Regulation of the BH3-only protein PUMA by growth factor signalling

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Abstract

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J.J. Sandow

P53 Upregulated Modifier of Apoptosis (PUMA), a pro-apoptotic member of the Bcl-2 family, is transcriptionally activated by p53 and is a key effector of p53-dependent apoptosis. We show that PUMA protein is subject to rapid post-translational regulation by phosphorylation at a conserved residue, serine 10, following serum or Interleukin-3 (IL-3) stimulation. Serine 10 is not within the BH3 domain and PUMA phosphorylated at serine 10 retained the ability to co-immunoprecipitate with anti-apoptotic Bcl-2 family members. However, phosphorylated PUMA was targeted for proteasomal degradation indicating that it is less stable than unphosphorylated PUMA. Importantly, we identified NEMO/IKK1/IKK2 as the kinase complex that interacts with and phosphorylates PUMA thereby also demonstrating that IL-3 activates NFκB signalling. This thesis therefore identified and characterised a novel survival pathway with important implications for IL-3 signalling and haemopoietic cell development.

Declaration

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Publications arising from this Thesis

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Table of contents

Abbreviations

4-OHT	4-hydroxytamoxifen
CHX	Cycloheximide
CID	Collision-induced dissociation
CMV	Cytomegalovirus
ESI	Electrospray ionization
ETD	Electron-transfer dissociation
FBS	Foetal Bovine Serum
FDM	Factor dependent myeloid
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HA	Haemagglutinin
IL-3	Interleukin-3
IL-5	Interleukin-5
IP	Immunoprecipitation
LPS	Lipopolysaccharide
MOMP	Mitochondrial outer membrane permeabilisation
TNFα	Tumour Necrosis Factor alpha
UV	Ultraviolet
Wt	Wild type

Chapter 1

Introduction

1.1 Introduction

The balance between survival and death in many types of haematopoietic cells is regulated by signals originating from cell surface receptors binding with specific cytokines. In the presence of cytokine cells are stimulated to survive, proliferate and activate whilst withdrawal of cytokine initiates a system of programmed cell death, known as apoptosis (Strasser, 2005). Recent data indicates that key mediators of the survival/death decision are members of the Bcl-2 family of proteins, in particular a sub-group of this family, the BH3-only proteins (Alves *et al.*, 2007). Investigations have shown a clear role for the BH3-only proteins in the mediation of an apoptotic response, however, the key players in this response are yet to be completely elucidated. While several of the BH3-only proteins have been proposed as important for an apoptotic response to cytokine withdrawal one member, PUMA, now appears to play a significant and vital role (Ekert *et al.*, 2006; Ekoff *et al.*, 2007; Jeffers *et al.*, 2003; Ming *et al.*, 2008).

1.2 Cytokine regulation of the haematopoietic system

A function essential to the body's ability to maintain a homeostatic balance of the haematopoietic system or to mount a successful immune/inflammatory response is the ability to control the behaviour of distinct subsets of immune cells which will respond to a particular situation. These actions are controlled by a group of haematopoietic cytokines with many having a multitude of functions. Each cytokine has a specific distinct receptor complex on the surface of a target cell although many cytokines within a family may share common elements of each receptor complex (Kitamura *et al.*, 1991; Metcalf and Nicola, 1995). A single cytokine can elicit a number of different responses signalling through the same receptor such as survival, proliferation or activation. The decision to take any particular course of action is determined by the regulation of domains and motifs specific for that response within the intra-cellular region of the receptor (Guthridge *et al.*, 2006; Santini *et al.*, 2003; Sato *et al.*, 1993).

The populations of cells required for the basal haematopoiesis are regulated by multiple cytokines with haematopoietic stresses promoting peaks in specific cytokines to elicit the required response. The haematopoietic stem cell (HSC) is a pluripotent population of cells

from which every cell in the haematopoietic system is derived, with proof of their pluripotency demonstrated by their ability to reconstitute the haematopoietic system when serially transplanted into irradiated animals (Osawa *et al.*, 1996; Zhu and Emerson, 2002). The HSC population is largely quiescent and is maintained in this state by signals from the bone marrow stroma such as intergrins, connexions, stem cell factor, fibroblast growth factor-1 and -2 and stromal-derived factor-1 (Fuchs and Segre, 2000). There are a number of cytokines that have been discovered which regulate the proliferation/activation of haematopoietic precursors. Cytokines that influence the differentiation and activation of cells from a myeloid lineage include erythropoietin, thrombopoietin, macrophage colony-stimulating factor, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and IL-5 (Lotem and Sachs, 2002). GM-CSF, IL-3 and IL-5 are often present during an allergic response or autoimmune disease effected by monocytes and have a significant role in regulating the haematopoietic response (Hamilton, 2002).

1.2.1 GM-CSF/IL-3/IL-5 regulate myeloid cell proliferation, differentiation and survival

GM-CSF, IL-3 and IL-5 are a unique subgroup of cytokines which act to link the innate immune response and the regulation of haematopoiesis (Metcalf, 2008). These cytokines are pleiotropic and stimulate proliferation, survival, and differentiation of myeloid haematopoietic cells and also activate terminally differentiated myeloid cells. While redundancy between the three cytokines has been observed in cells that respond to all three cytokines, there are specific roles for each cytokine in distinct populations of cells. The GM-CSF receptor is present in various myeloid cell lineages including most types of myeloid progenitors and is responsible for stimulating the production of granulocytes, macrophage, and dendritic cell populations (Arai *et al.*, 1990; Mellman and Steinman, 2001; Nicola, 1994). The IL-3 receptor is present on early haematopoietic progenitor cells and on certain populations of progenitor and mature myeloid cells (Arai *et al.*, 1990; Lopez *et al.*, 1986; Nicola, 1994). In addition to promoting differentiation, GM-CSF and IL-3 also work by inducing the effector function of their target cells, assisting in the response to pathogens (Arai *et al.*, 1990; Nicola, 1994). The IL-5 receptor is primarily expressed on eosinophils and is responsible for regulating their production and activation in response to parasites and has also been found to contribute to allergic reactions (Arai *et al.*, 1990;

Nicola, 1994). In addition to its role in haematopoiesis, GM-CSF has also been implicated in promoting the transformation and survival of a number of different forms of cancer including acute myeloid leukaemia, chronic myeloid leukaemia, juvenile myelomonocytic leukaemia and other cancers unrelated to the haematopoietic system such as melanoma, breast, lung and prostate cancers (Baldwin *et al.*, 1989; Baldwin *et al.*, 1991).

1.2.2 Signalling mechanisms of the GM-CSF, IL-3, IL-5 receptor family

The receptors for GM-CSF, IL-3, and IL-5 each consist of an α-chain which is specific to each cytokine and has low affinity binding (Gearing *et al.*, 1989; Kitamura *et al.*, 1991; Tavernier *et al.*, 1991), and a common β-chain (βc) which is shared amongst the three cytokines (in mice there is an additional βc which is specific to IL-3) (Hara and Miyajima, 1992; Hayashida *et al.*, 1990; Nicola *et al.*, 1997). The βc is unable to bind to cytokines alone and acts by associating with and converting the ligand-bound α-chain to a high affinity state, a process which appears to be necessary for most signalling from the receptor (Bagley *et al.*, 1997; Hayashida *et al.*, 1990; Kitamura *et al.*, 1991). Once assembled the receptor complex is capable of activating a number of different signalling pathways including the JAK/STAT, MAP kinase and phosphatidylinositol 3-kinase (PI3-K) pathways (de Groot *et al.*, 1998). In regard to GM-CSF, the receptor complex can form several conformations which are thought to be dependent on the concentration of ligand available. Under low concentrations (<10 pM) of GM-CSF a hexamer forms consisting of 2 GM-CSF molecules, 2 alpha chains, and 2 β chains. When higher concentrations of GM-CSF are available (>10 pM) two hexamer complexes interact to form a dodecamer (Hansen *et al.*, 2008). The decision to activate the different signalling pathways downstream of the two complexes is governed by the mutually exclusive phosphorylation of Ser585 and Tyr577 residues in a bidentate motif within the βc (Guthridge *et al.*, 2006; Hansen *et al.*, 2008). Concentrations of GM-CSF that generate a hexamer receptor complex lead to phosphorylation of Ser585 by PKA, the recruitment of 14-3-3 and the PI3-kinase subunit p85 resulting in the activation of downstream molecules such as Akt/PKB. The effect of Ser585 phosphorylation is to promote cell survival alone in TF-1 cells with the absence of cell proliferation (Figure 1.1). Treatment of cells with higher doses of GM-CSF generate a dodecamer receptor complex which leads to the activation of JAK2 and phosphorylation of

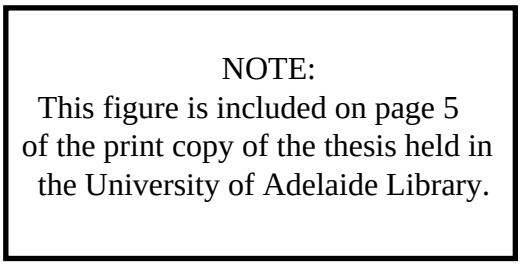


Figure 1.1 Model for the regulation of survival, proliferation and activation by the phosphotyrosine/phosphoserine binary switch. Low concentrations of GM-CSF lead to the phosphorylation of βc Ser585 and activation of the 14-3-3/PI-3 kinase survival-only pathway. Higher concentrations of GM-CSF trigger the formation of the receptor dodecamer complex promoting βc Tyr577 phosphorylation and leading to activation of the JAK/Stat, Ras/MAPK, and PI-3 kinase pathways supporting proliferation and activation of cells in addition to survival. (Figure adapted from Hercus et al., 2009)

tyrosine residues on the βc, including Tyr577. Phosphorylation of Tyr577 blocks the ability of PKA to phosphorylate Ser585, inhibiting the recruitment of 14-3-3 and instead promoting binding of Shc. While phosphorylation of Ser585 promotes signalling through PI3-kinase alone and is only able to promote cell survival, Tyr577 activates PI3-kinase in addition to STAT5 and MAP kinases such as ERK leading not only to cell survival but also promotes cell proliferation and activation (Figure 1.1). This mechanism of dose response allows for a pleiotropic response from a cell for stimulation with the same cytokine through the same receptor.

1.3 Apoptosis

Stimulation of haematopoietic cells with cytokines is able to promote the survival and proliferation of the cell, however, withdrawal of cytokines leads to the activation of a system of programmed cell death, known as apoptosis. Apoptosis is an essential process that occurs during development and in tissue homeostasis to remove superfluous, defective, damaged, or dangerous cells (Kerr *et al.*, 1972; Vaux and Strasser, 1996). There are two conserved signalling pathways that are responsible for the control of apoptosis in metazoans. The death receptor-induced apoptosis and the intrinsic death pathway. Of the two apoptotic pathways available to a cell, cytokine withdrawal is primarily controlled through the intrinsic (also known as the mitochondrial) pathway in a process that is regulated predominantly by members of the Bcl-2 family of proteins (Alves *et al.*, 2007; Strasser, 2005).

1.3.1 Mechanism of apoptosis through the intrinsic pathway

A diverse array of developmental and environmental stimuli such as ultraviolet and γ-irradiation, anoxia, drug treatment, DNA damage, loss of cell-matrix adhesion or growth factor deprivation can lead to cell death via the intrinsic, Bcl-2-regulated, apoptosis pathway (Adams, 2003; Cory *et al.*, 2003; Danial and Korsmeyer, 2004). While there is a diverse range of stimuli to initiate apoptosis, they all ultimately lead to the disruption of the outer mitochondrial membrane, known as mitochondrial outer membrane permeabilisation (MOMP). Disruption of the mitochondrial membrane leads to the release of several apoptogenic proteins such as cytochrome c, Smac/Diablo and HtrA2/Omi from the inner

membrane space to the cytosol and subsequent caspase activation (Green and Evan, 2002). Caspases are a family of aspartic-acid-specific cystine proteases that can be subdivided into two groups; the effector caspases (such as mammalian caspase-3, -6 and -7) which are responsible for most proteolysis of vital structural proteins and the initiator caspases (such as mammalian caspase-8 and -9), which work to activate the effector caspases by proteolysis (Green and Evan, 2002). The predominant mechanism for initiator caspase activation in the intrinsic apoptotic program is through cytochrome c mediated activation of caspase-9. Once cytochrome c is released into the cytosol by MOMP it binds to the cytosolic adapter protein Apaf-1 (apoptotic-protease-activating factor 1) to form a complex known as the apoptosome in the presence of dATP or ATP. The apoptosome then recruits procaspase-9 where it undergoes autocatalytic activation and can go on to activate the effector caspases (Adams, 2003; Danial and Korsmeyer, 2004; Shi, 2002). Once activated, the effector caspases cleave vital structural proteins, such as lamins or spectrin, and proteolytically activate enzymes that lead to a process of chromatin condensation, plasma membrane blebbing and cellular 'demolition' (Figure 1.2) (Degterev *et al.*, 2003; Shi, 2002).

1.3.2 The Bcl-2 family of proteins

Proteins in the Bcl-2 family are the regulators of the intrinsic apoptotic pathway and consist of both pro-survival and pro-apoptotic members. All members contain between one and four distinct regions of homology, which are known as Bcl-2 Homology domains (BH1-BH4) with the regions between the BH domains often being largely divergent (Chittenden *et al.*, 1995; Huang *et al.*, 1998; Yin *et al.*, 1994).

In mammals, the pro-survival family members include Bcl-2 (B-cell lymphoma 2), Bcl-x_L, Bcl-w, A1 and Mcl-1 (Figure 1.3) (Danial and Korsmeyer, 2004). These proteins contain all four of the BH domains except for Mcl-1 which has only two. The pro-survival nature of these proteins is demonstrated by overexpression in cultured cells leading to protection from a variety of apoptotic stimuli (Danial and Korsmeyer, 2004; Strasser *et al.*, 2000; Vaux *et al.*, 1988; Wang, 2001). Illustration of the importance of the different pro-survival proteins for maintenance of cell survival in distinct populations of cells is provided by

Figure 1.2 Model for caspase activation by the intrinsic apoptosis pathway. A variety of death stimuli leads to MOMP and release of cytochrome c. Cytochrome c then binds to APAF-1 which results in the activation of caspase-9 by cleavage of pro-caspase-9. Active caspase-9 then cleaves the effector caspases which cleave a number of vital proteins leading to cell death. (Figure adapted from Strasser, 2005)

Figure 1.3 The mammalian Bcl-2 family members. The Bcl-2 family contains both pro-survival and pro-apoptotic members. There are two sub-families of pro-apoptotic members, the multidomain and the BH3-only. (Figure adapted from Strasser, 2005)

gene-targeting experiments. Loss of Bcl-2 causes abnormal cell death in lymphocytes and melanocytes (Nakayama *et al.*, 1993; Veis *et al.*, 1993) while this occurs in erythroid progenitors and neurons in mice lacking Bcl-x_L (Motoyama *et al.*, 1995). Mice lacking Mcl-1 are unable to progress past an early stage of embryonic development and conditional deletion in B- or T-cell lineages demonstrates an inability to maintain mature lymphocyte populations (Opferman *et al.*, 2003), and inducible deletion of Mcl-1 in adult mice results in the loss of committed myelocytic progenitors and haematopoietic stem cells (Opferman *et al.*, 2005). Bcl-w knockout mice have demonstrated that it plays a niche role during spermatogenesis with no other processes affected by its loss (Print *et al.*, 1998; Ross *et al.*, 1998) while A1 knockout mice have defects in mast cell survival following allergic activation (Xiang *et al.*, 2001).

The pro-apoptotic Bcl-2 family members can be subdivided into two groups, the multidomain family of proteins which contain several BH domains and the BH3-only family of proteins which contain only the BH3 domain. In mammals there are several members of the multidomain family including Bax, Bak, Bok and Bcl-X_S, of which Bax and Bak play the predominant role in regulating apoptosis (Figure 1.3) (Strasser, 2005). Overexpression of the multidomain members leads to the initiation of apoptosis (Danial and Korsmeyer, 2004; Strasser *et al.*, 2000; Wang, 2001). The exact mechanism by which these multidomain proteins are activated is unclear, however, it is known to involve homotypic and heterotypic interactions with other Bcl-2 family members before the subsequent oligomerisation, the formation of pores in the mitochondrial membrane, induction of MOMP and caspase activation (Wei *et al.*, 2001). The essential role of these proteins for apoptosis is demonstrated by the finding that cells lacking both Bax and Bak are almost completely resistant to a diverse array of cellular stresses including DNA damage, growth factor deprivation, endoplasmic reticulum stress, or treatment with certain cytotoxic drugs (Lindsten *et al.*, 2000; Rathmell *et al.*, 2002; Wei *et al.*, 2001). Bax and Bak represent a critical step in the commitment to apoptosis as cells that have Bax and Bak removed retain proliferative capacity, clonogenic potential and long term survival after withdrawal of essential growth factors while loss of other factors in the apoptotic pathway such as Apaf-1 and Caspase-9 only delays the onset of apoptosis (Ekert *et al.*, 2004; Marsden *et al.*, 2002).

The second group of pro-apoptotic Bcl-2 proteins is the BH3-only family which contains upward of eight members in mice and humans and share little sequence similarity with each other except within a BH3 domain (9-16 amino acids) (Willis and Adams, 2005). The BH3-only family includes Bad (Bcl-2-antagonist of cell death) (Yang *et al.*, 1995), Bik (Bcl-2 interacting killer) (Boyd *et al.*, 1995; Han *et al.*, 1996; Hegde *et al.*, 1998), Bid (Wang *et al.*, 1996), Hrk (harakiri) (Imaizumi *et al.*, 1997; Inohara *et al.*, 1997), Bim (Bcl-2-interacting mediator of cell death) (Hsu *et al.*, 1998; O'Connor *et al.*, 1998), Noxa (Oda *et al.*, 2000), PUMA (p53 upregulated modulator of apoptosis; also known as Bbc3) (Han *et al.*, 2001; Nakano and Vousden, 2001; Yu *et al.*, 2001) and Bmf (Bcl-2-modifying factor)(Puthalakath *et al.*, 2001)(Figure 1.3). Like the multi-domain pro-apoptotic members, all BH3-only family members trigger apoptosis when overexpressed in a process that is dependent on the presence of Bax and Bak (Huang and Strasser, 2000; Lindsten *et al.*, 2000; Rathmell *et al.*, 2002; Wei *et al.*, 2001).

1.3.3 BH3-only family members engage their pro-survival counterparts

All BH3-only family members have the ability to bind to some, if not all, of the pro-survival Bcl-2 family members (Chen *et al.*, 2005). The binding of BH3-only family members to their pro-survival counterparts antagonises their pro-survival activity. The interaction between the BH3-only proteins and their pro-survival counterparts has been shown in a crystal structure of Bim/Bcl-x_L interaction with a hydrophobic pocket created by the BH1, BH2 and BH3 domain in Bcl-x_L binding to four conserved hydrophobic residues situated in the BH3-only domain of Bim (Liu *et al.*, 2003). This illustrates why a strict requirement for the interaction of BH3-only proteins with Bcl-2 like molecules is a functional BH3 domain. However, there is selectivity for binding partners with BH3-only members such as Bim, PUMA and tBid (a truncated form of Bid) able to engage all of the pro-survival Bcl-2 family members while other BH3-only family members have very selective binding such as Bad and Bmf binding only to Bcl-2, Bcl-x_L and Bcl-w while Noxa is only able to bind A1 and Mcl-1 (Figure 1.4)(Chen *et al.*, 2005). This differential binding is thought to aid the specificity of apoptotic response of cells to the diversity of stress stimuli by utilising different Bcl-2 family members for each response.

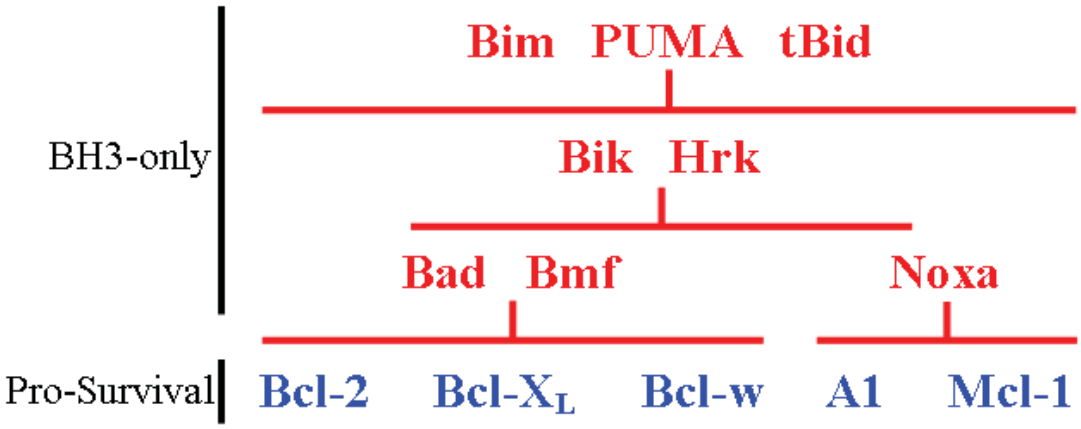


Figure 1.4 BH3-only binding preferences for their pro-survival counterparts. BH3-only proteins have different binding preferences for each of their Bcl-2 family pro-survival counterparts. Bim, PUMA and tBid are promiscuous and bind all of the pro-survival molecules while other BH3-only molecules have a more restricted binding profile.

1.3.4 Activation of Bax and Bak leads to mitochondrial outer membrane permeabilisation and caspase activation

Bak and Bax are required for apoptosis through the intrinsic apoptotic pathway and their activation is regulated by the BH3-only family members. Upon activation of Bak and Bax they oligomerise and associate with the mitochondrial outer membrane where they form pores leading to MOMP and release of apoptogenic proteins (Willis and Adams, 2005). It is presently unclear whether the BH3-only family members directly or indirectly activate Bak and Bax to induce apoptosis and several models have been suggested to explain this event.

In one model (displacement/indirect), the pro-survival proteins function as regulators of Bak and Bax by interacting with them. This model proposes that under normal survival conditions, the pro-survival Bcl-2 family members bind Bak and Bax, inhibiting their activation. Activation of the BH3-only family members leads to competition for binding to the pro-survival Bcl-2 family members, resulting in the release of Bak and Bax and subsequent oligomerisation, pore formation and disruption of the mitochondrial membrane leading to MOMP (Figure 1.5A). Evidence to support this model is provided by studies in MEFs that showed the pro-survival proteins Mcl-1 and Bcl-X_L constrain an active conformer of Bak by direct binding and this was able to inhibit apoptosis (Willis *et al.*, 2005). In order to induce apoptosis in these cells, Bak must be displaced from the two Bcl-2 family members by BH3-only proteins. This was achieved by using a combination of Noxa and Bad which have very selective binding and are only able to bind Mcl-1 and Bcl-x_L respectively. Expression of these proteins alone was ineffective at inducing apoptosis, presumably because the Bcl-2 family member that they are unable to bind to is still able to inhibit Bak activation. In cells lacking Bcl-x_L, Noxa alone is able to induce Bak mediated apoptosis. Further evidence to support this conclusion is the use of a promiscuous BH3-only protein such as PUMA which is able to interact with all of the pro-survival Bcl-2 family members. PUMA alone was able to induce apoptosis and did not require additional BH3-only family members. Experiments using BH3 domain peptides from a selection of BH3-only proteins displayed affinity for their pro-survival counterparts but could not be detected interacting with Bax or Bak. Use of full length BH3-only proteins also supported these findings with only the Bim_S isoform detected binding to Bax and loss of this ability

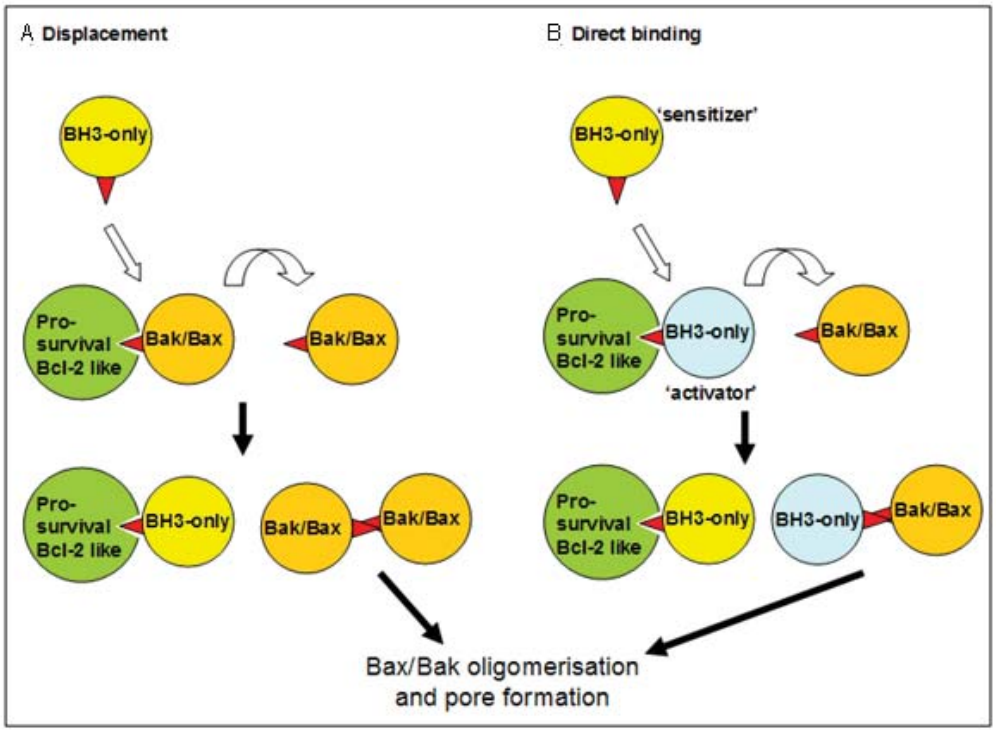


Figure 1.5 Models for the activation of Bax and Bak by BH3-only proteins. (A) The displacement model proposes that the active Bax and Bak are sequestered by the pro-survival Bcl-2 family members. BH3-only proteins are required to compete for binding to the pro-survival molecules to displace Bax and Bak before they undergo oligomerisation and pore formation. (B) The direct binding model proposes that there are two classes of BH3-only protein, 'sensitisers' and 'activators'. Sensitiser BH3-only proteins cannot interact with Bax and Bak and only serve to release activator BH3-only proteins that are sequestered by the pro-survival Bcl-2 family members. Once an activator BH3-only protein is released from the pro-survival molecules, it can directly interact with Bax and Bak to activate them leading to oligomerisation and pore formation. (Figure adapted from Willis and Adams, 2005)

by mutation had no measurable effect on its ability to induce apoptosis (Willis *et al.*, 2007). This model suggests that all BH3-only family members act to induce Bak/Bax activation in the same manner with regulation of the response being provided by the different affinities that the BH3-only proteins have for their pro-survival counterparts.

