life Sciences; SA-320), Actin (NeoMarkers; MS-1295-P1). Primary antibodies were prepared at concentrations between 1:1000 and 1:10,000 (v/v) in blocking solution supplemented with 0.5 μg/ml NaN3. α-Noxa was prepared at 1:1000 in HIKARI Secondary Signal Enhancer (Nacalai USA). Membranes were washed (3 x 10 minutes) in 1x TBS-T and incubated in blocking solution with 1:10,000 goat
anti-mouse (Molecular Probes; A-11005) or goat anti-rabbit HRP-conjugated secondary antibodies (Pierce; 31470). Membranes were washed (4 x 15 minutes) in 1x TBS-T and chemiluminescence was captured on Hyperfilm™ ECL (GE Healthsciences) using Western Lighting™ Chemiluminescence Reagent Plus (PerkinElmer). Films were developed using Kodak GBX developer and replenisher, followed by fixation in Kodak Rapid Fixer (Eastman Kodak Company).

**Transient transfection**

293rtTA cells were plated at 25% confluency in 10 cm dishes and allowed to grow at 37°C with 5% CO₂ for 24 hours. The following day media was aspirated and replaced with complete DMEM, and cells were allowed to incubate at 37°C with 5% CO₂ for 30 minutes prior to transfection. CaPO₄ transfection was completed as follows; 10 μg of plasmid DNA was diluted with 1:10 TE to a total volume of 100 μl (tube 1). Tube 1 was then added drop-wise to 338 μl dH₂O with 10 μl CaCl₂ (tube 2). 52 μl of 2M CaCl₂ was added to tube 2 drop-wise and mixed with gentle pipetting. Tube 2 was added drop-wise to 500 μl of 2x HBS (280 mM NaCl, 50 mM HEPES; pH 7.05, 1.5 mM Na₂HPO₄) while bubbling the 2x HBS solution (tube 3). Tube 3 was incubated at room temperature for 5 minutes. These steps were repeated for each plasmid used. After 5 minutes tube 3 was added drop-wise to the 293rtTA cells, the transfection reagent was mixed well by gentle swirling and cells were incubated at 37°C overnight.

**CHAPS immunoprecipitation**

Experimental samples were collected and pellets (~10 x 10⁶ cells) were resuspended by gentle pipetting in 200 μl of CHAPS lysis buffer (10 mM HEPES; pH 7.4, 5 mM MgCl₂, 1 mM
EGTA, 100 mM NaCl, 1% CHAPS), supplemented with in-house-prepared inhibitor cocktail. Lysates were incubated at 4°C on a rocker for 60 minutes and then centrifuged at 20,800 x g for 15 minutes at 4°C and soluble supernatant fraction from each sample was transferred to a separate 1.5 ml microcentrifuge tube. Protein concentration was determined using the BCA Protein Assay Kit. Soluble lysates (200 μg of protein) were transferred to new 1.5 ml centrifuge tubes and diluted to 1 μg/μl protein with CHAPS lysis buffer. Primary antibody was added to each sample at various concentrations, described later, and samples were incubated overnight at 4°C on a rocker. The following day 10 μl of either EZview™ Red Protein A Affinity Gel or EZview™ Red Protein G Affinity Gel (Sigma-Aldrich) was transferred to separate 1.5 ml microcentrifuge tubes for each sample. The beads were washed 3 times with 1 ml PBS and centrifuged at 10,600 x g for 2 minutes between each wash. After the third wash, PBS was completely removed from the beads and the overnight samples were transferred into microcentrifuge tubes containing washed beads. Samples were incubated for 60 minutes at 4°C on a rocker and subsequently centrifuged at 10,600 x g for 5 minutes. The supernatants were discarded and the beads were washed 4 times with 1 ml PBS and centrifuged at 10,600 x g for 2 minutes between each wash. After the fourth wash, PBS was completely removed and the beads were resuspended in 50 μl of 1x Laemmli loading buffer with complete inhibitors. The samples were denatured at 75°C for 10 minutes and prepared for analysis by diluting with 5x Laemmli loading buffer and heating at 75°C for 10 minutes. The samples were run on SDS-PAGE gels and western blotted as described previously.
Effect of hyperthermia on phosphorylation of MAPK/CDK targets

PErTA70 cells were grown as previously described to a density of 0.4 x 10^6 cells/ml and treated with 1 μg/ml doxycycline (ON). A separate sample of PErTA70 cells was grown as above and not treated with doxycycline as a control (OFF). Both OFF and ON cells were incubated for 24 hours at 37°C. The cells were then collected and resuspended in RPMI-1640 at a concentration of 1.0 x 10^6 cell/ml. The media was buffered for incubation outside of the CO₂ incubator by the addition of 20 mM HEPES, pH 7.2, and then exposed to 43°C by immersion in a circulating water bath for 30, 60, 90, or 120 minutes. One sample from both the OFF and ON cells was incubated at 37°C to serve as controls. The cells were collected and lysed with Triton X-100 lysis buffer as described previously. Both Triton-soluble and -insoluble lysates from both OFF and ON PErTA70 cells were prepared and examined by SDS-PAGE and western blotting as previously described.

To examine the effect of roscovitine on hyperthermia-induced changes in MAPK/CDK target phosphorylation, PErTA70 cells were grown as previously described to a density of 0.4 x 10^6 cells/ml and treated with 1 μg/ml doxycycline (ON). A separate sample of PErTA70 cells was grown as above but not treated with doxycycline (OFF). Both the OFF and ON cells were incubated for 24 hours at 37°C and then collected and resuspended in RPMI-1640 at a concentration of 0.8 x 10^6 cell/ml. The cells were either treated with 20 μM roscovitine (+) or were not treated (-) and were subsequently incubated at 37°C for 6 hours. All samples were then exposed to 43°C for 60 or 120 minutes in HEPES-buffered media. One sample from both the roscovitine-treated and non-treated OFF and ON cells was incubated at 37°C to serve as controls. The cells were collected and lysed with Triton X-100 lysis buffer as described previously. The
Triton-soluble lysates were analyzed by SDS-PAGE and western blotting as previously described.

**Effect of hyperthermia on CDK5**

PErTA70 cells were grown to a density of 0.4 x 10^6 cells/ml and treated with 1 μg/ml doxycycline (ON). A separate sample was incubated without doxycycline (OFF). Both the OFF and ON cells were incubated for 24 hours at 37°C and then collected and resuspended in RPMI-1640 at a concentration of 1.0 x 10^6 cell/ml. The cells were then exposed to 43°C for 30, 60, 90, or 120 minutes in HEPES-buffered media. One sample from the OFF and ON cells was incubated at 37°C to serve as non-heat shocked controls. The cells were collected and lysed with Triton X-100 lysis buffer as described previously. Both the Triton-soluble and -insoluble fractions were analyzed by SDS-PAGE and western blotting.

To examine the effect of recovery after hyperthermia on CDK5, PErTA70 cells (OFF and ON) were collected and resuspended in HEPES-buffered RPMI-1640 at a concentration of 1.0 x 10^6 cell/ml and exposed to 43°C for 60 minutes. The cells were then plated in 6-well plates at a concentration of ~0.5 x 10^6 cell/ml and allowed to incubate at 37°C for 2, 4, or 6 hours before being collected. One sample from both the OFF and ON PErTA70 cells was collected immediately after exposure to 43°C. Lastly, one sample from both the OFF and ON PErTA70 cells was incubated at 37°C without exposure to heat shock to serve as controls. Following treatment, the cells were collected and lysed with Triton X-100 lysis buffer and both Triton-soluble and -insoluble fractions were prepared for analysis by SDS-PAGE and western blotting.
**Alamar blue cell viability assay**

PErTA/Noxa clones (WT and S13A) were plated at a density of 0.25 x 10^6 cells/ml into 12-well plates and treated with 0.3 and 0.1 µg/ml doxycycline respectively in order to induce equal levels of protein expression. PErTA cells were treated with 1.0 µg/ml doxycycline as a control. In addition, each cell line was also incubated in the absence of doxycycline. To assess cell viability after Noxa protein expression, 100 µl from each sample was transferred into a 96-well plate, in triplicate, and 100 µl of a 50 µM resazurin solution was then added to each sample. Immediately after the addition of resazurin the fluorescence was measured using a fluorescence microplate reader (FLx800; BioTek Instruments) at an excitation of 516 (20) nm and emission of 590 (35) nm. This time zero reading represents the background fluorescence and was subtracted from the values recorded after incubation of the cells at 37°C for 5 hours. Plating into the 96-well plates from the 12-well plates, addition of 5 µM resazurin, and reading both 0 and 5 hour readings was repeated after 24, 48, and 72 hours of doxycycline induction. Growth curves were generated by comparing the relative growth of each clone as compared to the initial day zero reading.

**Caspase assay**

PErTA cells and the PErTA/Noxa clones (WT and S13A) were seeded at a density of 0.4 x 10^6 cells/ml in 6-well plates and cells and incubated with 0, 0.1, 0.3, or 1.0 µg/ml doxycycline for 24 hours at 37°C. The cells were then either collected immediately after induction or exposed to 43°C for 1 hour followed by 6 hours of incubation at 37°C. The cells were subsequently collected and lysed with caspase-3 lysis buffer (50 mM HEPES; pH 7.4, 0.1% CHAPS, 0.1 mM EDTA, 1 mM DTT). Lysates (25 µl) were mixed with caspase-3 assay buffer (50 mM HEPES;
pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol) containing the fluorogenic caspase-3 substrate Ac-DEVD-AMC (60 µM; Enzo Life Sciences) in a total volume of 100 µl per well of a 96 well plate. Fluorescence from the liberated AMC was measured at 37°C using a fluorescence microplate reader (FLx800; BioTek Instruments) at an excitation of 380 (20) nm and emission of 460 (40) nm. Readings were taken every 2 minutes for 1 hour. Relative DEVDase activity is expressed as the slope (RFU/min) divided by the total amount of protein in each sample (RFU/min/µg protein). Protein concentrations were measured using the BCA protein assay reagent (Thermo Scientific).

**Determination of Noxa half-life**

PErTA cells and the PErTA/Noxa clones (WT and S13A) were seeded at a density of 0.4 x 10^6 cells/ml in 6-well plates and incubated with 1.0, 0.3 and 0.1 µg/ml doxycycline respectively for 24 hours at 37°C. The cells were then treated with 200 µg/ml cyclohexamide (CHX) and samples were collected immediately and after incubation at 37°C for 30, 60, and 90 minutes. The samples were collected, lysed with Laemmli buffer, and analyzed by SDS-PAGE and western blotting.

**Characterization of pBI/Noxa-FF-ZZ expression**

293rtTA cells were transfected as described previously with 10 µg of pBI-EYFP-N1(ΔBsrG1)*tk/Noxa-WT-FF-ZZ/hygro plasmid and incubated overnight at 37°C. The following morning the media was aspirated and replaced with 10 ml of complete DMEM. Transfected cells were incubated with 0, 0.1, 0.3, 1.0, or 2.0 µg/ml doxycycline and incubated at 37°C for an additional 24 hours. As a control, one 10 cm dish of non-transfected 293rtTA cells
was treated with 1.0 μg/ml doxycycline and incubated as above. After 24 hours of induction cells were collected and lysed with Triton X-100 lysis buffer as described previously. Samples were prepared from both Triton-soluble and –insoluble lysates and western blotting was performed as previously described.

