Molecular and cellular roles of the N-end rule pathway in apoptotic cell death in mammalian-derived cell lines

by

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ABSTRACT

Cellular stresses and signaling that lead to the initiation of apoptotic pathways often result in the activation of proteases such as caspases which in turn culminate in the generation of proteolytically activated protein fragments with new or altered roles. Recent work has revealed that the activity of some of the caspase-generated activated pro-apoptotic protein fragments can be mitigated via their selective degradation by the N-end rule pathway. In this work, I report the investigations that examined the role of the N-end rule pathway in regulating apoptotic cell death via selective protein degradation of specific crucial cleaved pro- and anti-apoptotic fragments in mammalian cell lines. My investigation has focused on three cleaved protein fragments (cleaved fragments of Lyn kinase, BMX kinase and PKC-theta). Selective degradation of the C-terminal fragments of these three diverse protein kinases implicates an expansive role for the N-end rule pathway in the complex network of apoptotic pathways. The degradation of the anti-apoptotic cleaved Lyn kinase by the N-end rule pathway in K562 cell line revealed that N-end rule pathway may target anti-apoptotic protein fragments and thus regulates the apoptotic threshold in certain cells. Tellingly, the N-end-rule-mediated degradation of the pro-apoptotic cleaved BMX kinase in cancer-derived cell lines provides an example for an N-end rule-mediated anti-apoptotic response for cancer cell survival via destroying an endogenous caspase-generated pro-apoptotic protein fragment. Lastly, I have determined that the pro-apoptotic cleaved fragment of PKC-theta is unstable in mammalian cells as its N-terminal lysine targets it for proteasomal degradation via the N-end rule pathway and this degradation dampens the potent pro-apoptotic function of the cleaved fragment of PKC-theta. Collectively, this work delineates some of the functional scope of N-end rule pathway with respect to apoptotic cell death and supports the notion that targeting N-end rule machinery may have therapeutic implications.

Preface

This thesis is an original work by Mohamed Ali Eldeeb (MAE). As the primary author for the following manuscripts, MAE prepared most of the materials, performed and analyzed all experiments. MAE and RPF conceptualized the projects, designed the experiments and prepared the following manuscripts.

Part of Chapter 1 and Chapter 5 of this thesis has been published as Eldeeb, M. & Fahlman, R. (2016) The-N-end rule: The beginning determines the end. *Protein Pept. Lett. 23*, 343–348. MAE conceptualized the idea of the article and wrote the first draft of the article. MAE and RPF wrote the final draft of the article.

Chapter 2 of this thesis has been published as Eldeeb, M. A., and Fahlman, R. P. (2014) The anti-apoptotic form of tyrosine kinase Lyn that is generated by proteolysis is degraded by the N-end rule pathway. Oncotarget **5**, 2714–2722. MAE and RPF conceptualized the project, MAE and RPF designed the experiments, MAE performed experiments and analysed the data, MAE and RPF wrote the manuscript.

Chapter 3 of this thesis has been published as as Eldeeb MA, Fahlman RP (2016) Phosphorylation impacts N-end rule degradation of the proteolytically activated form of Bmx kinase. J Biol Chem 291: 22757-22768. MAE and RPF conceptualized the project, MAE and RPF designed the experiments, MAE performed experiments, MAE and RPF analysed the data, MAE and RPF wrote the manuscript.

Chapter 4 of this thesis has been prepared for publication as Eldeeb MA & Fahlman RP (2017). N-end-rule-mediated Degradation of the Proteolytically Activated Form of PKC-theta Kinase attenuates its Pro-Apoptotic Function. MAE and RPF conceptualized the project, MAE designed the experiments, MAE performed experiments and analysed the data, MAE wrote the manuscript, RPF supervised the study.

The seeker after truth is not one who studies the writings of the ancients and, following his natural disposition, puts his trust in them, but rather the one who suspects his faith in them and questions what he gathers from them, the one who submits to argument and demonstration and not the sayings of human beings whose nature is fraught with all kinds of imperfection and deficiency. Thus the duty of the man who investigates the writings of scientists, if learning the truth is his goal, is to make himself an enemy of all that he reads, and, applying his mind to the core and margins of its content, attack it from every side. He should also suspect himself as he performs his critical examination of it, so that he may avoid falling into either prejudice or leniency.

From Ibn al-Haytham's Doubts Concerning Ptolemy

Dedication

I would like to dedicate this work in memory of my mother.

This work is also dedicated to my father.

ACKNOWLEDGEMENTS

I am thankful to a lot of people that I have known during my Ph.D. studies. I am deeply indebted to my supervisor Dr. Richard Fahlman for his continuous support and guidance throughout my Ph.D. program. He gave me the opportunity and the independence to explore my scientific niche yet with a focus on the goal and steered me in the right direction. Dr. Fahlman is a diligent scientist, a respectful mentor, and an excellent supervisor. I learned a lot from him and glad to take those lessons with me. It was a great pleasure to have been part of this with you.

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I would like to express my sincerest gratitude to my father and my sister for their encouragement to follow my dreams and for their unlimited support.

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List of Abbreviations

A   Alanine

Ac   Acetylation

Arg   Arginylation / Arginylated

ATE1   Arginyltransferase 1

ATP   Adenosine Triphosphate

APAF1   Apoptotic protease activating factor 1

BCR   Breakpoint Cluster Region

BCR-ABL   Breakpoint Cluster Region-Abelson Protein Tyrosine Kinase-1 Fusion Protein

BMX   bone marrow kinase on chromosome X

BRCA1   breast cancer type 1 susceptibility protein

BID   BH3 interacting-domain death agonist

BIMEL   Bcl-2-interacting mediator

BIR   Baculovirus Inhibitor of apoptosis protein Repeat

C   Cysteine

C*   Cysteic Acid

CHX   Cycloheximide

CML   Chronic Myelogenous Leukemia

CARD   Caspase activation and recruitment domains

CrmA   cytokine response modifier A

CDB   Cyclin destruction box

ClpS	an essential regulator of ATP-dependent protease ClpAP
ClpP	a serine protease with a chymotrypsin-like activity
DR	Death receptor
DISC	Death-inducing signaling complex
DIAP1	Death-associated inhibitor of apoptosis 1
D	Aspartate
E	Glutamate
E1	E1 Ubiquitin Activating Enzyme
E2	E2 Ubiquitin Conjugating Enzyme
E3	E3 Ubiquitin Ligase Enzyme
F	Phenylalanine
FACS	Fluorescence Automated Cell Sorting
FADD	Fas-associated protein with death domain
FLICE	FADD-like IL-1β-converting enzyme
FLIP	(FADD-like IL-1β-converting enzyme)-inhibitory protein
G	Glycine
GFP	Green Fluorescent Protein
H	Histidine
HEK293T	Human embryonic kidney cells 293 T
I	Isoleucine
IAP	Inhibitor of apoptosis proteins
ICE	Interleukin-converting enzyme