The second model (direct binding) which has been proposed for Bak and Bax activation suggests that the BH3-only proteins do not all act in the same way to activate Bax and Bak and instead are assigned two distinct roles. In this model some BH3-only proteins act as 'sensitisers' which only serve to inactivate the pro-survival Bcl-2 family members, or 'activators' which are able to directly engage and activate Bax and Bak (Figure 1.5B). Under survival conditions the activators which include Bim, tBid and possibly PUMA are sequestered by the pro-survival Bcl-2 family members. Following apoptotic stimuli, the sensitiser BH3-only proteins which include all of the remaining BH3-only family members bind to their pro-survival counterparts, displace the activator molecules and allowing them to directly interact with Bax and Bak, promoting their activation, oligomerisation, pore formation and MOMP (Willis and Adams, 2005). Evidence to support this model was originally provided by the observation that isolated mitochondria which were exposed to BH3 domain peptides from Bim and Bid released Cytochrome c, however, BH3 domain peptides from Bad or Bik were only able to achieve this with limiting amounts of a Bim or Bid peptide (Certo *et al.*, 2006; Gavathiotis *et al.*, 2008; Kuwana *et al.*, 2005; Letai *et al.*, 2002; Lovell *et al.*, 2008; Walensky *et al.*, 2006). Direct activation of Bax and Bak by the activator proteins was supported using mutant Bax and Bak that were not able to bind to the pro-survival Bcl-2 family members, a factor which in the alternate model should lead to MOMP. Despite their inability to bind to the pro-survival proteins, they were only able to promote MOMP in the presence of the activator BH3-only proteins (Kim *et al.*, 2006b). However, the necessity of Bid and Bim to activate Bax and Bak was contested by the observation that PUMA expression can induce apoptosis in cell which lacks both Bid and Bim (Jabbour *et al.*, 2009). One possible explanation is that recent studies have implicated PUMA as a potential activator protein (Cartron *et al.*, 2004; Gallenne *et al.*, 2009; Kim *et al.*, 2006b) although studies from other groups go against these observations (Cartron *et al.*, 2004; Chipuk *et al.*, 2008; Jabbour *et al.*, 2009; Kuwana *et al.*, 2005; Willis *et al.*, 2007). A recent publication sought to investigate the two opposing models in a physiological setting by generating knock-in mutants of Bim that contained BH3 domains

from other BH3-only proteins such as Bad, Noxa and PUMA. These mutants bound the expected subsets of pro-survival molecules and lost the ability to bind Bax. Analysis of the mouse response to genetic and physiological stimuli indicated that the ability of Bim to induce apoptosis is not entirely due to interactions with pro-survival molecules nor is it solely due to interactions with Bax (Merino *et al.*, 2009). Therefore, while the two models have been largely presented as mutually incompatible it now seems likely that Bax and Bak oligomerisation induced by a diverse range of stimuli has aspects of each in the eventual response.

1.4 Growth factor regulation of the intrinsic apoptotic pathway

During development and in an adult, cytokine signalling regulates cell survival to maintain homeostasis and prevent neoplastic disease. Whilst stimulation of cells with cytokines promotes activation of pro-survival signalling pathways, withdrawal of cytokines leads to the removal of excess, auto-reactive and unwanted cells by initiating apoptosis (Marrack and Kappler, 2004; Raff, 1992). Bcl-2 family members were initially implicated in growth factor withdrawal induced apoptosis with the observation that Bcl-2 had anti-apoptotic properties and could protect cells from apoptosis (Vaux *et al.*, 1988). Later work supported this by demonstrating that apoptosis of cytokine dependent cells in response to cytokine withdrawal is controlled by regulating the intrinsic apoptosis pathway through Bcl-2 family members. These experiments established that apoptosis can be inhibited by combined deletion of pro-apoptotic Bax and Bak or overexpression of pro-survival Bcl-2 or Bcl-x_L (Rathmell *et al.*, 2002; Sentman *et al.*, 1991; Wei *et al.*, 2001). Because of their critical role in controlling apoptosis the Bcl-2 family members are tightly regulated both at a transcriptional and at a translational level.

1.4.1 Transcriptional regulation

There are a number of different signalling pathways downstream of an activated cytokine receptor. One of the best described pathways responsible for regulating cell survival is the PI3-K/Akt signalling pathway. When cytokines are present, Akt is activated and has been shown to increase expression of pro-survival Bcl-2, promoting T cell survival (Song *et al.*, 2004). Activated Akt is also responsible for phosphorylating Forkhead box (FoxO)

transcription factors, inhibiting their activity by promoting their exclusion from the nucleus (Bradley *et al.*, 2005; Brunet *et al.*, 2000; Maurer *et al.*, 2006; Tang *et al.*, 1999). Following cytokine withdrawal, Akt signalling is lost promoting dephosphorylation of FoxOs and enabling entry into the nucleus leading to transcription of target genes. FoxO3a has been demonstrated to promote apoptosis by upregulating *Bim* and *PUMA* expression in haematopoietic cells in response to cytokine withdrawal (Dijkers *et al.*, 2000; You *et al.*, 2006). Other transcription factors such as p53 and p73 are also utilised in response to growth factor and cytokine withdrawal in normal and leukaemia cells to upregulate *PUMA* expression (Jabbour *et al.*, 2010; Ming *et al.*, 2008).

1.4.2 Post-translational regulation

Transcriptional controls regulate the production of new protein, however, there are also post-translational mechanisms that are employed to regulate the activity and stability of proteins that are already present in cells. Bid is translated as an inactive protein and requires truncation of the protein by cleavage (tBid) for it to gain apoptotic activity (Li *et al.*, 1998; Luo *et al.*, 1998). The activity of Bad and Bim is restricted by IL-3 signalling in a process that involves sequestration of the phosphorylated Bad and Bim by 14-3-3 proteins (Qi *et al.*, 2006; Zha *et al.*, 1996). Withdrawal of IL-3 or mutation of the serine residues in Bad or Bim prevented their interaction with 14-3-3 and promotes binding to, and inhibition of, the pro-survival Bcl-2 family members leading to increased apoptosis (Datta *et al.*, 1997; del Peso *et al.*, 1997; Qi *et al.*, 2006). However, in seizure-injured hippocampi of rats Bid is truncated and promoted to interact with 14-3-3 (Shinoda *et al.*, 2003) indicating that 14-3-3 may sequester both active and inactive BH3-only family members. In addition to interactions with 14-3-3, Bim can undergo phosphorylation at several different sites in response to growth factor signalling leading to changes in stability and interactions with pro-survival Bcl-2 family members (Hubner *et al.*, 2008). Bad can also be phosphorylated within its BH3-domain following growth factor treatment, inhibiting its ability to antagonise the pro-survival Bcl-2 family members and prevent apoptosis (Datta *et al.*, 2000; Virdee *et al.*, 2000). The stability of pro-survival proteins such as Bcl-2 and Mcl-1 is also regulated by cytokine signalling (Deng *et al.*, 2004; Maurer *et al.*, 2006). Cytokine signalling is known to promote activation of Akt which in turn inactivates GSK-3α and GSK-3β (Cross *et al.*, 1995). IL-3 dependent murine cell lines rely on activated Akt from cytokine signalling to survive. Following IL-3 withdrawal,

GSK-3 is activated and can phosphorylate Mcl-1, a modification that promotes Mcl-1 degradation and subsequent increase in apoptosis (Maurer *et al.*, 2006).

1.4.3 Important regulators of apoptosis in growth factor and cytokine dependent cells

The BH3-only proteins are key mediators of the intrinsic apoptotic pathway in response to a variety of apoptotic stimuli, including withdrawal of cytokines such as IL-3 (Willis and Adams, 2005). Several members of the BH3-only family have been implicated as potential regulators including Bad and Bim which were found to be phosphorylated on serine residues by Akt in response to IL-3 signalling, a modification that results in their association with 14-3-3 (Qi *et al.*, 2006; Yang *et al.*, 1995; Zha *et al.*, 1996). These results suggest a potential mechanism for cytokine regulation of apoptosis in haematopoietic cells mediated by BH3-only proteins, however, these studies often used over-expression systems to demonstrate their effects leading to doubts about their physiological relevance. Further evidence to implicate Bim in the response to cytokine withdrawal is provided by knockout studies where bone marrow cultures from *Bim*$^{-/-}$ mice grew a larger number of lymphoid and myeloid colonies after cytokines were restored when compared to the wild-type cultures (Bouillet *et al.*, 1999). PUMA has also been implicated in the apoptotic response to cytokine withdrawal as it was found that *PUMA*$^{-/-}$ MEF cells were able to survive longer than wild type MEFs after starvation from cytokines (Villunger *et al.*, 2003). Further evidence demonstrated primary myeloid progenitors from *PUMA*$^{-/-}$ mice were able to survive significantly longer when cultured without IL-3 than their *Bim*$^{-/-}$ or wild type counterparts (Jeffers *et al.*, 2003). In a study that sought to clarify the role of each BH3-only protein in the apoptotic response to cytokine withdrawal, IL-3 dependent myeloid cells were generated from mice lacking *PUMA*, *Bad*, both *Bad* and *Bim*, or both *Bax* and *Bak* (Ekert *et al.*, 2006). As expected the *Bax*$^{-/-}$*;Bak*$^{-/-}$ cells showed long term survival after cytokine withdrawal, however, in contrast to what has been suggested from previous evidence only the *PUMA*$^{-/-}$ and not *Bad*$^{-/-}$ or *Bad*$^{-/-}$*;Bim*$^{-/-}$ cells were able to form colonies on soft agar containing IL-3 after a period of IL-3 deprivation. It was also found that in response to IL-3 withdrawal, Cytochrome c release from the mitochondria was significantly inhibited in the *PUMA*$^{-/-}$ cells while both *Bad* and *Bim* had to be removed together to have a similar effect. When regulation of the endogenous BH3-only proteins in these IL-3 dependent cells was investigated it demonstrated that there was little regulation of the serine residues responsible for the Bad/14-3-3 interaction suggesting that Bad

regulation by this mechanism has limited significance in these cells. It was also shown that *PUMA*$^{-/-}$ cells but not *Bad*$^{-/-}$ or *Bim*$^{-/-}$ had impaired activation of Bax in response to cytokine withdrawal (Ekert *et al.*, 2006). Additional studies have also discovered the importance of PUMA in primary Mast cells which are dependent on IL-3 signalling for survival. Loss of PUMA significantly protected Mast cells from apoptosis when IL-3 was withdrawn (Ekoff *et al.*, 2007). These results raise questions about the role of Bad or Bim which were previously thought to mediate apoptosis in IL-3 dependent cells and instead suggest that PUMA is a key mediator of apoptosis in these cells.

1.5 PUMA

PUMA was initially discovered by three independent groups. 2 groups identified it as a transcriptional target of p53 through gene expression profiling and named it PUMA (p53 upregulated modulator of apoptosis)(Nakano and Vousden, 2001; Yu *et al.*, 2001) and the third identifying it as an interacting partner of Bcl-2 through a yeast two-hybrid screen naming it Bbc3 (Bcl-2 binding component 3)(Han *et al.*, 2001). PUMA DNA and protein has high sequence similarity between human and rodents and is conserved as far back as Zebrafish (Sidi *et al.*, 2008; Yu *et al.*, 2001). There are two major isoforms of PUMA which contain a BH3 domain, PUMA-α and PUMA-β, although most publications to date focus on PUMA-α (Nakano and Vousden, 2001; Yu *et al.*, 2001). Most PUMA protein within a cell localises to the mitochondria due to uncharacterised regions within the C-terminus downstream of the BH3 domain with both regions reported to be important for PUMA activity (Yee and Vousden, 2008; Yu *et al.*, 2003b).

1.5.1 Regulation of PUMA in the response to apoptotic stimuli

PUMA was originally identified as a novel protein that was transcriptionally upregulated in response to p53 activation (Nakano and Vousden, 2001; Yu *et al.*, 2001). In response to DNA damaging agents such as a large variety of genotoxic agents or UV and γ-irradiation, p53 upregulates *PUMA* by binding to sites within the *PUMA* promoter (Jeffers *et al.*, 2003; Kaeser and Iggo, 2002; Sidi *et al.*, 2008; Wang *et al.*, 2007; You *et al.*, 2006; Yu *et al.*, 2003b; Yu *et al.*, 2001). Other agents such as proteasome inhibitors, microtubule poisons and neurotoxins are also responsible for p53-dependent upregulation of *PUMA* (Ding *et*

al., 2007; Giannakakou *et al.*, 2002; Gomez-Lazaro *et al.*, 2005; Yu *et al.*, 2003a). The same p53-response element can also be utilised by p73 to regulate *PUMA* expression in response to a number of different stimuli (Matallanas *et al.*, 2007; Melino *et al.*, 2004; Ming *et al.*, 2008).

As described previously, the forkhead box family of proteins, particularly FoxO3a, regulates *PUMA* expression in response to growth factor and cytokine withdrawal (You *et al.*, 2006). The *PUMA* promoter is also a direct target of NFκB following TNFα treatment (Wang *et al.*, 2009). CHOP, E2F1 and c-Myc have also been reported to upregulate *PUMA* expression in response to diverse stimuli (Fernandez *et al.*, 2003; Futami *et al.*, 2005; Hershko and Ginsberg, 2004; Li *et al.*, 2006; Zou *et al.*, 2009). Other transcription factors have been discovered to downregulate *PUMA* expression including Slug (Zilfou *et al.*, 2005), splice varients of *p73* (Melino *et al.*, 2004; Nyman *et al.*, 2005) and *p63* (Rocco *et al.*, 2006). Post-transcriptional regulation of PUMA mRNA stability and translation has also been reported to be regulated by MAP4K3 signalling (Lam *et al.*, 2009) and a virally encoded microRNA, miR-BART5 (Choy *et al.*, 2008).

1.5.2 The role of PUMA in Cancer

The role of apoptosis as an essential process to remove cancerous cells and reduce oncogenesis has been clearly established with impaired apoptosis contributing to a lack of responsiveness of some malignancies to anticancer therapies (Adams and Cory, 2007; Johnstone *et al.*, 2002). Common treatments for cancer such as irradiation and chemotherapeutic drugs rely, in part, on an upregulation of *PUMA* in a p53-dependent manner. However, with more than half of human tumours containing *p53* mutations this response can often be attenuated or absent (Vogelstein and Kinzler, 2004; Yu and Zhang, 2005). In numerous cancers, *PUMA* itself does not appear to be the direct target of genetic inactivation (Ahn *et al.*, 2008; Hoque *et al.*, 2003; Kim *et al.*, 2007; Yoo *et al.*, 2007). However, deletion of PUMA has been demonstrated to co-operate with other oncogenic lesions, such as deregulated c-myc expression, to promote tumour development (Garrison *et al.*, 2008; Hemann *et al.*, 2004; Michalak *et al.*, 2009) and PUMA is often silenced in paediatric ALL (Davidsson *et al.*, 2009). In addition to the inhibition of pro-apoptotic activity, the development and progression of tumours is often accompanied by the over-

expression of pro-survival Bcl-2 family members which would serve to actively oppose any PUMA tumour-suppressing activity (Adams and Cory, 2007). Animal studies have also highlighted a potential role for PUMA in the development of cancer because of an increased risk for spontaneous malignancies when PUMA and Bim are knocked out in mice when compared to Bim alone, however, it must be noted that no increased risk is seen for PUMA alone (Erlacher *et al.*, 2005; Jeffers *et al.*, 2003; Villunger *et al.*, 2003).

1.6 Aims of this Thesis

The overall aim of my thesis is to identify mechanisms of cell survival which are regulated by cytokine signalling, in particular members of the IL-3/IL-5/GM-CSF family of cytokines.

Numerous types of cells require signals from growth factors and cytokines to remain viable with loss of these signals initiating a series of events that eventually leads to a cell undergoing apoptosis. PUMA has been identified as a key regulator of apoptosis in response to growth factor and cytokine withdrawal, particularly in response to the loss of IL-3 signalling in cells from a myeloid lineage (Ekert *et al.*, 2006; Ekoff *et al.*, 2007; Jeffers *et al.*, 2003).

Transcriptional regulation of proteins is a slow response and can take several hours for any change to be effected. By directly regulating existing protein through post-translational modification a cell can respond to any new stimuli rapidly over the course of seconds to minutes. Given the importance of PUMA for regulating apoptosis and the devastating result for a cell should it fail to regulate PUMA appropriately we hypothesised that PUMA protein is the likely candidate to undergo post-translational modification, particularly phosphorylation. Therefore, the first aim of this thesis was to determine if PUMA is post-translationally regulated in response to growth factor and cytokine signalling. The investigation of this regulation involved using a variety of established cell lines such as HEK 293T cells which are dependent on foetal calf serum for survival and an immortalised primitive myeloid cell line which is dependent on IL-3 for survival. The global regulation of endogenous and over-expressed PUMA in these lines was investigated upon survival

factor treatment or withdrawal. Having identified if PUMA is post-translationally modified I also sought to identify the sites within the PUMA protein sequence where this regulation occurs. To achieve this, motif scans and proteomics were utilised to identify specific sites of regulation within PUMA. Identified sites were then confirmed by site-specific mutational analysis of PUMA. A site-specific antibody was developed to allow closer analysis of the residue within PUMA that I have been found to be regulated.

Post-translational modification of the Bcl-2 family of proteins has been demonstrated to control their activity by several means. They can prevent the interaction of BH3-only proteins with pro-survival Bcl-2 family members by sequestering them in other compartments of the cells or providing steric inhibition of any interaction (Datta *et al.*, 2000; Qi *et al.*, 2006; Virdee *et al.*, 2000; Zha *et al.*, 1996). Signalling can also affect the abundance of Bcl-2 family members in a cell by regulating the stability of the protein (Hubner *et al.*, 2008; Maurer *et al.*, 2006). Post-translational regulation of sites within PUMA is likely to regulate its apoptotic activity. Therefore, the second aim of this thesis was to demonstrate the effect that post-translational modification has on PUMA. This was investigated by mutational analysis of the identified sites within PUMA to determine if removing the ability of these sites to be regulated by growth factors and cytokines has an effect on the behaviour of PUMA within a cell. Experiments investigated any changes in the localisation, apoptotic potency, stability and interactions with known binding partners.

There are a number of signalling pathways that are activated in response to growth factor and cytokine signalling (de Groot *et al.*, 1998; Hercus *et al.*, 2009). Identification of specific sites within the PUMA protein sequence allowed analysis of the mechanisms responsible for regulating that site, for example, identifying the kinase that phosphorylates a specific residue. Initially bioinformatics and motif scans were used to identify kinases that are likely to phosphorylate a particular site. Kinases that are known to be important in the response to growth factor and cytokine signalling were shortlisted. Biochemical analysis of PUMA regulation was then performed using protein purification and kinase inhibitor libraries to determine if the kinase that regulates specific sites within PUMA can be identified. Kinases for particular sites were then validated biochemical approaches.

The results showed that PUMA was phosphorylated at a specific serine residue, serine 10, following treatment of cells with either serum or IL-3. This phosphorylation does not regulate interactions with known binding partners or localisation within a cell but instead regulates PUMA protein degradation through the proteasome. The kinase responsible for phosphorylation of PUMA following IL-3 treatment was IKK1 as part of an IKK1/IKK2/Nemo signalling complex.

Chapter 2

Materials and Methods

2.1 Cells

2.1.1 HEK 293T cells

The HEK 293T cell line was derived from the adenovirus type transformed human embryonic kidney 293 cell line, containing simian virus 40 large tumour antigen (Dubridge *et al.*, 1987) were maintained in DMEM supplemented with 10% v/v HI FBS.

2.1.2 Factor Dependent Myeloid (FDM) Cells

Factor dependent myeloid cell lines from a variety of genetic backgrounds were obtained have been described previously (Ekert *et al.*, 2006). The cells were maintained in DMEM supplemented with 10% v/v HI FBS and 0.5 ng/ml mIL-3.

2.1.3 Mouse Embryonic Fibroblast (MEF) cells

Mouse Embryonic Fibroblast cell lines from a variety of genetic backgrounds were obtained from Dr. Vinay Tergaonkar, A*STAR, Singapore. The cells were maintained in DMEM supplemented with 10% v/v HI FBS.

2.2 Antibodies

2.2.1 Development of new antibodies

Polyclonal rabbit antibodies against PUMA phosphorylated at serine 10 (pSer10) were raised by immunising rabbits with the peptide ARARQEGS(pS)PEPVEGLC (residues 2-18 of human PUMA-α) coupled to Keyhole Limpet Hemocyanin (Millipore, North Ryde, NSW). Antibodies were affinity purified using a PUMA pSer10 peptide column. The antibody was used for immunoblotting and was selected for giving a strong signal in Western blotting.

2.2.2 Commercial antibodies

The antibody against Puma was from ProScience (Sapphire Bioscience, Redfern, NSW). The antibody against β-actin was from Calbiochem (Merck, Darmstadt, Germany). Antibodies against Bcl-x_L, VDAC, IκBα, IKK1, IKK2 (L570), phospho-IKK1/2, Hsp70 were from Cell Signaling (Genesearch, Arundel, QLD). The antibody against Mcl-1 was

from Rockland (Gilbertsville, USA). The antibody against EF-1α (clone CBP-KK1) was from Upstate (Billerica, USA). The antibody against phosphotyrosine (clone 4G10) was from Upstate. The antibody against hemagglutinin (HA) (clone 12CA5) was from MAbSA (Gillies Plains, SA). The antibody against Cytochrome C was from BD Pharmingen (North Ryde, NSW).

2.3 Cytokines

Recombinant mouse IL-3 was purchased from Shenendoah Biotechnology (Warwick, USA).

2.4 Mutagenesis of human PUMA-α and plasmid constructs

Serine residues were substituted with alanine, aspartate or glutamate in the human PUMA-α cDNA using oligonucleotide-directed mutagenesis as described previously (Woodcock *et al.*, 1994). Mutagenesis primers (5'-3'): S9A, Forward: GCA CGC CAG GAG GGC GCC TCC CCG GAG CCC GTA GAG, Reverse: CTC TAC GGG CTC CGG GGA GGC GCC CTC CTG GCG TGC; S10A, Forward: GCA CGC CAG GAG GGC AGC GCC CCG GAG CCC GTA GAG, Reverse: CTC TAC GGG CTC CGG GGC GCT GCC CTC CTG GCG TGC. The mutations were confirmed by nucleotide sequencing and the mutant PUMA cDNAs subcloned into the eukaryotic expression vector pEF-HA-hygro and pF 5XUAS SV40 puromycin. All constructs were sequenced for authenticity and purified using QIAGEN maxiprep kits prior to use (QIAGEN, Clifton Hill, Australia).

2.5 Transient Transfection

HEK 293T were grown till they were around 80% confluence. These cells were transfected with PUMA cDNA in a pEF-HA-hygro vector using Lipofectamine 2000 following the manufacturer's instructions (Invitrogen, Carlsbad, USA). Cell medium was replaced 6 hours after addition of transfection reagents.

2.6 Lentiviral particle production and transduction of cells

The lentiviral inducible expression system used has two components. The first is a fusion protein of Gal4 with a modified oestrogen receptor. The fusion protein is constitutively expressed from an ubiquitin promoter (GEV16). In the absence of 4-OHT, this fusion protein is held in an inactive state in the cytosol bound to heat-shock protein-90 (HSP90). The second component is the gene-of-interest downstream of a promoter consisting of five UAS sites (pF 5xUAS SV40 puromycin). In the presence of 4-OHT, the Gal4-ER fusion protein dissociates from HSP90 and translocates to the nucleus to drive expression (Jabbour *et al.*, 2009). Lentivirus particles were generated by transfecting HEK 293T cells with pCMV-ΔR8 and pVSV-G lentiviral packaging constructs in addition to the pF 5xUAS SV40 puromycin containing the gene-of-interest. After 48 hours supernatants were collected, filtered, and either stored -80°C or mixed 1:1 in the presence of 4 µg/ml polybrene and added to cells. Stably infected cells were selected using puromycin and hygromycin.

2.7 Metabolic labelling of cells

To metabolically label cells using ^{32}P orthophosphate, cells were starved into phosphate free medium for 4-24 hrs before the addition of ^{32}P orthophosphate (Perkin Elmer, Waltham, USA) for 4 hours followed by a variety of experiments. To metabolically label cells with methionine and cystine that has been labelled with ^{35}S, cells were starved into methionine and cystine free medium for 30 mins before the addition of EXPRE^{35}S^{35}S protein labelling mix (Perkin Elmer, Waltham, USA) at 100 µCi/ml for one hour. Cells were washed into fresh medium and samples taken at various time points. Cells were then lysed using NP40 lysis buffer before immunoprecipitation, precipitates resolved by SDS-PAGE and transferred to nitrocellulose before being exposed to x-ray film or storage phosphor screen.

2.8 Cell lysis

Total cell lysates were prepared by lysing either directly into SDS load buffer (250mM Tris-HCl, 4% glycerol, 5% SDS, 0.25% Bromophenol Blue), NP40 lysis buffer (10mM Tris-HCl pH7.4, 137mM NaCl, 10% Glycerol, 1% Nonidet P-40), CHAPS lysis buffer (20mM Tris-HCl pH 7.4, 135mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 10% glycerol, 1%

CHAPS), RIPA buffer (150mM NaCl, 50mM Tris-HCl, 0.5% Sodium Deoxycholate, 0.1% SDS, 1% NP40) supplemented with Complete protease inhibitor cocktail tablets (Roche, Mannheim, Germany) and phosphatase inhibitors (2mM sodium fluoride, 10mM β-glycerophosphate, 2mM Na_3VO_4) at a density of $5x10^7$ - $1x10^8$ cells/mL. Cells were fractionated into cytosolic and membrane fractions at a density of $1x10^8$ cells/mL. Cytosolic fractions were generated by incubating cells in digitonin (stock solution 20 mg/mL) diluted 1:125 in HMKEE (20 mM HEPES pH 7.4, 5 mM $MgCl_2$, 10 mM KCL, 1 mM EDTA, 1 mM EGTA) with 250 mM Sucrose and Complete protease inhibitor cocktail tablets on ice for 15 min. Supernatant (cytosolic fraction) was collected after centrifugation at 500 g for 10 min. The pellet was resuspended in HMKEE, 250 mM Sucrose and 0.5% Triton X-100 and incubated on ice for 30 min. Supernatant (membrane fraction) was collected after centrifugation at 5000 g for 10 min.

2.9 Immunoprecipitation and Pull-down of proteins

Following a variety of different treatment conditions cells were lysed in NP40 lysis buffer, HMKEE with 1% CHAPS or HMKEE with 0.5% Triton X100 for 30 mins at 4°C followed by centrifugation of the lysate for 15 mins at 13,000g 4°C. Supernatant was incubated for 16 hours with anti-HA (12CA5) or a variety of peptides conjugated to sepharose beads. The beads were then washed 3 times in the same buffer as the lysis performed before boiling for 5 min in SDS load buffer either in the presence or absence of β-mercaptoethanol (reducing or non-reducing) before separating proteins by SDS-PAGE.