**Determination of Noxa phosphorylation status in pBI-EYFP/Noxa-FF-ZZ transfected cells**

293rtTA cells were transfected as described previously with 10 μg of either pBI-EYFP-N1(ΔBsrG1)*tk/Noxa-WT-FF-ZZ/hygro or pBI-EYFP-N1(ΔBsrG1)*tk/Noxa-S13A-FF-ZZ/hygro plasmid and incubated overnight at 37°C. The following morning the media was aspirated and replaced with 10 ml of complete DMEM. Transfected cells were treated with 0.1 μg/ml doxycycline and incubated at 37°C for 24 hours. The cells were then treated with 20 μM MG132 and 5 nM calyculin A for 6 and 2 hours respectively at 37°C and subsequently collected and lysed with Triton X-100 lysis buffer as described previously. Protein concentration was determined using the BCA Protein Assay Kit. The tap-tagged Noxa proteins were purified using IgG Sepharose and then TEV cleavage was performed to remove the IgG-binding domains from the FLAG-tagged Noxa. IgG Sepharose 6 Fast Flow bead slurry (10 µl; GE Healthcare) was washed 3x with 1 ml TBS-TxG (50 mM Tris-HCl pH 6.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) with centrifugation at 1000 x g for 2 minutes between each wash. After the third wash all buffer was removed and 100 μg of protein from the Triton-soluble lysates was added to the IgG beads. Each sample was diluted to 1 μg/μl with Triton X-100 lysis buffer and samples were incubated at 4°C on a shaker for 3 hours. Samples were then centrifuged at 1000 x g for 5 minutes and supernatants were removed. The beads were washed three times as above with TBS-TxG and all traces of buffer were removed after the third wash. AcTEV protease (Invitrogen)
was prepared by diluting 1.0 μl of 10U/μl AcTEV with 7.5 μl 20x TEV buffer (Invitrogen), 1.5 μl 0.1M DTT, 6 μl of phosphatase inhibitors, and 134 μl dH2O. 150 μl of prepared AcTEV protease (10U/sample) was then added to each bound-IgG bead sample and samples were incubated overnight at 4°C on a shaker. The following day the samples were centrifuged at 1000 x g for 5 minutes and the supernatants were transferred to new 1.5 ml microcentrifuge tubes. The beads were then washed 4 times with TBS-TxG as above and all buffer was removed after the fourth wash. Laemmli buffer (37.5 μl of 5x buffer) was added to each 150 μl TEV cleavage supernatant (containing Noxa-FF). IgG beads (bound to ZZ domain of Noxa-FF-ZZ) were resuspended in 187.5 μl 1x Laemmli buffer. Both supernatant and bead samples were heated at 75°C for 10 minutes. Triton-soluble lysates were also prepared by dilution in Laemmli buffer. Samples were analyzed by SDS-PAGE and western blotting.

**Determination of Noxa phosphorylation status in pBI/Noxa-nFLAG transfected cells by immunoprecipitation**

293rtTA cells were transfected as described previously with 10 μg of pBI-EYFP-N1(ΔBsrG1)*tk/Noxa-nFLAG/hygro plasmid (Noxa-nFLAG), or 10 μg of pBI-EYFP-N1(ΔBsrG1)*tk /hygro plasmid (pBI) as a control, and allowed to incubate overnight at 37°C. The following morning the media was aspirated and replaced with 10 ml of complete DMEM. Transfected cells were treated with 1.0 μg/ml doxycycline and incubated at 37°C for 24 hours. Following this, one 10 cm dish of Noxa-nFLAG transfected cells was treated with 20 μM MG132 and 5 nM calyculin A for 6 and 2 hours respectively at 37°C. Another dish of Noxa-nFLAG transfected cells was exposed to 43°C for 1 hour in HEPES-buffered media. The controls included one dish of Noxa-nFLAG transfected and pBI transfected cells that were
maintained at 37°C. Samples were collected and a CHAPS IP was performed as previously described using α-FLAG-M2 primary mouse IgG\textsubscript{1} antibody (Sigma-Aldrich; F3165) and EZview\textsuperscript{TM} Red Protein G Affinity beads (Sigma-Aldrich). Samples were run on SDS-PAGE gels and western blotting was performed as previously described.

**Determination of Noxa phosphorylation status in pBI/Noxa-nFLAG transfected cells by Pro-Q Diamond staining**

293rtTA cells were transfected with either pBI-EYFP-N1(ΔBsrG1)*tk/hygro (pBI) or pBI-EYFP-N1(ΔBsrG1)*tk/Noxa-nFLAG/hygro plasmids (Noxa-nFLAG) and treated as described above. A CHAPS IP was completed as previously described using α-FLAG primary mouse IgG\textsubscript{1} antibody (Sigma-Aldrich; F3165) and EZview\textsuperscript{TM} Red Protein G Affinity beads (Sigma-Aldrich). Samples were run on SDS-PAGE gels and transferred on to PVDF membranes as previously described. Blots were allowed to dry completely overnight at room temperature. Once dried, blots were stained with the Pro-Q diamond phosphoprotein blot stain kit (Molecular Probes) and allowed to dry completely. Phosphoproteins were visualized using a Typhoon 9400 Variable Mode Imager (Amersham Biosciences) with an excitation of 532 nm and bandpass emission of 580 nm.

**Effect of Noxa serine-13 phosphorylation on Noxa-induced Mcl-1 degradation**

PErTA/Noxa clones (WT and S13A) were grown as previously described to a density of 0.5 x 10\textsuperscript{6} cells/ml in 6-well plates and treated with 0.3 and 0.1 µg/ml doxycycline respectively. PErTA cells were grown to an equal density and treated with 1.0 µg/ml as a control. Cells were incubated for 24 hours at 37°C and then exposed to 43°C for 1 hour in HEPES-buffered media.
Cells were either collected immediately after exposure to hyperthermia or were allowed to incubate at 37°C for 6 hours before collection. Control cells were incubated at 37°C throughout the experiment. Cells were lysed in Laemmli lysis buffer and western blotting was completed as described previously.
Results

In silico prediction of Noxa phosphorylation sites

It has been previously demonstrated that exposure of PErTA cells, a human acute lymphoblastic T cell line derived from the PEER cell line that expresses rtTA, to 43°C results in Mcl-1 degradation and activation of apoptosis (Stankiewicz et al., 2009). Interestingly, depletion of Noxa by shRNA prevents hyperthermia-induced Mcl-1 degradation and activation of apoptosis in PErTA cells (Stankiewicz et al., 2009). Additionally, it has been observed that JNK activity is increased in cells exposed to hyperthermia, and that JNK is known to regulate the function of multiple Bcl-2 family members through phosphorylation (Mosser et al., 1997; Mosser et al., 2000; Liu and Lin, 2005; Beere, 2004). Therefore at the beginning of my study I was interested in whether the function of Noxa during hyperthermia-induced apoptosis was regulated by JNK, though at the time it was unknown whether Noxa was phosphorylated. To investigate whether Noxa is a target for phosphorylation I performed in silico analysis of the amino acid sequence of Noxa using the phosphorylation prediction server NetPhos 2.0 and the kinase prediction program GPS to determine which sites on Noxa could potentially be phosphorylated and by what kinases (Figure 8). In silico analysis revealed a high probability that serine-13 of Noxa could be phosphorylated. Analysis of Noxa by the NetPhos 2.0 server revealed that serine-13 obtained the highest probability score (0.991), as compared to serine-47 and serine-52 (0.002 and 0.009 respectively). Additionally, analysis by the GPS program revealed that the cyclin-dependent kinase CDK5 obtained a probability score of 9.895, while other top ranking kinases included JNK (9.429) and GSK3β (7.041), when determining the probability that these kinases could phosphorylate Noxa. Interestingly, 8 of the 10 most probable kinases to phosphorylate Noxa,
Figure 8: Determination of Noxa phosphorylation in silico. (A) Analysis of Noxa amino acid sequence by the phosphorylation prediction server NetPhos 2.0 reveals that Noxa is highly likely to be phosphorylated on serine-13. Higher scores correlate with a higher probability of site-specific phosphorylation. (B) Analysis of Noxa amino acid sequence by the kinase prediction program GPS reveals that Noxa serine-13 is likely to be phosphorylated by the cyclin-dependent kinase CDK5 or the stress-activated c-jun N-terminal kinase JNK. Higher scores correlate with higher probability of kinase-specific phosphorylation of Noxa on the indicated amino acid.
identified by the GPS program, were kinases that were predicted to phosphorylate Noxa on serine-13, further supporting my in silico analysis by the NetPhos 2.0 server. My in silico analysis of Noxa phosphorylation was performed early during my study, and at the time there had been no studies completed that identified that Noxa is phosphorylated in vivo. Though, after obtaining my in silico results, a separate group published a study successfully demonstrating that Noxa is phosphorylated in vitro on serine-13 by the cyclin-dependent kinase CDK5 (Lowman et al., 2010).

**Effect of hyperthermia on phosphorylation of MAPK/CDK targets**

With both in silico and published evidence that Noxa is phosphorylated by CDK5 on serine-13 I wanted to determine if hyperthermia had an effect on CDK5 kinase activity. However, I first wanted to investigate the broader effect of hyperthermia on the phosphorylation of MAPK and CDK targets. In order to do this I utilized a rabbit phospho-serine antibody that recognizes phospho-serine when phosphorylated by either MAPKs or CDKs (PXS*P or S*PXR/K, Cell Signaling; #2325). When I exposed PErTA70 cells to 43°C I observed both increases and decreases in phosphorylation of various MAPK/CDK targets (Figure 9a). I also observed increases in insoluble phosphoproteins when PErTA70 cells were exposed to 43°C. These changes in phosphorylation and solubility were enhanced with longer durations of exposure to 43°C (Figure 9a). Additionally, the change in phosphorylation of some MAPK/CDK targets was mild, whereas others demonstrated a severe change when exposed to 43°C. These results demonstrate that exposure to hyperthermia results in changes in phosphorylation of MAPK/CDK targets, suggesting a potential role of various MAPKs and CDKs in hyperthermia-induced apoptosis.
**Figure 9**

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**pSer**

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**pSer**
Figure 9: Effect of hyperthermia on phosphorylation of MAPK/CDK targets. (A) Changes in phosphorylation of MAPK/CDK targets in cells exposed to hyperthermia and overexpressing Hsp70. PErTA70 cells either not overexpressing (OFF) or overexpressing Hsp70 (ON) were exposed to 43°C for 30, 60, 90, or 120 minutes. Control cells were incubated at 37°C with 5% CO₂ (0). Cells were lysed with Triton X-100 lysis buffer and loading samples were prepared from both the Triton-soluble and -insoluble (pellet) lysates. Samples were run on SDS-PAGE gels and western blotting was completed using a phospho-serine antibody targeted to MAPK/CDK targets. MAPK/CDK targets that demonstrated reduced phosphorylation with exposure to 43°C are indicated by a single asterisk (*). Conversely, MAPK/CDK targets that demonstrated increased phosphorylation when exposed to 43°C are indicated by double asterisks (**). The approximate size of detected proteins in kDa is shown on the right side of the blots. (B) Roscovitine inhibits hyperthermia-induced increases in specific MAPK/CDK targets. PErTA70 cells either not overexpressing (OFF) or overexpressing Hsp70 (ON) were exposed to 43°C for 60 or 120 minutes. Additionally, both OFF and ON PErTA70 cells were either treated with 20 µM roscovitine (+) or not treated (-) for 6 hours at 37°C with 5% CO₂ prior to exposure to 43°C. Control cells were incubated at 37°C with 5% CO₂ (0). Cells were lysed with Triton X-100 lysis buffer and loading samples were prepared from the Triton-soluble lysate. Samples were run on SDS-PAGE gels and western blotting was completed using a phospho-serine antibody targeted to MAPK/CDK targets. MAPK/CDK targets that demonstrated increased phosphorylation with exposure to 43°C that was reduced when cells were treated with roscovitine are indicated by a single asterisk (*). The approximate size of detected proteins in kDa is shown on the right side of the blot.