K	Lysine
K562	Chronic Myelogenous Leukemia Cell Line K562
L	Leucine
LynΔN	Truncated Lyn Tyrosine Kinase Reporter Protein
M	Methionine
MARCH6	E3 Ubiquitin-Protein Ligase MARCH6
MetAP	Methionine Aminopeptidase
MG132	26S Proteasome Inhibitor MG132
mRNA	Messenger RNA
N	Asparagine
NAT	N-terminal Acetyltransferase
NTAN1	Protein N-terminal asparagine amidohydrolase
NTAQ1	Protein N-terminal glutamine amidohydrolase
P	Proline
P1'	Position 1
PTM	Post-translational modification
PEST	sequence that is rich in proline (P), glutamic acid (E), serine (S), and threonine (T)
PKC-θ	Protein-Kinase C-theta
PARP	Poly (ADP-ribose) polymerase
PIDDosome	A protein complex implicated in activation of caspase-2
Q	Glutamine
R	Arginine

RFP	Red Fluorescent Protein
RING	(Really Interesting New Gene) finger domain
S	Serine
SH3	Src Homology 2
SH2	Src Homology 3
T	Threonine
TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain
TNF	Tumor necrosis factor
TRAIL-R1	TNF-related apoptosis-inducing ligand-receptor 1
TL1A	TNF-family ligand for DR3
UBE2A/B	E2 Ubiquitin Conjugating Enzymes A and B
UBR1	E3 Ubiquitin Ligase UBR1
UBR2	E3 Ubiquitin Ligase UBR2
V	Valine
W	Tryptophan
XIAP	X-linked inhibitor of apoptosis protein
Y	Tyrosine

Chapter 1: General Introduction

1.1 Thesis Overview

The objective of this thesis is to explore the role of the N-end rule degradation pathway in caspase-dependent apoptotic cell death in mammalian-derived cell models. Specifically, the aims of this work are:

1. Investigate whether caspase activated Lyn kinase, BMX kinase, and PKC-θ are bona fide substrates for the N-End Rule Pathway in mammalian-derived cell models.
2. Examine the impact of altering the metabolic stability of these activated proteolytic kinase fragments (Lyn kinase, BMX kinase, and PKC-θ) on cellular death in specific cancer-cell line models.
3. Evaluate the impact of partial ablation of the N-end rule pathway on cell death by a range of apoptotic cell death-inducing agents in specific cancer-cell line models.

In multicellular organisms, cellular homeostasis is maintained through a fine-tuned balance between cell proliferation and cell death (1). Deregulation of this "life versus death" balance may result in several human diseases and disorders such as cancer, neurodegenerative disorders, and autoimmune diseases (2–5). Mounting lines of evidence, over the last two decades, suggest that either hyper-regulation or hypo-regulation of apoptosis (a format of programmed cell death (PCD)) may underlie the previous pathologies (2–5). Tellingly, apoptosis itself is often a balance between pro- and anti-apoptotic molecules(6). Limited proteolytic processing (restricted proteolysis) is at the nexus of crucial regulatory networks controlling apoptotic cell death (7). Many proteinases such as caspases and calpains cleave ~500 to ~1000 cellular proteins that are crucial for the proper execution of apoptotic cellular demise (8–10). By executing sequence –specific cleavages in many cellular proteins, activated caspases or calpains either abrogate or alter the functions of these proteins (6, 11–14). Some of the generated protein fragments can have deleterious (pro-apoptotic) effects such as the cleaved fragments of Bax (14), BID (13), BIMEL (12) and BRCA1 (11), whereas other cleaved protein fragments may have anti-apoptotic functions such as the cleaved fragments of Lyn kinase (15), synphilin 1 (16), P27kip1 (17) and RasGAP (18). Thus, this dichotomy of restricted proteolytic cleavages, in addition to the necessity of apoptosis in multicellular eukaryotes might account for the acquisition and conservation of caspase cleavage sites (and some of calpain cleavage sites) in many proteins during evolution (10, 19, 20). So, if

restricted proteolysis can generate pro-and anti-apoptotic fragments during apoptosis, how do cells regulate and control the output of such proteolytic (caspase /calpain) cascade which regulates the apoptotic execution reactions and hence impacts cellular fate. A possible avenue for regulating the output of such proteolytic cascade reactions (let alone the proportion of pro-and anti-apoptotic fragments produced in each case) would stem from the difference in metabolic stability between different proteolytically-activated protein fragments generated in each case (pro-apoptotic and anti-apoptotic fragments). Consequently, this would result in pro-apoptotic and anti-apoptotic signaling imbalance and thus leads to either cell death or cell survival. In sum, given the irreversible nature of limited proteolysis and the presence of feedback loops (either pro- or anti-apoptotic) originating from the generated proteolytic fragments (6, 10) and the generation of proteolytically-active protein fragments with neo N-termini, One possibility for regulation of limited proteolytic cascades during apoptotic signalling is selective protein degradation of specific proteolytic fragments via the N-end rule degradation pathway which may play a significant role in determining cell fate upon induction of apoptosis (10, 21).

1.2 Introduction: an overview of programmed apoptotic cell death

Despite the fact that cell death can occur occasionally subsequent to an overwhelming damage, most cell death programmes in mammals take place in an active manner, as a result of a specific cascade of cellular signaling events (22, 23). Tellingly, there are three general types of cell death defined, at least in part, by the morphological features of the dying cell: apoptosis (also designated as type I cell death), autophagic cell death (type II cell death), and necrosis (type III cell death) (22, 23).

Apoptotic cell death is a genetically programmed form of cellular demise that permits the cell to commit molecular-controlled cellular suicide while avoiding inflammation and damage to neighboring cells and environment (22–26). Tellingly, Apoptosis is characterized by distinct biochemical features including membrane blebbing, cellular shrinkage, chromatin condensation (pyknosis) and DNA fragmentation (26). It can be further defined molecularly as a cellular death format that accompanied by caspases (proteases) activation (27). In general, apoptosis plays a crucial role in the survival of multicellular organisms via eliminating and removal of damaged, abnormal or any otherwise infected cells that may disrupt the normal physiological functions of

the organism (28). Although diverse apoptotic pathways exist that can be distinguished by the identity of molecular mediators that include adapters and initiator caspases involved. Most apoptotic programmes can be categorized into either the extrinsic (death-receptor pathway) or intrinsic (mitochondrial) pathway (22, 27).