2.10 SDS-PAGE

Proteins, immunoprecipitates and pull-downs were analysed by one-dimentional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions. Molecular weights were estimated using the commercially available MW markers Benchmark Pre-stained Protein Markers (Invitrogen, Carlsbad, USA). Proteins could then be stained using either Coomassie brilliant blue or silver stain as described previously (Morrissey, 1981).

2.11 Immunoblot and ECL

Proteins separated by SDS-PAGE were transferred to nitrocellulose membrane by electroblotting. Routinely, nitrocellulose membranes were blocked in a solution of TBS/0.05% (v/v) Tween 20 containing 1% (w/v) blocking reagent (Roche, Mannheim, Germany) then probed overnight 4°C with a variety of antibodies followed by the appropriate horseradish peroxidise secondary antibody. Proteins were detected by chemiluminescence using an ECL kit (Amersham, Little Chalfont, UK) following manufacturers instructions.

2.12 Analysis of cell viability by flow cytometry

Cell viability was determined by staining cells with FITC-coupled annexin V (Roche) in balanced salt solution including 5 mM $CaCl_2$ and propidium iodide (PI; 5µg/mL, Sigma) followed by flow cytometric analysis (Becton Dickinson, San Jose, USA). Viable cells were identified by not binding Annexin V and their ability to exclude PI. All cell death experiments were independently repeated at least 3 times.

2.13 Mass Spectrometry

HA-PUMA was overexpressed in HEK 293T or FDM cells before lysis, HA immunoprecipitation, SDS-PAGE and stained with Coomassie Brilliant Blue. The bands of interest were excised from the gel, destained and digested with 100 ng of trypsin per sample according to the "low salt" protocol. One microlitre of each sample was applied to a 600 mm AnchorChip (Bruker Daltonik GmbH, Bremen, Germany), washed with 10mM dihydroammonium phosphate and overlaid with a-cyano-4-hydroxycinnamic acid (HCCA) matrix (0.8µl of a 0.5g/L solution in 90% ACN/0.1% TFA). 10 µl of an Immobilised Metal Affinity Chromatography (IMAC, Bruker Bruker Daltonik GmbH) magnetic bead suspension was pre-treated with 50µl MB-IMAC Fe wash solution 1 three times. The beads were suspended in 20µl MB-IMAC Fe wash solution 1, plus 20µl MB-IMAC binding solution prior to adding 1µl of the peptide sample and incubating for 45 mins at ambient temperature with low agitation. The supernatant was removed and the beads washed three times with 100µl MB-IMAC Fe wash solution 2 and eluted using 3µl 50% acetonitrile (ACN) with 0.1% phosphoric acid for 10 mins to enhance sensitivity. 1 µl of

this eluate was subsequently applied to an AnchorChip with 1 µl 2, 5-dihydroxy benzoic acid (DHB - 10mg/mL) in 50% acetonitrile (ACN) with 0.1% phosphoric acid for MALDI-TOF MS analysis. Half of each IMAC-purified sample was diluted to 5·5mL with 1% FA in an autosampler vial and 5mL chromatographed using an Agilent Protein ID Chip column assembly (40 nL trap column with 0·075 x 43 mm C-18 analytical column) housed in an Agilent HPLC-Chip Cube Interface connected to an a HCT ultra 3D-Ion-Trap mass spectrometer (Bruker Daltonik GmbH). The column was equilibrated with 4% acetonitrile (ACN) / 0·1% FA at 0·5 mL/min and the samples eluted with an ACN gradient (4%-31% in 32 min). Ionizable species (300 < m/z < 1,200) were trapped and one or two of the most intense ions eluting at the time were fragmented by collision-induced dissociation (CID) and electron-transfer dissociation (ETD). A specific inclusion list was used to preferentially fragment ions that corresponded in *m/z* to potential phosphopeptides (Condina *et al.*, 2009).

2.14 IKK *in vitro* kinase assay

Wild type or S10A HA-PUMA was over expressed in HEK 293T cells overnight before lysis in NP40 lysis buffer. Over expressed protein was immunoprecipitated from lysates using an anti-HA antibody coupled to sepharose beads. The beads were then washed twice in NP40 lysis buffer, washed once in CIP buffer (50mM Tris-HCl pH7.9, 100mM NaCl, 10mM $MgCl_2$, 1mM DTT) before being treated with 5U Alkaline Phosphatase (Promega, Sydney) for 30 minutes at 37°C. Beads were then washed twice with NP40 lysis buffer before being washed once in Reaction Buffer (8mM MOPS/NaOH pH7.0, 200µM EDTA, 5mM β-glycerophosphate). Beads or 1µg recombinant IκBα were then incubated in an IKK *in vitro* kinase assay buffer (8mM MOPS/NaOH pH7.0, 200µM EDTA, 5mM β-glycerophosphate, 0.5% glycerol, 0.001% Triton X-100, 0.01% 2-mercaptoethanol, 0.1mg/mL BSA, 10mM Magnesium Acetate, 0.1mM ATP, 2µCi [γ-^{32}P]ATP, 100ng enzyme (recombinant human IKK1 (US biological, Swampscott, USA)/recombinant human IKK2 (Millipore)) or equivalent volume of Reaction Buffer). Beads were then washed twice in NP40 lysis buffer, boiled in SDS load buffer before being subjected to SDS-PAGE and Western Blot analysis to detect protein and exposed to x-ray film to detect incorporated ^{32}P.

Chapter 3

Growth factors and cytokines regulate the post-translational modification of PUMA

3.1 Introduction

Growth factors and cytokines are critical for regulating cell homeostasis, activation in response to challenge and development. Signalling pathways initiated by these factors act to regulate the activity of proteins that control cell survival at a post-translational level by several mechanisms. One critical mechanism is the chemical modification of proteins within a cell such as phosphorylation, acetylation, methylation and numerous other examples. Enzymes that catalyse the addition and removal of these moieties allow the cell to finely control a protein's activity. Phosphorylation of proteins is heavily utilised by eukaryotic organisms with 30% of proteins within a cell predicted to be phosphorylatable on the side-chains of tyrosine, threonine and serine residues (Kreegipuu *et al.*, 1999).

Phosphorylation of proteins within a cell occurs through the actions of enzymes called kinases. The catalytic domains of most kinases are very similar with substrate specificity determined by the conformation of the active site surrounding the catalytic domain, a region complementary to the 7 to 12 residues that flank the potential phospho-acceptor site on substrate proteins (Zhou *et al.*, 1994). It is challenging to manually inspect a protein sequence and predict the probability that any particular amino acid residue in that protein may be susceptible to phosphorylation by a kinase. Therefore, artificial neural networks have been developed which are able to classify complex sequence patterns and correlate which positions are important in relation to the acceptor residue. Use of these networks allows a probability for phosphorylation of each potential phospho-acceptor site to be generated (Blom *et al.*, 1999; Wu, 1997). Once likely phosphorylation sites have been identified from a protein sequence, mass spectrometry can be utilised to confirm predictions. In recent years a number of mass spectrometric techniques have been developed to study post-translational modifications including electrospray ionization (ESI), collision-induced dissociation (CID) and electron-transfer dissociation (ETD) mass spectrometry. The most common technique to identify phosphorylation of proteins is tandem mass spectrometry which involves using ESI and CID, however, this can lead to the cleavage of phosphate groups from proteins and make identification of phosphorylated residues difficult when there are multiple, closely assembled potential phosphorylation sites (Reinders and Sickmann, 2005). In many cases a combinatorial approach is taken utilising CID and ETD. In this situation ETD has advantages as it allows for a protein to be

fragmented along the polypeptide backbone while retaining any phosphate groups associated with residues in a peptide (Mikesh *et al.*, 2006).

Post-translational modification of the pro-apoptotic BH3-only family of proteins by growth factors and cytokines is critical for promoting or inhibiting their apoptotic activity and regulating cell survival. Bad was the first BH3-only member demonstrated to be post-translationally modified by phosphorylation on two serine residues following IL-3 stimulation in FL5.12 cells (Zha *et al.*, 1996). Other members have since been shown to be phosphorylated including Bim (Ley *et al.*, 2005), Bik (Verma *et al.*, 2001), Bid (Desagher *et al.*, 2001) and Bmf (Lei and Davis, 2003). Phosphorylation of BH3-only proteins is utilised to promote or inhibit their apoptotic activity and in many circumstances PUMA has been shown to be important for regulating cell survival and apoptosis (Ekert *et al.*, 2006; Ekoff *et al.*, 2007; Jeffers *et al.*, 2003; Ming *et al.*, 2008; Villunger *et al.*, 2003). Transcriptional regulation of PUMA by growth factor and cytokine signalling is well established, however, no post-translational regulation has ever been identified. In this chapter it is shown that PUMA can be phosphorylated in response to growth factor and cytokine signalling in several cell types. Bioinformatic analysis of PUMA demonstrated the presence of several amino acid residues that can undergo phosphorylation that are conserved across a number of different species. Mass spectrometric and mutational analysis of PUMA positively identified one of these residues, serine 10, as being phosphorylated in response to growth factor and IL-3 signalling.

3.2 Results

3.2.1 PUMA is phosphorylated in response to growth factor and cytokine signalling

In order to determine if PUMA is phosphorylated in response to growth factor signalling HA-tagged PUMA was expressed in HEK 293T cells followed by overnight serum and phosphate starvation. Cells were then radioactively labelled by addition of ^{32}P orthophosphate which is rapidly taken into the cell due to the phosphate starvation. Stimulation of HEK 293T cells was performed by re-addition of serum over 60 minutes. Cells were then lysed and the HA-PUMA purified by immunoprecipitation and subjected to SDS-PAGE before western blot analysis and exposure to X-ray film. Western blot analysis using a monoclonal antibody that detects HA-tagged proteins confirmed that expression of the HA-PUMA mutants was equivalent and that there was no expression in the mock transfected HEK 293T cells. Incorporation of the ^{32}P into a protein indicates phosphorylation. Analysis of the autoradiograph demonstrated that incorporation of ^{32}P into PUMA could be observed at basal levels in the absence of serum. Following re-addition of serum, increased PUMA phosphorylation was observed after 5 minutes, peaked at 15 minutes and reverted back to basal levels by 30 min (Figure 3.1A).

The previous result demonstrated that growth factor signalling from serum treatment can promote PUMA phosphorylation in HEK 293T cells. Additional experiments were performed using IL-3 dependent, HoxB8-transformed primitive myeloid (Factor Dependent Myeloid, FDM) cells to determine if IL-3 starvation and re-addition could elicit a similar response. FDM cells which have the pro-apoptotic, multi-domain Bcl-2 family members Bax and Bak removed (FDM *Bax*$^{-/-}$*;Bak*$^{-/-}$) were used to avoid the induction of apoptosis following PUMA over-expression (Jabbour *et al.*, 2009). Preventing apoptosis allows upstream events such as growth factor, receptor-regulated post-translational modification of PUMA to be investigated without potential confusion from events initiated by the activation of the intrinsic apoptotic pathway. HA-PUMA expression was induced in FDM cells overnight using 4-OHT followed by IL-3 and phosphate starvation for 24 hrs. Cells were then radioactively labelled by addition of ^{32}P orthophosphate. Stimulation of FDM cells was performed by re-addition of IL-3 at a final concentration of 8ng/ml over a 60 minute time course. Cells were then lysed and the HA-PUMA purified by immunoprecipitation before western blot analysis and exposure to X-ray film. Western blot

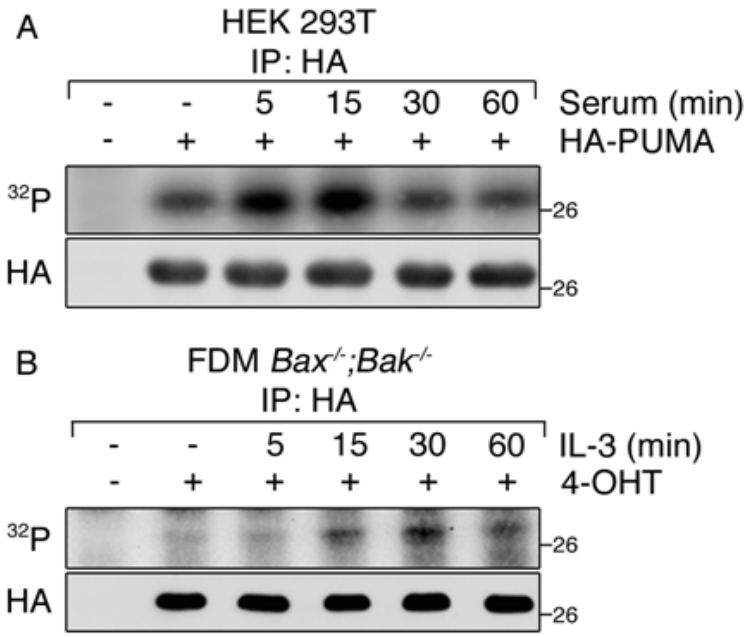


Figure 3.1 Growth factor and cytokine stimulation promotes phosphorylation of PUMA. (A) HA-PUMA protein was transiently expressed in HEK 293T cells 16 hrs before starving cells of serum and phosphate for 24 hrs. Cells were then metabolically labelled with 250 µCi ^{32}P orthophosphate for 4 hrs prior to stimulation with 10% serum for the indicated period of time. HA-PUMA was immunoprecipitated using anti-HA (12CA5) Sepharose gel. Immunoprecipitates were resolved by SDS-PAGE and then either immunoblotted using anti-HA (12CA5) antibody or exposed to x-ray film. (B) HA-PUMA protein expression was induced in FDM *Bax*$^{-/-}$*;Bak*$^{-/-}$ cells using 100 nM 4-hydroxy tamoxifen (4-OHT) 16 hrs before starving cells of IL-3 and phosphate for 24 hrs. Cells were then metabolically labelled with 250 µCi ^{32}P orthophosphate for 4 hrs prior to stimulation with 8 ng/ml IL-3 for the indicated period of time. HA-PUMA was immunoprecipitated using anti-HA (12CA5) Sepharose gel. Immunoprecipitates were resolved by SDS-PAGE and then either immunoblotted using anti-HA (12CA5) antibody or exposed to an autoradiograph.

analysis using an anti-HA antibody confirmed that expression of the HA-PUMA mutants was equivalent and that there was no expression in the non-induced FDM cells. Analysis of the autoradiograph demonstrated that PUMA phosphorylation was evident as a diffuse band following 15 minutes of IL-3 stimulation and peaked after 30 minutes before returning to near basal levels by 60 minutes (Figure 3.1B)

3.2.2 Phosphorylated tyrosine residues cannot be detected in PUMA in response to serum stimulation

Kinases that phosphorylate proteins can be roughly divided into two groups. Those specific for phosphorylation of tyrosine residues and those specific for phosphorylation of serine and threonine residues (Manning *et al.*, 2002). To determine if the observed phosphorylation of PUMA potentially occurs on any of the three tyrosine residues in the human PUMA protein sequence, HA-PUMA was expressed in HEK 293T cells followed by overnight serum starvation. Stimulation of HEK 293T cells was performed by re-addition of serum for 20 minutes. HEK 293T cells treated with sodium orthovanadate, a potent tyrosine phosphatase inhibitor, before serum stimulation was used to maximise any potential tyrosine phosphorylation of PUMA in addition to acting as a positive control for tyrosine phosphorylation of other tyrosine kinase substrates. Cells were then lysed and the HA-tagged PUMA was purified by immunoprecipitation before western blot analysis. Western blot analysis was performed using the pan-phosphotyrosine monoclonal antibody 4G10 which detects many, but not all, proteins phosphorylated on tyrosine residues. Analysis of the data indicated that no tyrosine phosphorylation of the immunoprecipitated PUMA was detected by the antibody with a successful blot confirmed by the presence of a large dark smear of phospho-tyrosine protein in the sodium orthovanadate control lane from the input samples. Probing with anti-HA confirmed that expression of the HA-PUMA mutants was successful and that there was no expression in the mock transfected HEK 293T cells (Figure 3.2). This result demonstrates that it is highly likely the PUMA phosphorylation that is observed is on serine or threonine residues as no phosphorylation of tyrosine residues could be detected.

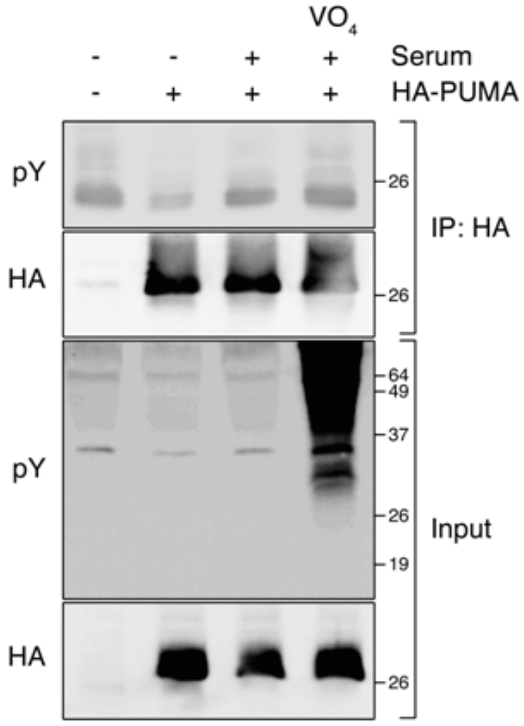


Figure 3.2 PUMA is not phosphorylated on tyrosine residues. HA-PUMA protein was transiently expressed in HEK 293T cells 16 hrs before starving cells of FBS. Cells were then stimulated ±10% FBS, ±Sodium Orthovanadate for 20 minutes. HA-PUMA was immunoprecipitated using anti-HA (12CA5) Sepharose gel and immunoblotted using anti-HA (12CA5) and anti-phosphotyrosine (4G10).

3.2.3 PUMA serine 10 is phosphorylated in response to growth factor and cytokine signalling

Having established that PUMA is a protein phosphorylated on serine or threonine residues, bioinformatic analysis of the PUMA protein sequence was undertaken to help determine which residues are likely to undergo phosphorylation. Conservation of amino acid residues can suggest sites in a protein which may be critical for regulation such as phosphorylation sites. Therefore, a sequence alignment of PUMA proteins was performed from Human, Rat, Mouse, Dog and Opossum. This demonstrated a number of phosphorylatable residues which are conserved across these species including 7 serines and 2 tyrosines in addition to the conserved BH3 domain (Figure 3.3). Additional bioinformatic methods to predict phosphorylation sites within a protein sequence are available from a variety of institutions. The publically available database NetPhos, hosted by the Technical University of Denmark (DTU), is a neural network-based method for predicting potential phosphorylation sites at serine, threonine or tyrosine residues in independent protein sequences. A score ranging from 0 to 1 is attributed to each residue in a protein sequence based on the likelihood that a particular residue may be phosphorylated with scores closer to 1 indicating a higher chance of phosphorylation (Blom *et al.*, 1999). Analysis of the human PUMA protein sequence using NetPhos demonstrated 6 serine residues scoring over 0.5, the threshold for predicted phosphorylation, with none of the tyrosine or threonine residues within the PUMA sequence scoring over 0.5. These predicted sites include serine 9, 10, 83, 98, 106 and 166 with serine 10 scoring the highest at 0.996 (Table 3.1).

The bioinformatic analysis of PUMA suggested a number of potential phosphorylation sites within the PUMA sequence. To determine which of these sites is specifically phosphorylated, proteomic analysis of PUMA protein was undertaken. Initially HA-PUMA was expressed in HEK 293T cells followed by overnight serum starvation. Stimulation of HEK 293T cells was performed by re-addition of serum for 20 minutes. In addition to HEK 293T cells, HA-PUMA expression was induced in FDM cells overnight using 4-OHT followed by IL-3 starvation for 24 hrs. Cells were then stimulated by re-addition of IL-3 for 15 minutes. In both cell lines an untransfected/non-induced sample was used as a negative control. Cells were lysed and the HA-PUMA purified by immunoprecipitation before being subjected to SDS-PAGE and protein stained with Coomassie Brilliant Blue. A

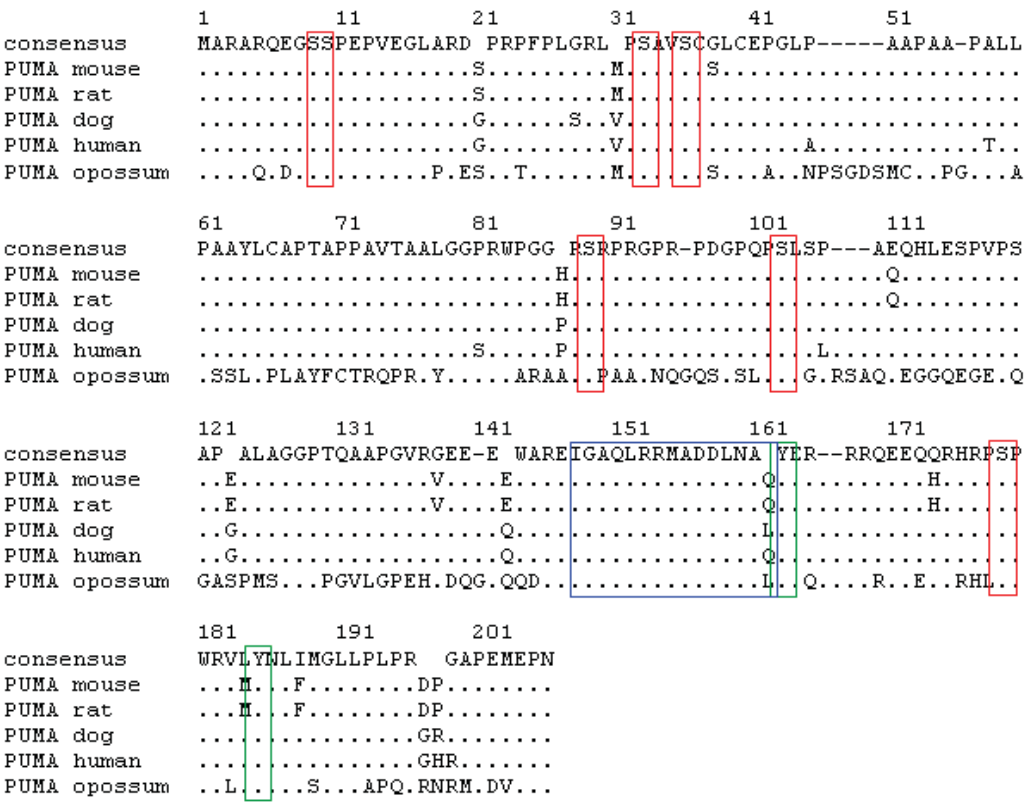


Figure 3.3 PUMA sequence alignment demonstrates conserved residues. Alignment of the PUMA protein sequences from 5 species allows a consensus sequence to be determined. Conserved residues that can undergo phosphorylation are indicated in red (serine) and green (tyrosine). Blue identifies the conserved BH3 domain.

Serine predictions

Name	Pos	Context	Score	Pred
		v		
PUMA	9	RQEGSSPEP	0.696	*S*
PUMA	10	QEGSSPEPV	0.996	*S*
PUMA	33	RLVPSAVSC	0.012	.
PUMA	36	PSAVSCGLC	0.015	.
PUMA	75	ALGGSRWPG	0.009	.
PUMA	83	GGPRSRPRG	0.885	*S*
PUMA	96	GPQPSLSLA	0.145	.
PUMA	98	QPSLSLAEQ	0.953	*S*
PUMA	106	QHLESPVPS	0.924	*S*
PUMA	110	SPVPSAPGA	0.117	.
PUMA	166	RHRPSPWRV	0.953	*S*
		^		

Threonine predictions

Name	Pos	Context	Score	Pred
		v		
PUMA	52	PAAPTLLPA	0.445	.
PUMA	63	LCAPTAPPA	0.046	.
PUMA	69	PPAVTAALG	0.055	.
PUMA	120	AGGPTQAAP	0.044	.
		^		

Tyrosine predictions

Name	Pos	Context	Score	Pred
		v		
PUMA	58	LPAAYLCAP	0.053	.
PUMA	152	LNAQYERRR	0.138	.
PUMA	172	WRVLYNLIM	0.017	.
		^		

Table 3.1 Netphos predicted phosphorylation sites. Summary of predicted phosphorylation sites from Netphos analysis of human PUMA protein sequence. A specific residue is predicted to be phosphorylated if the program calculates a score above 0.5 (Blom *et al.*, 1999).

band corresponding to HA-PUMA could be seen in both the HEK 293T cells and FDM cells that had expressed HA-PUMA with the band absent in the immunoprecipitions where HA-PUMA was not expressed (Figure 3.4). The HA-PUMA bands were excised from the gel, digested with trypsin and subjected to Immobilised Metal Affinity Chromatography (IMAC) to enrich for peptides containing phosphorylated residues. MALDI-TOF mass spectra were acquired from HEK 293T and FDM samples following IMAC enrichment using MALDI mass spectrometry (MS) and positively identified several PUMA peptides (Figure 3.5). Potential phospho-peptides identified along with other peaks that did not match to the theoretical sequence were chosen for MALDI tandem mass spectrometry (MS/MS). For each sample, MS/MS data were combined and matched to an *in silico* digest to determine the phospho-peptides present. This analysis identified a phosphorylated HA-PUMA N-terminal peptide, $[M+H]^+$ 1762.9, encompassing PUMA serine 9 and serine 10 but could not definitively determine which of these serine residues was phosphorylated (Table 3.2). In order to isolate more specifically which serine residue is phosphorylated, the IMAC purified peptides were subjected to electrospray ionization (ESI) mass spectrometry (MS & MS/MS). Ionizable species ($300 < m/z < 1{,}200$) were trapped and one or two of the most intense ions eluting at a time were fragmented by collision-induced dissociation (CID) in conjunction with electron-transfer dissociation (ETD), a process which should improve phospho-peptide annotation. CID can lead to the loss of phosphate groups from peptides leading to poor MS/MS fragmentation. The ETD fragmentation process involves cleavage of the N-C_α bonds along the peptide, a process that generates complementary c- and z-type fragment ions and leaves side chains and modifications such as phosphorylations intact (Mikesh *et al.*, 2006). A specific inclusion list was used to preferentially fragment ions that correspond in *m/z* (mass-to-charge ratio) to potential phospho-peptides (Table 3.3). Several ions corresponding to peptides that include phosphorylation at either PUMA serine 9 or 10 (serine 23 and 24 respectively due to the HA tag) were observed in the HEK 293T and FDM samples in addition to other peptides in the HEK 293T sample which are likely to have co-purified artifactually during IMAC purification due to their acidity (Figure 3.6). Phosphorylation of serine 10 (24) was demonstrated by the ETD fragmentation spectra for both the HEK 293T (Figure 3.7) and FDM (Figure 3.8) samples where fragmentation produced c-ions at 646.2 and 910.3 and not 813.7 which can only occur when serine 10 (24) is phosphorylated and not serine 9 (23).