When investigating the effect of hyperthermia on various molecular mechanisms it is also important to examine the role of heat shock proteins, such as HSP70. HSP70 belongs to a large family of heat-shock proteins that are expressed when cells undergo stress, namely hyperthermia. It has been previously shown that hyperthermia leads to expression of HSP70 via activation of heat shock factor protein 1 (HSF1; Morimoto et al., 1992), and that HSP70 is able to increase the resistance of hyperthermia-stressed cells to subsequent exposure to lethal hyperthermia through inhibition of apoptosis (Mosser and Martin, 1992) This state of thermotolerance is due to the protective ability of HSP70 by which HSP70 is able to bind to hyperthermia-induced misfolded proteins and refold them into their native confirmation (Mayer and Bukau, 2005). From my results I again observed changes in phosphorylation of MAPK/CDK targets when PErTA70
cells, overexpressing HSP70, were exposed to various lengths of hyperthermia (Figure 9a). Interestingly, overexpression of HSP70 both enhanced the hyperthermia-induced increase in phosphorylation of specific MAPK/CDK targets and, conversely, impaired the hyperthermia-induced decrease in phosphorylation of other MAPK/CDK targets (Figure 9a). These results suggest that HSP70 may be an important regulator of phosphorylation of various MAPK/CDK targets during hyperthermia.

Once I identified that hyperthermia induced changes in phosphorylation of MAPK and CDK targets I wanted to investigate more specifically if any of these changes were due to changes in CDK5 activity. In order to do this I exposed PErTA70 cells to 43°C while being treated with the CDK5 inhibitor roscovitine (Figure 9b). I specifically observed one MAPK/CDK target phosphoprotein, indicated in Figure 9b by the asterisk and with an approximate size of 26 kDa, that when exposed to 43°C for 120 minutes demonstrated an increase in phosphorylation that was inhibited with roscovitine. Furthermore, when PErTA70 cells overexpressing HSP70 were exposed to 43°C for 120 minutes I observed an enhanced increase in phosphorylation of this specific MAPK/CDK phosphoprotein, further supporting my observations in Figure 9a. Interestingly, in cells that are overexpressing HSP70, roscovitine was not as effective at reducing the hyperthermia-induced increase in phosphorylation of this specific MAPK/CDK phosphoprotein in PErTA70 cells. I also observed other MAPK/CDK targets that demonstrated hyperthermia-induced changes in phosphorylation using the phospho-Ser (MAPK/CDK) antibody, although treatment with roscovitine during exposure to hyperthermia did not affect these changes (Figure 9b). From these results I observed a specific MAPK/CDK target that exhibits hyperthermia-induced changes in phosphorylation that is further affected by
treatment with roscovitine, overall suggesting that CDK5 activity may be altered in cells exposed to hyperthermia.

**Effect of hyperthermia on solubility and phosphorylation of CDK5**

After identifying a potential role of CDK5 activity in hyperthermia-induced changes on the phosphorylation of MAPK/CDK targets, I needed to determine specifically if CDK5 activity is altered during hyperthermia. In order to accomplish this I first assessed whether hyperthermia has an effect on CDK5 protein levels and phosphorylation of CDK5 (Figure 10a). When PErTA70 cells not overexpressing HSP70 were exposed to 43°C I observed a decrease in both CDK5 and phospho-CDK5 levels that began as early as 30 minutes and continued to decrease up to 120 minutes. Overexpression of HSP70 reduced the loss of both CDK5 and phospho-CDK5 in PErTA70 cells exposed to 43°C at all recorded time points. I also investigated whether the loss of CDK5 and phospho-CDK5 was due to heat-induced insolubility (Figure 10b). As in Figure 10a, I observed the same decrease in both CDK5 and phospho-CDK5 levels following exposure to 43°C in the Triton-soluble fraction, though I also observed that the presence of CDK5 in the Triton-insoluble fraction increased with exposure to 43°C. Furthermore the presence of insoluble CDK5 increased with longer exposure to 43°C. Conversely, I did not see any increase in phospho-CDK5 in the Triton-insoluble fraction, indicating that the hyperthermia-induced loss of phospho-CDK5 is not due to increased insolubility of phospho-CDK5. When PErTA70 cells were induced to overexpress HSP70 I observed not only a decrease in the loss of both CDK5 and phospho-CDK5 from the Triton-soluble fraction, but also a mild decrease in the generation of insoluble CDK5. These results suggest that following hyperthermia CDK5 becomes insoluble and phospho-CDK5 levels are reduced, possibly due to dephosphorylation or proteasomal
Figure 10: Hyperthermia results in the loss of phospho-CDK5 and soluble CDK5. (A) Comparison of CDK5 and phospho-CDK5 protein levels over different lengths of hyperthermia. PErTA70 cells either not overexpressing (OFF) or overexpressing HSP70 (ON) were exposed to 43°C for 30, 60, 90, or 120 minutes. Both OFF and ON PErTA70 cells were incubated at 37°C with 5% CO₂ as control cells (C). Cells were collected after exposure to 43°C and lysed in Triton X-100 lysis buffer. Loading samples were prepared from Triton-soluble lysates and western blotting was completed using antibodies targeted to total CDK5, phospho-Tyr¹⁵ CDK5, and HSP70. (B) Comparison of CDK5 and phospho-CDK5 protein levels in soluble and insoluble (pellet) fractions during hyperthermia. PErTA70 cells either not overexpressing (OFF) or overexpressing HSP70 (ON) were exposed to 43°C for 30, 60, 90, or 120 minutes. Both OFF and ON PErTA70 cells were incubated at 37°C with 5% CO₂ as control cells (C). Cells were collected after exposure to 43°C and lysed in Triton X-100 lysis buffer. Loading samples were prepared from both Triton-soluble and –insoluble (pellet) lysates and western blotting was completed using antibodies targeted to total CDK5, phospho-Tyr¹⁵ CDK5, and HSP70.
degradation. Additionally, overexpression of HSP70 reduces the heat-induced loss of both CDK5 and phospho-CDK5 in PErTA70 cells.

I next wanted to determine if recovery at 37°C after exposure to hyperthermia had an effect on CDK5 and phospho-CDK5 levels (Figure 11a). PErTA70 cells that were not overexpressing HSP70 and were exposed to 43°C for 1 hour demonstrated reduced CDK5 and phospho-CDK5 levels immediately after heat shock in accordance with previous results (Figure 10). In addition levels of both CDK5 and phospho-CDK5 continued to decrease during recovery at 37°C up to 6 hours. Conversely, overexpression of HSP70 resulted in the recovery of phospho-CDK5 and prevented the loss of total CDK5 in PErTA70 cells that were exposed to 43°C for 1 hour. I also investigated the effect of recovery after hyperthermia on heat-induced insolubility of CDK5 (Figure 11b). I observed a mild recovery of CDK5 in cells not overexpressing HSP70 in the Triton-soluble fraction with a correlative decrease of CDK5 in the insoluble fraction. Furthermore I observed a very mild recovery of phospho-CDK5 in the soluble fraction, though again there was no phospho-CDK5 present in the insoluble fraction as observed previously (Figure 10b). Overexpression of HSP70 prevented the insolublization of CDK5 and enhanced the 6 hour recovery of phospho-CDK5 in PErTA70 cells exposed to 43°C for 1 hour. Overall these results suggest that CDK5 remains insoluble during recovery at 37°C following exposure to 43°C, though levels of insoluble CDK5 do decrease with increased duration of recovery at 37°C. Furthermore, HSP70 is able to prevent the hyperthermia-induced insolublization of CDK5 and allows for recovery of phospho-CDK5 following exposure to 43°C.
**Figure 11**: Recovery of phospho-CDK5 and CDK5 in HSP70 overexpressing cells exposed to hyperthermia. (A) Comparison of CDK5 and phospho-CDK5 protein levels after exposure to hyperthermia and recovery at 37°C. PErTA70 cells either not overexpressing (OFF) or overexpressing HSP70 (ON) were exposed to 43°C for 60 minutes and either collected immediately after heat shock (0) or were incubated at 37°C for 2, 4, or 6 hours prior to collection. Both OFF and ON PErTA70 cells were incubated at 37°C with 5% CO₂ as control cells (C). Collected cells were lysed in Triton X-100 lysis buffer and loading samples were prepared from Triton-soluble lysates. Samples were run on SDS-PAGE gels and western blotting was completed using antibodies targeted to total CDK5, phospho-Tyr₁⁵ CDK5, and HSP70. (B) Comparison of CDK5 and phospho-CDK5 protein levels in soluble and insoluble (pellet) fractions after exposure to hyperthermia and recovery at 37°C. PErTA70 cells either not overexpressing (OFF) or overexpressing HSP70 (ON) were exposed to 43°C for 60 minutes and either collected immediately after heat shock (0) or were incubated at 37°C for 2, 4, or 6 hours prior to collection. Both OFF and ON PErTA70 cells were incubated at 37°C with 5% CO₂ as control cells (C). Collected cells were lysed in Triton X-100 lysis buffer and loading samples were prepared from both Triton-soluble and –insoluble (pellet) lysates. Samples were run on SDS-PAGE gels and western blotting was completed using antibodies targeted to total CDK5, phospho-Tyr₁⁵ CDK5, and HSP70.
Effect of hyperthermia on CDK5/p35 interaction

I wanted to further investigate the effect of hyperthermia on CDK5 activity by measuring the interaction of CDK5 and its direct activator p35. Though p35 was originally identified as a neuronal-specific activator of CDK5 (Lalioti et al., 2010), recent studies have identified that not only is p35 present, but that p35 also can be found associated with CDK5 in non-neuronal cells (Liebl et al., 2011). In order to determine the effect of hyperthermia on the interaction between CDK5 and p35 I exposed PErTA70 cells to 43°C for 1 hour and completed a CHAPS IP using a CDK5 primary rabbit antibody (Figure 1). Examination of the total cell extracts showed that in cells not overexpressing HSP70 the protein levels of CDK5 were decreased immediately after exposure to 43°C, further demonstrating previously discussed observations (Figure 11). Interestingly, I observed that p35 levels are increased in PErTA70 cells when exposed to 43°C. Furthermore the hyperthermia-induced increase in p35 was slightly decreased when PErTA70 cells were overexpressing HSP70. However, when looking specifically at samples prepared from the CDK5 immunoprecipitation I was unable to detect any association of CDK5 and p35. Therefore, since I have identified that p35 is expressed in PErTA70 cells, it will be essential to further investigate whether CDK5 and p35 are able to interact in PErTA70 cells by completing immunoprecipitations for p35 and blotting for CDK5 interaction.