The extrinsic apoptotic cell death pathway is initiated via extracellular signals that are transferred to the internal cellular environment via the binding of specific ligands (death ligands) to specific receptors (death receptors (DRs)) (22, 27). Tellingly, death receptors are membrane protein receptors and members of tumor necrosis factor (TNF) superfamily and encompass TNF receptor-1 (TNFR1), Fas, death receptor 3 (DR3), DR4 (TRAIL-R1), and DR5 (TRAIL-R2)(22, 29). Death receptor ligands include TNF, CD95-ligand (Fas-L; also, called CD95-L), TRAIL, and TL1A (22, 29). Following the binding to its ligand, the death receptor transduces the signal to promote the recruitment of the monomeric procaspase-8 protein, via its DED motif, to the death-inducing signaling supra-molecular complex (DISC) assembled at its cytoplasmic terminal that also involves the adapter molecules as FAS-associated death domain (FADD) or TNFR-associated death domain (TRADD)(22). Further, recruitment of caspase-8 monomers culminates in dimerization and activation and then subsequently leads to activation of downstream effector caspases (22, 29).

Intrinsic apoptosis is also designated as mitochondrial apoptotic pathway because it hinges on mitochondrial-derived factors. Importantly, this pathway is activated by a vast spectrum of cellular stress cues, including the withdrawal of a growth factor, partial ablation of cytoskeleton, aggregation of unfolded proteins, DNA damage, and many others. In addition, developmental cues (such as hormones) also can result in inducing apoptotic cell death (30).Tellingly, caspase-9 has been demonstrated to act as the initiator caspase for the intrinsic apoptotic cell death pathway. Caspase-9 activation proceeds via dimerization-dependent mechanism, which is induced upon interaction between the caspase-9 CARD domain and APAF1(31).

Although it was demonstrated that both caspase-9 and APAF1 exist in non-stressed cell as inert cytosolic monomers (30, 31). Upon induction of apoptotic cell death pathway or exposure to cellular stresses, cytochrome *c* is released from the mitochondria. The cytochrome *c* subsequently binds APAF1's WD domain which results in initiating a cascade of conformational changes that

ultimately lead to the assembly of seven of activated APAF1 monomers to subsequently form an oligomeric supra-molecular complex, the core of which encompass the CARDs that recruit and activate caspase 9 (32). The resulting complex apoptosome, which encompasses cytochrome c, caspase 9, and APAF- 1, mediates the activation of caspase 9 and the subsequent activation the downstream effector caspases (32, 33).

Since the role of cytochrome *c* in electron transport chain reactions has long been established, it was revealed that mammalian cells devoid of cytochrome *c* couldn't activate caspases in response to induction of mitochondrial apoptotic cell death pathway (34). However, recent work has demonstrated that the role of cytochrome *c* in electron transport operates independent of its ability to interact with APAF1 and induce caspase activation and apoptosome formation (35). Concomitantly, cells derived from knock in mouse mutant in which residue 72 (Lysine residue), a crucial residue for APAF1 interaction, of cytochrome *c* was mutated were able to promote electron transport yet not apoptotic cell death (35).

It is imperative to mention that the extrinsic and intrinsic pathways cross-talk via caspase-8 cleavage of the BH3-only protein BH3-interacting domain death agonist (BID), where this cleavage event produces the active, truncated pro-apoptotic form of BID (tBID) that triggers MOMP (13) and subsequently amplify the apoptotic cell death signalling (13,22,23,48).

Activation of effector caspases can result in processing and activation of additional signalling molecules including other proteases, leading to an amplified proteolytic cascade. It is also noteworthy to mention that the activation of these or related proteases contributes to the activation of another protease, calpain, that is often stimulated during apoptotic cell death (22,23,48,32,35). The overall result may be an escalating cascade of proteolytic processing. Proteolytic cleavage of specific substrates may further contribute to the process of apoptotic cell death through different ways: via structural changes, by activation of other effector signalling molecules such as nucleases (e.g., processing of proforms of a protein), or by removal of an inhibitor molecule (22,23,48).

Among the most characteristic changes associated with apoptotic cell death chromatin condensation and nuclear changes, and proteolytic processing could play a pivotal role in this

context. For instance, lamin B1 degradation during apoptotic progression could lead to collapse of the chromatin due to the severe loss of attachment points on the nuclear matrix (22,23,48). Other characteristic alterations and changes during apoptotic cell death are related to the plasma membrane and cytoskeleton, as cells lose attachment, undergo blebbing, and fragment. Membrane blebbing and cellular fragmentation into apoptotic bodies depend upon actin polymerization; thus, targeted proteolytic cleavage of actin (48) and of the actin-associated protein fodrin is possibly related to these membrane structural alterations (22,23, 48).