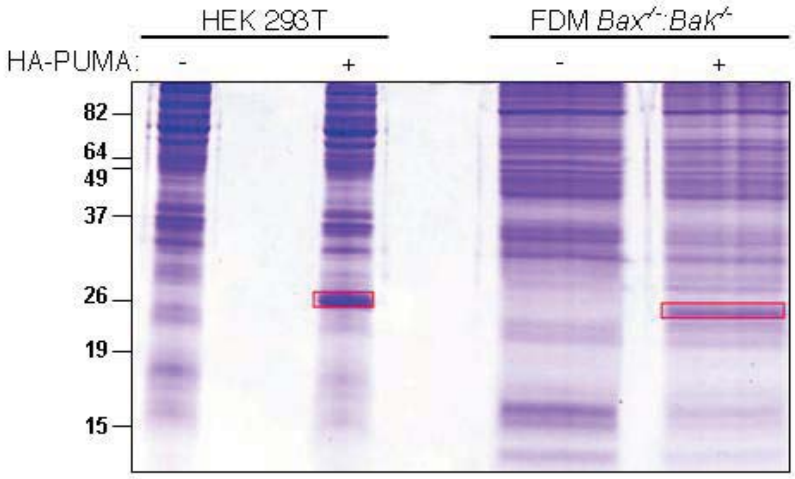
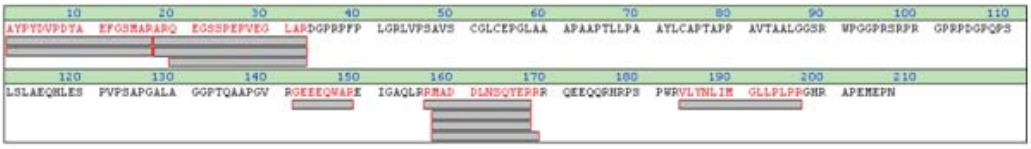


Figure 3.4 Expression of HA-PUMA protein for mass spectrometry. HA-PUMA was overexpressed in HEK 293T before starvation of serum overnight. 10% serum was then reintroduced for 20 min before lysis of cells. HA-Puma was also overexpressed in FDM *Bax*$^{-/-}$;*Bak*$^{-/-}$ cells before starvation of IL-3 for 24 hrs. 8 ng/ml IL-3 was then reintroduced for 15 min before lysis of cells. HA-Puma was immunoprecipitated using anti-HA (12CA5) Sepharose gel, resolved by SDS-PAGE and protein stained with Coomassie Brilliant Blue. Red boxes denote HA-PUMA and indicate the area that was excised.

A



B

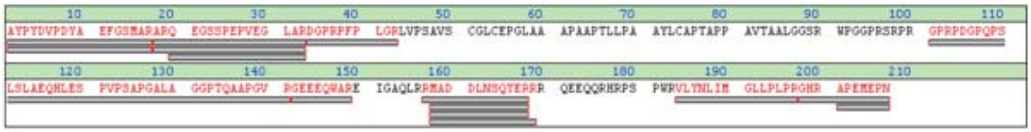


Figure 3.5 Sequence coverage from MALDI-TOF analysis of HA-PUMA following IMAC enrichment. (A) Tryptic HA-PUMA peptides from HEK 293T cells were subjected to IMAC enrichment before analysis from MALDI-TOF mass spectrometry. The observed peptides are indicated by grey bars. (B) As for (A) except tryptic peptides were obtained from FDM *Bax*$^{-/-}$*;Bak*$^{-/-}$ cells.

Peptide matched to sequence	MS/MS analysis	Cell line	Sequence
1762.908	Yes	HEK 293T FDM $Bax^{-/-};Bak^{-/-}$	ARQEGpSSPEPVEGLAR or ARQEGSpSPEPVEGLAR

Table 3.2 Observed peptide following IMAC purification with potential phosphorylation sites. MS/MS analysis identified an ion corresponding to a phosphorylated form of the indicated peptide.

		m/z		
Sequence number	Sequence	Range for *z*=2	Range for *z*=3	Range for *z*=4
18-33	ARQEGSSPEPVEGLAR	881.6-882.6	588-589	
		921.6-922.6	614.6-615.6	
20-33	QEGSSPEPVEGLAR	768-769	512.3-513.3	
		808-809	538.9-539.9	
91-100	WPGGPRSRPR	623-624	415.6-416.6	
101-141	GPRPDGPQPSLSLAEQHLESPV PSAPGALAGGPTQAAPGVR			1012-1013
177-183	HRPSPWR	507.9-508.9	338.9-339.9	

Table 3.3 Inclusion list of ions that correspond in *m/z* to potential phospho-peptides. Summary of ions to be included in CID and ETD fragmentation following ESI. Ranges for *m/z* are given based of potential charge following ESI. Sequence numbers have an additional 14 residues due to the presence of the N-terminal HA-tag.

A

B

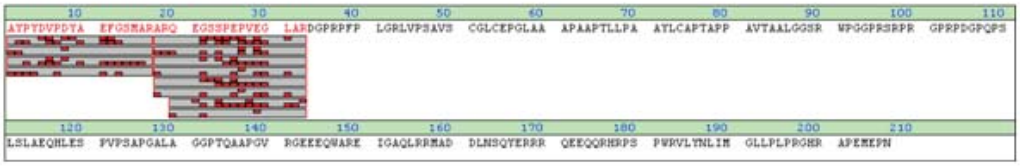


Figure 3.6 Sequence coverage for IMAC-purified PUMA peptides by ESI CID/ ETD MS. (A) Tryptic HA-PUMA peptides from HEK 293T cells were subjected to IMAC enrichment before analysis from ESI CID/ETD mass spectrometry. The observed peptides are indicated by grey bars. The red boxes show the amino acids that were sequenced by MS/MS analysis of the parent ion, whereby the upper series of boxes represents the *b*-ions and the lower the *y*-ions (B) As for (A) except tryptic peptides were obtained from FDM *Bax*$^{-/-}$*;Bak*$^{-/-}$ cells.

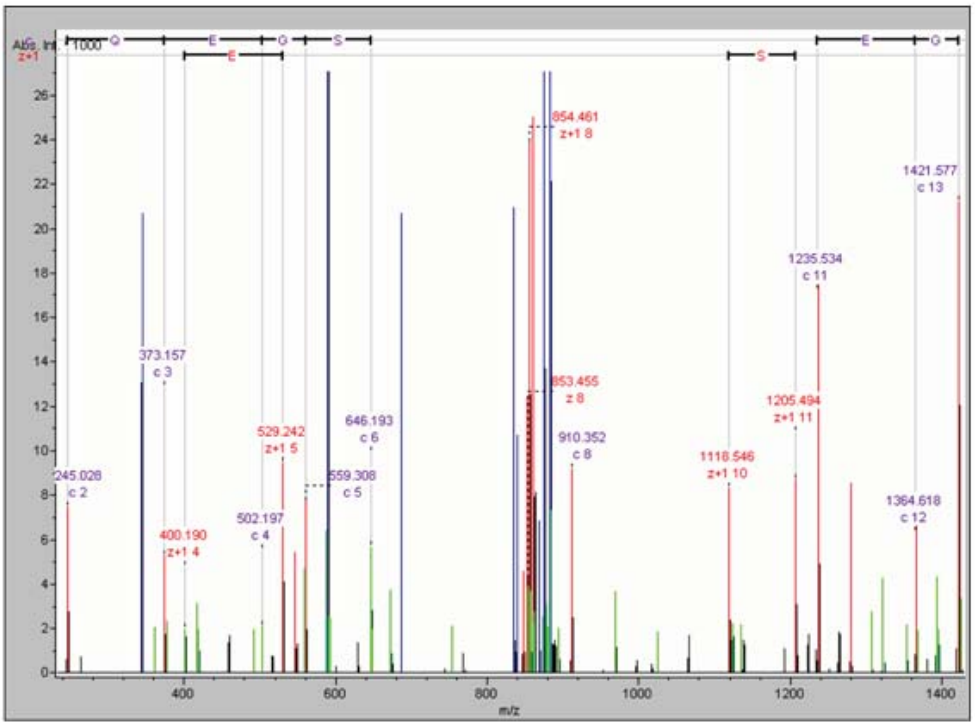


Figure 3.7 ETD spectrum for HA-PUMA peptide from HEK 293T cells. ETD spectrum for the 18-33 HA-PUMA phospho-peptide from IMAC-purified tryptic digest of HA-PUMA from HEK 293T cells. 18-33 sequence – ARQEGSSPEPVEGLAR.

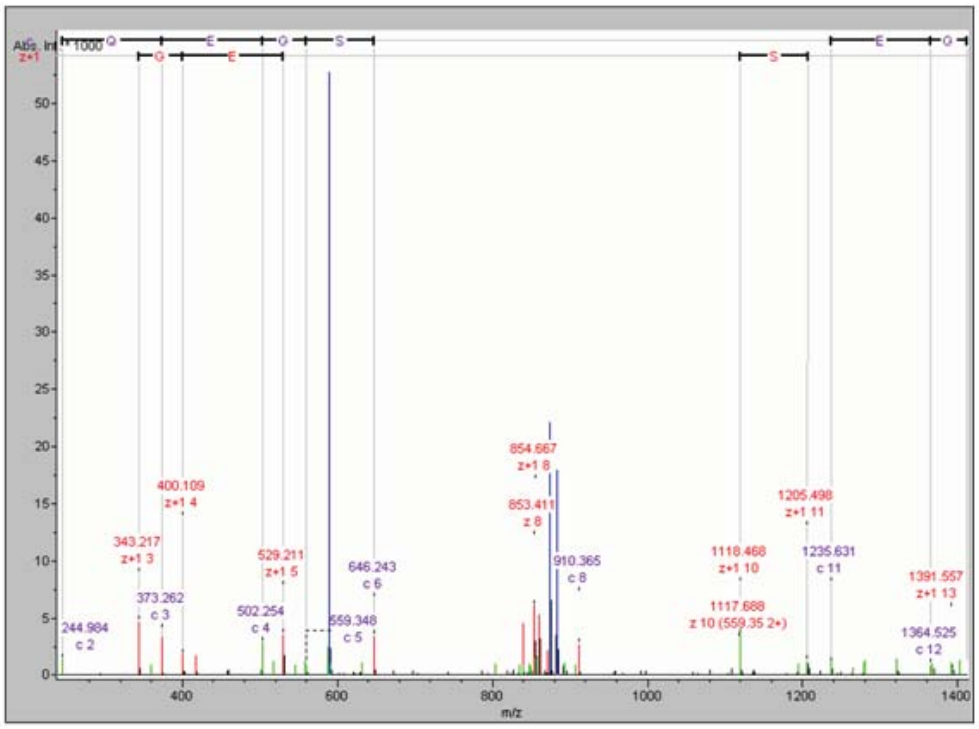


Figure 3.8 ETD spectrum for HA-PUMA peptide from FDM cells. ETD spectrum for the 18-33 HA-PUMA phospho-peptide from IMAC-purified tryptic digest of HA-PUMA from FDM *Bax*$^{-/-}$;*Bak*$^{-/-}$ cells. 18-33 sequence – ARQEGSSPEPVEGLAR.

3.2.4 Mutation of PUMA serine 10 greatly reduces the ability of PUMA to be phosphorylated in response to growth factor and cytokine stimulation

In order to determine the role of PUMA serine 10 in the phosphorylation seen following serum stimulation in HEK 293T cells and IL-3 stimulation of FDM cells and to demonstrate biochemically that serine 10 was the phosphorylated residue identified by mass spectrometry , HA-tagged human PUMA with serine 9 or serine 10 to alanine (HA-PUMA^{S9A}, HA-PUMA^{S10A}) point mutations were generated. Initially wild type HA-PUMA, HA-PUMA^{S9A} and HA-PUMA^{S10A} were expressed in HEK 293T cells followed by overnight serum and phosphate starvation. Cells were then radioactively labelled by addition of ^{32}P orthophosphate. Stimulation of HEK 293T cells was performed by re-addition of serum at a final concentration of 10% for 20 minutes. Cells were then lysed and HA-PUMA purified by immunoprecipitation before western blot analysis and exposure to x-ray film. Western blot analysis using an anti-HA antibody confirmed that expression of the HA-PUMA mutants was equivalent and that there was no expression in the mock transfected HEK 293T cells. Analysis of the autoradiograph demonstrated that wild type PUMA and PUMA^{S9A} were phosphorylated in response to serum stimulation, however, PUMA^{S10A} was not phosphorylated following serum stimulation (Figure 3.9A).

To determine if this observation is also true for FDM cells stimulated with IL-3, wild type HA-PUMA and HA-PUMA^{S10A} expression was induced in FDM cells overnight using 4-OHT followed by IL-3 and phosphate starvation for 24 hrs. Cells were then radioactively labelled by addition of ^{32}P orthophosphate. Stimulation of FDM cells was performed by re-addition of IL-3 for 15 and 30 minutes. Cells were then lysed and the HA-PUMA was purified by immunoprecipitation before being subjected to western blot analysis and exposure to x-ray film. Western blot analysis using an anti-HA antibody confirmed that expression of the HA-PUMA mutants was equivalent and that there was only negligible expression in the non-induced FDM cells. Analysis of the autoradiograph demonstrated that wild type PUMA was phosphorylated in response to IL-3 stimulation, however, the PUMA^{S10A} mutation significantly reduces the ability of PUMA to be phosphorylated this time following IL-3 stimulation. The phosphorylated PUMA in FDM cells resolves as two bands with the upper band disappearing upon mutation of serine 10 while the lower bands intensity is reduced but still clearly present and regulated by IL-3 stimulation (Figure 3.9B).

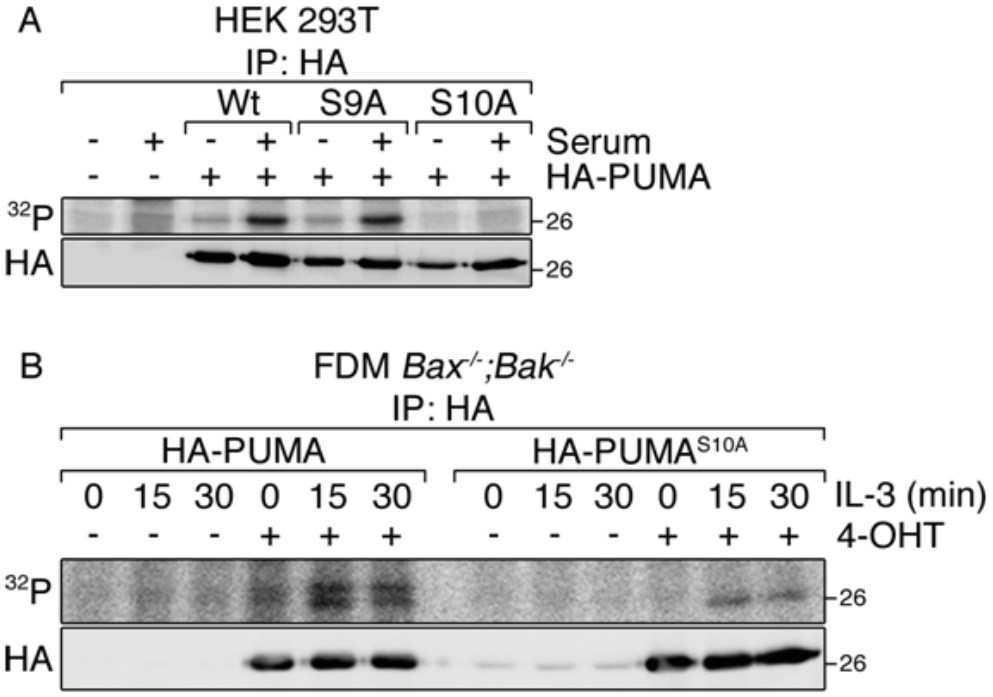


Figure 3.9 Mutation of PUMA serine 10 greatly reduces phosphorylation of PUMA in response to serum and IL-3 signalling. (A) HA-PUMA wild type or S10A were expressed in HEK 293T cells for 16 hrs before starving cells of serum and phosphate for 4 hrs. Cells were then metabolically labelled with ^{32}P orthophosphate for 4 hrs prior to stimulation with 10% serum for 20 min. HA-PUMA was immunoprecipitated using anti -HA (12CA5) Sepharose gel. Immunoprecipitates were resolved by SDS-PAGE and then either immunoblotted using anti-HA (12CA5) antibody or exposed to an autoradiograph. (B) FDM *Bax*$^{-/-}$*;Bak*$^{-/-}$ cells containing an inducible expression cassette for HA-tagged PUMA Wt or S10A mutant were induced using 100 nM 4-hydroxy tamoxifen (4-OHT) 16 hrs before starving cells of IL-3 and phosphate for 24 hrs. Cells were then metabolically labelled with ^{32}P orthophosphate for 4 hrs prior to stimulation with IL-3 at 8 ng/ml for the indicated period of time. HA-PUMA was immunoprecipitated, resolved and visualised as described in (A).

3.3 Discussion

In this chapter it has been demonstrated that PUMA undergoes phosphorylation on serine residues in response to serum or IL-3 treatment in HEK 293T and FDM cells respectively. Furthermore, it has been identified using mass spectrometric analysis that serine 10 in the N-terminal region of PUMA was phosphorylated in both cell lines. These results demonstrate that PUMA can be phosphorylated on serine 10 in human and mouse cells, however, an alignment analysis of PUMA protein sequence across a wide variety of species suggests that this phosphorylation is likely to be conserved. The alignment also demonstrates that the amino acid residues flanking serine 10 are also highly conserved suggesting that the N-terminal region of PUMA may serve as a critical functional motif. Interestingly, serine 10 is also conserved in the protein sequence of another mammalian BH3-only protein, Bim_{EL}, and in the *C. elegans* BH3-only protein homolog, Egl-1 (Figure 3.10). However, while serine 10 in these proteins is present there is no data available to suggest that they are phosphorylated in the same manner as PUMA.

It is known that PUMA can be regulated at a transcriptional level by cytokine signalling (Han *et al.*, 2001; Jabbour *et al.*, 2010; You *et al.*, 2006). Transcriptional regulation is a relatively slow process that can take a number of hours to respond to stimuli such as cytokine and growth factor treatment or withdrawal. This regulation is an essential component of a cells response to growth factors and cytokines, however, signalling molecules downstream of cytokine receptors such as kinases are utilised to allow a rapid and specific response to stimuli providing a mechanism for post-translational modification of existing proteins. It has previously been demonstrated that cytokine signalling is able to regulate the phosphorylation of a number of different BH3-only proteins (Desagher *et al.*, 2001; Lei and Davis, 2003; Ley *et al.*, 2005; Verma *et al.*, 2001; Zha *et al.*, 1996). The observations in this chapter now demonstrate that PUMA is also able to rapidly undergo phosphorylation in response to survival signals when a cell is 'recovering' from growth factor and cytokine deprivation. This is a significant observation as it is the first post-translational regulation of PUMA to be reported. Both serum and IL-3 are requisite components of the growth medium for each cell line indicating that the phosphorylation of PUMA is likely to occur under conditions where a cell is intending to survive and prevent or inhibit the induction of apoptosis. The pattern of phosphorylation seen in response to the

	4	5	6	7	8	9	10	11	12	13	14	15	16
H. sapiens PUMA	A	R	Q	E	G	S	S	P	E	P	V	E	G
M. musculus PUMA	A	R	Q	E	G	S	S	P	E	P	V	E	G
R. norvegicus PUMA	A	R	Q	E	G	S	S	P	E	P	V	E	G
C. familiaris PUMA	A	R	Q	E	G	S	S	P	E	P	V	E	G
M. domestica PUMA	A	Q	Q	D	G	S	S	P	E	P	V	E	G
H. sapiens BimEL	Q	P	S	D	V	S	S	E	C	D	R	E	G
M. musculus BimEL	Q	P	S	D	V	S	S	E	C	D	R	E	G
C. elegans EGL-1	V	F	D	V	Q	S	S	V	F	Y	N	E	K

Figure 3.10 PUMA N-terminal sequence alignment. Alignment of the PUMA protein sequences from 5 species, BimEL and EGL-1 demonstrate conservation of serine 10.

different factors indicates a common degree of regulation between the two cell lines and it would be reasonable to expect that this pattern may extend to other cell types that are capable of phosphorylating PUMA.

The PUMA protein sequence contains 2 conserved tyrosine residues and a number of conserved serine residues across a variety of species. The observation that no tyrosine phosphorylation could be detected, under conditions where PUMA was known from orthophosphate labelling experiments to be phosphorylated, strongly suggests that the phosphorylation occurs exclusively on serine residues within PUMA. This conclusion is also supported by evidence that the residues predominantly phosphorylated in Bcl-2 family proteins are serine and threonine with no verified tyrosine phosphorylation sites (Kutuk and Letai, 2008). Bioinformatic analysis suggested several potential serine residues that are likely to undergo phosphorylation. The data from these networks is useful for reducing the number of putative sites of regulation, however, the results are ultimately predictions and must be experimentally verified. Mass spectrometry is a powerful tool that is increasingly utilised to study post-translational modifications such as phosphorylation (Morelle, 2009). Combining the bioinformatic analysis with mass spectrometry allowed targeting of specific sites within PUMA that are predicted to be phosphorylated. Netphos predicted 6 serine residues were likely to be phosphorylated but only one of these, serine 10, was positively detected using mass spectrometric analysis. The phospho-peptide initially identified using MALDI-TOF MS/MS encompassing serine 9 and 10 could be confirmed as containing a single phosphate group but the existence of two serine residues within its sequence did not allow for a definitive site of regulation to be determined. By using ESI MS/MS and applying ETD, the isolated peptide containing a phosphorylated serine 9 or 10 could be fragmented to separate the two residues while ensuring that the phosphate group is retained on the serine side-chain. The ETD results suggest that serine 9 is unlikely to be phosphorylated, particularly when serine 10 phosphorylation is present, as the predicted c-ions were not recorded in the ETD spectra. However, this does not rule out the possibility of other serine residues within PUMA also being phosphorylated as this technique relies heavily on the successful purification of phosphorylated peptides from the tryptic digest in addition to the peptides requiring properties that allow for ionisation and analysis in a mass spectrometer.

To demonstrate the significance of PUMA serine 10 on the total phosphorylation of PUMA that was observed, characterisation of serine 10 and mutational analysis of PUMA was performed. Orthophosphoric labelling of PUMA protein which had a serine 10 to alanine mutation confirmed that PUMA serine 10 was the predominate residue within PUMA that was phosphorylated in response to serum or IL-3 stimulation. The PUMA^{S10A} mutation inhibited the ability of PUMA to be phosphorylated in HEK 293T cells, however, there were two visible phosphorylation bands in FDM cells and only one disappeared following mutation of serine 10. This indicates that either a non-specific protein was immunoprecipitated in FDM cells which undergoes phosphorylation in response to IL-3 re-addition, or alternatively, a protein specifically interacting with PUMA in a serine 10 independent manner. Additionally, while only phosphorylated serine 10 was identified by mass spectrometric analysis there are potentially other sites within PUMA that can also be phosphorylated. Multiple sites of regulation from the same stimuli are present in many BH3-only proteins such as Bim with each residue playing a specific role in regulating Bim activity (Hubner *et al.*, 2008). It is possible that there were several phosphorylation bands in HEK 293T cells that did not resolve apart enough to visualise both, however, it would then be expected that there would be a decrease in the bands intensity rather than a complete loss when the site was mutated.

The additional phosphorylation site(s) that appear in FDM cells in response to IL-3 signalling may not be regulated by serum treatment of HEK 293T cells and, therefore, would not be phosphorylated in response to serum treatment. Additionally, phosphorylation sites in other BH3-only proteins such as Bad Ser112 are known to play a 'gatekeeper' role in that they must be dephosphorylated before subsequent regulation of other sites can occur (Chiang *et al.*, 2003). Some kinases such as GSK-3, CKII and PDK1 require a phospho-serine docking sites in order to phosphorylate additional sites within a protein (Fiol *et al.*, 1987; Frodin *et al.*, 2002). PUMA serine 10 may play a similar role in HEK 293T cells in response to serum stimulation where it is required to be phosphorylated before additional sites can be phosphorylated through conformational changes or creation of kinase docking sites. Each site of regulation within PUMA is likely to have a specific role in regulating PUMA activity. To determine the specific role of PUMA serine 10 an analysis of its regulation and the behaviour of serine 10 mutants should be preformed. This is addressed in chapter 4.

Chapter 4

Biological significance of PUMA serine 10 phosphorylation

4.1 Introduction

Signalling from the IL-3 receptor initiates an array of different signalling cascades utilising a number of kinase signalling pathways that are reliant on serine/threonine phosphorylation for their successful progression and regulation of effector proteins (de Groot *et al.*, 1998; Hercus *et al.*, 2009). There are numerous ways in which cytokine signalling elicits the desired downstream effect. At the level of the receptor, the dose of cytokine a receptor is exposed to can determine which signalling pathways are initiated and lead to the regulation of different downstream proteins as is the case for the GM-CSF receptor and likely to be the case for IL-3 and IL-5 which all utilise the common β-chain receptor. Low doses of GM-CSF promote only cell survival by, in part, regulating Bcl-2 family members through PI-3K activation while higher doses promote cell proliferation in addition to survival by activating the full complement of signalling pathways from the receptor (Guthridge *et al.*, 2006; Hansen *et al.*, 2008). Downstream of the receptor, signalling kinases can cause different effects dependent on the duration of their activation. A well characterised example of this type of regulation is ERK kinase. Treatment of PC12 cells, a neuronal cell line, with Nerve Growth Factor (NGF) causes sustained activation of ERK and differentiation into sympathetic neurons, however, treatment with Epidermal Growth Factor (EGF) causes transient activation of ERK and promotes survival and proliferation of cells without differentiation (Gotoh *et al.*, 1990; Marshall, 1995; Nguyen *et al.*, 1993). In quiescent fibroblasts, sustained but not transient ERK activation is required for initiation of proliferation in response to thrombin or Platelet-derived Growth Factor (PDGF) signalling (Balmanno and Cook, 1999; Dobrowolski *et al.*, 1994; Murphy *et al.*, 2002; Vouretcraviari *et al.*, 1993). Therefore, it is important when characterising the regulation of a protein by serine/threonine phosphorylation to determine the spatiotemporal setting in which this regulation occurs as this may play a significant role in determining the outcome of the signalling event.