Effect of hyperthermia on Noxa phosphorylation

Once I identified that hyperthermia reduces both the phosphorylation and solubility of CDK5, and that hyperthermia increases total protein levels of p35, I next needed to identify whether hyperthermia induces changes in the CDK5-mediated phosphorylation of Noxa
serine-13. To accomplish this I first designed an expression system in which I fused to the C-terminus of Noxa to a tap-tag cassette containing two FLAG domains (FF) and two protein A IgG binding domains (ZZ) that are separated by a TEV protease cleavage domain. I designed the expression of Noxa-FF-ZZ to be under the control of a tetracycline response element, so that expression would only be activated in doxycycline-induced transfected cells, by inserting the Noxa-FF-ZZ sequence into the pBI-EYFP-N1(ΔBsrg1)*tk/hygro plasmid (Figure 13a). Once I constructed both wild-type Noxa (Noxa-WT) and non-phosphorylatable Noxa (Noxa-S13A)
Figure 13
Figure 13: Effect of hyperthermia on Noxa phosphorylation. (A) Schematic diagram of Noxa-FF-ZZ expression system. Shown is Noxa fused at the C-terminal to two FLAG domains (FF) and two protein A IgG binding domains (ZZ). The FF and ZZ domains are separated by a TEV cleavage domain (TEV), allowing for separation of the FF and ZZ domains by TEV protease cleavage. Expression of Noxa-FF-ZZ is under the control of a tetracycline response element (TRE) with the direction of transcription represented by the arrow. (B) Optimization of pBI-EYFP/Noxa-FF-ZZ. 293rtTA cells were transfected with pBI-EYFP/Noxa-WT-FF-ZZ (WT) and induced for 24 hours with 0.1, 0.3, 1.0, or 2.0 µg/ml doxycycline. One sample of pBI-EYFP/Noxa-WT-FF-ZZ transfected cells was not treated with doxycycline (0) as a control. Additionally, 293rtTA cells were transfected with pBI-EYFP-N1(ΔBsrG1)*tk/hygro and treated with 1.0 µg/ml doxycycline for 24 hours as a negative control (C). Cells were lysed in Triton X-100 lysis buffer and protein samples were prepared from both the Triton-soluble and -insoluble (pellet) fractions. Samples were run on SDS-PAGE gels and western blotting was completed. (C) Failure to detect phospho-Noxa in pBI-EYFP/Noxa-FF-ZZ transfected cells. 293rtTA cells were transfected with either pBI-EYFP/Noxa-WT-FF-ZZ (WT) or pBI-EYFP/Noxa-S13A-FF-ZZ (S13A) and induced for 24 hours with 0.1 µg/ml doxycycline. After 24 hours of induction cells were treated with 20 µM MG132 and 5 nM calyculin A for 6 and 2 hours respectively at 37°C with 5% CO₂. Cells were lysed in Triton X-100 lysis buffer and samples were cleaved with TEV protease. Protein samples from both WT and S13A transfected cells were obtained after the TEV cleavage that contained either the protein A IgG binding domains (IgG) or Noxa-FF (FLAG). Loading samples were also prepared from non-cleaved samples (total). Samples were run on SDS-PAGE gels and western blotting was completed. (D) Schematic diagram of Noxa-nFLAG expression system. Shown is Noxa fused at the N-terminal to a single FLAG domain (F). Expression of Noxa-nFLAG is under the control of a tetracycline response element (TRE) with the direction of transcription represented by the arrow. (E) Optimization of pBI-EYFP/Noxa-nFLAG. 293rtTA cells were transfected with pBI-EYFP/Noxa-nFLAG and either induced for 24 hours with 1.0 µg/ml doxycycline (+) or not treated with doxycycline (-) as a control. Cells were lysed with Triton X-100 lysis buffer and western blotting was completed. Relative location of Noxa-nFLAG is represented by the asterisk. (F) Failure to detect phospho-Noxa in pBI-EYFP/Noxa-nFLAG transfected cells by immunoprecipitation. 293rtTA cells were transfected with pBI-EYFP/Noxa-nFLAG and induced for 24 hours with 1.0 µg/ml doxycycline. After 24 hours of induction cells were treated with 20 µM MG132 and 5 nM calyculin A for 6 and 2 hours respectively at 37°C with 5% CO₂ (MC). Additionally, Noxa-nFLAG transfected cells were exposed to 43°C for 1 hour and collected immediately (43). One sample of Noxa-nFLAG transfected cells was incubated at 37°C with 5% CO₂ as a control (37). Lastly, 293rtTA cells were also transfected with pBI-EYFP-N1(ΔBsrG1)*tk/hygro and treated with 1.0 µg/ml doxycycline for 24 hours as a negative control (C). Cells were lysed in CHAPS lysis buffer and a CHAPS IP was completed using α-FLAG-M2 primary mouse antibody (Sigma-Aldrich; F3165) and EZView™ Red Protein G Affinity beads. Loading samples were run on SDS-PAGE gels and western blotting was completed. Relative location of Noxa-nFLAG is represented by the asterisk. (G) Failure to detect phospho-Noxa in pBI-EYFP/Noxa-nFLAG transfected cells by ProQ stain. Loading samples prepared in Figure 13f were also run on SDS-PAGE gels, transferred to PVDF membranes, and membranes were stain with ProQ diamond phosphoprotein blot stain kit. Phosphoproteins were visualized using a Typhoon 9400 Imager with an excitation of 532 nm and bandpass emission of 580 nm. Relative location of Noxa-nFLAG is represented by the asterisk.
tagged sequences I next optimized the doxycycline-inducibility of my pBI-EYFP/Noxa-FF-ZZ plasmids in 293rtTA cells (Figure 13b). I observed that treatment of transfected 293rtTA cells with pBI-EYFP/Noxa-WT-FF-ZZ with 0.1 µg/ml doxycycline was sufficient to induce expression of Noxa-WT-FF-ZZ. Furthermore, increasing the doxycycline concentration resulted in increased expression of Noxa-WT-FF-ZZ as compared to cells induced with 0.1 µg/ml doxycycline. I also investigated whether high concentrations of doxycycline resulted in production of insoluble Noxa-WT-FF-ZZ as I wished to avoid the production of such insoluble proteins. To do this I prepared protein samples from the Triton-insoluble lysate and performed western blotting. I observed that treatment with 1.0 or 2.0 µg/ml of doxycycline resulted in the production of insoluble Noxa-WT-FF-ZZ in 293rtTA cells transfected with pBI-EYFP/Noxa-WT-FF-ZZ. From this optimization experiment I determined that an optimal concentration of doxycycline for induction of Noxa-WT-FF-ZZ was 0.1 µg/ml, and I subsequently utilized this concentration for all other experiments using both Noxa-WT-FF-ZZ and Noxa-S13A-FF-ZZ. I next needed to determine if I could detect phosphorylation of Noxa-WT-FF-ZZ. To do this I transfected 293rtTA cells with either pBI-EYFP/Noxa-WT-FF-ZZ or pBI-EYFP/Noxa-S13A-FF-ZZ and doxycycline-induced expression for 24 hours. I then utilized the phospho-Ser (MAPK/CDK) antibody that is targeted towards MAPK/CDK targets. *In silico* analysis suggested that this antibody should detect serine-13 phosphorylated Noxa, which has the sequence ARKNAQPSPARAPAE. Unfortunately the phospho-Ser (MAPK/CDK) antibody (specific for *SPXR/K or PX*SP, where *S denotes phosphorylated serine) detected phosphorylation in both Noxa-WT-FF-ZZ and Noxa-S13A-FF-ZZ, whereas Noxa-S13A-FF-ZZ should not be able to be phosphorylated (data not shown). After further *in silico* analysis of the Noxa-FF-ZZ amino acid sequence I determined that the phospho-Ser (MAPK/CDK) antibody
was able to phosphorylate the protein A IgG binding domain (ZZ) of both Noxa-WT-FF-ZZ and Noxa-S13A-FF-ZZ. To correct for this issue I treated lysates from 293rtTA cells, transfected with either pBI-EYFP/Noxa-WT-FF-ZZ or pBI-EYFP/Noxa-S13A-FF-ZZ, with TEV protease in order to separate Noxa-FF from the ZZ domain. Though, when I performed western blotting on TEV cleavage products I was unable to detect phosphorylation of Noxa-WT-FF (data not shown). As phosphorylation of Noxa may enhance its proteasomal degradation, or that Noxa may be quickly dephosphorylated, I further treated transfected cells with MG132 and calyculin A in order to prevent proteasomal degradation and dephosphorylation of Noxa respectively (Figure 13c). From my results I observed that I was able to isolate both Noxa-WT-FF and Noxa-S13A-FF from their respective ZZ domains. Though, I was unable to detect phosphorylation of Noxa-WT-FF using the phospho-Ser (MAPK/CDK) antibody. I did however observe binding of the phospho-Ser (MAPK/CDK) antibody to the ZZ domain of both Noxa-WT-FF-ZZ and Noxa-S13A-FF-ZZ, again demonstrating that TEV cleavage was required in order to verify Noxa phosphorylation using the pBI-EYFP/Noxa-FF-ZZ expression system.

One possible reason that I was unable to detect phosphorylation of Noxa-WT-FF was due to steric interference of the FF-ZZ tag with CDK5 that might have interfered in the phosphorylation of Noxa in vitro. Therefore, I generated a new expression system in which I fused a single FLAG domain to the N-terminus of Noxa. I again designed the expression of Noxa-nFLAG to be under the control of a tetracycline response element by inserting the Noxa-nFLAG sequence into the pBI-EYFP-N1(ΔBsrG1)*tk/hygro plasmid (Figure 13d). Since my goal was to detect phosphorylation of Noxa I generated only Noxa-nFLAG with the wild-type cDNA Noxa sequence. Once I successfully generated pBI-EYFP/Noxa-nFLAG I performed an initial experiment where I transfected 293rtTA cells and induced expression of Noxa-nFLAG for
24 hours (Figure 13e). From my results I was able to successfully overexpress Noxa-nFLAG. I additionally detected multiple bands using a FLAG antibody in cells overexpressing Noxa-nFLAG, presumably due to ubiquitination of Noxa-nFLAG. Unfortunately, I was unable to detect phosphorylation of Noxa-nFLAG using the phospho-Ser (MAPK/CDK) antibody. Though, in this initial experiment I did not treat the pBI-EYFP/Noxa-nFLAG transfected 293rtTA cells with MG132 or calyculin A, and therefore I performed another experiment whereby I treated doxycycline-induced pBI-EYFP/Noxa-nFLAG transfected 293rtTA cells with MG132 and calyculin A. Additionally, I exposed induced transfected cells to 43°C to investigate if phosphorylation of Noxa-nFLAG is reduced when cells were exposed to hyperthermia. I performed a CHAPS immunoprecipitation using a FLAG primary mouse antibody in order to enrich for Noxa-nFLAG for western blotting (Figure 13f). I successfully immunoprecipitated Noxa-nFLAG and observed high levels of overexpression in total lysates. However, I was unable to detect phosphorylated Noxa-nFLAG in samples loaded from the total lysate or from the immunoprecipitated samples.

Another possibility as to why I was unable to detect phosphorylated Noxa-nFLAG could be because the phospho-Ser (MAPK/CDK) antibody is unable to recognize phosphorylated serine-13 of Noxa. To test for this possibility I re-ran samples from the above FLAG immunoprecipitation on separate SDS-PAGE gels, transferred samples onto PVDF membranes, and stained the membrane with Pro-Q Diamond phosphoprotein blot stain (Figure 13g). Pro-Q Diamond stain is a phospho-specific stain that can be used to detect phosphorylated proteins that have been transferred onto PVDF membranes. Since I completed a FLAG immunoprecipitation and enriched for Noxa-nFLAG in my immunoprecipitation samples, I was able to specifically identify if Noxa-nFLAG was phosphorylated. From my results I was unable to detect
phosphorylated Noxa-nFLAG from my immunoprecipitation samples. Furthermore, I was unable to detect either phosphorylated Noxa-nFLAG or endogenously phosphorylated Noxa in samples prepared from total lysates. Overall I was unable to detect phosphorylated Noxa using a combination of a phospho-Ser (MAPK/CDK) antibody and a phosphor-specific stain. Given that it has been previously shown that Noxa is phosphorylated on serine-13 by the cyclin-dependent kinase CDK5 (Lowman et al., 2010) it will be essential in the future for us to demonstrate that Noxa is phosphorylated in lymphoid cells.