In addition to the intrinsic and extrinsic apoptotic pathways, granzymes, granule-secreted enzymes, can mediate apoptotic cell death programme through caspase-dependent and -independent molecular pathways (36, 37) (Figure 1). In granzyme-mediated apoptotic cell death, granzyme B (GrB) and perforin are released from the granules of cytotoxic T-cells. Granzyme B, which has access to the cytoplasm of target cells by a perforin-mediated endocytosis process, cleaves a number of signalling substrates, including vitronectin, fibronectin and laminin (38), and initiates apoptotic programme via caspase-dependent and -independent mechanisms (37). Although it was demonstrated that GrB effectively elicits apoptotic cell death in target host cells via mediating caspase activation through caspase-10, there is significant evidence that GrB may still activate caspases through caspase-7 or through caspase-3 if the target cells lack functional caspase-10 (36-38). It was also unveiled that GrB has the redundant capacity to initiate caspase activation despite the absence of specific caspases. Importantly, it was revealed that in MCF7 cells, which express very low levels of caspase-3 and -10, microinjection of GrB results in rapid apoptotic cell death (36-38). The ability of granzyme B to induce apoptotic cell death in the presence of a partially or completely inactivated caspase refelects the robustness of this important cellular host defense system.
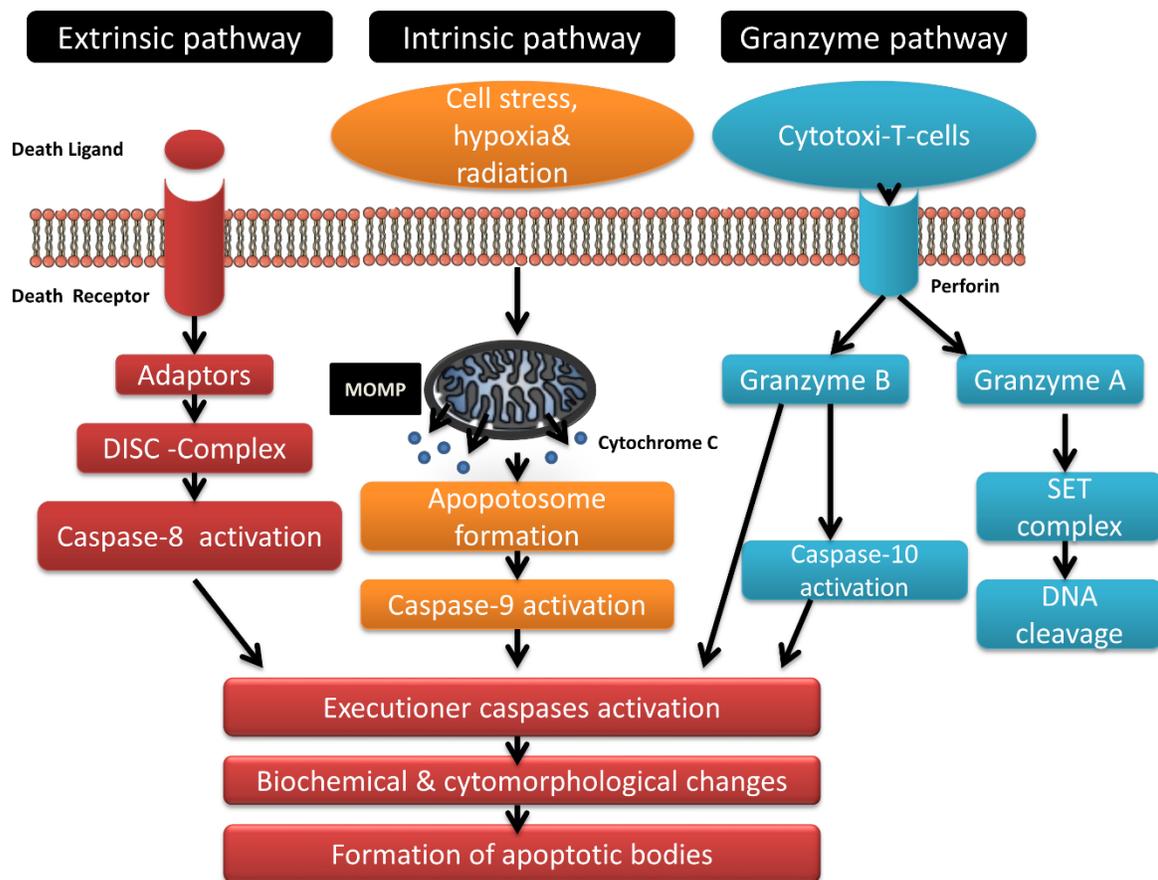
Figure 1.1: The three apoptotic pathways (Extrinsic, intrinsic and granzyme B pathway). In the extrinsic pathway, the extrinsic apoptotic signaling pathway is triggered by ligation of a death receptor, followed by the assembly of a caspase activation platform called the DISC complex. This activation platform recruits and activates caspase-8 via adaptor molecules and then subsequently leads to caspase 8-mediated activation of effector downstream caspases. In the intrinsic pathway, in response to various cellular stresses, pro-apoptotic proteins of the Bcl2 family mediate mitochondrial outer membrane permeabilization (MOMP), permitting the release into the cytosol of pro-apoptotic factors such as cytochrome c and others. Cytochrome c then binds to Apaf 1, mediates its oligomerization and recruits caspase 9 to form apoptosome that subsequently leads to caspase 9 activation then leads to activation of the downstream effector caspases. In the granzyme B-pathway, Granzyme B may lead to activation of caspase 10 which subsequently lead to activation of effector caspases.

1.3 Restricted proteolysis and apoptotic cell death: regulating death and preventing danger

Before I discuss limited proteolysis and proteases, their roles in regulating apoptotic cell death, and how limited proteolysis can be regulated via targeted protein degradation during apoptosis, it is worth highlighting some of the crucial consequences of failing to tightly-regulate the intricate proteolytic cascades during apoptotic cell death. For long time, it has been recognized that necrosis (that is, type III cell death) is associated with a significant loss of the integrity of the cellular plasma membrane and the subsequent release of various molecular cellular components into the extracellular space (22, 23, 26, 27). Beside the undesirable destruction to neighbouring cells that such release may provoke, there are also various lines of evidence that suggest that the immune system responds to apoptotic and necrotic cells in distinctly different ways (39, 40). Necrotic cells can invariably trigger inflammation by neutrophils, macrophages and other cells of the innate immunity, and previous work demonstrated that this may be attributed to the release of some molecules (collectively designated as danger-associated molecular patterns (DAMPs) or alarmins) that subsequently induce pattern-recognition receptors on macrophages, dendritic cells and natural killer cells (41). It was revealed that stimulation of pattern-recognition receptors on innate immunity cells, particularly dendritic cells, may ultimately lead to initiation of immune responses (42). Thus, the presence of necrotic cells in a tissue may act as a signal to initiate immune responses (43).

Because apoptotic cells exhibit proteases-mediated cellular signalling cascades including plasma membrane alterations and the subsequent formation of membrane-bound apoptotic bodies which facilitate their rapid removal from tissues before rupture and release of their cytoplasmic contents (44), such cell death formats typically do not attract the attention of innate immune cells. Therefore, apart from limiting direct cell damage due to release of cytoplasmic contents, one of the major benefits of tightly control of apoptotic pathways, via proteolytic signalling cascades and other interconnected networks, could be to prevent the unmasking of hidden self, thereby halting unwanted immune responses. Tellingly, it has been found that alarmins such as genomic DNA and heat shock proteins are typically not released from apoptotic cells unless they appear in enormous levels that overwhelm the phagocytes capacity to dispose them quickly (39, 45). Therefore, what happens to a cell within apoptotic cell death signalling, including proteases-mediated structural packaging of cellular contents and caspases-mediated alterations in the internal environment of the

dying cell appears to be elegantly geared towards preventing provoking unnecessary immune response as it is towards terminating the life of the cell (44).