There are a number of changes in the structure and behaviour of proteins that can be driven by phosphorylation of serine/threonine residues. A phosphate group attached to an amino acid side-chain carries two negative charges and can therefore initiate major conformational changes by attracting clusters of positively charged amino acids within the protein or repelling a particular part of the protein away from the phosphate group. These structural changes can have allosteric effects at other sites within the protein allowing, for

example, the activation/inhibition of an active or catalytic site within a protein. The regulation can often be reversed by removal of the phosphate group by phosphatases (Alberts *et al.*, 2002; Walsh and Jefferis, 2006). In addition to binding and repelling domains within the phosphorylated protein the negatively charged group can also bind to similar sites within other proteins facilitating the interaction of binding partners or causing steric hindrance of an interaction thus allowing regulation of the assembly and disassembly of protein complexes (Walsh and Jefferis, 2006; Yaffe and Elia, 2001). It is these consequences of serine/threonine phosphorylation that permit the transduction of signals down a kinase signalling cascade and regulate the activity and stability of effector proteins at the end of these cascades. This form of regulation occurs in an enormous array of proteins and is one of the most important mechanisms for regulating the activity of the Bcl-2 family proteins.

Phosphorylation of Bcl-2 family members has been shown to promote or inhibit protein activity with many proteins having multiple sites of regulation. Phosphorylation of Bcl-2 within a flexible loop between the BH3 and BH4 domains, conserved amongst Bcl-2, Bcl-x_L and Mcl-1, has been demonstrated as an essential site of regulation in response to survival and apoptotic stimuli (Fang *et al.*, 1998; Ito *et al.*, 1997; Srivastava *et al.*, 1999). There is conflicting data from studies of Bcl-2 phosphorylation within this loop as phosphorylation of multiple sites (Ser70, Thr69, Ser87) can both promote and inhibit apoptosis. Phosphorylation of Ser70 alone or in conjunction with other site in response to IL-3, etoposide or paclitaxel treatment is able to inhibit the progression of apoptosis by increasing Bcl-2 protein stability and preventing degradation (Deng *et al.*, 2004; Deng *et al.*, 2000; Ruvolo *et al.*, 2001). These results are supported by the observation that Bcl-2 dephosphorylated at Ser87 becomes susceptible to ubiquitin-dependent degradation (Breitschopf *et al.*, 2000). However, in a number of different cell lines, paclitaxel treatment was also shown to induce mono- and multisite phosphorylation in the flexible loop and inhibit Bcl-2 pro-survival activity (Srivastava *et al.*, 1999; Yamamoto *et al.*, 1999). Therefore, Bcl-2 phosphorylation appears to be heavily dependent on the cellular context of the regulation when determining the impact on its activity.

Another pro-survival family member, Bcl-x_L, is also regulated by phosphorylation at several sites. As opposed to the complex regulation of Bcl-2, addition of phosphates to Bcl-x_L has only been found to inhibit its pro-survival activity and in some cases is required for the progression of apoptosis, however, the mechanism of this regulation is not entirely clear (Basu and Haldar, 2003; Kazi *et al.*, 2002; Kharbanda *et al.*, 2000; Vijapurkar *et al.*, 2007). Mcl-1 has also been shown to have multiple sites of regulation and like Bcl-2 they can be both pro-survival or pro-apoptotic. The stability of Mcl-1 protein is significantly less than either Bcl-2 or Bcl-x_L and is heavily regulated by phosphorylation (Iglesias-Serret *et al.*, 2003; Kozopas *et al.*, 1993; Schubert and Duronio, 2001). Phorbol ester treatment stimulates Mcl-1 phosphorylation at Thr163, promoting the stability of Mcl-1 protein and inhibiting apoptosis (Domina *et al.*, 2004). However, when cytokine is withdrawn from IL-3 dependent cells Mcl-1 becomes phosphorylated on Ser159, facilitating its ubiquitin mediated degradation and promoting apoptosis (Maurer *et al.*, 2006). Mcl-1 is regulated at a functional level by stimuli such as oxidative stress where phosphorylation reduces its pro-survival activity (Inoshita *et al.*, 2002). Mcl-1 can also be regulated to promote survival. TRAIL treatment of KMCH-1 cells, a cholangiocarcinoma cell line, leads to phosphorylation of Ser64 and has no effect on Mcl-1 stability but instead increases the pro-survival activity of Mcl-1 by enhanced binding to the pro-apoptotic proteins Bak, Bim and Noxa (Kobayashi *et al.*, 2007).

In the same manner as the pro-survival Bcl-2 family members, the BH3-only family are also regulated by phosphorylation leading to a diverse array of regulatory responses. Bad was the first BH3-only protein shown to be regulated. Growth factor and cytokine stimulation leads to the phosphorylation of Bad at Ser112 and Ser136 creating a binding site for the phospho-serine binding protein 14-3-3 and leading to the sequestration of Bad in the cytoplasm, preventing it from antagonising the pro-survival Bcl-2 proteins (Peruzzi *et al.*, 1999; Shimamura *et al.*, 2000; Zha *et al.*, 1996). This interaction with 14-3-3 can be disrupted by phosphorylating Bad at Ser128 (Donovan *et al.*, 2002). The pro-apoptotic activity of Bad can also be ablated by steric inhibition with phosphorylation of Bad at Ser155, a site within the BH3 domain, preventing an interaction with the pro-survival Bcl-2 family members (Datta *et al.*, 2000; Tan *et al.*, 2000; Zhou *et al.*, 2000). Phosphorylation of Bad at Thr201 was also found to inhibit binding to Bcl-x_L but is located outside of the BH3 domain (Yu *et al.*, 2004). Bim is also regulated by phosphorylation with most

modifications regulating protein stability. Bim has 3 major splice variants; Bim_{EL}, Bim_{L} and Bim_{S} (Bouillet *et al.*, 2001). Phosphorylation of Bim_{EL} at multiple sites by survival stimuli has been demonstrated to regulate protein stability through ubiquitin-mediated degradation, leading to an inhibition of apoptosis (Fukazawa *et al.*, 2004; Hubner *et al.*, 2008; Ley *et al.*, 2003; Luciano *et al.*, 2003). Apoptosis can also be inhibited by IL-3 signalling promoting the phosphorylation of Bim at Ser87 and leading to an interaction with 14-3-3 in a similar manner to Bad (Qi *et al.*, 2006). Other sites within Bim_{EL} have been shown to regulate its interaction with Bcl-2 family members when phosphorylated, inhibiting apoptosis (Hubner *et al.*, 2008). Bid requires caspase cleavage to be targeted to the mitochondrial membrane and induce apoptosis (Wei *et al.*, 2000). Phosphorylation of serine and threonine residues also plays a role in this event with the process tightly controlled by phosphorylation of residues surrounding the caspase cleavage site (Esposti *et al.*, 2003).

The multidomain, pro-apoptotic Bcl-2 family members are also regulated by phosphorylation. Bax activation requires the exposure of a C-terminal transmembrane domain to allow oligomerisation and insertion into the outer mitochondrial membrane. GM-CSF treatment was found to phosphorylate Ser184 within this critical domain and prevent apoptosis by inactivating Bax (Gardai *et al.*, 2004). However, activation of GSK-3β leads to phosphorylation of Bax at Ser163, enhancing the conformational change required for Bax activation and promoting apoptosis (Linseman *et al.*, 2004). JNK and p38 can also play a similar role by phosphorylating Thr167, again, enhancing the Bax conformational change and promoting apoptosis (Kim *et al.*, 2006a).

The regulation of proteins by phosphorylation is critical for the control of cellular systems including the intrinsic apoptosis pathway. Cells have utilised phosphorylation in a large variety of mechanisms to regulate, both positively and negatively, the activity of Bcl-2 family members. PUMA has now been identified as a phosphorylated protein, however, the result of this phosphorylation on PUMA activity is unknown. In this chapter I characterise the biological significance of phosphorylation of PUMA serine 10 in response to serum and IL-3 signalling. Upon phosphorylation of PUMA serine 10 the localisation and association of PUMA with other Bcl-2 family members remains unchanged, however,

the rate of PUMA degradation was found to be increased. These novel observations demonstrate how serum and IL-3 signalling promotes the regulation of PUMA to rapidly inhibit PUMA apoptotic activity and promote the survival of cells that are yet to commit to apoptosis.

4.2 Results

4.2.1 Development and characterisation of a PUMA phospho-serine 10 antibody

In order to specifically study PUMA serine 10 phosphorylation, a rabbit polyclonal PUMA phospho-serine 10 (PUMA pS10) antibody was developed for the purposes of western blot analysis and potentially immunoprecipitation. The antibody serum from a number of rabbits which underwent a PUMA pS10 peptide immunisation program were tested by dot blot analysis for their affinity toward a synthetic PUMA pS10 peptide and their lack of reactivity toward both the non-phosphorylated peptide and a peptide phosphorylated at serine 9. A pool of antibodies was purified which showed significant specificity toward PUMA peptide phosphorylated at serine 10 at a variety of dilutions (Figure 4.1). Specificity toward PUMA pS10 in the context of the total protein was determined by immunoprecipitation of wild type HA-PUMA and HA-PUMA^{S10A} that had been overexpressed in FDM cells ± IL-3 stimulation at a variety of concentrations after a period of IL-3 starvation, followed by SDS-PAGE and western blot analysis probing with the PUMA pS10 antibody and anti-HA as a load control. The result demonstrated that while there is some non-specific binding to the non-phosphorylated PUMA a significantly stronger band can be seen following treatment of FDM cells containing wild type HA-PUMA with 7.5 ng/mL IL-3 while this signal is not seen when HA-PUMA^{S10A} is treated under the same conditions (Figure 4.2).

Using the PUMA pS10 antibody for Western blot analysis will be an invaluable tool, however, an ability to purify PUMA phosphorylated at serine 10 by immunoprecipitation would also be extremely useful, particularly for co-immunoprecipitation studies. To determine if the PUMA pS10 antibody can also be used for immunoprecipitation of PUMA pS10, HA-PUMA was overexpressed in FDM cells followed by 24 hours of IL-3 starvation and re-addition of IL-3 for 20 mins. Lysates were then subjected to immunoprecipitation using either the PUMA pS10 or anti-HA antibody before Western blot analysis probing with either anti-HA or PUMA pS10. The blot probed with anti-HA demonstrates equal PUMA expression with no expression in the non-induced cells in the input and HA IP lanes, however, a weak band appears in the +IL-3 PUMA pS10 IP lane at the correct size for PUMA which is not present in the –IL-3 or non-induced lanes. Probing the blot with the PUMA pS10 antibody confirms that the IL-3 stimulation was successful as induction of

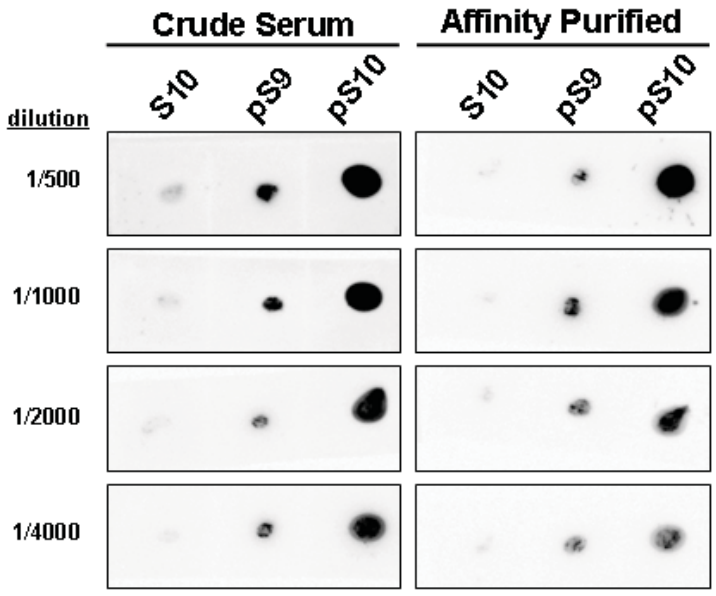


Figure 4.1 Affinity purification of PUMA phospho-serine 10 antibody. Crude antibody serum from a rabbit immunised with a phospho-serine 10 peptide from PUMA was collected and subjected to affinity purification against the immunogen. The crude antibody serum and affinity purified antibody was tested by serial dilution targeting 2 ug of either a non-phosphorylated peptide (ARARQEGSSPEPVEGLC), a peptide phosphorylated at serine 9 (ARARQEG(pS)SPEPVEGLC) or on serine 10(ARARQEGS(pS)PEPVEGLC).

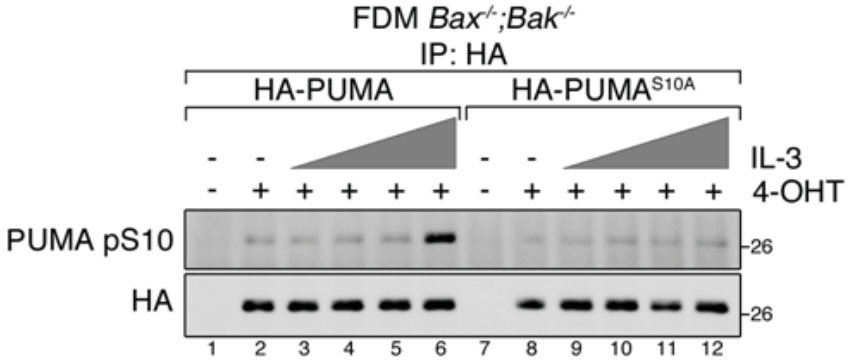


Figure 4.2 PUMA phospho-serine 10 antibody does not detect PUMA mutated at serine 10. *Bax*$^{-/-}$*;Bak*$^{-/-}$ FDM cells containing the lentiviral constructs encoding HA-PUMA wild type or HA-PUMA^{S10A} described earlier were induced using 100 nM 4-OHT 16 hours before starving cells of IL-3 for 24 hours. Cells were then stimulated by IL-3 re-addition at increasing concentrations for 15 min (lanes 3 and 9, $1.5x10^{-4}$ ng/mL; lanes 4 and 10, $1.5x10^{-3}$ ng/mL; lanes 5 and 11, $1.5x10^{-2}$ ng/mL; lanes 6 and 12, 7.5 ng/mL). HA-PUMA was immunoprecipitated using anti-HA before immunoprecipitates were resolved by SDS-PAGE and immunoblotted using PUMA pS10 or anti-HA antibodies.

phosphorylation can be seen in the input and HA IP lanes. Unfortunately the PUMA pS10 IP lanes have too many background bands to visualize the protein using this antibody and the immunoprecipitation of PUMA pS10 was too weak to be useful for any practical application (Figure 4.3).

4.2.2 PUMA serine 10 is phosphorylated in a dose and time dependent manner

It has been established that PUMA serine 10 is phosphorylated in response to IL-3 stimulation. This experiment aimed to characterise the dose of IL-3 required to visualise PUMA serine 10 phosphorylation and the timeframe over which the phosphorylation occurs. To determine the dose and time, FDM *Bax*$^{-/-}$*;Bak*$^{-/-}$ cells were starved of IL-3 for 24 hrs before re-addition of IL-3 for either a range of doses up to 10 ng/ml or a time course over 120 minutes at a concentration of 10 ng/ml. FDM *PUMA*$^{-/-}$ cells treated with IL-3 for 15 min were used as a negative control and FDM wild type cells treated with IL-3 for 15 min were used to demonstrate that this regulation can occur in the presence of Bax and Bak. Cells were lysed, protein collected and separated by SDS-PAGE before western blot analysis. The results showed that at concentrations of IL-3 at or below 1 ng/ml there is only a faint band seen after probing with the PUMA pS10 antibody indicating very weak phosphorylation of PUMA or potentially the antibody binding non-specifically to the unphosphorylated protein. However, when cells were treated with 10 ng/ml IL-3 a more intense band appeared suggesting that this dose of IL-3 is sufficient to allow visualisation of endogenous PUMA phosphorylation by Western blot analysis using the PUMA pS10 antibody (Figure 4.4). The results of the time course demonstrated when FDM cells were treated with 10 ng/ml IL-3, phosphorylation of PUMA can begin to be seen at 5 mins, however, the peak of phosphorylation is not seen until 15 min post-treatment after which the degree of phosphorylation diminishes over 30 and 60 min and is no longer observed at 120 min. No bands were seen in the FDM *PUMA*$^{-/-}$ cells before or after IL-3 treatment confirming that the regulated band which was observed is PUMA. The FDM wild type cells confirmed that this regulation also occurs in the presence of Bax and Bak. Probing the Western blot with a total PUMA antibody confirms equal amounts of PUMA were present in the FDM *Bax*$^{-/-}$*;Bak*$^{-/-}$ and wild type lanes and that no PUMA can be seen in the FDM *PUMA*$^{-/-}$ cell controls (Figure 4.4).

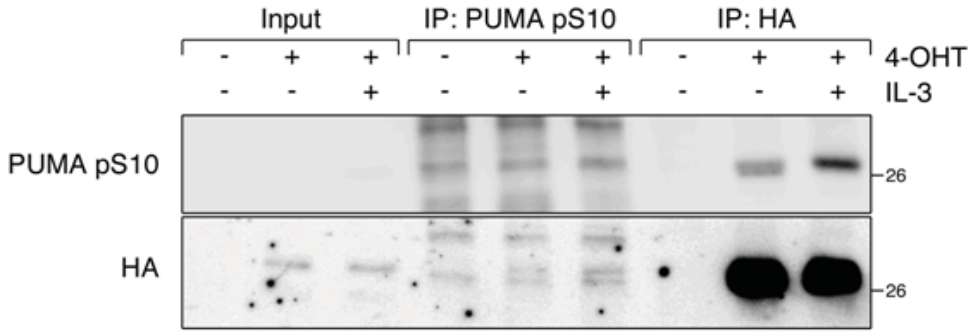


Figure 4.3 PUMA phospho-serine 10 antibody can immunoprecipitate PUMA phosphorylated at serine 10. FDM *Bax*$^{-/-}$;*Bak*$^{-/-}$ cells containing an inducible expression cassette for HA-tagged PUMA Wt were induced using 100 nM 4-hydroxy tamoxifen (4-OHT) 16 hrs before starving cells of IL-3 for 24 hrs. Cells were then stimulated by IL-3 at 8 ng/ml for 15 mins. HA-PUMA was immunoprecipitated using either phospho-PUMA serine 10 antibody or anti-HA (12CA5) Sepharose gel. Immunoprecipitates were resolved by SDS-PAGE and then immunoblotted using antibodies against PUMA pS10 or an anti-HA (12CA5) antibody.

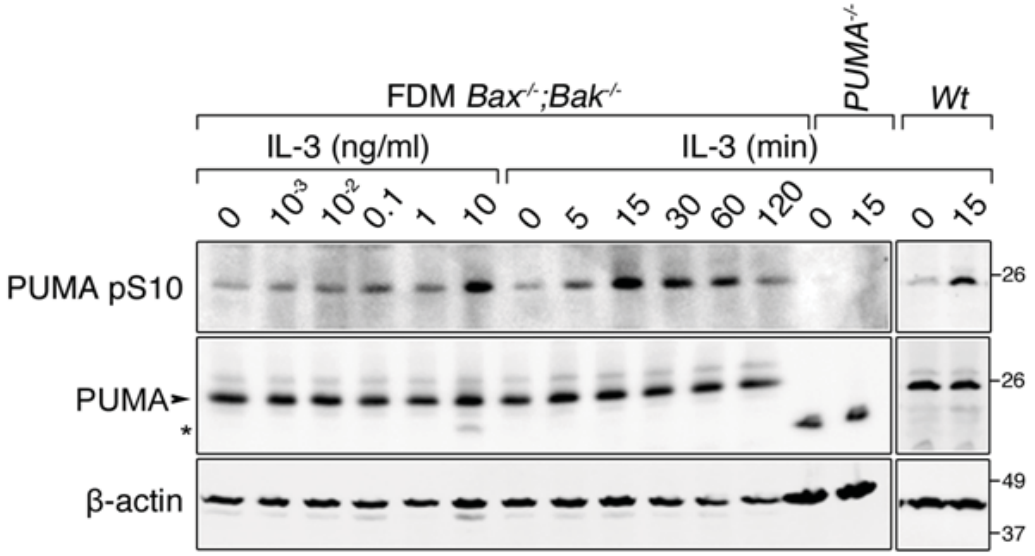


Figure 4.4 Endogenous PUMA is phosphorylated at serine 10 by IL-3 signalling. FDM *Bax*$^{-/-}$;*Bak*$^{-/-}$, FDM *PUMA*$^{-/-}$ or FDM wild type cells were starved of IL-3 for 24 hrs. Cells were then stimulated with IL-3 for 15 mins at the indicated concentrations or were stimulated with 10 ng/ml IL-3 for the indicated periods of time. Cells were then lysed, proteins resolved by SDS-PAGE and then immunoblotted using antibodies against PUMA pS10, total PUMA (* indicates non-specific band) or β-actin.

4.2.3 PUMA phosphorylated at serine 10 remains at the mitochondria

PUMA is known to localise to the mitochondrial outer membrane (Yee and Vousden, 2008). To examine the possibility that phosphorylation of PUMA at serine 10 is responsible for regulating PUMA localisation, FDM *Bax*$^{-/-}$;*Bak*$^{-/-}$ cells were starved of IL-3 for 24 hrs before re-addition of IL-3 in a time course over 60 minutes at a concentration of 10 ng/ml. Cells were then lysed and resolved into membrane and cytosolic fractions before proteins were separated by SDS-PAGE and subjected to Western blot analysis. Probing with the PUMA pS10 antibody demonstrated that phosphorylation of PUMA serine 10 is highest at 15 mins and occurs entirely in the membrane fraction of the cell (Figure 4.5). Probing the blot with total PUMA confirms that PUMA remained in the membrane fraction over the IL-3 treatment time course. At no stage in this time course treatment experiment was PUMA seen in the cytosolic fraction indicating that the observed event occurs entirely in the membrane fraction of the cell. Probing the blot with EF-1α, a cytosolic protein, and VDAC, a mitochondrial membrane bound protein, confirmed that fractionation was successful and loading in each lane was equal (Figure 4.5).

4.2.4 PUMA serine 10 mutants induce apoptosis and bind to anti-apoptotic Bcl-2 family members

Our data indicated that PUMA phosphorylation may be associated with inactivation of the protein since PUMA phosphorylation increased following the re-addition of IL-3, when cells are "recovering" from cytokine deprivation. To explore the functional effects of serine 10 phosphorylation on PUMA, we generated mutations of serine 10. In addition to using the PUMA^{S10A} mutant to prevent phosphorylation of PUMA at serine 10 we also generated PUMA mutants in which serine 10 was substituted with an aspartate (PUMA^{S10D}) or glutamate (PUMA^{S10E}) in an effort to enforce a negative charge at position 10 and mimic serine 10 phosphorylation. These constructs were inducibly expressed in *Bax*$^{-/-}$;*Bak*$^{-/-}$ FDM cells before immunoprecipitation of the HA-tagged protein. Both wild type PUMA and all PUMA serine 10 mutants co-immunoprecipitated Bcl-x_L and Mcl-1 (Figure 4.6). The ability of wild type PUMA and the serine 10 mutants to immunoprecipitate more Mcl-1 over time after IL-3 addition followed the levels of Mcl-1 present in the cells. Bcl-x_L levels remained steady in the presence or absence of IL-3 over

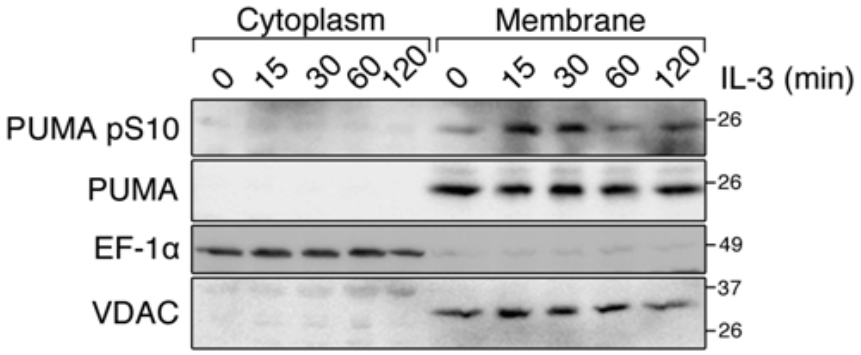


Figure 4.5 PUMA phosphorylated at serine 10 remains at the mitochondria. FDM *Bax*$^{-/-}$;*Bak*$^{-/-}$ cells were starved of IL-3 for 24 hrs. Cells were then stimulated with 10 ng/ml IL-3 for the indicated periods of time. Cells were then resolved into cytoplasm and membrane fractions, proteins resolved by SDS-PAGE and then immunoblotted using antibodies against PUMA pS10, total PUMA, EF-1α and VDAC.

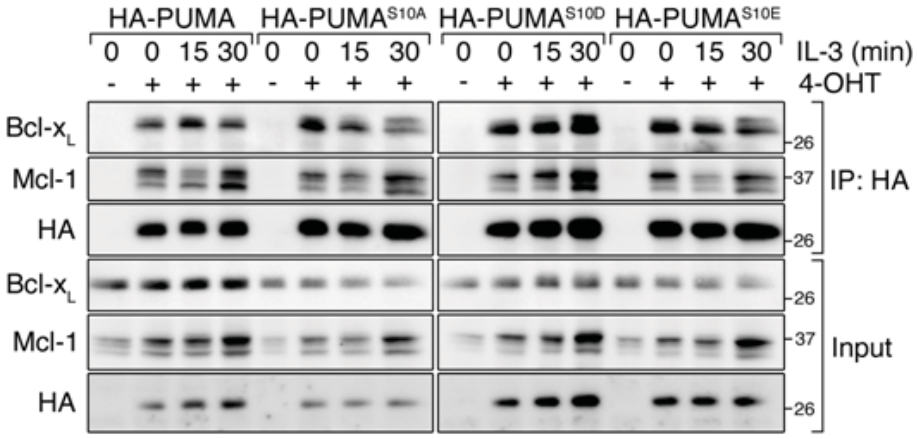


Figure 4.6 PUMA mutated at serine 10 retains its ability to interact with pro-survival Bcl-2 family members. *Bax*$^{-/-}$*;Bak*$^{-/-}$ FDM cells containing the wild type HA-PUMA, HA-PUMA^{S10A}, HA-PUMA^{S10D}, HA-PUMA^{S10E} constructs were induced using 100 nM 4-OHT 16 hours before starving cells of IL-3 for 24 hours. Cells were then stimulated by IL-3 (10ng/mL) for 15 and 30 min. HA-PUMA was immunoprecipitated using anti-HA before immunoprecipitates were resolved by SDS-PAGE and immunoblotted using antibodies against HA, Mcl-1 and Bcl-x_L.

the time course examined, and, as a consequence, little change in the amount of Bcl-x_L immunoprecipitated by PUMA was observed. This result indicated that mutation of serine 10 did not influence the ability of PUMA to bind to Bcl-x_L or Mcl-1 which suggests that phosphorylation of this residue regulates PUMA function independently of the ability of PUMA to bind anti-apoptotic Bcl-2 family members.