**Generation of Tet-ON PErTA/Noxa clones**

Though I was unable to demonstrate Noxa phosphorylation I still wanted to investigate the role of Noxa serine-13 phosphorylation on hyperthermia-induced apoptosis. To investigate this I first generated stably transfected clones. PErTA cells, a cell line derived from the acute T-cell lymphoblastic leukemia cell line PEER that has constitutive overexpression of rtTA, were transfected with plasmids containing wild-type Noxa (Noxa-WT), non-phosphorylatable Noxa (Noxa-S13A), or a phospho-mimic Noxa (Noxa-S13E) under the control of a tetracycline response element (Figure 7). Stably transfected clones were generated and doxycycline-inducible overexpression was measured by western blotting (Figure 14a). Clones that demonstrated doxycycline-inducible overexpression were further compared and relative levels of overexpression between each clone were measured by western blotting and densitometry (Figure 14b). It was determined that PErTA/Noxa-WT clone WT1A7, PErTA/Noxa-S13A clone A1B6, and PErTA/Noxa-S13E clone E2G7 were optimal due to similar levels of overexpression, and henceforth were used in all experiments using my PErTA/Noxa clones (Figure 14c). Due to minor differences in levels of overexpression I induced my selected PErTA/Noxa clones with
Figure 14

A

B

C

D

E

F

Noxa

Mcl-1

Actin

Noxa

C 24 48 72

Noxa

PErTA WT S13A S13E

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**Figure 14:** PErTA/Noxa clone selection. (A) Analysis of stably transfected PErTA cells with the tet-regulated dicistronic expression plasmid pTR5-DC/Noxa-GFPQ*TK/hygro. PErTA cells were transfected with wild-type Noxa (WT), Noxa-S13A, or Noxa-S13E by electroporation and selected with G418 and hygromycin at 37°C. Once established, stable cell lines were treated with 1 μg/ml doxycycline (+) and incubated at 37°C with 5% CO₂ for 24 hours in order to induce Noxa and GFPQ expression. Cells were lysed in Laemmli lysis buffer and inducibility was analyzed by western blotting. Control cells were not induced with doxycycline (-). (B) Comparison of stable PErTA/Noxa clones that had doxycycline-inducible overexpression of Noxa-WT, Noxa-S13A, or Noxa-S13E. All PErTA/Noxa clones and PErTA cells were treated with 1 μg/ml doxycycline and incubated at 37°C with 5% CO₂ prior to being collected and lysed with Laemmli lysis buffer. Protein levels were analyzed by western blot. Relative overexpression of each PErTA/Noxa clone, as compared to PErTA, is shown at the bottom of each respective lane. (C) Comparison of doxycycline-inducible overexpression of selected PErTA/Noxa clones. Cells were treated with 1 μg/ml doxycycline (+) and incubated at 37°C with 5% CO₂ for 24 hours. Control cells were not treated with doxycycline (-). Cells were lysed with Laemmli lysis buffer and samples western blotted was completed (D) Determination of doxycycline-inducible overexpression using different doxycycline concentrations. PErTA/Noxa-WT-1A7 (WT), PErTA/Noxa-S13A-1BG (S13A) and PErTA/Noxa-S13E-2G7 (S13E) cells were treated with 0.1, 0.3, or 1.0 μg/ml doxycycline respectively and incubated at 37°C with 5% CO₂ for 24 hours. Control cells were not treated with doxycycline (0). Cells were lysed with Laemmli lysis buffer and western blotting was completed. (E) Determination of optimal doxycycline-induction duration. PErTA/Noxa-WT cells were treated with 1 μg/ml doxycycline and incubated at 37°C with 5% CO₂ for either 24, 48, or 72 hours. Control cells were not treated (C). Cells were lysed with Laemmli lysis buffer and western blotting was completed. (F) Comparison of the rate of ectopic doxycycline-inducible overexpression of selected PErTA/Noxa clones. PErTA, PErTA/Noxa-WT, PErTA/Noxa-S13A, and PErTA/Noxa-S13E cells were treated with 1 μg/ml doxycycline and incubated at 37°C with 5% CO₂ for 24 hours. Cells were collected immediately after induction (C) or were treated with 10 µM MG132 and collected after 3 or 6 hours of incubation at 37°C with 5% CO₂. Cells were lysed with Laemmli lysis buffer and western blotting was completed.

Various concentrations of doxycycline for 24 hours in order to determine optimal doxycycline concentration to produce equivalent levels of overexpression (Figure 14d). I observed that induction of PErTA/Noxa-WT, PErTA/Noxa-S13A, and PErTA/Noxa-S13E cells with 0.3, 0.1, and 1.0 μg/ml doxycycline respectively resulted in the most similar levels of overexpression. I also determined that 24 hours of doxycycline-induction resulted in optimal overexpression, whereas 48 or 72 hours of induction resulted in lower levels of overexpression (Figure 14e).
Since induction of my PErTA/Noxa-WT, PErTA/Noxa-S13A, and PErTA/Noxa-S13E clones with equal concentrations of doxycycline resulted in different levels of overexpression I wanted to determine if this was due to differences in their rates of synthesis (Figure 14f). To accomplish this I treated PErTA cells and induced my PErTA/Noxa clones with 1.0 µg/ml doxycycline and subsequently treated them with the proteasome inhibitor MG132 to prevent protein degradation. I observed different rates of Noxa protein accumulation in my PErTA/Noxa clones when treated with MG132 for 6 hours. Specifically, accumulation of Noxa-S13A by 6 hours was greater than both Noxa-WT and Noxa-S13E, whereas Noxa-S13E had the lowest levels of accumulation by 6 hours of proteasomal inhibition, suggesting that the difference in expression between my PErTA/Noxa clones was due to differences in protein synthesis. Overall these results established and characterized novel stably transfected cell lines with doxycycline-inducible overexpression of Noxa-WT, Noxa-S13A, or Noxa-S13E. Once characterized these cell lines were used to determine the mechanistic role of Noxa phosphorylation in lymphoid cells under standard growing conditions and during hyperthermia. Unfortunately, my PErTA/Noxa-S13E clone was excluded from further studies due to low levels of overexpression as compared to both PErTA/Noxa-WT and PErTA/Noxa-S13A clones (data not shown). Therefore, all subsequent experiments were performed with only PErTA/Noxa-WT and PErTA/Noxa-S13A cells, though it will be important in the future for us to generate a PErTA/Noxa-S13E with acceptable levels of overexpression in order to assess the role of Noxa phosphorylation during hyperthermia.

**Effect of Noxa serine-13 phosphorylation on the degradation of Noxa**

As it has yet to be determined what regulates the degradation of Noxa I wanted to determine if phosphorylation of Noxa serine-13 has an effect on Noxa degradation. Though I
previously determined that my selected PErTA/Noxa clones have different rates of protein synthesis (Figure 14f) I was still unsure whether the differences in levels of overexpression might also be due to differences in the rate of degradation between Noxa-WT and Noxa-S13A. Therefore I induced both PErTA/Noxa-WT and PErTA/Noxa-S13A cells for 24 hours with 1.0 µg/ml doxycycline and subsequently treated the induced cells with cycloheximide to determine the half-life of Noxa-WT and Noxa-S13A. Analysis by western blotting demonstrated that I obtained unequal overexpression of Noxa-WT and Noxa-S13A when cells were both treated with 1 µg/ml doxycycline (Figure 15a). Further analysis by densitometry revealed that ectopically overexpressed Noxa-WT has a half-life of approximately 30 minutes, and that both Noxa-WT and Noxa-S13A have similar rates of degradation (Figure 15b). I then tested the rate of degradation of Noxa-WT and Noxa-S13A when I induced my PErTA/Noxa clones to produce equal levels of protein. Western blotting revealed that treatment of PErTA/Noxa-WT and PErTA/Noxa-S13A cells with 0.3 and 0.1 µg/ml doxycycline respectively results in similar levels of overexpression (Figure 15c). Analysis by densitometry revealed again that ectopically overexpressed Noxa-WT and Noxa-S13A have similar rates of degradation when doxycycline-induced PErTA/Noxa-WT and PErTA/Noxa-S13A cells were treated with cycloheximide (Figure 15d). Though when induced with 0.3 µg/ml doxycycline Noxa-WT demonstrated an approximate half-life of 60 minutes as compared to a 30 minute half-life when induced with 1.0 µg/ml (Figure 15b). Overall these results suggest that serine-13 phosphorylation is not required for Noxa degradation as prevention of Noxa serine-13 phosphorylation has no effect on the degradation of Noxa in PErTA cells incubated at 37°C.
Figure 15

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Noxa

B

[C] Relative protein levels versus time (min) for WT and S13A.

C

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Noxa

D

[C] Relative protein levels versus time (min) for WT and S13A.
Figure 15: The half-life of Noxa is unaffected by phosphorylation. (A) PErTA/Noxa-WT (WT) and PErTA/Noxa-S13A (S13A) cells were treated with 1 µg/ml doxycycline and incubated at 37°C with 5% CO₂ for 24 hours. PErTA cells (P) were also treated with 1 µg/ml doxycycline as above as a control. Following incubation cells were either collected (0) or treated with 0.2 mg/ml CHX and incubated for 30, 60, or 90 minutes at 37°C with 5% CO₂ prior to collection. Cells were lysed with Triton X-100 lysis buffer and samples from the Triton-soluble fraction were western blotted. (B) Analysis of relative Noxa protein levels for both PErTA/Noxa-WT (WT) and PErTA/Noxa-S13A (S13A) cells induced with 1µg/ml doxycycline. Noxa protein levels for each time point are compared to Noxa levels immediately after 24 hours of doxycycline-induction (0). (C) PErTA/Noxa-WT (WT) and PErTA/Noxa-S13A (S13A) cells were treated with 0.3 or 0.1 µg/ml doxycycline respectively and incubated at 37°C with 5% CO₂ for 24 hours. PErTA cells (P) were also treated with 1 µg/ml doxycycline as above as a control. Following incubation cells were either collected (0) or treated with 0.2 mg/ml CHX and incubated for 30, 60, or 90 minutes at 37°C with 5% CO₂ prior to collection. Cells were lysed with Triton X-100 lysis buffer and samples from the Triton-soluble fraction were western blotted. (D) Analysis of relative Noxa protein levels for both PErTA/Noxa-WT (WT) and PErTA/Noxa-S13A (S13A) cells induced with either 0.3 or 0.1 µg/ml doxycycline respectively. Noxa protein levels for each time point are compared to Noxa levels immediately after 24 hours of doxycycline-induction (0).