1.4 Proteases as an essential elements of apoptotic cell death
In the early 1990s, different lines of evidence have supported a pivotal role for proteases in regulating apoptotic cell death. First, In C. elegans, genetic and biochemical studies unveiled that apoptotic cell death was dependent on an intracellular protease (*ced-3*) bearing significant homology to the human interleukin-1b converting enzyme (ICE), which converts the 33 kDa protease form of IL-113 to the active 17.5 kDa form (46). Interestingly, ectopic expression of ICE in fibroblasts leads to apoptotic cell death (47). A second line of evidence for the crucial role of proteases in apoptotic cell death revealed from investigations that have examined the role of diverse protease inhibitors on apoptosis-induced by various agents. Some of these studies have implicated calpain I, a calcium-dependent protease, in activation-induced apoptotic cell death (48, 49). Other studies that examined the effects of CrmA, an inhibitor of ICE encoded by the cowpox virus, on apoptotic cell death further support the role of protease ICE in induction and regulation of apoptosis. For instance, it was demonstrated that CrmA expressing Rat1 fibroblasts were protected from apoptotic death owing to serum withdrawal (50). Moreover, a number of different intracellular proteins, including poly(ADP-ribose) polymerase (PARP) (51), lamin B1(52) and topoisomerase I (53) have been reported to be cleaved during the onset of apoptosis, thereby supporting a direct function for the activation of one or more proteases during the process of apoptotic cell death. Subsequent and more recent studies have led to the identification of 14 mammalian proteases that are related to ICE, and these proteases, along with ICE, have been termed caspases proteases (caspase-1 to caspase-14) (54). Although previous work have revealed that other proteases, such as cathepsins, calpains, granzymes and other non processive proteases may be involved in regulatory roles in apoptotic cell death (55), this body of work will focus mainly on caspases-dependent apoptotic cell death.

1.5 Caspases: Activation, specificity, function and regulation.
The core element of apoptotic cell death machinery is a proteolytic system involving a family of proteases known as caspases. The term caspase dictates 2 pivotal features of these proteases: (i) they are cysteine proteases and exploit cysteine as the active nucleophilic moiety for cleavage of their target substrates and (ii) they are specific aspases and cleave the peptide bond C-terminal to aspartic acid residues (56).Tellingly, the notion that caspases play a pivotal role in apoptotic cell

death is based on three key lines of evidence. First, inhibitors of caspases effectively halt apoptotic cell death induced by diverse apoptosis-inducing agents (57). Second, animals lacking certain caspases exhibit prominent ablations in apoptotic cell death (58). Third, it has been demonstrated that caspases mediate most of proteolytic cleavage events that lead to the characteristic biochemical and morphological features of apoptotic cell death (11–13, 30).

Human caspases can be classified based on their reported function and location in cellular signaling pathways and networks. Additional criteria encompass: favoring specific target substrates, and the length of pro-domain (54). Accordingly, caspases were divided into (apoptotic) and (pro-inflammatory) (54). Nonetheless, most apoptotic candidates (caspase-2, 3, 6, 7, 8, 9, 10) have had at least one non-apoptotic role related to them (30) .

Inside the apoptotic-related group, caspases have been classified into (initiators) caspases and (executioners) or (effector) caspases to distinguish between the caspases that initiate the cascade (caspase-8, 9, 10) from those that are induced and activated via the initiators to promote the execution of apoptotic cell death programme (caspase-3, 6, 7) (30). Initiator caspases have been categorized into caspases participating in the intrinsic apoptotic cell death pathway (caspase-9) or extrinsic (caspase-8 and -10) (30).

Like other multi-step proteolytic cascade reactions, downstream caspases are activated by limited proteolysis, yet upstream ones, having no protease (Upstream) to them, should respond to an activating cue via an alternative mechanism (59). Even though the initial thought was that all caspases were activated by limited proteolysis, yet recently it has become clear that this is one of the mechanisms in caspase activation, related mainly, at least in humans, to the three executioner caspases 3, 6 and 7 (59). In general, two main mechanisms are involved in caspases activation, dimerization-dependent activation and proteolytic cleavage-dependent activation (Figure 2).

1.5.1 Initiator caspases – Dimerization-dependent activation

In the resting state, initiator caspases are inert monomers that needs homodimerization for activation (59). In response to an apoptotic-initiating signal, caspase-dimerization is brought about through caspase recruitment to supra-molecular complex that serve as an activation platform (59). Certain molecules-derived from the supra-molecular complex (adaptors) interact with caspase recruitment domains (CARDs) of caspase- 1, 2 and 9, and caspase pro-domains like death effector

domains (DEDs) of caspase-8 and 10 (60). Consequently, this recruitment and subsequent interaction mediate an augmentation in caspase levels and promotes activity via proximity-induced dimerization (60). Each initiator caspase has its own supra-molecular complex or activation platform: the DISC (death inducing signaling complex) recruits and activates caspase-8 and 10, the apoptosome activates caspase-9, while the PIDDosome may be involved in the activation of caspase-2 (54).

1.5.2 Executioner caspases –Proteolytic cleavage-dependent activation.

Executioner caspases exist as inert dimers that need proteolytic cleavage of the catalytic domain to become active (Figure 2). Tellingly, zymogens are sequestered via the action of a short-motif (linker or junction) that separates the large and small subunits of the catalytic domain(59). Structural data of the zymogen form of caspase-7 (61, 62) unveil some of the molecular principles of activation-induced catalytic groove formation. Interestingly, Limited proteolysis of the linker or junction permits rearrangement of some mobile loops favoring catalytic site formation (59). It was demonstrated that, in vivo, the initiators caspases (caspase-8, 9, 10) and the lymphocyte-specific serine protease Granzyme B can induce the activation of the downstream executioner caspases (54).