According to indirect and direct activation models for the initiation of apoptosis through the mitochondrial pathway, PUMA protein that can bind and antagonize the pro-survival Bcl-2 family members should be able to successfully promote apoptosis. To test the functional role of PUMA phosphorylated at serine 10, we overexpressed wild type PUMA, $PUMA^{S10A}$, $PUMA^{S10D}$ or $PUMA^{S10E}$ in *PUMA*$^{-/-}$ FDM cells so that the PUMA expressed was exclusively wild type or mutant PUMA. Viability was determined using Annexin V staining and propidium iodide exclusion measured by flow cytometry after induction of PUMA expression (Figure 4.7). Wild type and all PUMA mutants could induce apoptosis with only a subtle increase in the amount of apoptosis induced by $PUMA^{S10A}$ compared to wild type PUMA. $PUMA^{S10D}$ and $PUMA^{S10E}$ mutants were also able to induce a similar amount of apoptosis as wild type PUMA. These data demonstrated that mutation of PUMA serine 10 does not significantly affect the ability of PUMA to induce apoptosis when overexpressed.

4.2.5 PUMA serine 10 regulates PUMA protein stability

To determine whether mutation of PUMA serine 10 could regulate PUMA stability, we probed Western blots of lysates from *Bax*$^{-/-}$*;Bak*$^{-/-}$ FDM cells or *Bax*$^{-/-}$*;Bak*$^{-/-}$ MEFs expressing wild type PUMA, $PUMA^{S10A}$, $PUMA^{S10D}$ or $PUMA^{S10E}$ which had been subjected to a period of IL-3 or serum deprivation followed by re-stimulation in the presence of cycloheximide (CHX). PUMA expression over time was followed by Western blot (Figure 4.8). In FDM cells following IL-3 and CHX stimulation, wild type PUMA levels steadily declined over sixty minutes before reaching baseline levels. In contrast, levels of the PUMA proteins mutated at serine 10 remained elevated over the two hour time course of the experiment. Interestingly, the $PUMA^{S10D}$ and $PUMA^{S10E}$ mutants had the same effect on PUMA stability as mutating serine 10 to a non-phosphorylatable

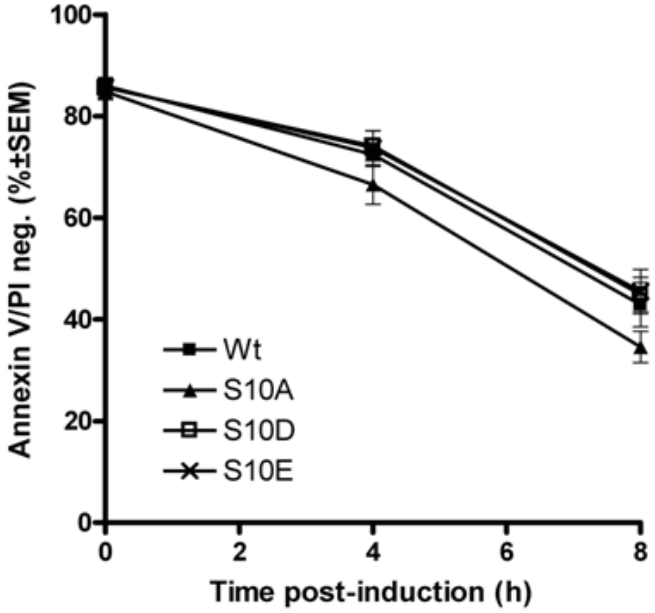


Figure 4.7 Overexpression of wild type PUMA and serine 10 mutants induces apoptosis. Multiple independent clones of *PUMA*$^{-/-}$ FDM cells expressing wild type HA-PUMA (6 clones), HA-PUMA^{S10A} (7 clones), HA-PUMA^{S10D} (6 clones), HA-PUMA^{S10E} (6 clones) were induced over a time course using 200 nM 4-OHT. Cell viability was determined at the indicated times by analyzing FITC-conjugated Annexin V and PI uptake by flow cytometry. Results show mean ±SEM of 5 independent experiments.

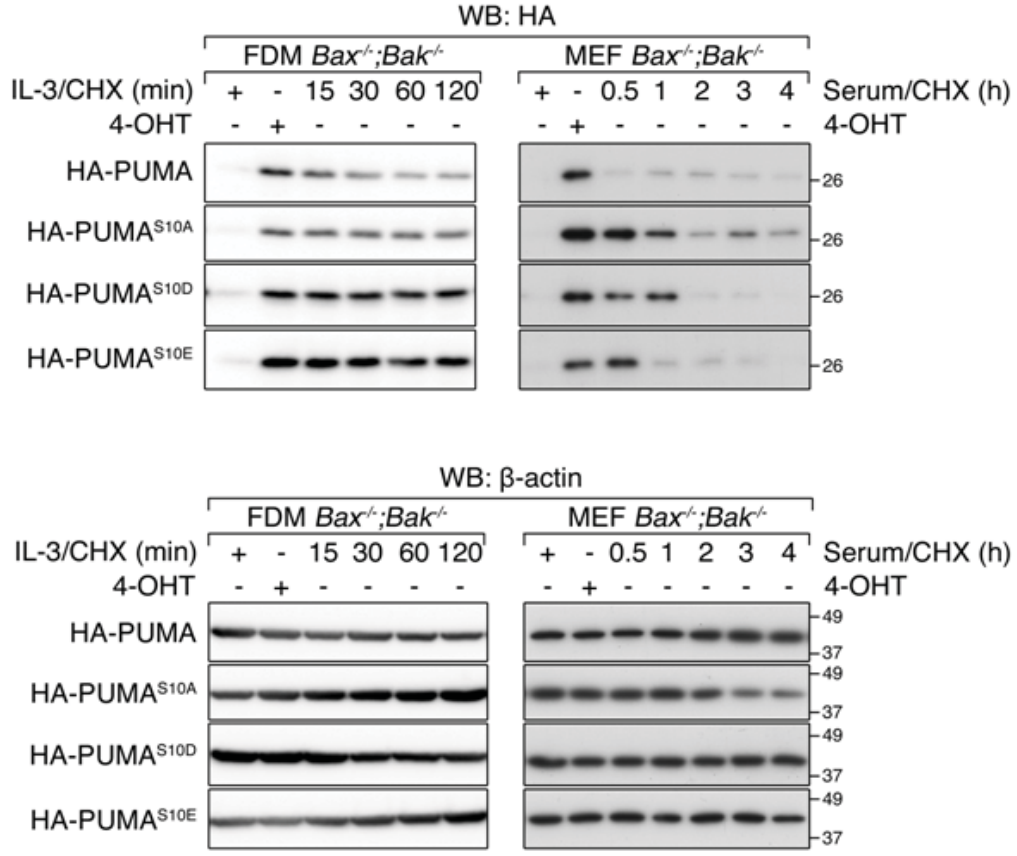


Figure 4.8 Mutation of PUMA at serine 10 increases PUMA protein stability. Expression of wild type HA-PUMA, HA-PUMA^{S10A}, HA-PUMA^{S10D} or HA-PUMA^{S10E} was induced in *Bax*$^{-/-}$;*Bak*$^{-/-}$ FDM cells (for 4 hours) or *Bax*$^{-/-}$;*Bak*$^{-/-}$ MEFs (for 2 hours) during a period of serum or IL-3 deprivation for 4 hours. Cells were re-stimulated with IL-3 (10ng/mL) or serum and cycloheximide (20μg/mL) for the indicated periods of time. Cell lysates were resolved by SDS-PAGE and probed with an anti-HA antibody to detect induced HA-PUMA expression and β-actin as a loading control.

alanine. In MEF cells following serum and CHX stimulation, wild type PUMA levels were observed to quickly fall to baseline levels over thirty minutes. As was the case in FDM cells, the PUMA proteins mutated at serine 10 had significantly longer half lives than wild type PUMA, however, in contrast to FDM cells, their levels ultimately declined over the course of the experiment. We also compared wild type PUMA and PUMA^{S10A} after IL-3 stimulation in *Bax*$^{-/-}$;*Bak*$^{-/-}$ FDM cells cultured in the presence of ^{35}S-methionine and ^{35}S-cysteine for one hour in the absence of IL-3. After IL-3 was added back, ^{35}S-methionine/^{35}S-cysteine-labeled PUMA^{S10A} expression was elevated over a twelve-hour period after IL-3 re-stimulation, whereas wild type-labelled PUMA declined within two hours of IL-3 re-addition (Figure 4.9). These results indicate that PUMA^{S10A} has a significantly longer half life than wild type PUMA, and suggests serine 10 phosphorylation of PUMA targets PUMA for degradation. The observation that aspartate and glutamate mutations of serine 10 have a similar effect to alanine mutation suggests that regulation of PUMA stability by phosphorylation of serine 10 is not a matter of an acidic, negatively charged residue in that position but specifically requires a serine residue.

We next used the phospho-specific anti-PUMA antibody to investigate effect of IL-3 signalling on endogenous PUMA stability. Lysates from wild type FDM cells stimulated with IL-3 were probed with the phospho-specific PUMA antibody and a total PUMA antibody. Following IL-3 stimulation, a peak of PUMA serine 10 phosphorylation was observed after fifteen minutes before returning to baseline levels by thirty minutes (Figure 4.10). Interestingly, the disappearance of PUMA phosphorylated at serine 10 after thirty minutes coincided with a decline in total PUMA protein levels. This observation confirms the data from overexpressed PUMA, demonstrating that IL-3 regulated phosphorylation of endogenous PUMA at serine 10 regulates total PUMA levels in wild type cells.

It has been observed that PUMA is rapidly degraded by caspases upon over expression in cells containing Bax and Bak (Callus *et al.*, 2008). To determine if PUMA serine 10 is involved in the acute degradation of PUMA following over expression, HA-PUMA wild type and HA-PUMA^{S10A} were expressed in FDM cells over a 6 hour timecourse. Cells were then lysed, protein collected and separated by SDS-PAGE before western blot analysis. The blot probed with anti-HA demonstrates that there is observable expression of

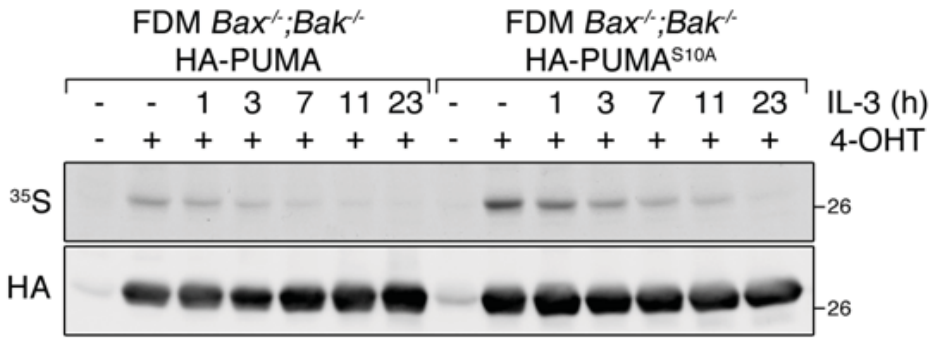


Figure 4.9 The stability of PUMA protein is reduced when PUMA is phosphorylated at serine 10. FDM *Bax*$^{-/-}$*;Bak*$^{-/-}$ cells containing an inducible expression cassette for HA-PUMA Wt or S10A mutant were induced using 100 nM 4-hydroxy tamoxifen (4-OHT) in addition to starving of IL-3 for 16 hours. Cells were then starved of cystine and methionine before being metabolically labelled with ^{35}S labelled cystine and methionine for 1 hrs. Cells were then washed into medium containing cold methionine and cystine for one hour before the addition of IL-3 at 10 ng/ml. Cells were lysed at several timepoints before HA-PUMA was immunoprecipitated using anti-HA (12CA5) Sepharose gel. Immunoprecipitates were resolved by SDS-PAGE and then either immunoblotted using anti-HA (12CA5) antibody or exposed to x-ray film.

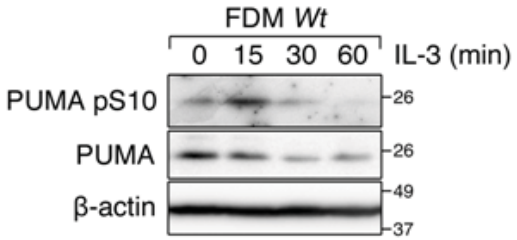


Figure 4.10 Phosphorylation of PUMA at serine 10 is accompanied by a loss of total PUMA protein. Wild type FDM cells were starved of IL-3 for 16 hours. Cells were then stimulated with IL-3 (10ng/mL) for the indicated periods of time. Protein lysates were resolved by SDS-PAGE before being immunoblotted using antibodies against PUMA pS10, total PUMA or β-actin.

HA-PUMA wild type and HA-PUMA^{S10A} at 1 hour post-induction with expression peaking at 2 hours and almost completely gone by 4 and 6 hours. In this experiment both wild type PUMA and PUMA^{S10A} are quickly degraded, likely through the action of caspase cleavage, following the peak of expression at 2 hours, demonstrating that PUMA serine 10 phosphorylation does not regulate the caspase cleavage of PUMA (Figure 4.11).

To investigate whether phosphorylated PUMA was degraded by the proteasome, we made lysates from *Bax*$^{-/-}$*;Bak*$^{-/-}$ FDM cells stimulated with IL-3 in the presence or absence of the proteasome inhibitor MG132. These lysates were probed with an anti-PUMA antibody and the phospho-specific anti-PUMA antibody. MG132 substantially prolonged the half-life of phosphorylated PUMA without significantly affecting total PUMA levels (Figure 4.12). Endogenous PUMA is expressed to much higher levels in *Bax*$^{-/-}$*;Bak*$^{-/-}$ FDM cells compared to wild type cells, presumably because *Bax*$^{-/-}$*;Bak*$^{-/-}$ FDM cells are completely resistant to apoptosis induced by PUMA expression, and PUMA may accumulate without causing cell death. This likewise indicates that the capacity of the normal mechanisms regulating total PUMA levels are exceeded by the levels of PUMA in these cells. This is the most probable explanation for the observation that total PUMA levels do not decline after IL-3 stimulation in *Bax*$^{-/-}$*;Bak*$^{-/-}$ FDM cells as they do in wild type cells (Figure 4.10). The results showing an increase in endogenous phosphorylated PUMA, together with a decline in total endogenous PUMA levels (Figure 4.10) most accurately reflects what occurs physiologically. This result suggests that phosphorylated PUMA undergoes proteasomal degradation in a manner that is blocked by MG132. Because PUMA contains no lysine residues, proteasomal degradation of PUMA might occur by ubiquitination of the N-terminus of the protein, however this remains to be determined.

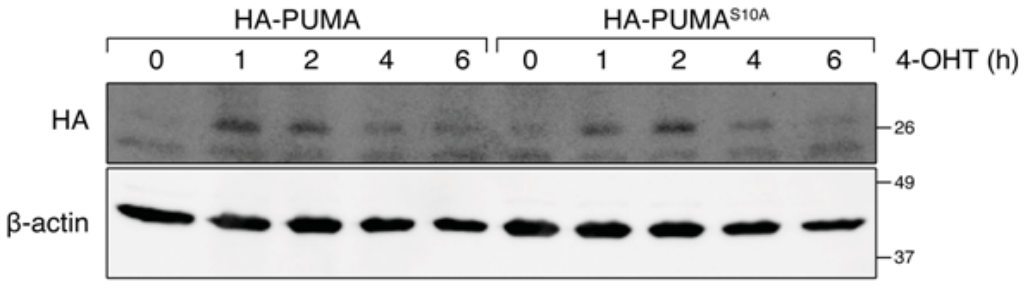


Figure 4.11 PUMA^{S10A} remains susceptible to degradation by caspase activation. FDM wild type cells containing an inducible expression cassette for HA-PUMA Wt or S10A mutant were induced using 1 μM 4-hydroxy tamoxifen (4-OHT) with cells lysed at the indicated time points over a 6 hour time course. Lysates were resolved by SDS-PAGE and immunoblotted using either anti-HA (12CA5) or anti-β-actin antibody.

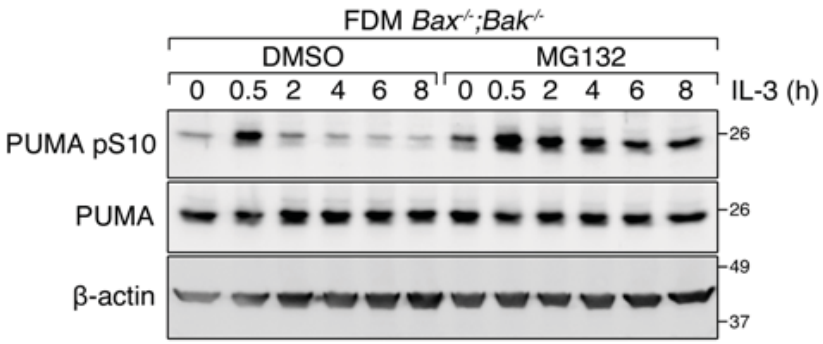


Figure 4.12 PUMA serine 10 phosphorylation regulates PUMA degradation through the proteasome. *Bax*$^{-/-}$*;Bak*$^{-/-}$ FDM cells were starved of IL-3 for 16 hours. Cells were then pre-treated with either 0.1% (v/v) DMSO or 25 μM MG132 for 30 minutes before re-addition of IL-3 at 10 ng/mL for the indicated periods of time. Cells were then lysed, proteins resolved by SDS-PAGE and then immunoblotted using antibodies against PUMA pS10, total PUMA or β-actin.

4.3 Discussion

The regulation of PUMA serine 10 phosphorylation from signalling induced by serum and IL-3 treatment and the effect of such regulation on the ability of a cell to regulate PUMA stability has been demonstrated by the results in this chapter. Development of a phospho-specific polyclonal antibody that detects PUMA phosphorylated at serine 10 allowed for regulation of both over-expressed and endogenous PUMA serine 10 to be characterised. Upon IL-3 stimulation of FDM cells, PUMA serine 10 was phosphorylated in a dose and time dependent manner in a pattern that mimicked the phosphorylation of PUMA seen in the ^{32}P orthophosphate labelling experiments. This supports the suggestion that a majority of the phosphorylation that was originally observed can be attributed to phosphorylation of PUMA at serine 10.

Growth factor and cytokine stimulated phosphorylation of the BH3-only family members Bad and Bim can lead to sequestration and re-localisation within the cell (Qi *et al.*, 2006; Zha *et al.*, 1996), however, following IL-3 treatment neither the phosphorylated nor non-phosphorylated PUMA was observed to be outside of the mitochondrial, membrane fraction. Other means of regulating BH3-only proteins are known to involve inhibition of interactions with binding partners such as the pro-survival Bcl-2 family members. The BH3 domain is critical for the interaction between BH3-only proteins and their pro-survival binding partners. The PUMA BH3 domain forms a stable alpha-helix upon binding to the hydrophobic groove in the pro-survival proteins (Smits *et al.*, 2008). PUMA serine 10 does not lie within the BH3 domain, however, residues known to be involved in regulating interactions with pro-survival proteins can be within or outside of the BH3 domain (Datta *et al.*, 2000; Hubner *et al.*, 2008; Virdee *et al.*, 2000). When co-immunoprecipitation experiments were performed, PUMA was able to bind to the pro-survival proteins Bcl-x_L and Mcl-1 in the presence or absence of IL-3 and this interaction was not disrupted when PUMA serine 10 was mutated to an alanine, preventing phosphorylation, or an aspartic or glutamic acid to potentially mimic phosphorylation at serine 10. Other novel PUMA binding partners may be promoted or disrupted by PUMA serine 10 phosphorylation, however, it is unclear how PUMA would be affected structurally by phosphorylation of serine 10 as many BH3-only proteins, including PUMA, are intrinsically unstructured outside of the BH3 domain (Hinds *et al.*, 2007).

Mutation of serine 10 to alanine, aspartic acid or glutamic acid all resulted in a prolonged PUMA half-life following either IL-3 or serum stimulation. The substitution of a negatively charged, acidic residue in place of serine 10 did not recapitulate constitutive serine phosphorylation. This is not surprising given that such mutations do not necessarily function as phosphomimics. Our results clearly show the specific requirement for a phosphorylated serine residue at this position to promote PUMA degradation. This may be because the phosphorylated serine co-ordinates a protein-protein interaction which ultimately results in PUMA degradation, but the nature of any such interaction remains to be determined.

When wild type FDM cells were starved of IL-3, PUMA was seen to peak before falling to baseline levels. The fall in PUMA phosphorylated at serine 10 was accompanied by a concomitant fall in total PUMA levels demonstrating that phosphorylation of PUMA at serine 10 my earmark PUMA protein for degradation. When *Bax*$^{-/-}$*;Bak*$^{-/-}$ FDM cells were starved of IL-3 and treated with a proteasome inhibitor the PUMA phospho-serine 10 levels remained elevated for at least 8 hours following IL-3 re-stimulation while the PUMA in cells without proteasome inhibition demonstrated a loss of phospho-serine 10 by 2 hrs. This observation demonstrates that PUMA phosphorylated at serine 10 is degraded through the proteasome, however, the result was intriguing as PUMA does not contain any lysine residues which are normally required for the covalent attachment of poly-ubiquitin molecules, a necessary step to drive a protein to the proteasome for degradation (Haglund and Dikic, 2005). Recently it has been discovered that ubiquitin can also be attached to the N-terminus of proteins and does not require the presence of lysine residues in the target protein (Ciechanover and Ben-Saadon, 2004). Therefore, it is likely that any ubiquitination of PUMA occurs at the N-terminus.

The results in this chapter demonstrate that the role of PUMA serine 10 phosphorylation in response to IL-3 signalling is to regulate PUMA protein stability and not cellular localisation or interactions with known binding partners. The regulation of PUMA abundance within a cell is an important finding as it has been demonstrated that a critical factor regulating the induction of apoptosis within a cell is the balance of pro-survival and pro-apoptotic proteins (Callus *et al.*, 2008; Mason *et al.*, 2007). Using IL-3 as the survival

stimuli has allowed us to define the regulation to a single receptor, however, there are numerous kinase signalling pathways that are activated upon ligand binding. Because PUMA serine 10 is a novel site of regulation the specific kinase responsible for phosphorylation is unknown. The identification of the PUMA serine 10 kinase is addressed in chapter 5.

Chapter 5

Identification of the kinase responsible for PUMA serine 10 phosphorylation in response to serum and IL-3 signalling

5.1 Introduction

The Bcl-2 family of proteins are responsible for regulating apoptosis in response to a diverse range of stimuli. The signalling pathways and kinases utilised to regulate this process are equally as diverse (Table 5.1). To complicate matters further, many kinases have been found to both inhibit and promote apoptosis depending on the stimuli. For example, JNK has been found to phosphorylate numerous Bcl-2 family members. In response to paclitaxel treatment, microtubule-damaging agents, oxidative stress and ionising radiation JNK phosphorylates the pro-survival proteins Bcl-2, Bcl-x_L and Mcl-1 at multiple sites, promoting apoptosis (Basu and Haldar, 2003; Inoshita *et al.*, 2002; Kharbanda *et al.*, 2000; Srivastava *et al.*, 1999; Yamamoto *et al.*, 1999). However, in response to TRAIL treatment, JNK in conjunction with CDK1/2 phosphorylates Mcl-1 at an additional site and instead promotes the ability of Mcl-1 to inhibit apoptosis (Kobayashi *et al.*, 2007). CDK1 alone is responsible for opposing the activity of growth factors in post-mitotic neurons by acting to promote Bad apoptotic activity (Konishi *et al.*, 2002). Pro-apoptotic proteins have also been found to be targets of JNK phosphorylation. In response to stimuli such as UV radiation, staurosporine, H_2O_2, etoposide, and transcriptional regulation Bad, Bim and Bax are phosphorylated by JNK promoting their ability to induce apoptosis (Becker *et al.*, 2004; Donovan *et al.*, 2002; Kim *et al.*, 2006a; Lei and Davis, 2003). However, in response to IL-3 signalling JNK phosphorylates Bad at an additional site and prevents apoptosis (Yu *et al.*, 2004). Other MAPK kinase family members have more defined roles when regulating apoptosis. Following growth factor and cytokine treatment ERK1/2 phosphorylation promotes the ability of Bcl-2 and Mcl-1 to inhibit apoptosis while also antagonising the apoptotic activity of Bim (Deng *et al.*, 2000; Domina *et al.*, 2004; Harada *et al.*, 2004; Luciano *et al.*, 2003). Another MAPK, p38 kinase, promotes apoptosis by phosphorylating Bim and Bax in response to a vast array of cellular insults (Cai *et al.*, 2006; Kim *et al.*, 2006a). Following cytokine starvation and Fas-ligand treatment other members of the CMGC kinase family such as GSK-3 and CK1/2 phosphorylate all 3 classes of Bcl-2 family members, Bid, Bax and Mcl-1, to promote apoptosis (Desagher *et al.*, 2001; Ding *et al.*, 2007; Linseman *et al.*, 2004; Maurer *et al.*, 2006).

Kinase	Protein	Residue	Outcome	Reference
ERK1/2	Bcl-2	Ser70	Inhibition of apoptosis	Deng *et al.*, 2000
	Mcl-1	Thr163	Inhibition of apoptosis	Domina *et al.*, 204
	Bim_{EL}	Ser55, Ser65, Ser100	Inhibition of apoptosis	Harada *et al.*, 2004; Luciano *et al.*, 2003
p38	Bim_{EL}	Ser65	Promote apoptosis	Cai *et al.*, 2006
	Bax	Thr167	Promote apoptosis	Kim *et al.*, 2006
JNK	Bcl-2	Ser70, Thr69, Ser87	Promote apoptosis	Srivastava *et al.*, 1999; Yamamoto *et al.*, 1999
	$Bcl\text{-}x_L$	Ser62, Thr47, Thr115	Promote apoptosis	Basu *et al.*, 2003; Kharbanda *et al.*, 2000
	Mcl-1	Ser121, Thr163	Promote apoptosis	Inoshita *et al.*, 2002
	Mcl-1	Ser64	Inhibition of apoptosis	Kobayashi *et al.*, 2007
	Bad	Thr201	Inhibition of apoptosis	Yu *et al.*, 2004
	Bad	Ser128	Promote apoptosis	Donovan *et al.*, 2002
	Bim_L	Ser44, Thr56, Ser58	Promote apoptosis	Lei *et al.*, 2003
	Bim_{EL}	Ser65	Promote apoptosis	Becker *et al.*, 2004
	Bax	Thr167	Promote apoptosis	Kim *et al.*, 2006
CDK-1/-2	Mcl-1	Ser64	Inhibition of apoptosis	Kobayashi *et al.*, 2007
	Bad	Ser128	Promote apoptosis	Konishi *et al.*, 2002
PKC-α	Bcl-2	Ser70	Inhibition of apoptosis	Ruvolo *et al.*, 2001
PKC-ζ	Bax	Ser184	Inhibition of apoptosis	Xin *et al.*, 2007
Akt	Bad	Ser136, Ser155	Inhibition of apoptosis	Datta *et al.*, 2000; Peruzzi *et al.*, 1999
	Bim	Ser87	Inhibition of apoptosis	Qi *et al.*, 2006
	Bax	Ser184	Inhibition of apoptosis	Gardai *et al.*, 2004
PKA	Bad	Ser155	Inhibition of apoptosis	Tan *et al.*, 2000; Zhou *et al.*, 2000
RSK1	Bad	Ser112, Ser155	Inhibition of apoptosis	Tan *et al.*, 1999
Pim1/2	Bad	Ser112	Inhibition of apoptosis	Aho *et al.*, 2004; Yan *et al.*, 2003
CK1/2	Bid	Ser61, Ser64	Promote apoptosis	Desagher *et al.*, 2001
ATM	Bid	Ser61, Ser78	Promote apoptosis	Kamer *et al.*, 2005; Zinkel *et al.*, 2005
GSK-3	Mcl-1	Ser155, Ser159, Thr163	Promote apoptosis	Ding *et al.*, 2007; Maurer *et al.*, 2006
	Bax	Ser163	Promote apoptosis	Linseman *et al.*, 2004

Table 5.1 Kinases responsible for regulation of Bcl-2 family members.