Effect of Noxa serine-13 phosphorylation on cell viability

Though prevention of Noxa serine-13 phosphorylation had no effect on the degradation of Noxa I was interested in whether phosphorylation has a role in cell viability. It has been previously shown that Noxa is able to engage and inhibit the anti-apoptotic Bcl-2 family protein Mcl-1 (Willis et al., 2005). Therefore to test whether Noxa serine-13 phosphorylation has an effect on cell viability I induced PErTA/Noxa-WT and PErTA/Noxa-S13A cells to overexpress equal levels of protein and performed alamar blue assays over 72 hours to determine cell growth. I also performed alamar blue assays on PErTA/Noxa-WT and PErTA/Noxa-S13A cells that were not induced to determine whether low-level leaky expression in these cells affected cell growth. When comparing PErTA/Noxa-WT and PErTA/Noxa-S13A cells that were not doxycycline-induced I observed that both PErTA/Noxa-WT and PErTA/Noxa-S13A cells demonstrated reduced cell growth after 72 hours of growth at 37°C as compared to control PErTA cells (Figure
16a). The difference of cell growth seen in both non-induced PErTA/Noxa-WT and PErTA/Noxa-S13A cells was observable after 48 hours, whereas there was no observable difference in cell growth between PErTA, PErTA/Noxa-WT, and PErTA/Noxa-S13A cells after 24 hours of growth at 37°C. When comparing doxycycline-induced PErTA/Noxa-WT and PErTA/Noxa-S13A cells I observed that both PErTA/Noxa-WT and PErTA/Noxa-S13A expressing cells demonstrated reduced cell growth after 72 hours of doxycycline-induction at 37°C as compared to control doxycycline-treated PErTA cells (Figure 16b). In addition differences in cell growth between PErTA and PErTA/Noxa-S13A cells can be observed as early as after 24 hours of doxycycline-induction, whereas differences between PErTA and PErTA/Noxa-WT cells cannot be observed until after 48 hours. Comparison of the 72 hour cell growth of PErTA/Noxa-WT, PErTA/Noxa-S13A, and PErTA cells revealed that overexpression of Noxa resulted in statistically different cell growth (Figure 16c). Specifically, analysis by a one-tailed t-test revealed that non-induced PErTA/Noxa-WT and PErTA/Noxa-S13A cells both have statistically reduced cell growth as compared to non-doxycycline-treated PErTA cells ($P = 0.0002$ and 0.025 respectively; $n = 3$). Additionally, doxycycline-induced overexpression of Noxa-S13A significantly reduced cell growth as compared to either overexpression of Noxa-WT or treatment of PErTA cells with doxycycline as a control ($P = 0.04$ and 0.018 respectively; $n = 3$). Overexpression of Noxa-S13A also resulted in a significant decrease in cell growth as compared to PErTA/Noxa-S13A cells that were not doxycycline-induced ($P = 0.006; n = 3$). Conversely, doxycycline-induced overexpression of Noxa-WT did not result in a significant decrease in cell growth as compared to doxycycline-treated PErTA cells ($P = 0.190; n = 3$). These results suggest that both non-induced PErTA/Noxa-WT and PErTA/Noxa-S13A cells have
Figure 16

A

B

C

Relative cell growth

Time (hours)

Dox-

PEETA

WT

S13A

Dox +

PEETA

WT

S13A

Relative cell growth

Dox -

Dox +

*
significantly reduced cell growth as compared to PErTA cells, possibly due to low levels of uncontrolled leaky expression. Additionally, overexpression of non-phosphorylatable Noxa (Noxa-S13A) significantly reduced cell growth after 72 hours of overexpression at 37°C as compared to overexpression of wild-type Noxa (Noxa-WT), overall suggesting that dephosphorylation of Noxa results in decreased cell viability of PErTA cells at 37°C, possibly due to increased activation of apoptosis. I also performed alamar assays on PErTA and PErTA/Noxa cells that were doxycycline-induced and exposed to 43°C to investigate whether Noxa phosphorylation has an effect on cell viability following exposure to hyperthermia. Unfortunately, early results were difficult to decipher due to experimental error (data not shown), and therefore it will be important in the future to repeat these experiments at 43°C in order to investigate the role of Noxa serine-13 phosphorylation on cell viability following hyperthermia.
Effect of Noxa serine-13 phosphorylation on caspase activation

One of the limiting factors of the alamar blue assay is that any observed changes in cell viability are directly related to cell growth. As such, it is important to decipher whether observed changes in cell viability are due to changes in activation of apoptosis or due to changes in rate of cell replication. In order to determine if my observed decrease in cell viability in cells overexpressing Noxa-S13A was due to increased activation of apoptosis I performed caspase-3 assays, which measure the activity of caspase-3 to cleave a fluorogenic substrate that can be measured by a fluorescence reader (Nicholson et al., 1995; Lee et al., 2003). I first assessed whether overexpression of Noxa-S13A results in increased caspase-3 activation in my PErTA/Noxa cells when induced at 37°C for 24 hours with various concentration of doxycycline (Figure 17a). I observed that PErTA, PErTA/Noxa-WT, and PErTA/Noxa-S13A cells not treated with doxycycline exhibited relatively equal caspase-3 activity. Furthermore, when these cell lines were treated with doxycycline I observed that capase-3 activity was increased in both PErTA/Noxa-WT and PErTA/Noxa-S13A cells, and that the caspase-3 activity was increased in these two cell lines with greater concentration of doxycycline. As a control I did not see any increase in caspase-3 activity in PErTA cells treated with 1.0 µg/ml doxycycline as compared to PErTA cells not treated with doxycycline. When I directly compared PErTA/Noxa-WT and PErTA/Noxa-S13A cells induced to overexpress equal levels of protein with 0.3 and 0.1 µg/ml doxycycline respectively I observed that both PErTA/Noxa-WT and PErTA/Noxa-S13A cells had increased caspase-3 activity as compared to both non-treated and PErTA cells treated with 1.0 µg/ml doxycycline (Figure 17b). Analysis by one-tailed t-test revealed that overexpression of Noxa-S13A significantly increased caspase-3 activity as compared to PErTA cells treated with 1.0 µg/ml doxycycline and PErTA/Noxa-S13A cells that were not doxycycline-induced.
Figure 17
Figure 17: Overexpression of Noxa-S13A results in increases caspase-3 activity. PErTA, PErTA/Noxa-WT (WT), and PErTA/Noxa-S13A (S13A) cells were treated with 0.1, 0.3, and 1.0 µg/ml doxycycline and incubated at 37°C with 5% CO₂ for 24 hours. Cells were not treated with doxycycline as controls (0). Cells were either collected immediately after induction, or were exposed to 43°C for 1 hour prior to collection, and a caspase-3 assay was completed. DEVDase activity was measured using a fluorescence microplate reader (FLx800; BioTek Instruments) at an excitation of 380 (20) nm and emission of 460 (40) nm. (A) Caspase-3 activity in doxycycline-induced cells incubated at 37°C. Shown is the mean DEVDase activity (RFU/min/µg) with SE (n=3) for each cell line induced with the indicated concentration of doxycycline. (B) Comparison of doxycycline-induced (Dox+) and non-induced (Dox-) caspase-activity in cells grown at 37°C. WT and S13A cells were induced with 0.3 and 0.1 µg/ml doxycycline respectively in order overexpress equal levels of protein. PErTA cells were treated with 1.0 µg/ml doxycycline as a control. Shown is the mean DEVDase activity with SE (n=3). *P<0.05 for both S13A (Dox+) vs. S13A (Dox-) and PErTA (Dox+). There is no significant difference between WT (Dox+) and PErTA (Dox+). (C) Caspase-3 activity in doxycycline-induced cells incubated exposed to 43°C for 1 hour. Shown is the mean DEVDase activity with SE (n=3) for each cell line induced with the indicated concentration of doxycycline. (D) Comparison of doxycycline-induced (Dox+) and non-induced (Dox-) caspase-activity in cells exposed to 43°C for 1 hour. WT and S13A cells were induced with 0.3 and 0.1 µg/ml doxycycline respectively in order overexpress equal levels of protein. PErTA cells were treated with 1.0 µg/ml doxycycline as a control. Shown is the mean DEVDase activity with SE (n=3). *P<0.05 for WT (Dox+) vs. WT (Dox-). There is no significant difference between both WT (Dox+) and S13A (Dox+) vs. PErTA (Dox+).

(P = 0.032 and 0.030 respectively; n = 3). Overexpression of Noxa-WT did not result in a significant increase in caspase-3 activity as compared to PErTA cells treated with 1.0 µg/ml doxycycline (P = 0.069; n = 3). Overall these results suggest that prevention of Noxa phosphorylation results in increased activation of caspase-3, and subsequent increased activation of apoptosis, in PErTA cells incubated at 37°C.

Once I determined that overexpression of Noxa-S13A significantly induces caspase-3 activation in PErTA cells incubated at 37°C I next wanted to determine if overexpression of Noxa-S13A results in increased caspase-3 activation when PErTA cells are exposed to hyperthermia (Figure 17c). I observed no difference in the activation of caspase-3 in non-treated PErTA and non-induced PErTA/Noxa-S13A cells that were exposed to 43°C for 1 hour. When
doxycycline-induced PErTA/Noxa-WT and PErTA/Noxa-S13A cells were exposed to 43°C for 1 hour. I observed that caspase-3 activity was increased in both cell lines, and that caspase-3 activity was further increased with higher concentrations of doxycycline. As a control, I did not see any increase in caspase-3 activity in PErTA cells exposed at 43°C for 1 hour and treated with various concentrations of doxycycline and as compared to PErTA cells not treated with doxycycline (Figure 17c). When I directly compared PErTA/Noxa-WT and PErTA/Noxa-S13A cells that were exposed to 43°C for 1 hour and doxycycline-induced to overexpress equal levels of protein with 0.3 and 0.1 µg/ml doxycycline respectively, I observed that both PErTA/Noxa-WT and PErTA/Noxa-S13A cells had decreased caspase-3 activity as compared to doxycycline-treated PErTA cells (Figure 17d). Though, statistical analysis by one-tailed t-test revealed that the decrease in caspase-3 activity in both doxycycline-induced PErTA/Noxa-WT and PErTA/Noxa-S13A cells was not statistical when compared to caspase-3 activity of doxycycline-treated PErTA cells that were exposed to 43°C for 1 hour ($P = 0.158$ and $0.177$ respectively; $n = 3$).