1.5.3 Caspases maturation events

Caspase activation is often proceeded by (auto)proteolytic cleavage events designated as maturation events. Tellingly, maturation events involve proteolytic excision of the pro-domain or the linker (junction) region. Notably, activation process is essential for maturation and the subsequent induction of enzymatic activity (59, 63). Importantly, maturation has a crucial impact at the cellular level. For instance, active caspase-8 dimer, which has not experienced maturation, can mediate signalling of T cell proliferation and activation, yet not apoptotic cell death, which requires mature cleaved caspase-8 (64). In sum, caspase maturation is a distinct process from proteolytic activation, crucial for producing caspase metabolic stability or mediating specific downstream regulatory events.
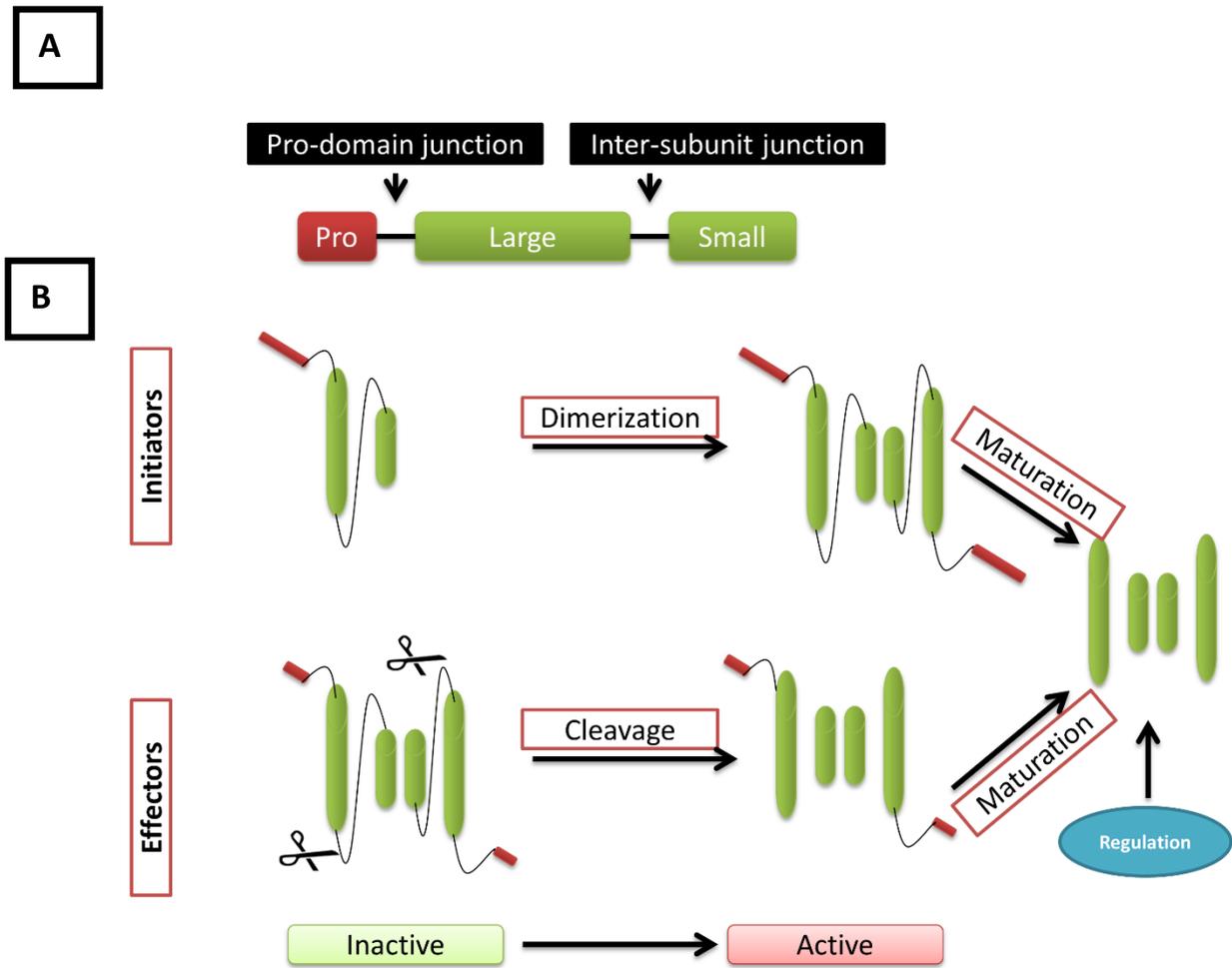
Figure 1.2: Caspases organization and activation

A- Human caspases domain organization. An N-terminal prodomain and C-terminal catalytic domain, consists of two covalently attached subunits. Locations for (auto)proteolytic events at Asp residues are labelled.

B- General mechanism of activations. Initiator capsases are monomers that can be activated by pro-domain-dependent dimerization. Effector or executioner caspases are dimers that activate by the cleavage of inter-subunit junctions (linkers). Following activation, additional proteolytic events mediate the maturation process of the caspases to more stable versions.

1.5.4 Caspases Specificity

Although the most prominent feature of caspase specificity is that caspases cleave after Asp residues (59). Nevertheless, other recongtion elements, in most cases, need to be fulfilled to turn a peptide/protein into a genuine caspase target substrate (65–67). For instance, a peptide of sequence P4-P3-P2-P1-P1', with P1-P1' representing scissile bond, is a genuine caspase substrate when1) the P1 residue is Asp (66); 2) the P1' residue is uncharged and small (Ala, Gly, Ser) (67); and 3) the three amino acid residues (P4-P3-P2) are complementary for interactions with the catalytic groove which promote optimal interaction and cleavage (66). For example, it was found that executioner caspases cleave very efficiently DEVD/G peptides yet in much less efficient manner WEHD/G peptides (65).

1.5.5 Caspases regulation

Since limited proteolysis is irreversible, activation of caspases in cells must be tightly regulated. To curb undesirable cellular responses that may result from pre-mature activation of caspases, cells exploit three counteracting responses to activated caspases: inhibition of activated caspases, targeted degradation of activated caspases and decoy inhibitors(68–71). One of the cellular strategies to inhibit activated caspases is often targeting the substrate-binding site, blocking it with a segment that mimics a genuine caspase target substrate (54, 57). One of the best characterized caspase inhibitors, CrmA (cytokine response-modifier A) from the cowpox virus that has an active site directed "suicide" inhibitors. Although it was demonstrated to mediate a rapid inhibition of caspases, this CrmA-mediated inhibition of caspases was revealed to proceed in a relatively non-specific manner (68). Conversely, XIAP (X-linked inhibitor of apoptosis) mediates an efficient and specific inhibition of caspase-9 (via its BIR3 domain), caspase-3 and 7 (via its BIR2 domain) (72). Tellingly, XIAP's BIR3 and BIR2 domains mediate 2 specific, yet relatively weak, interactions with their target caspases, to ultimately and selectively inhibit activated caspases, via a two-site mechanism that is mechanistically distinct to the viral caspase-inhibitors discussed above (72). Interestingly, inhibition by decoy proteins employs proteins structurally related to caspase pro-domains, competing for the same adaptors within the activation platforms. Thus, they are not considered as direct inhibitors but rather activation preventers. For instance, FLIP (FLICE inhibitory protein), a pseudo-caspase-8 with a non-functional catalytic domain, halt caspase-8 recruitment to the DISC complex (54, 68). The final mechanism of caspase regulation involves targeted proteasomal degradation. Tellingly, activated caspases exhibited more dynamic metabolic

stability profile with respect to their inert counterparts or zymogens (73), and IAPs have been suggested as proteins that mediate selective degradation of activated-capsases. In addition to bearing BIR domain, many IAPs also contain RING and UbA domains that are involved in ubiquitin ligation activity (69, 70). Tellingly, IAPs are currently thought to be the main players responsible for removal of active caspases before they reach an apoptotic threshold. In line with this is the finding that mice harboring ablation in XIAP RING domain activity revealed an elevated caspase activity in specific cell types, suggesting a pivotal cellular role of XIAP ubiquitin ligase activity in dampening activated caspase (71).

1.5.6 Caspase-generated proteolytic fragments: functions and regulation
Following the induction of apoptotic cell death in a mammalian cell, around 1,000 different proteins are cleaved by caspases (8, 9). Although a biologically significant subset of the resulting proteolytic fragments encompasses proteins with proapoptotic (Pro-death) or anti-apoptotic (Pro-life) activity (6), it is remarkable that our understanding is little regarding the molecular mechanisms that regulate the stabilities and functions of such activated proteolytic fragments.