Kinases within the AGC family are also involved in regulating the intrinsic apoptosis pathway, typically to inhibit apoptosis. Akt is a kinase commonly associated with survival signalling from a large variety of stimuli and acts directly on the Bcl-2 family by phosphorylating Bad, Bim and Bax (Datta *et al.*, 1997; Gardai *et al.*, 2004; Peruzzi *et al.*, 1999; Qi *et al.*, 2006). Activation of other AGC kinases such as PKA or RSK inhibits the ability of Bad to induce apoptosis by disrupting 14-3-3 binding (Tan *et al.*, 2000; Tan *et al.*, 1999; Zhou *et al.*, 2000). PKC-α and PKC-ζ have also been found to inhibit the activation of apoptosis by phosphorylating Bcl-2 and Bax respectively (Ruvolo *et al.*, 2001; Xin *et al.*, 2007). Other, more atypical, kinase families have recently been implicated in regulation of Bcl-2 family proteins. In response to pro-survival cytokine signalling Pim1/2 have been found to phosphorylate Bad and promote interaction with 14-3-3, inhibiting apoptosis, while in response to DNA damage ATM kinases phosphorylate Bid around the N-terminus and promote truncation, driving tBid toward the mitochondria to induce apoptosis (Aho *et al.*, 2004; Kamer *et al.*, 2005; Yan *et al.*, 2003; Zinkel *et al.*, 2005).

Another distinct kinase family that has been implicated in promoting cell survival and proliferation is the IκB Kinase (IKK) family (Kucharczak *et al.*, 2003; Luo *et al.*, 2005; Rocha *et al.*, 2003; Westerheide *et al.*, 2001). IKK family members are primarily involved in regulation of the NFκB pathway of transcription factors by phosphorylating inhibitor of NFκB (IκB) proteins via both canonical and non-canonical signalling pathways (Hayden and Ghosh, 2004). The canonical signalling pathway can be activated by several factors including engagement of the T-cell receptor, or treatment with IL-1, TNFα or LPS (Hayden and Ghosh, 2004). Functional IKK signalling induced by the canonical signalling pathway occurs by the activation of a complex composed of two IKK subunits that contain kinase domains, IKK1 and IKK2 (also known as IKKα and IKKβ), and a third, non-catalytic, subunit called NFκB essential modifier (NEMO, also known as IKKγ)(Hayden and Ghosh, 2004). NEMO is a requisite component of the IKK complex activated via the canonical pathway by regulating the phosphorylation and activation of IKK1 and IKK2 through recruitment of kinases such as TGFβ-activated kinase and ataxia telangiectasia mutated kinase to the IKK complex (Burns and Martinon, 2004; Chen, 2005; Krappmann and Scheidereit, 2005; Perkins, 2006; Wu *et al.*, 2006). The non-canonical signalling pathway can be activated by factors such as B-cell-activating factor, LPS and latent

membrane protein-1 and has been suggested to signal using IKK1 homodimers independent of the larger IKK complex (Bonizzi and Karin, 2004; Perkins, 2003).

IKK proteins are known to phosphorylate the IκB family of proteins, however, IKK proteins have increasingly been shown to target additional substrates which regulate cell survival and proliferation that are unrelated to the NFκB family. The transcription factor FoxO3a regulates apoptosis by, in part, controlling the transcription of Bim and PUMA (Gilley *et al.*, 2003; You *et al.*, 2006). The inhibition of FoxO3a activity is typically controlled by Akt phosphorylation to promote cell survival, however, in a number of tumours it was shown that IKK2 can also phosphorylate FoxO3a and inhibit its activity, contributing to tumourigenesis (Hu *et al.*, 2004). IKK2 has also been shown to phosphorylate 14-3-3β on Ser132 when it is bound to tristetraprolin, an AU-rich element (ARE) binding protein, to inhibit this interaction and promote mRNA stability (Gringhuis *et al.*, 2005). AREs are known to be widely used to regulate expression of a number of factors involved in cell survival. In addition to directly regulating protein activity, IKK2 can also regulate kinase signalling cascades. The MEK kinase TPL2 is known to form a complex with p105. IKK2 phosphorylation of p105 releases TPL2 from this complex and leads to downstream activation of the MAP kinase pathway (Beinke *et al.*, 2004; Waterfield *et al.*, 2004). Conversely, IKK2 can also downregulate MAP kinase signalling through phosphorylation of the adapter protein, DOK1 (Lee *et al.*, 2004). IKK1 has also been shown to have NFκB independent activity. In breast cancer, IKK1 phosphorylation of the oestrogen receptor-α and its co-activator SRC3 leads to activation of cell cycle and proliferation, contributing to tumourigenesis (Park *et al.*, 2005; Wu *et al.*, 2002).

There are a myriad of kinases and pathways known to be involved in regulating cell survival. Many of these have been shown to act either directly on the Bcl-2 family of proteins or indirectly by impacting on their transcriptional expression. I have demonstrated that PUMA serine 10 is responsible for regulating the stability and degradation of PUMA protein, however, the kinase and pathways regulating its phosphorylation remain to be discovered. There are numerous methods that can be utilised to identify the kinase of a known substrate including bioinformatic analysis, large scale screening and direct analysis of specific candidates. When these methods were employed to analyse the PUMA serine 10 motif the IKK family of proteins, and more specifically IKK1, was identified as a kinase that directly phosphorylates PUMA serine 10. Inhibitors of IKK are able to block

the phosphorylation of PUMA serine 10 in response to IL-3 signalling. To support this finding, PUMA phosphorylation in MEF cells that were generated from *IKK1*$^{-/-}$ and *IKK2*$^{-/-}$ genetic backgrounds was analysed and found to be inhibited by the loss of either kinase. However, when co-immunoprecipitation experiments were performed only IKK1 was observed interacting with PUMA and only IKK1 could directly phosphorylate PUMA at serine 10 in an *in vitro* kinase assay, suggesting that while both IKK1 and IKK2 are required for successful PUMA phosphorylation ultimately the direct phosphorylation of PUMA occurs through the action of IKK1.

5.2 Results

5.2.1 Prediction of the kinase responsible for PUMA serine 10 phosphorylation

The consensus substrate sequence motifs for numerous kinases within a cell have been characterised. This data can be utilised to predict a possible kinase when the linear substrate sequence is known such as PUMA serine 10. The bioinformatic program NetPhorest covers 179 of 518 known kinases integrating artificial neural networks and position-specific scoring matrices to produce a probability figure that the substrate will be phosphorylated by a particular kinase (Miller *et al.*, 2008). When the PUMA serine 10 linear motif was analysed using NetPhorest a number of kinases and kinase groups were predicted with high probability (Table 5.2). Many of these were members of the MAPK family due to many of the kinases in these groups having a strong preference for a proline at +1. However, despite scoring a high probability of being the PUMA serine 10 kinase these results are only predictions and must be experimentally verified.

5.2.2 Inhibition of IKK prevents PUMA serine 10 phosphorylation following IL-3 stimulation

In order to determine which kinase may be responsible for phosphorylation of PUMA serine 10 in response to IL-3 stimulation in FDM cells a panel of kinase inhibitors was used. These inhibitors include U0126 (ERK, 10 µM), SB203580 (p38, 10 µM), SP600125 (JNK, 10 µM), Roscovitine (CDKs, 100 µM), Akt inhibitor VIII (Akt, 10 µM), Rapamycin (mTOR, p70 S6K, cdc2, 10 µM), LY294002 (PI3K, 20 µM), H89 (PKA, 10 µM), TG003 (Clk, 10 µM), IKK inhibitor VII (IKK1/2, 5 µM), D4476 (CK1, 10 µM), CK2 inhibitor II (CK2, 10 µM) and Staurosporine (broad range, 1 µM). To test the effect of these inhibitors on PUMA serine 10 phosphorylation, FDM cells were starved of IL-3 for 24 hrs before treatment with the inhibitors or vehicle control (DMSO) for 30 minutes followed by re-addition of IL-3 for 15 minutes at a concentration of 10 ng/ml. Cells were then lysed and analysed by Western blot. Probing with the PUMA pS10 antibody demonstrated significant induction of PUMA pS10 in response to IL-3 treatment when compared to untreated cells as has been seen previously (Figure 5.1). Most inhibitors appeared to have little ability to inhibit the phosphorylation of PUMA at serine 10. The only inhibitors that were reproducibly able to prevent the IL-3 induced phosphorylation of PUMA serine 10 were

Residue	Kinase	Motif	Probability
S10	CDK2_CDK3_group	RQEGSsPEPVE	0.2928
S10	JNK_group	RQEGSsPEPVE	0.259
S10	p38_group	RQEGSsPEPVE	0.2545
S10	CDK1	RQEGSsPEPVE	0.1017
S10	CLK_group	RQEGSsPEPVE	0.0647
S10	RCK_group	RQEGSsPEPVE	0.0605
S10	MAPK3_MAPK1_MAPK7_NLK_group	RQEGSsPEPVE	0.0443
S10	CDK4_CDK6_group	RQEGSsPEPVE	0.0441
S10	GSK3_group	RQEGSsPEPVE	0.0363
S10	CK2_group	RQEGSsPEPVE	0.0324
S10	CDK5	RQEGSsPEPVE	0.0302
S10	mTOR	RQEGSsPEPVE	0.028
S10	CDK7	RQEGSsPEPVE	0.0272
S10	PLK1	RQEGSsPEPVE	0.0241

Table 5.2 Netphorest predictions of PUMA serine 10 kinase

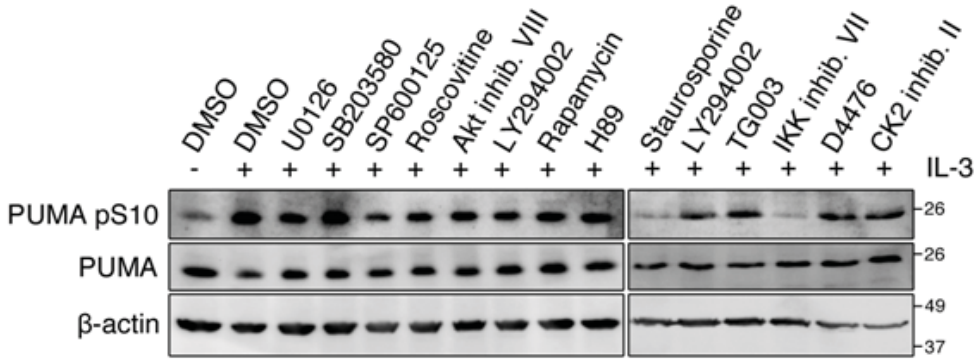


Figure 5.1 Phosphorylation of PUMA at serine 10 is blocked by an IKK inhibitor
FDM wild type cells were starved of IL-3 for 16 hours. Cells were then pre-treated for 30 minutes with either 0.2 % (v/v) DMSO or the kinase inhibitors U0126 (10 μM), SB203580 (10 μM), SP600125 (10 μM), Roscovitine (100 μM), Akt inhibitor VIII (10 μM), Rapamycin (10 μM), LY294002 (20 μM), H89 (10 μM), TG003 (10 μM), IKK inhibitor VII (5 μM), D4476 (10 μM), CK2 inhibitor II (10 μM) and Staurosporine (1 μM). Cells were then stimulated with 10 ng/mL IL-3 for 15 min. Cells were then lysed, proteins resolved by SDS-PAGE and then immunoblotted using antibodies against PUMA pS10, total PUMA or β-actin.

IKK inhibitor VII, which is known to inhibit IKK1 and IKK2, and the broad range inhibitor Staurosporine.

5.2.3 IKK and PUMA are phosphorylated in response to IL-3 signalling

This data somewhat surprisingly suggested that IL-3 receptor signalling induced IKK activity, given that IKK is typically associated with signalling from other classes of receptor such as TNF and Toll receptors. To test whether IL-3 activated IKK, Western blots of lysates from *Bax*$^{-/-}$;*Bak*$^{-/-}$ FDM cells that were stimulated with different doses of IL-3 or IL-3 stimulation over a time-course were probed with antibodies against the unphosphorylated and phosphorylated forms of IKK. Consistent with the IKK inhibitor data, an increase in phosphorylated IKK was observed in response to IL-3 signalling. Phosphorylated IKK could be detected in cells that were treated with 1ng/mL IL-3, and phosphorylation increased with higher doses of IL-3 (Figure 5.2). Interestingly, the phosphorylation and activation of IKK was blocked when a JAK inhibitor was used during IL-3 stimulation suggesting that the activation of IKK by IL-3 signalling requires JAK activation by the IL-3 receptor, consistent with JAK2 being the tyrosine kinase required for IL-3 receptor signalling (Parganas *et al.*, 1998). When the FDM cells were stimulated with IL-3 over a time-course, IKK phosphorylation was observed within 5 minutes of stimulation (Figure 5.2). The increase in phosphorylated IKK was not explained by an increase in total levels of either IKK1 or IKK2. When these same lysates were probed with antibodies to detect phosphorylated PUMA, we observed a concomitant increase in phosphorylated PUMA. Total PUMA levels remained consistent, as we have observed previously in *Bax*$^{-/-}$;*Bak*$^{-/-}$ FDM cells.

5.2.4 PUMA is phosphorylated at serine 10 by the IKK complex.

In order to further establish the role of IKK on PUMA phosphorylation, we expressed wild type PUMA in MEFs derived from wild type mice or mice lacking either of the kinase components of the IKK complex, IKK1 or IKK2. PUMA was immunoprecipitated from lysates prepared from these cells and the blots probed with anti-PUMA and PUMA pS10 antibodies. We observed that phosphorylated PUMA was not detectable in MEFs lacking either IKK1 or IKK2 despite PUMA expression being induced to similar levels (Figure

5.3). Immunoprecipitated PUMA^{S10A} served as a negative control for the PUMA pS10 antibody. This data shows that both IKK1 and IKK2 are required in the IKK enzyme complex responsible for PUMA phosphorylation.

My data provided strong evidence that IKK activity was required for PUMA phosphorylation. In order to determine whether there was a direct interaction between PUMA and either IKK1 or IKK2 were directly responsible for PUMA phosphorylation, we immunoprecipitated over-expressed PUMA from MEFs and probed the immunoprecipitates with antibodies against IKK1 and IKK2. We used either a full length wild type PUMA construct or a loss-of-function PUMA^{4E} mutant in which four of the key residues of the BH3 domain are mutated to glutamate (Chen *et al.*, 2005; Jabbour *et al.*, 2009) in *Bax*$^{-/-}$*;Bak*$^{-/-}$ MEF cells (Figure 5.4). We observed that IKK1, but not IKK2 co-immunoprecipitated with wild type PUMA or PUMA^{4E}. The specificity of this interaction was confirmed by expressing PUMA^{4E} in wild-type or *IKK1*$^{-/-}$ MEFs, where we again observed an interaction between PUMA and IKK1 and this was lost when IKK1 was not present (Figure 5.5). This data demonstrates that IKK1 directly binds PUMA and is therefore responsible for PUMA phosphorylation. Together with the data indicating loss of PUMA phosphorylation in *IKK2*$^{-/-}$ MEFs (Figure 5.3), these data strongly suggest that the IKK complex responsible for PUMA phosphorylation is a heteromeric complex of IKK1 and IKK2, together with IKKγ/NEMO, the adaptor molecule required for PUMA phosphorylation.

To determine the subcellular localisation of activated IKK, *Bax*$^{-/-}$*;Bak*$^{-/-}$ FDM cells were stimulated with IL-3 over a time-course before being fractionated into cytoplasmic and membrane fractions. Western blot analysis of the lysates revealed that activated IKK is present at five and fifteen minutes following IL-3 stimulation in the cytosolic fraction of the cell while both phosphorylated and non-phosphorylated PUMA localised to the membrane fraction which contains mitochondrial proteins (Figure 5.6). The fractionation results do not, however, preclude PUMA on the outer mitochondrial membrane from being exposed to cytosolic proteins such as activated IKK. A similar published example would be phosphorylation of mitochondrial bound Mcl-1 by GSK-3β (Maurer *et al.*, 2006). Therefore, although PUMA is anchored to the mitochondria it remains exposed to the cytosol which allows interaction with IKK. These data revealed a correlation between IKK

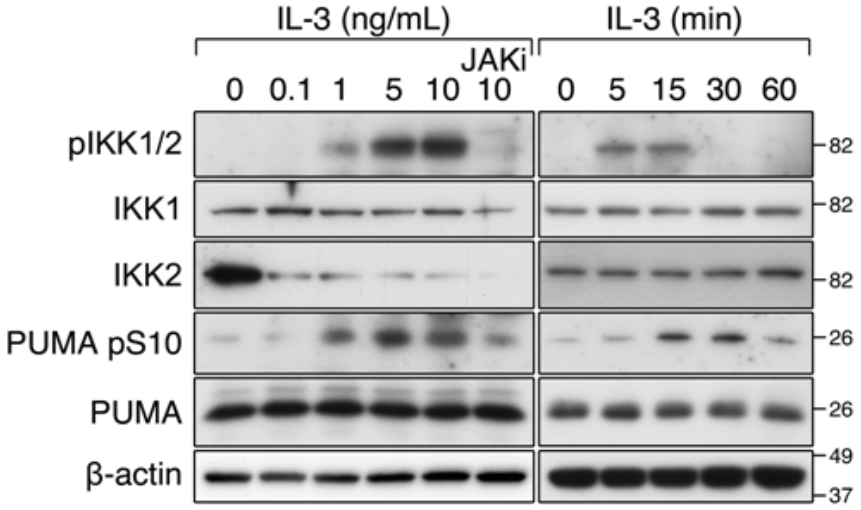


Figure 5.2 PUMA and IKK are phosphorylated in response to IL-3 stimulation. *Bax*$^{-/-}$*;Bak*$^{-/-}$ FDM cells were starved of IL-3 for 16 hours. Cells were then stimulated with IL-3 for 5 min at the indicated concentrations in the presence or absence of JAK inhibitor I (JAKi, 1μM) or were stimulated with IL-3 (10ng/mL) for the indicated periods of time. Protein lysates were resolved by SDS-PAGE before being immunoblotted using antibodies against phospho-IKK1/2, total IKK1, total IKK2, PUMA pS10, PUMA and β-actin.

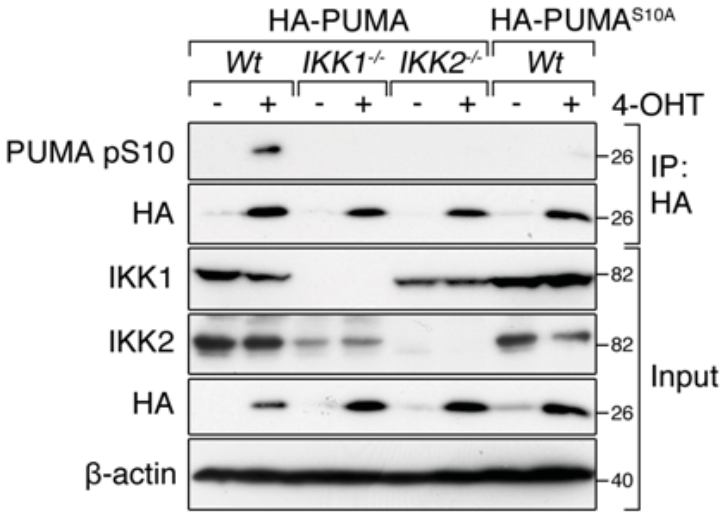


Figure 5.3 Loss of IKK1 or IKK2 prevents phosphorylation of PUMA at serine 10. An HA-tagged PUMA was expressed in wild type, *IKK1*$^{-/-}$ or *IKK2*$^{-/-}$ MEFs under the control of a 4-OHT-inducible promoter. PUMA was immunoprecipitated from protein lysates made in the presence or absence of 4-OHT and lysates and immunoprecipitates resolved using SDS-PAGE. Blots were probed with antibodies against HA, PUMA pS10, IKK1, IKK2 and β-actin.

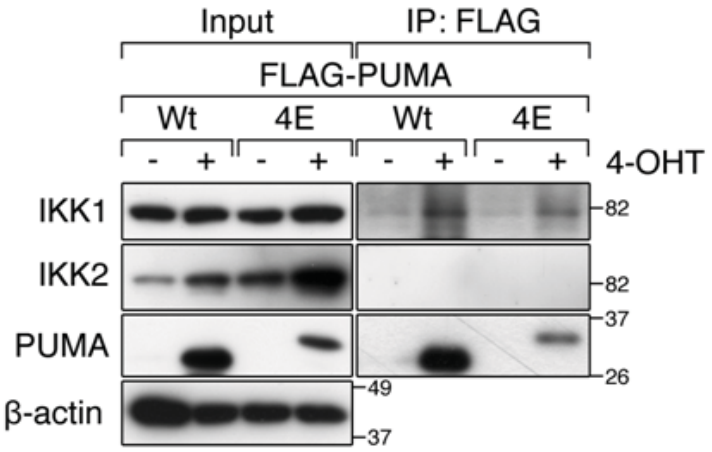


Figure 5.4 PUMA interacts with IKK1 but not IKK2 in MEF cells. A Flag-tagged PUMA or PUMA^{4E} was expressed in *Bax*$^{-/-}$;*Bak*$^{-/-}$ MEFs under the control of a 4-OHT-inducible promoter. PUMA was immunoprecipitated from protein lysates made in the presence or absence of 4-OHT and lysates and immunoprecipitates resolved using SDS-PAGE. Blots were probed with antibodies against PUMA, IKK1, IKK2 and β-actin.

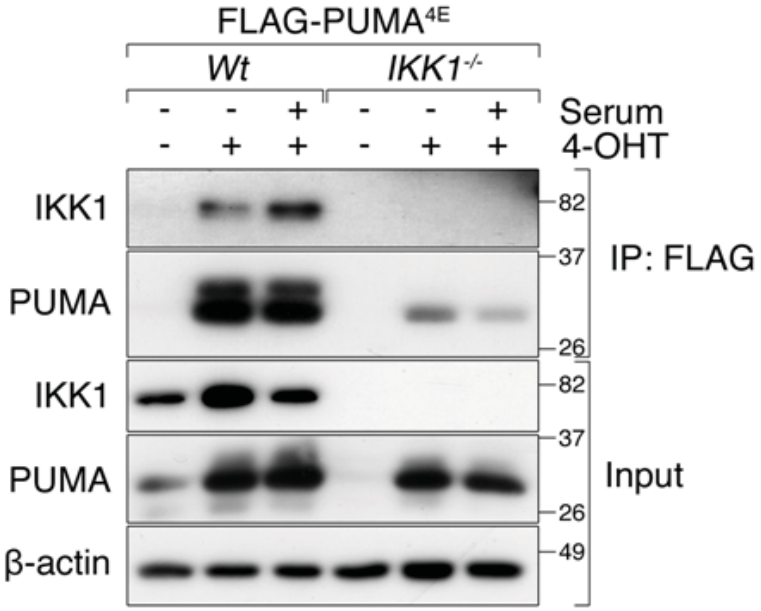


Figure 5.5 IKK1 specifically interacts with FLAG-PUMA^{4E} in wild-type but not *IKK1*$^{-/-}$ MEFs. A Flag-tagged PUMA^{4E} was expressed in wild-type or *IKK1*$^{-/-}$ MEFs under the control of a 4-OHT-inducible promoter in the absence of serum. Cells were then treated with or without 10% serum for 20 min. PUMA was immunoprecipitated from protein lysates made in the presence or absence of 4-OHT and lysates and immunoprecipitates resolved using SDS-PAGE. Blots were probed with antibodies against PUMA, IKK1 and β-actin.

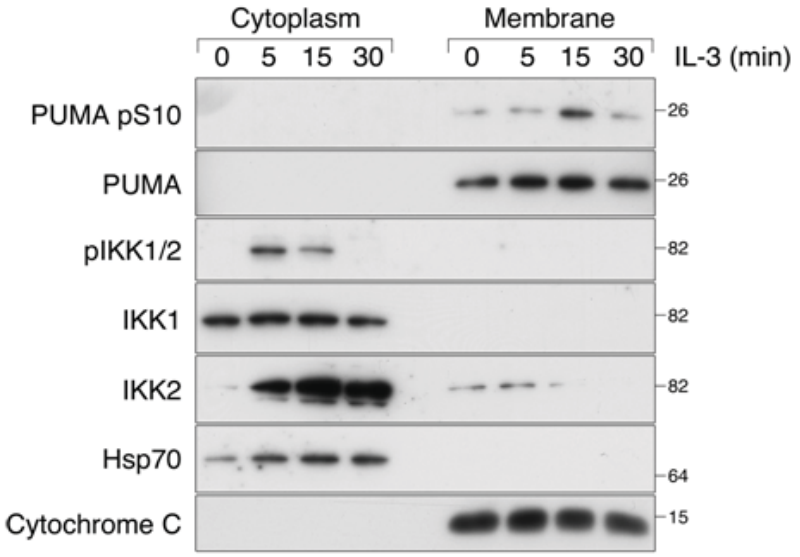


Figure 5.6 PUMA and IKK are localized to different subcellular fractions. *Bax*$^{-/-}$*;Bak*$^{-/-}$ FDM cells were starved of IL-3 for 16 hours. Cells were then stimulated with IL-3 (10 ng/mL) for the indicated periods of time. Cells were then fractioned into cytoplasm and membrane fractions using digitonin and lysates resolved by SDS-PAGE before immunoblotting using antibodies against PUMA pS10, total PUMA, phospho-IKK1/2, total IKK1, total IKK2, Hsp70 and Cytochrome C. Hsp70 and Cytochrome C as used as controls for fractionation and loading.

activation in response to IL-3 stimulation and PUMA phosphorylation.