Additionally, overexpression of Noxa-WT did result in a statistically significant increase in caspase-3 activity as compared to PErTA/Noxa-WT cells that were not doxycycline-induced ($P = 0.028$; $n = 3$). Conversely, overexpression of Noxa-S13A did not result in a significant increase in caspase-3 activity as compared to PErTA/Noxa-S13A cells that were not doxycycline-induced ($P = 0.057$; $n = 3$), though this might be due to experimental variance between each experimental repeat. Overall, these results suggest that overexpression of either wild-type Noxa or non-phosphorylatable Noxa does not have an additive effect on hyperthermia-induced caspase-3 activation. Further experimentation will be required to assess the role of Noxa serine-13 phosphorylation on apoptosis during hyperthermia.
Effect of Noxa serine-13 phosphorylation on Noxa-induced Mcl-1 degradation

After observing that overexpression of Noxa-S13A at 37°C resulted in increased caspase-3 activity I next wanted to investigate how prevention of Noxa serine-13 phosphorylation is able to induce apoptosis. It has been previously observed that overexpression of Noxa results in the Noxa-dependent proteasomal degradation of Mcl-1 (Willis et al., 2005, Morales et al., 2008, Czabotar et al., 2007). Therefore I speculated that phosphorylation of Noxa serine-13 might play a role in Noxa-induced Mcl-1 degradation. To investigate this I overexpressed Noxa-WT and Noxa-S13A in my PErTA/Noxa clones and analyzed the effect of Noxa-WT and Noxa-S13A overexpression on Mcl-1 levels in cells incubated at 37°C and exposed to 43°C (Figure 18). From my results I observed that overexpression of both Noxa-WT and Noxa-S13A reduced Mcl-1 levels in PErTA/Noxa-WT and PErTA/Noxa-S13A cells incubated at 37°C as compared to PErTA cells treated with 1.0 µg/ml doxycycline. Furthermore, overexpression of Noxa-S13A resulted in a greater decrease in Mcl-1 levels as compared to overexpression of Noxa-WT. When doxycycline-induced PErTA/Noxa cells were exposed to 43°C for 1 hour I observed a greater decrease in Mcl-1 levels in PErTA/Noxa cells overexpressing either Noxa-WT or Noxa-S13A as compared to doxycycline-treated PErTA cells exposed to 43°C. Additionally, overexpression of Noxa-S13A resulted in a greater decrease in Mcl-1 levels, as compared to overexpression of Noxa-WT, in PErTA/Noxa-S13A and PErTA/Noxa-WT cells, respectively, that were exposed to 43°C. When doxycycline-induced PErTA/Noxa-WT and PErTA/Noxa-S13A cells were allowed to recover at 37°C after exposure to 43°C I again observed that overexpression of either Noxa-WT or Noxa-S13A resulted in greater decrease in Mcl-1 levels as compared to doxycycline-treated PErTA cells that were allowed to recover at 37°C after exposure to 43°C. Furthermore overexpression of Noxa-S13A resulted in a greater decrease in Mcl-1 levels as compared to
Figure 18: Effect of Noxa serine-13 phosphorylation on Noxa-induced Mcl-1 degradation. PErTA/Noxa-WT (WT) and PErTA/Noxa-S13A (S13A) cells were treated with 0.3 and 0.1 µg/ml doxycycline, respectively, and incubated at 37°C with 5% CO₂ for 24 hours. PErTA cells were treated with 1.0 µg/ml doxycycline as a control and incubated as above. After 24 hours cells were exposed to 43°C for 1 hour and either collected immediately after (0) or were allowed to incubate at 37°C with 5% CO₂ for 6 hours after exposure to 43°C (6). Control cells were incubated at 37°C with 5% CO₂ for 6 hours (C). Cells were lysed in Laemmli lysis buffer and western blotting was completed.

Overexpression of Noxa-WT in PErTA/Noxa-S13A and PErTA/Noxa-WT cells, respectively, that were allowed to recover at 37°C after exposure to 43°C. Interestingly, levels of Noxa-S13A were greater than that of Noxa-WT in PErTA/Noxa-S13A and PErTA/Noxa-WT cells respectively after recovery at 37°C. Though this result may suggest differences in degradation between Noxa-WT and Noxa-S13A, my previous results suggest that phosphorylation of Noxa serine-13 has no effect on the degradation of Noxa (Figure 15), and therefore these differences in Noxa recovery in my PErTA/Noxa clones may be due to clonal variation in doxycycline inducibility following exposure to hyperthermia. Additionally, I was only able to complete this experiment once, and therefore additional experimental repeats will be required to further investigate the effect of Noxa serine-13 phosphorylation on Mcl-1 degradation. Overall these
results suggest that overexpression of non-phosphorylatable Noxa is able to induce degradation of Mcl-1 more effectively than overexpression of wild-type Noxa in PErTA cells incubated at 37°C and during exposure to hyperthermia.
Discussion

Regulation of apoptosis is critical for cell survival during mild stress and for proper removal of damaged cells during severe stress. Hyperthermia is one type of stress that is able to induce apoptosis through activation of the intrinsic pathway via release of cytochrome c from the mitochondria (Mosser et al., 2000). Knockdown of the BH3-only protein Noxa prevents hyperthermia-induced Mcl-1 degradation and Bax activation (Stankiewicz et al., 2009). Noxa is a member of the pro-apoptotic BH3-only protein subfamily of Bcl-2 family proteins, and is able to selectively bind to anti-apoptotic Mcl-1, leading to their mutual degradation (Oda et al., 2000; Willis et al., 2005; Chen et al., 2005). Additionally, Noxa is phosphorylated on serine-13 by the cyclin-dependent kinase CDK5, and dephosphorylation of Noxa is required to activate its apoptotic potential (Lowman et al., 2010). In this study I hypothesized that hyperthermia is able to induce apoptosis by preventing Noxa phosphorylation, due to reduced CDK5 activity, leading to activation of Noxa and subsequently Mcl-1 degradation. Overall my results regarding CDK5 phosphorylation and solubility, alongside my results concerning the apoptotic potential of non-phosphorylatable Noxa, support this hypothesis. However, I was unable to detect in vivo phosphorylation of Noxa serine-13 in lymphoid cells, providing support to my null hypothesis that hyperthermia does not have an effect on Noxa serine-13 phosphorylation.

My initial results were completed prior to the publication that Noxa was phosphorylated on serine-13 by CDK5 (Lowman et al., 2010). Initial In silico analysis of the Noxa amino acid sequence revealed a high probability that Noxa could be phosphorylated on serine-13 by CDK5. Interestingly both CDK5 and JNK demonstrated high probability scores from the kinase
prediction program GPS, indicating that JNK has a high probability of phosphorylating Noxa on serine-13, though initial results (not shown) suggested that JNK is not responsible for phosphorylating Noxa. Though, given that JNK has been implicated in hyperthermia-induced apoptosis, it would be advisable to re-investigate JNK as a potential hyperthermia-activated kinase that phosphorylates Noxa.

In order to investigate whether hyperthermia results in changes in Noxa serine-13 phosphorylation I first assessed the broader effect of hyperthermia on the phosphorylation of MAPK/CDK targets. I have shown that hyperthermia affects serine phosphorylation of many MAPK/CDK targets in PErTA70 cells, suggesting a potential role of various MAPKs and CDKs in hyperthermia-induced apoptosis. However, since the phospho-Ser (MAPK/CDK) antibody I used is unable to distinguish between the different MAPK/CDK targets based on identity it will be important in the future to determine the identity of these phosphoproteins that have demonstrated hyperthermia-induced changes in phosphorylation. I also investigated whether CDK5 activity is altered under hyperthermia by treating hyperthermia-exposed PErTA70 cells with the CDK5 inhibitor roscovitine and measuring changes in the phosphorylation of MAPK/CDK targets. I observed that the hyperthermia-induced phosphorylation of one specific ~26 kDa MAPK/CDK target was decreased when cells were treated with roscovitine. This result suggests that CDK5 activity is altered during hyperthermia. However, changes in phosphorylation of this specific MAPK/CDK target may also be due to hyperthermia-induced changes in specific phosphatases responsible for dephosphorylation of this MAPK/CDK target. Additionally, roscovitine is not a specific inhibitor of CDK5, and is able to also inhibit CDK1/2 and Erk1/2 (Liebl et al., 2011). Given that the phospho-serine antibody is able to detect targets of
CDK1/2 and Erk1/2 it will be important in the future to determine the identity of this specific ~26 kDa MAPK/CDK target in order to determine if hyperthermia-induced changes in CDK5 activity are responsible for phosphorylation of this specific MAPK/CDK target.

With results suggesting that CDK5 activity might be altered during hyperthermia I next investigated the effect hyperthermia on both the phosphorylation of CDK5 and on the protein levels of CDK5. Overall, I observed that exposure to hyperthermia resulted in the loss of phosphorylated CDK5 and generation of insoluble CDK5. Interestingly, the effect of hyperthermia on the loss of phosphorylated CDK5 was more dramatic than the effect on CDK5 insolubility. Since phosphorylation of CDK5 on tyrosine-15 results in increased enzymatic activity of CDK5 (Lalioti et al., 2010), it will be important in the future to investigate whether changes in insolubility or loss of phosphorylated CDK5 result in reduced CDK5 activity. Additionally, it will be important to determine what effect hyperthermia has on the activity of c-Abl and Fyn, the kinases responsible for phosphorylating CDK5 (Zukerberg et al., 2000; Sasaki et al., 2002). In addition to the overall effects of hyperthermia, if PErTA70 cells are allowed to recover at 37°C after exposure to hyperthermia I observed a mild recovery of soluble CDK5 and phosphorylated CDK5 after 4-6 hours of recovery. One reason for the recovery of both phosphorylated and total CDK5 may be due to increased levels of the protein chaperone HSP70, which increases thermotolerance by binding to hyperthermia-induced misfolded proteins and refolding them into their native confirmation (Mosser and Martin, 1992; Mosser et al., 1997; Mayer and Bukau, 2005). To further demonstrate the restorative role of HSP70 to both phosphorylated and total CDK5 I exposed PErTA70 cells that were overexpressing HSP70 to hyperthermia. As expected overexpression of HSP70 not only prevented the insolubilization of
CDK5, but also enhanced the recovery of phosphorylated CDK5. These results suggest that HSP70 is able to reduce the effect of hyperthermia on CDK5, opening up novel clinical approaches to diseases that involve alterations in CDK5 function and activity. Overall my results demonstrate that exposure to hyperthermia reduces solubility and phosphorylation of CDK5, and that these hyperthermia-induced effects are diminished by the protective ability of HSP70. Therefore I hypothesize that hyperthermia reduces CDK5 activity, though this is still to be determined.

One limitation of my study was that I was unable to demonstrate changes in phosphorylation of known CDK5 targets. Of interest is the role of CDK5-mediated phosphorylated of members of the Bcl-2 family. In neuronal cells it has been demonstrated that CDK5 is able to phosphorylate Bcl-2, enabling its anti-apoptotic function (Cheung et al., 2008). One possible role for CDK5 in hyperthermia-induced apoptosis may be through phosphorylation of Bcl-2, whereby hyperthermia-induced reduction in CDK5 activity results in reduced Bcl-2 phosphorylation, preventing the anti-apoptotic function of Bcl-2 and enabling hyperthermia-induced apoptosis. Further experiments will be required in order to determine if hyperthermia-induced changes in CDK5 activity results in changes in Bcl-2 phosphorylation. The BH3-only protein Noxa has also been identified as a target of CDK5 in non-neuronal cells (Lowman et al., 2010) though I was unable to detect Noxa phosphorylation in 293rtTA cells, as will be discussed later. I hypothesized that hyperthermia-induced apoptosis is regulated through phosphorylation of Noxa, and that hyperthermia prevents phosphorylation of Noxa due to reduced activity of CDK5. In order to fully assess the effect of hyperthermia on CDK5 activity, and subsequently on
Noxa phosphorylation, it will be essential to further investigate CDK5-mediated phosphorylation of known non-neuronal CDK5 targets during hyperthermia in lymphoid cells.