As in other proteolytic activation cascade, caspase-mediated proteolytic activation of downstream signalling substrates may lead to amplification of caspase proteolytic signalling (positive feed-back loops) to promote the progression of apoptotic cell death (6, 12–14). In addition, proteolytic cleavage/activation of specific signalling substrates may further contribute to the biochemical and structural changes associated with apoptotic cell death in several ways: through specific structural alterations, via activation of other crucial effector signalling proteins such as nucleases (e.g., processing of pro-forms of a protein) (48, 74), or via mediating the disposal of an inhibitor signalling protein (48).

Although it was thought that caspases activation and the subsequent caspase-mediated-generation of proteolytic fragments during the apoptotic programme is a sign of impending cellular death. However, interestingly, recent work suggested that some mammalian cells and cancer cell lines can survive beyond caspase proteolytic activation if the apoptotic cell death stimulus is ceased (75–77). Moreover, previous reports have demonstrated the presence of basal activation of caspases in living cells (77). In line with this observation, previous studies revealed that the same caspases that orchestrate apoptotic cell death programme have been involved in diverse normal cellular roles, such as fine-tuning of neuronal activity (78), molecular mechanisms regulating

learning and memory (79), and the process of spermatid individualization (80). So, given the irreversible nature of limited proteolysis, turning off the caspase proteolytic cascades presents a distinct cellular challenge. How then, do cells evade apoptosis when unscheduled apoptosis takes place? In contrast to activated caspases which can be curbed, through targeted proteasome-dependent degradation, via binding to inhibitor of apoptosis (IAP) proteins (81), little is known about the molecular counteracting responses to the toxic caspase-generated proteolytic products. These anti-apoptotic molecular responses might contribute to the survival of cells and enable cells to continue to proliferate in culture under basal caspase activation.

Previous studies have unveiled that many proteolytic fragments (around 40 % of total caspase-generated proteolytic fragments)generated by caspases and documented in two databases, MEROPS and the degrabase, are short-lived (having a short half-life; less than 3 hours) (9). Tellingly, it is estimated that more than 15 % of the total caspase-generated fragments bear N-terminal destabilizing residues according to the current understanding of Arg-N-end rule degradation pathway (9). Furthermore, it has been indicated, recently, that the destabilizing feature of the N-terminal residues of a cellular significant subset of caspase-generated proteolytic fragments is fully conserved in vertebrates (the metabolic instability nature of Nt-residue is conserved, rather than the exact identity of the N-terminal residue of the proteolytic fragment) (10, 20) So, this evolutionary conserved pattern of destabilizing N-terminal residues (P1' residue in the precursor protein), suggests that their metabolic instability is imperative for their functional attribute. Indeed, in insects, it was shown that the inhibitor of apoptosis proteins (IAPs) (anti-apoptotic molecules) require priming in order to function as negative regulator of executioner caspase (55, 60). Consistently, active caspases initially process IAPs resulting in the exposure of destabilizing N-terminal residues. As a result, the N-end rule degradation machinery can target IAPs for degradation through the proteasome. Thus, somewhat paradoxically, even though the N-end rule degradation pathway decreases the level of these key anti-apoptotic molecules (IAPs) and therefore would be expected to reduce the apoptotic threshold, it was demonstrated that the N-end rule-mediated degradation of insect IAPs is important for IAPs anti-apoptotic function (inhibiting apoptosis via targeting active caspases for degradation). These observations strongly suggested that the Arg-N-end rule degradation pathway may play a pivotal role in curbing the caspase-mediated proteolytic generation of pro-apoptotic and anti-apoptotic fragments that bear N-destabilizing amino acid residues.

1.6 Protein degradation by ubiquitin proteasome system (UPS) and the N-end rule degradation pathway.

In eukaryotes, regulated protein degradation of intracellular proteins is mediated mainly by the ubiquitin proteasome system (UPS) (20, 82). UPS-mediated protein degradation regulates virtually all crucial aspects of cellular physiology such as cell proliferation (83), cell division (84) cell differentiation (85) and cell death (10). UPS-mediated protein degradation is carried out via two sequential steps: the substrate is first recognized and covalently conjugated to ubiquitin by specific ubiquitin E3 ligases. After the initial ubiquitination additional ubiquitin molecules are conjugated in series at lysine 48 of the ubiquitin to form a polyubiquitin structure which is then recruited to the 26S proteasome for degradation (82, 86). Targets of the UPS system are ubiquitinated by a cascade of three enzymes: E1 (ubiquitin activating enzyme, using ATP), E2 (ubiquitin conjugating-enzyme or carrier enzyme) and E3 (ubiquitin protein-ligase) (82). Since protein degradation by UPS is an energy-dependent and irreversible process, tight regulation is essential to ensure specificity and to avoid random, uncontrolled degradation. E3 ubiquitin protein ligases endow the UPS system with selectivity and specificity by direct recognition of degradation signals on a target protein (82).

The feature (a region of the protein amino acid sequence and /or a conformational determinant) that renders proteins their metabolic instability is called primary degradation signal or primary degron (20, 82). Tellingly, primary degradation signals may take diverse forms; for instance, some primary degrons can be very simple such as the N-terminal amino acid residue in the protein (i.e. the N-end rule), while others can be a complex structural feature such as; (i) PEST sequence , a sequence motif that is rich in proline (P), glutamic acid (E), serine (S), and threonine (T) or (ii) the cyclin destruction box (CDB), a 9- amino acid residue sequence that was originally discovered in sea urchin cyclin B (87, 88). The secondary degron is the poly ubiquitin chain, as ubiquitin is conjugated to protein substrates that have a primary degradation signal (82). Primary degrons may be masked or conditional so that recognition necessitates that they first be exposed, for example by proteolytic cleavage, local unfolding or subunit separation (89–91). Another aspect of recognition of primary degrons is that it can be induced by the translocation from one compartment to another, for example the degradation of p53, p27Kip1 and cyclin D when they are translocated from the nucleus to the cytosol (92–94).

The first discovered degradation signals that confer metabolic instability to proteins were destabilizing N-terminal amino acids (21). The N-end rule relates the in vivo half-life of a protein to the identity of its N-terminal amino acid (20), where the underlying pathway is called the N-end rule pathway. Variations of the N-end rule pathway are present in prokaryotes (95), fungi (96), plants (97) and mammals (96). The recognition elements of the eukaryotic N-end rule pathway are E3 ubiquitin ligases and they are called N-recognins (20). In eukaryotes, The N-end rule mediated-protein degradation regulates a myriad of biological functions, including the elimination of misfolded proteins (20), the control of subunit stoichiometry (through degradation) (98), the regulation of chromosome repair, transcription and cohesion/segregation (99), DNA damage response (100), the regulation of G proteins (20), the regulation of apoptosis (10, 101), cardiovascular development(102), peptide import (103) and other processes in plants (97). Three branches of the N-end rue pathway exist in eukaryotes, the recently identified Pro-N-end rule pathway(104), Ac-N-end rule pathway which targets N-terminal-acteylated residues (105), and the classical Arg-N-end rule pathway which targets specific unacetylated –N-terminal residues (20) (Figure 1).