In order to determine whether IKK1 or IKK2 could directly phosphorylate PUMA serine 10, an *in vitro* kinase assay was performed. HA-tagged wild type PUMA or $PUMA^{S10A}$ were overexpressed in HEK 293T cells before being lysed and the HA-PUMA immunoprecipitated using an anti-HA antibody. The immunoprecipitated protein was then incubated with recombinant IKK1 or IKK2 in a radiometric *in vitro* kinase reaction. Incubation of IKK1 and IKK2 with recombinant IκBα, a known IKK2 substrate, was used as a positive control. The levels of phosphorylation of IκBα by IKK1 represent the background phosphorylation in the assay. The reaction was then resolved by SDS-PAGE and analyzed by exposure to x-ray film and Western blotting. Analysis of the autoradiograph demonstrated that wild type PUMA was phosphorylated by IKK1 but not by IKK2 (Figure 5.7). IKK2 was able to phosphorylate IκBα. The incorporation of ^{32}P into $PUMA^{S10A}$ by IKK1 and into wild type PUMA or $PUMA^{S10A}$ in the presence of IKK2 were all very similar to the background phosphorylation represented by IκBα phosphorylation by IKK1. Wild type PUMA phosphorylation by IKK1 and IκBα phosphorylation by IKK2 were similar and substantially greater. This *in vitro* kinase assay demonstrates that IKK1 but not IKK2 can directly phosphorylate PUMA at serine 10.

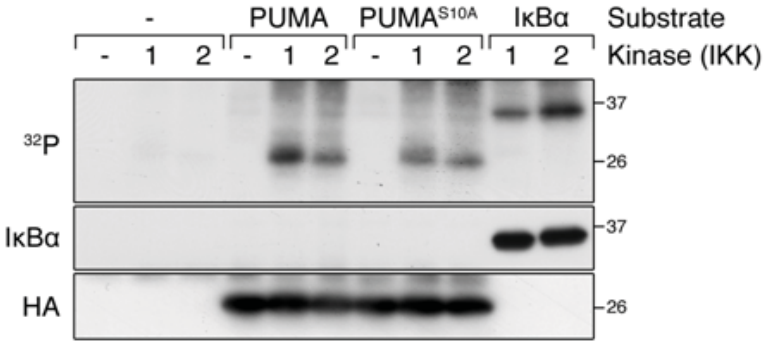


Figure 5.7 IKK1 directly phosphorylates PUMA at serine 10. HA-PUMA wild type or HA-PUMA^{S10A} were expressed in HEK 293T cells. HA-tagged proteins were immunoprecipitated and phosphorylated *in vitro* by performing a radiometric kinase assay using recombinant IKK1 or IKK2 (see materials and methods). Recombinant IκBα was used as a positive control substrate for IKK2 phosphorylation. The kinase reaction was resolved using SDS-PAGE with blots probed with antibodies against IκBα and HA. Incorporation of radioactive phosphates into proteins was visualized by autoradiography.

5.3 Discussion

I have demonstrated that IL-3 signalling leads to the phosphorylation and degradation of PUMA protein. In this chapter I have sought to characterise this novel anti-apoptotic pathway and identity of the kinase/s responsible for phosphorylating PUMA. The data provides compelling evidence that IKK1 is the kinase responsible for PUMA phosphorylation at serine 10. This evidence includes the blocking of PUMA phosphorylation by an IKK inhibitor, disruption of PUMA phosphorylation in MEFs derived from *IKK1*$^{-/-}$ or *IKK2*$^{-/-}$ mice, the demonstration of a physical interaction between IKK1 and PUMA, and importantly, the direct phosphorylation of PUMA by IKK1 in an *in vitro* kinase assay. IKK is a complex of closely related kinases, IKK1/IKKα and IKK2/IKKβ, and a regulatory subunit IKKγ or Nemo (reviewed in Perkins, 2007). In canonical TNFR1 signaling, the IKK complex is a heteromeric complex consisting of IKK1, IKK2 and Nemo. Activation of kinase activity is critically dependent on Nemo (Rudolph *et al.*, 2000). Lysine-63 polyubiquitination of Nemo recruits other kinases to the signalling complex which in turn phosphorylate and activate IKK2 (Xia *et al.*, 2009). The IKK complex phosphorylates IκB, which targets this protein for proteasomal degradation and as a consequence NFκB is activated. Our results suggest the form of IKK responsible for PUMA phosphorylation is remarkably similar to the IKK complex activated in canonical TNFR1 signaling, a heteromeric complex of IKK1 and IKK2, together with the regulatory subunit Nemo, since deletion of either IKK1 or IKK2 blocked PUMA phosphorylation.

The identification of IKK as the PUMA kinase is intriguing as raises the important question as to how IKK is activated in response to a cytokine such as IL-3. Our results clearly indicate that IKK activation is dependent on JAK kinase, since a JAK inhibitor could block both IKK activation and PUMA phosphorylation. This is entirely consistent with IL-3 signalling as JAK2 is the receptor tyrosine kinase required for IL-3 signalling (Parganas *et al.*, 1998). Only scant information exists regarding the activation of the IKK complex by the IL-3 and GM-CSF family of receptors (Hercus *et al.*, 2009). In terms of GM-CSF receptor signalling, immunoprecipitation data suggest IKK2 is recruited to the GM-CSF receptor α-chain in the presence of GM-CSF ligand or an activating mutation of βc (Ebner *et al.*, 2003; Perugini *et al.*, 2010). The stage is now set to map the molecular mechanisms that link IL-3 receptor engagement to IKK activation in myeloid cells.

Substrates of IKK1 and IKK2 other than IκB have been identified which indicate that these enzymes have functions independent of NFκB activation (reviewed inChariot, 2009). Until now, no published evidence suggested Bcl-2 family members are substrates of IKK1 or IKK2. Interestingly, the enforced expression of a dominant negative IKKγ/Nemo construct in keratinocytes increased levels of PUMA protein, even in the absence of an apoptotic stimulus (Leis *et al.*, 2007). It is important to consider how PUMA and IKK might come into contact. Although PUMA and IKK separated to different subcellular fractions, this does not preclude PUMA on the outer mitochondrial membrane being exposed cytosolic proteins such as IKK. A similar example is the phosphorylation of mitochondrial membrane bound Mcl-1 by GSK-3β (Maurer *et al.*, 2006). My data is the first to identify PUMA as an IKK substrate and expands our understanding of the mechanisms by which pathways resulting in NFκB activation can regulate Bcl-2 family members and cell survival.

The results in this chapter demonstrate that the kinase responsible for phosphorylation of PUMA at serine 10 is IKK1 as part of an IKK1/IKK2/Nemo signalling complex activated by IL-3 signalling. Other than IKK inhibitor VII, phosphorylation of PUMA at serine 10 following IL-3 receptor activation was not blocked by individual kinase inhibitors downstream of the JAK-regulated tyrosine phosphorylation of the IL-3R βc. This indicates that the individual pathways previously thought to be responsible for most of the signals from the receptor such as PI3-K and MAPK signalling pathways are not likely to be involved in activation of IKK. This suggests that activation of IKK may involve multiple components downstream of the IL-3 receptor, or, alternatively, activation of IKK may involve potentially unknown and novel components of the IL-3 receptor signalling complex. This question will be important to address in future studies.

Chapter 6

General Discussion

6.1 General discussion

During development and in an adult the differentiation, proliferation and survival of cells from a myeloid lineage are dependent on cytokines signalling through the IL-3/GM-CSF/IL-5 receptor family. Signals from these cytokines regulate haematopoietic homeostasis and are also responsible for activation and expansion of cells in response to haematopoietic stresses (Hamilton, 2002; Lotem and Sachs, 2002). When the homeostatic balance is upset or a challenge to the immune system has receded, cytokines are withdrawn from the environment leading to the induction of apoptosis and removal of superfluous, defective, damaged, or dangerous cells (Alves *et al.*, 2007; Kerr *et al.*, 1972; Vaux and Strasser, 1996). Control of apoptosis by cytokines is primarily exerted through the intrinsic apoptosis pathway which is regulated by the Bcl-2 family of proteins (Strasser, 2005). Cytokine regulation of Bcl-2 family members is exerted at both a transcriptional and post-translational level, however, the role of different members of the family in regulating the final outcome is still subject to intense investigation. The regulation of apoptosis following cytokine withdrawal in myeloid cells has been shown to rely heavily on the BH3-only protein PUMA (Ekert *et al.*, 2006; Ekoff *et al.*, 2007; Jabbour *et al.*, 2009; Jeffers *et al.*, 2003). PUMA is known to be regulated at a transcriptional level, however, the post-translational regulation of PUMA has never been reported. The results contained within this thesis demonstrate that PUMA is post-translationally regulated at a specific serine residue by serum and IL-3 signalling.

In ^{32}P orthophosphate labelling experiments in HEK 293T cells PUMA serine 10 appeared to be the predominant residue that was phosphorylated when cells were re-stimulated with serum. A doublet of phosphorylated PUMA bands was observed in FDM cells, however, the same doublet of bands did not appear after Western blot analysis. One possible explanation for this second band is the presence of a second protein. This could be a protein which has inadvertently immunoprecipitated with PUMA or potentially a protein that interacts with PUMA in a serine 10 independent manner and is regulated by phosphorylation with similar kinetics. A second possibility may be that in FDM cells PUMA phosphorylated at serine 10 may represent only a small proportion of the total PUMA within a cell, particularly when using an overexpression system. The second, upper band, representing PUMA phosphorylated at serine 10 may not be visible in Western blot analysis due to its low abundance and the overwhelming presence of PUMA not

phosphorylated at serine 10. This explanation would suggest that there are multiple residues in PUMA that may be phosphorylated. Multiple sites of regulation is a common theme for Bcl-2 family members and it is not unexpected that PUMA may also have multiple sites (Kutuk and Letai, 2008). Mass spectrometric analysis was only able to positively identify PUMA serine 10 and did not detect any other modifications, however, this is potentially due to the technical limitations of the IMAC phospho-peptide purification and mass spectrometric analysis rather than a lack of further modifications of PUMA. This view is also supported by the Netphos predictions indicating that several serine residues other than serine 10 in the PUMA sequence are likely to be phosphorylated.

There is a diverse array of responses following the phosphorylation of various Bcl-2 family members. Phosphorylation can promote or inhibit apoptotic activity with many members having multiple sites of regulation. The interactions between Bcl-2 family members is a critical event for the regulation of apoptosis and is utilised by several family members. Phosphorylation of Mcl-1 can inhibit apoptosis by binding BH3-only proteins with a greater affinity while phosphorylation of Bad can inhibit its interaction with pro-survival proteins and re-localise it within the cell by promoting/preventing its interaction with 14-3-3 (Datta *et al.*, 2000; Donovan *et al.*, 2002; Kobayashi *et al.*, 2007; Tan *et al.*, 2000; Yu *et al.*, 2004; Zhou *et al.*, 2000). Mutational analysis of PUMA demonstrated that the localisation and interaction with Bcl-2 family members was not affected by phosphorylation of serine 10. Instead, phosphorylation of serine 10 targets PUMA for degradation. This is similar to the effect of serum stimulation on the stability of the BH3-only protein Bim. Although multiple serine and threonine phosphorylation sites in Bim have been described, phosphorylation of three serine residues encoded in exon 3, in a manner dependent on MAPK signalling, targets Bim for proteasomal degradation (Hubner *et al.*, 2008). In this instance MEFs derived from mice in which these serine residues have been deleted, fail to down-regulate Bim expression in response to serum stimulation after a period of serum starvation and more cells undergo apoptosis. This indicates that there are several kinase-dependent mechanisms by which BH3-only proteins are degraded in cells that do not commit to apoptosis after a potential apoptotic insult such as growth factor or serum deprivation. These mechanisms provide for the rapid clearance of PUMA and Bim. Recently, PUMA phosphorylation at serine 10 was independently described (Fricker *et al.*, 2010). This study demonstrated that phosphorylation of overexpressed PUMA at serine 10

is associated with increased PUMA turnover. In contrast, I have identified the activating stimuli and the kinase pathway responsible. In addition, because I developed a phospho-specific antibody, I demonstrate serine 10 phosphorylation of endogenous PUMA.

While I have only investigated the regulation of PUMA serine 10 in respect to serum and IL-3 signalling it is likely that other signalling pathways emanating from stimuli such as nutrients or other cytokines could regulate PUMA in a similar manner. IKK2 has been found to interact with GM-CSFα chain when the receptor is activated by ligand or a mutation (Ebner *et al.*, 2003; Perugini *et al.*, 2010). This would suggest that GM-CSF should also be capable of promoting PUMA serine 10 phosphorylation. TNFα is another candidate for regulating PUMA serine 10 phosphorylation as it is known to signal through the TNFR1 receptor to activate the IKK signalling complex (Perkins, 2007). However, the relationship between TNFα signalling and regulation of PUMA abundance within a cell is likely to be complex as TNFα induced activation of IKK and the subsequent activation of the NFκB pathway has been shown to increase PUMA transcription, raising PUMA protein levels and promoting apoptosis (Wang *et al.*, 2009). This would occur at the same time as we hypothesise that IKK signalling should be phosphorylating PUMA and promoting degradation, suggesting the presence of a negative feedback loop where increases in PUMA protein through transcription are attenuated by post-translational regulation of PUMA through phosphorylation. Interestingly, a recent publication observed that GM-CSF, which is normally thought to activate pathways responsible for suppression of Bim protein expression, can upregulate Bim transcription through NFκB in Neutrophils to prime cells for TNFα induced apoptosis (Cowburn *et al.*, 2010). Our data demonstrates that IL-3 signalling also activates IKK and the NFκB pathway suggesting that, although clearly responsible for suppression of PUMA expression in most cell types, IL-3 signalling may upregulate PUMA expression through the NFκB pathway under certain situations. In addition to starvation from cytokines, PUMA is also responsible for regulation of apoptosis in response to stimuli such as DNA damage from genotoxic agents or UV and γ-irradiation (Jeffers *et al.*, 2003; Kaeser and Iggo, 2002; Sidi *et al.*, 2008; Wang *et al.*, 2007; You *et al.*, 2006; Yu *et al.*, 2003b; Yu *et al.*, 2001). It will be vital to investigate if stimuli that promote PUMA expression will also actively inhibit PUMA phosphorylation.

It is clear from the experiments utilising the JAK inhibitor that activation of IKK from IL-3 signalling requires that tyrosine kinase activity of JAK proteins at the IL-3 receptor. However, the events downstream of JAK activation that lead to IKK phosphorylation are unclear. IKK activation is known to occur downstream of multiple receptors. In addition to IKK activation through TNFα signalling, components of pathogens such as bacterial lipopolysaccharide are known to bind to Toll-like receptors and promote the activation of IKK and NFκB (Haziot *et al.*, 1996; Nagai *et al.*, 2002). IL-1 binding to IL-1RI and the engagement of the B-cell receptor are also events which have been shown to lead to IKK activation (Greenfeder *et al.*, 1995; Huang *et al.*, 1997; Thome, 2004). While these signalling pathways ultimately converge on IKK phosphorylation and activation, the components of the signalling cascades that leads to this phosphorylation are diverse and often specific to a single receptor or family of receptors. It is unclear which of the components, if any, may be utilised by the IL-3 receptor. Future experiments are likely to involve a duel approach by interrogating proteins known to lead to IKK phosphorylation in addition to experiments identifying potentially novel proteins linked to the IL-3 receptor.

The result of PUMA serine 10 phosphorylation is the regulation of PUMA protein stability and degradation, however, while degradation of PUMA appears to proceed through the proteasome the mechanism involved has yet to be fully characterised and is likely to involve an interaction between novel binding partners and the phosphorylated serine 10 motif. The stability of numerous Bcl-2 family members is regulated through a ubiquitin-mediated, proteasome dependent pathway (Kutuk and Letai, 2008). Mcl-1 is known to interact with the E3 ubiquitin ligases β-TrCP and MULE to promote the addition of ubiquitin leading to proteasome mediated degradation of Mcl-1 and the initiation of apoptosis (Ding *et al.*, 2007; Zhong *et al.*, 2005). Conversly, the deubiquitinase USP9X removes ubiquitin from Mcl-1, inhibiting degradation of the protein and promoting cell survival (Schwickart *et al.*, 2010). It is unclear if PUMA stability may be regulated by a similar mechanism as the PUMA sequence does not contain any lysine residues which are normally required for the attachment of ubiquitin to a protein. However, recent studies have identified the N-terminus of proteins as a novel site of ubiquitination (Kirisako *et al.*, 2006). The proximity of PUMA serine 10 to the N-terminus of PUMA suggests a possible role for recruiting E3 ligases for ubiquitination of PUMA. Unfortunately, the mechanisms

involved in N-terminal ubiquitination have yet to be fully elucidated and it is unclear if the proximity of a site of regulation to the site of ubiquitination is important for this process.

The data I present has demonstrated the biochemical regulation of PUMA at serine 10, however, future work will focus on the physiological outcome of PUMA phosphorylation in development, normal homeostasis and disease. PUMA is not required for normal embryonic or post natal development since gene-deleted mice are born at the normal mendelian ratio and develop and function normally (Jeffers *et al.*, 2003; Villunger *et al.*, 2003). This observation does not, however, exclude the possibility that post-translational regulation of PUMA levels is important in development. Loss of PUMA post-translational regulation would potentially result in accumulation of PUMA protein and may initiate inappropriate apoptosis. Therefore, regulation of PUMA levels by post-translational modification may be part of the signalling pathways which suppress the activation of apoptosis under normal conditions. Direct evidence from experimental tumour models, and indirect evidence from human leukaemia samples, support a tumour suppressor role for PUMA. For example, deletion of PUMA co-operates with other oncogenic lesions, such as the deregulated expression of c-myc, to promote tumour development (Garrison *et al.*, 2008; Hemann *et al.*, 2004; Michalak *et al.*, 2009) and PUMA is frequently silenced in paediatric ALL (Davidsson *et al.*, 2009). The activation of signalling pathways that result in IKK activation and which lead to phosphorylation and degradation of PUMA, would effectively act to mimic loss of PUMA expression. Constitutive activation of the IKK complex contributes to oncogenesis, primarily thought to be through NFκB activation (reviewed in Lee and Hung, 2008). However, the regulation of PUMA may also contribute to this effect. Determining whether mutations in tyrosine kinases which confer a hypersensitivity to cytokine signals or which promote cytokine independence, for example JAK2 activating mutations (Levine *et al.*, 2007), or CD123 overexpression (IL-3 alpha chain), which is common in acute myeloid leukaemia (Jin *et al.*, 2009; Munoz *et al.*, 2001), activate IKK and promote PUMA degradation may shed more light on the role of this new pathway in disease.

In conclusion, I have described a new mechanism for the post-translational regulation of PUMA, a BH3-only protein previously thought to be regulated primarily by transcriptional

mechanisms (summarised in Figure 6.1). In cells starved of growth factors such as serum or cytokines, PUMA protein levels rise until such point where the anti-apoptotic Bcl-2 family members are overcome and apoptosis is initiated through activation of Bax and Bak. If growth factors are reintroduced before the initiation of apoptosis, PUMA is phosphorylated and rapidly degraded, preventing apoptosis and ensuring cell survival. Under normal homeostasis where cytokines are present this mechanism is also likely aid in the suppression of apoptosis through repression of PUMA expression. Interestingly, the phosphorylation of PUMA appears to directly result from the activation of the IKK complex. This is an entirely novel pathway downstream of the IL-3 receptor and this finding raises new questions regarding the broader role of the IKK complex in regulating cell survival in IL-3 dependent cells. This finding also presents new therapeutic targets which may be targeted in diseases where deregulation of cytokine signalling has occurred.

6.2 Future Investigations

The work published in this thesis advances our knowledge on IL-3 regulation of cell growth and survival. However, there are a number of experiments that must be performed if the post-translational regulation of PUMA in response to IL-3 signalling is to be thoroughly characterised. The role of serine 10 phosphorylation in the initiation of PUMA degradation through the proteasome has been established, however, the components responsible for the attachment of ubiquitin and transportation of PUMA to the proteasome are unclear. Given the absence of lysine residues in PUMA for the attachment of poly-ubiquitin chains, it will be imperative to establish if PUMA is N-terminally ubiquitinated in addition to identification of the ubiquitin lygases that are involved in the recognition and attachment of the ubiquitin moiety.

The pursuit of additional regulators of PUMA serine 10 phosphorylation will also be essential to increase our understanding of how PUMA controls apoptosis and cell survival. It has been published that PUMA is responsible for regulating apoptosis in response to nutrient (glucose) deprivation (Zhao *et al.*, 2008). It would be interesting to investigate if

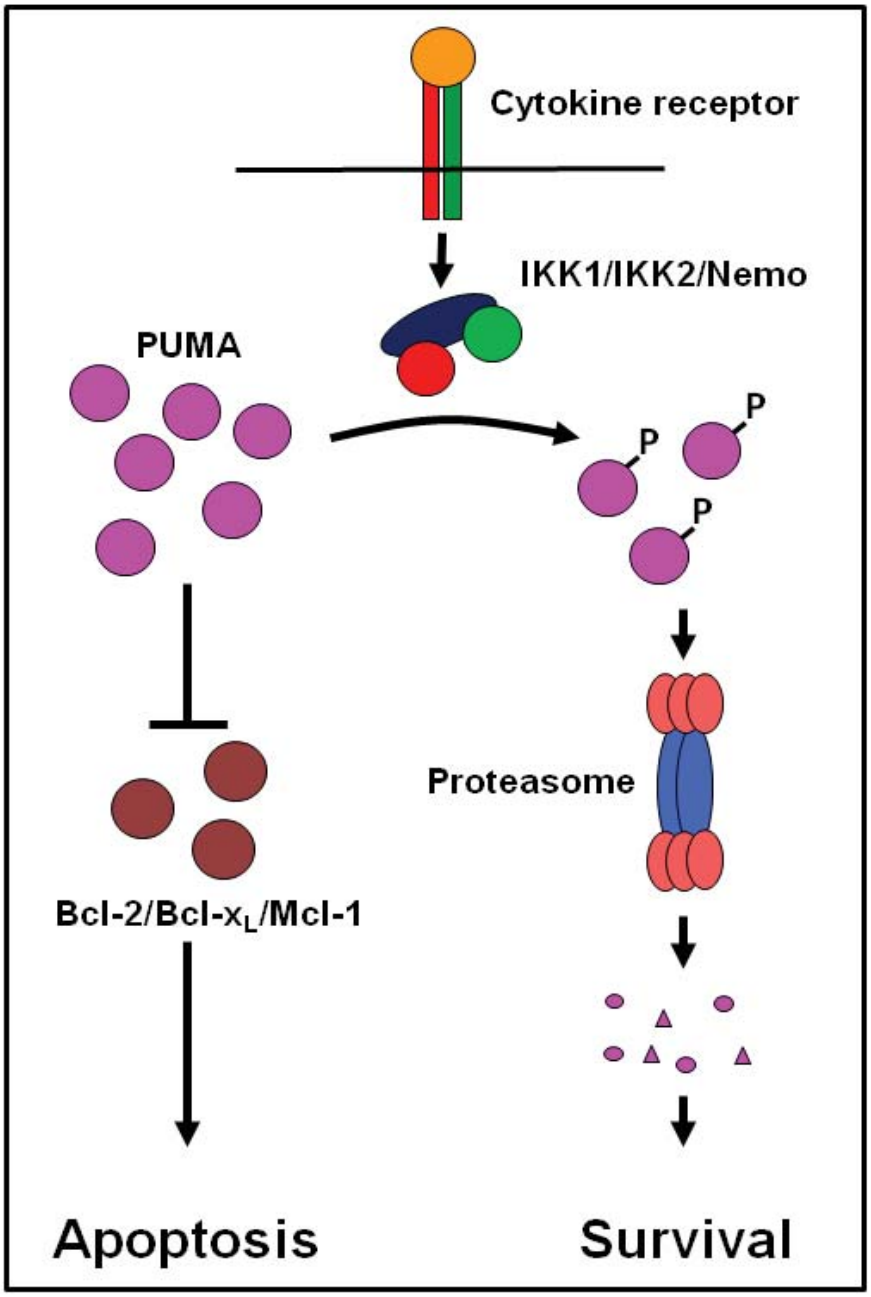


Figure 6.1 Model for cytokine regulation of PUMA degradation. PUMA protein within a cell acts to inhibit the activity of the pro-survival Bcl-2 family members which include Bcl-2, Bcl-x_L and Mcl-1 leading to the induction of apoptosis. Upon activation of cytokine receptor signalling, the IKK1/IKK2/Nemo complex is activated leading to the phosphorylation of PUMA at serine 10. Phosphorylated PUMA is then earmarked for degradation through the proteasome, preventing PUMA from antagonising the pro-survival Bcl-2 family members and leading to cell survival.

readdition of glucose following a period of deprivation would elicit a similar effect to IL-3 on PUMA serine 10 phosphorylation. It would also be of importance to investigate if stimuli that promote PUMA activity such as DNA damage from genotoxic agents or UV and γ-irradiation are responsible for inhibiting PUMA serine 10 phosphorylation.

While my data has clearly established that PUMA serine 10 is a phospho-regulatable residue, the data from my work and the published work from collaborators suggests that there are additional phosphorylation sites within PUMA. As the data presented in the thesis specifically focuses on PUMA serine 10, little is known about how these additional sites may be regulated and the stimuli required for their phosphorylation. A thorough investigation, similar to the project presented in this thesis, would need to be undertaken in order to establish the significance of any additional phosphorylation sites.

Another exciting discovery from my research is the identification of a novel signalling pathway downstream of the IL-3 receptor. Evidence that IKK is activated downstream of the GM-CSF receptor is very limited and although the IL-3 and GM-CSF receptors share a common β-chain, the observation of IKK activation initiated by IL-3 signalling has not previously been reported. A thorough dissection of the IL-3 receptor and the mechanisms responsible for IKK activation would be of great interest to the scientific community. IL-3 is also strongly associated with regulation of cell survival and it will be important to investigate if activation of IKK plays a role in the regulation of cell survival outside of PUMA phosphorylation.

6.3 Addendum

During the production of this thesis a scientific group published work that identified the phosphorylation of overexpressed PUMA at serine 10 in addition to another site within the PUMA sequence (Fricker *et al.*, 2010). The data produced by this group is complementary to my findings as it identifies PUMA serine 10 as a site of phosphorylation and demonstrates that this site regulates PUMA protein stability and degradation. However, the data presented in this thesis goes much further than these published findings in that it establishes the initial stimuli required to promote PUMA phosphorylation, the

demonstration that this regulation occurs on both overexpressed and endogenous PUMA, the spatio-temporal regulation of PUMA serine 10 phosphorylation in response to IL-3 signalling, a more comprehensive analysis of PUMA stability and degradation and I establish the kinase that is directly responsible for phosphorylation of PUMA at serine 10. While the work I have presented is comprehensive, their work provides independent confirmation of the findings in this thesis.

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Appendix

Jabbour, A.M., Heraud, J.E., Daunt, C.P., Kaufmann, T., Sandow, J., O'Reilly, L.A., Callus, B.A., Lopez, A., Strasser, A.,Vaux, D.L. and Ekert, P.G. (2009) Puma indirectly activates Bax to cause apoptosis in the absence of Bid or Bim.
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