In addition to my results regarding phosphorylated CDK5 levels and insolubility of CDK5 I also performed co-immunoprecipitations to determine if hyperthermia affected CDK5/p35 interaction. I was unable to detect p35 associated with CDK5, and since the lack of observable CDK5/p35 association might be due to experimental error regarding methodology, it will be essential in the future to determine if p35 is able to associate with CDK5 in lymphoid cells by performing co-immunoprecipitations using a p35 antibody. However, I was able to detect p35 in PErTA70 cells and observed an increase in total p35 levels immediately after hyperthermia that was reduced when PErTA70 cells were overexpressing HSP70. Given that p35 is a direct activator of CDK5, and that the activity of CDK5 is directly related to cellular levels of p35 (Lalioti et al., 2010; Dhariwala and Rajadhyaksha, 2008; Liebl et al., 2011), this result suggests that CDK5 activity is increased in cells exposed to hyperthermia. However, it has been previously demonstrated that CDK5 is responsible for phosphorylating p35, which results in the degradation of p35 in a negative feedback loop (Patrick et al., 1998; Kamei et al., 2007). If hyperthermia results in decreased CDK5 activity, which is yet to be determined, then I would predict that p35 levels would increase due to reduced CDK5-mediated phosphorylation. Further experiments will be required to assess the levels and interaction of CDK5 and p35 during hyperthermia, and what role this dynamic has on CDK5 activity in cells exposed to hyperthermia.
As discussed above my original hypothesis was that hyperthermia reduces phosphorylation of Noxa, due to reduced activity of CDK5, resulting in activation of apoptosis. In contrast to previously published results demonstrating Noxa serine-13 phosphorylation (Lowman et al., 2010) I was unable to demonstrate that Noxa is phosphorylated in either PErTA or 293rtTA cells. In order to assess whether hyperthermia prevents phosphorylation of Noxa I utilized a phoso-serine antibody that is able to recognize phospho-serine that has been phosphorylated by either MAPKs or CDKs. Examination of the amino acid sequence surrounding serine-13 on Noxa (ARKNAQPSPARAPAE) and of the putative recognition sites for the phospho-Ser (MAPK/CDK) antibody (PXS*P or S*XPR/K) revealed a 100% match. Initial experiments performed in PErTA cells demonstrated that I was unable to detect endogenously phosphorylated Noxa using the phospho-Ser (MAPK/CDK) antibody (data not shown). One possibility for why I could not detect endogenous phosphorylated Noxa using the phospho-Ser (MAPK/CDK) antibody is due to the constitutively low levels of Noxa expression in PErTA cells. Additionally, it is currently unknown to what degree the total amount of Noxa is phosphorylated in healthy cells. It is possible that only a small percentage of the total Noxa is phosphorylated at one time, making it difficult or impossible to detect endogenous phosphorylated Noxa in PErTA cells using the phospho-Ser (MAPK/CDK) antibody.

In order to remedy this issue I enriched for Noxa by transient overexpression of a TAP-tagged Noxa, containing two FLAG and two protein A IgG binding domain, and immunoprecipitation. My results failed to demonstrate that Noxa is phosphorylated on serine-13 by using the phospho-Ser (MAPK/CDK) antibody. One possible explanation for why I was unable to demonstrate Noxa serine-13 phosphorylation is because of possible steric interference
due to the large size (24 kDa) of the tap-tag relative to Noxa (6 kDa). To test this I generated N-terminal FLAG-Noxa (7 kDa) but was still unable to detect Noxa serine-13 phosphorylation despite producing large amounts of N-terminal FLAG-Noxa. Though Lowman et al. (2010) identified that Noxa is phosphorylated by CDK5, there have been no studies investigating how readily phosphorylated Noxa is dephosphorylated or whether Noxa phosphorylation promotes the degradation of Noxa. To determine if Noxa is rapidly dephosphorylated or degraded, I inhibited all phosphatase activity and proteasome mediated degradation of Noxa. I again was unable to detect Noxa serine-13 phosphorylation either using the phospho-Ser (MAPK/CDK) antibody or using a phospho-specific stain.

Since I originally wanted to investigate the effect of hyperthermia on Noxa phosphorylation, it is necessary to demonstrate that Noxa is phosphorylated on serine-13 in PErTA cells. There are two possibilities as to why I was unable to observe Noxa phosphorylation. Firstly, it is possible that the methods I utilized (a phospho-Ser (MAPK/CDK) antibody or a phospho-specific stain) were not able to detect Noxa phosphorylation. The second possibility is that Noxa is not phosphorylated by CDK5 on serine-13. Lowman et al. (2010) demonstrated that Noxa is phosphorylated by CDK5 on serine-13 through the use of in vitro kinase assays and an in-house generated phospho-Ser-13-Noxa rabbit antibody. Though in vitro kinases assays are an effective and efficient way to demonstrate protein phosphorylation by a specific kinase, these assays are performed with recombinant proteins and are not completed in vivo. Therefore, there is no guarantee that the proteins identified to be phosphorylated through in vitro kinase assays are phosphorylated by the same kinase in vivo. One feature that can produce differences between in vivo phosphorylation and phosphorylation observed in in vitro kinase
assays is the subcellular localization of the kinase and target protein in question. With respect to Noxa it has been previously demonstrated that Noxa contains a mitochondrial targeting domain that preferentially directs Noxa to the mitochondria where it is able to bind and disable Mcl-1 (Seo et al., 2003; Ploner et al., 2009), whereas the subcellular localization of CDK5 is dependent on the activator in which CDK5 is bound to. Specifically, CDK5 when not bound to any activators remains primarily cytosolic and inactive (Paglini and Cáceres, 2001), whereas interaction with either p35 or p39 results in localization of CDK5 primarily to the plasma membrane and perinuclear region, due to myristoylation of p35 and p39 (Asada et al., 2008). Given that active CDK5/p35 primarily localizes to the plasma membrane and Noxa primarily localizes to the mitochondrial membrane the likelihood of these two proteins interacting in vivo is questionable. One way to demonstrate that CDK5 and Noxa are able to associate together in vivo would be to perform co-immunoprecipitations, which is currently underway. To date all experiments that have been published that demonstrate Noxa serine-13 phosphorylation have been completed in cells overexpressing FLAG-tagged Noxa, and therefore the possibility exists that Noxa is able to be non-specifically phosphorylated by CDK5 when Noxa is exogenously overexpressed. One option to measure phosphorylation of endogenous Noxa would be by mass spectrometry, though this would prove difficult without overexpressing Noxa due to low levels of Noxa expression in many types of cells (Ploner et al., 2009). Overall it will be important in the future to determine if endogenous Noxa is truly phosphorylated by CDK5 in vivo.

Though I was unable to demonstrate that hyperthermia reduces Noxa phosphorylation, I was able to investigate a functional role for Noxa phosphorylation in PErTA cells. In order to measure the effect of Noxa phosphorylation on Noxa degradation, cell viability, activation of
caspase-3, and on Mcl-1 degradation I generated stably transfected PErTA cells that had doxycycline-dependent overexpression of either wild-type Noxa or non-phosphorylatable Noxa-S13A. I generated stable cells lines in order to reduce variation between experimental repeats. The first experiment I performed with my stable PErTA/Noxa clones was to measure the effect of Noxa phosphorylation on Noxa degradation. When I overexpressed either wild-type Noxa or Noxa-S13A I was unable to detect any difference in the rate of degradation between the two forms of Noxa, suggesting that Noxa phosphorylation has no effect on Noxa degradation.

I also investigated the potential role of Noxa serine-13 phosphorylation on cell viability, by alamar blue and caspase-3 activity assays. I observed that overexpression of Noxa-S13A in my PErTA/Noxa clones resulted in a statistically significant decrease in cell viability as compared to overexpression of wild-type Noxa, suggesting that prevention of Noxa phosphorylation results in reduced cell viability. One limitation to performing alamar blue assays is that any observed changes in cell viability are directly related to cell growth. As such, it is important to decipher whether observed changes in cell viability are due to changes in activation of apoptosis or due to changes in the rate of cell replication. Therefore, another possibility for my cell viability observation is that overexpression of non-phosphorylatable Noxa may result in increased activation of apoptosis as compared to overexpression of wild-type Noxa. Lowman et al. (2010) investigated the role of Noxa serine-13 phosphorylation on activation of apoptosis by the use of annexin-V staining and flow cytometry and found that overexpression of non-phosphorylatable Noxa resulted in a greater increase of annexin-V uptake as compared to overexpression of wild-type Noxa. This demonstrated that overexpression of non-
phosphorylatable Noxa results in a greater induction of apoptosis as compared to overexpression of wild-type Noxa, suggesting that phosphorylation of Noxa reduces its apoptotic potential.

Another approach to measuring apoptosis is through caspase-3 assays that measure the activity of caspase-3, isolated from cells or in intact cells undergoing apoptosis, to cleave a fluorogenic substrate (Nicholson et al., 1995; Lee et al., 2003). From my results I demonstrated that overexpression of Noxa-S13A induced apoptosis more effectively than overexpression of wild-type Noxa under standard growth conditions. Interestingly I observed no significant effect of overexpression of either wild-type or Noxa-S13A on caspase-3 activity, as compared to control doxycycline-treated PErTA cells, when cells were exposed to 43°C. I speculate that I did not see a difference in caspase-3 activity because the endogenous pool of Noxa is sufficient to induce caspase-3 activity in cells exposed to 43°C, and that overexpression of Noxa does not have a significantly additive effect on caspase-3 activation in cells exposed to 43°C.

Noxa has been identified as an important regulator of the anti-apoptotic Bcl-2 family protein Mcl-1. Given this important role I investigated the role of Noxa serine-13 phosphorylation on the degradation of Mcl-1 and observed that overexpression of Noxa-S13A resulted in a greater loss of Mcl-1 as compared to overexpression of wild-type Noxa in PErTA cells. This result demonstrates that non-phosphorylatable Noxa is able to engage Mcl-1 more readily then phosphorylated Noxa in order to induce Mcl-1 degradation and activation of apoptosis, and is supported by previously published results where it has been demonstrated that overexpression of non-phosphorylatable Noxa induces apoptosis more effectively than overexpression of wild-type Noxa (Lowman et al., 2010). Lowman et al. (2010) speculated that, when phosphorylated, Noxa is able to associate with additional proteins that sequester Noxa in
the cytosol, preventing Noxa from engaging Mcl-1 and subsequently preventing the pro-apoptotic function of Noxa. To date no studies have been completed as to mechanism of how phosphorylation of Noxa prevents Noxa/Mcl-1 interaction and prevents the apoptotic function of Noxa. Therefore I plan to generate N-terminal FLAG-tagged Noxa, containing either the cDNA sequence for wild-type Noxa or Noxa-S13A, and complete co-immunoprecipitations for FLAG-Noxa. I speculate Noxa-S13A will interact with Mcl-1 more readily than wild-type Noxa, providing support for my results regarding the effect of Noxa phosphorylation on Mcl-1 degradation. Additionally, recent research has revealed that Noxa overexpression results in increased Mcl-1/Mule interaction with a correlative decrease in Mcl-1/USP9X interaction, resulting in increased Mcl-1 ubiquitination and degradation (Gomez-Bougie et al., 2011). I speculate that hyperthermia-induced dephosphorylation of Noxa allows Noxa to localize to the mitochondria and to interact with Mcl-1, promoting Mcl-1/Mule interaction, leading to ubiquitination of Mcl-1 and activation of apoptosis. Further experimentation is needed to examine the interactions between Mcl-1, Mule, USP9X, and Noxa during hyperthermia.

In this study I investigated the role of Noxa serine-13 phosphorylation on hyperthermia-induced apoptosis. From my results I have established a potential role for the cyclin-dependent kinase CDK5 in hyperthermia-induced apoptosis, though further investigation is required to determine the effects of hyperthermia on CDK5 activity and on the function of CDK5 targets. Furthermore, I established a role for Noxa phosphorylation by observing that overexpression of non-phosphorylatable Noxa is able to induce Mcl-1 degradation and caspase-3 activation more effectively than overexpression of wild-type Noxa. Lastly, I was unable to demonstrate that Noxa is phosphorylated on serine-13 by CDK5 in PErTA cells, suggesting that Noxa may not be
phosphorylated by CDK5 in vivo, and subsequently was unable to determine whether
phosphorylation of Noxa is altered during hyperthermia. Further studies will reveal whether
Noxa is phosphorylated on serine-13 in vivo, allowing for future investigation into whether Noxa
phosphorylation can be used as a therapeutic target for diseases such as cancer and
neurodegenerative disorders.
References


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