Ac-N-End rule pathway: An emerging major branch on N-termini dependent protein degradation is dependent on N-terminal acetylation, and has been referred to as the Ac-N-End rule pathway. This selective targeted degradation has been demonstrated to be via E3 ubiquitin ligases that included Doa10 in yeast (105) and MARCH6 in mammals (105). As it has been estimated that ~60% of the yeast proteome and ~90% of the mammalian proteomes are N-terminally acetylated (105), this pathway is predicted to have a widespread impact on cellular proteostasis. Emerging lines of evidence are revealing that the selective degradation of proteins by the Ac-N-End Rule may be more complex than initially appreciated. This apparent complexity may be rationalized in several ways, which include sufficient residual acetylation in the mutant, additional unforeseen sequence specificity of the Ac-N-End Rule pathway or that many of the N-termini are inaccessible for the Ac-N-End rule pathway as this pathway has been reported to degrade protein subunits that are in excess of their target complex (105).

Pro-N-end rule pathway: We now learn about yet another branch of N-termini dependent protein degradation, the N-terminal Proline-degron, that targets the gluconeogenic enzymes fructose-1, 6-bisphosphatase (Fbp1), isocitrate lyase (Icl1), malate dehydrogenase (Mdh2), and phosphoenolpyruvate carboxykinase (Pck1) for N-termini dependent degradation (104). In this report, Varshavsky and colleagues demonstrated that Gid4 subunit of the GID E3 ubiquitin ligase is the major recognin responsible for targeting substrates with an N-terminal proline. The recent work by Chen et. al. (104) now delineates the specificity of Gid4 as a Pro-N-recognin and elucidated the molecular elements that bring about the binding of Gid4 to its target substrates of Fbp1 and other gluconeogenic enzymes.

The major focus of this thesis is studying the role of Arg-N-end rule pathway with respect to capsase-generated proteolytic fragments in mammalian cell lines. In the mammlian Arg-N-end rule pathway, N-terminal amino acid residues are categorized into stabilizing, primary (1º) destabilizing (Arg, Lys, His, Leu, Phe, Tyr, Trp and Ile), secondary (2º) destabilizing (Asp and Glu) and tertiary (3º) destabilizing residues (Asn and Gln) (also Cys, under specific conditions) (18). Proteins bearing a 1ºdestabilizing Nterminal amino acid residue are recognised by N-recognins (including UBR1, UBR2, UBR4 and UBR5) (106), whereas the secondary and tertiary residues are destabilizing due to their preliminary modification via Nt-deamidation (via NTAN or NTAQ amidases) and Nt-arginylation (via ATE1 transferase) (20, 107) (Figure 3).
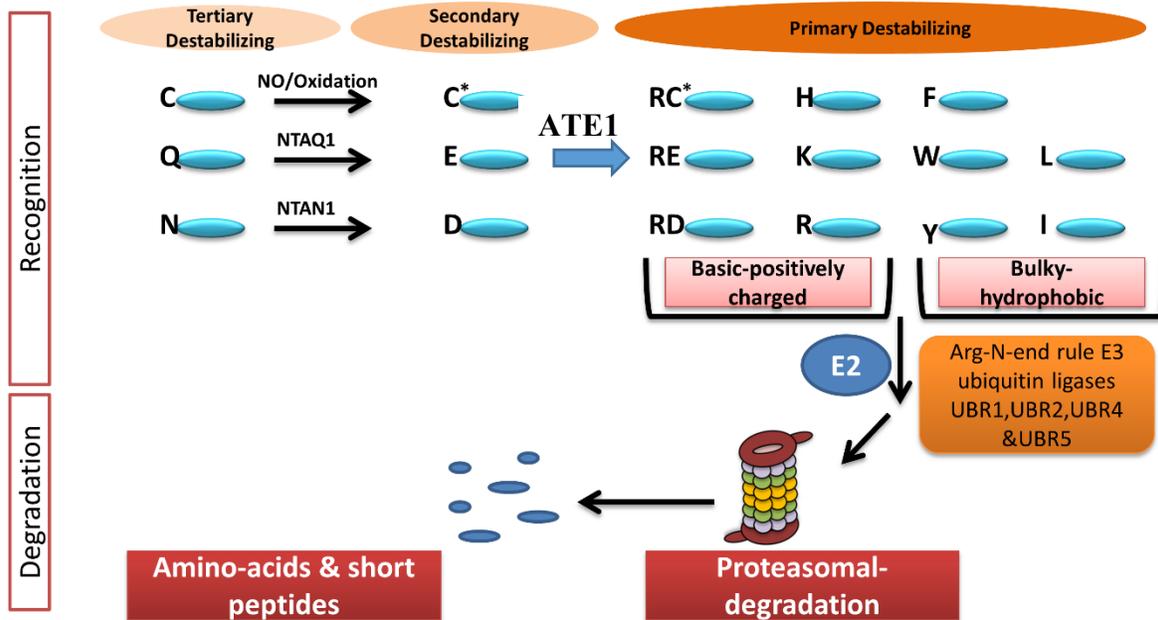
Figure 1.3: The mammalian canonical Arg/N-end rule pathway. N-terminal destabilizing residues are indicated by single-letter abbreviations for amino acid residues. Blue ovals denote the rest of a protein substrate. Primary, secondary, and tertiary dictate mechanistically distinct subsets of N-terminal destabilizing amino acid residues. C* denotes oxidized N-terminal Cys (Cys-sulfinate, Cys-sulfonate or Cys-sulfinic acid). The main Arg-N-end rule pathway E3 ubiquitin ligases are the UBR1, UBR2, UBR4, and UBR5. They mediate the recognition of the N-terminal destabilizing residue then subsequently mediate the polyubiquitination of the target substrate. Following polyubiquitination, the protein substrate is then subsequently targeted for proteasomal degradation.

1.6.1 Protein recognition by the UBR-box E3 ubiquitin ligases of the Arg-N-end rule pathway

In eukaryotes, a set of primary N-dergons that include positively charged (type 1: arginine, lysine, histidine) and bulky hydrophobic (type 2: phenylalanine, tyrosine, tryptophan, leucine, isoleucine) N-terminal amino acid residues can be directly recognized by N-recognins [108] . Previous work unveiled [106] a family of mammalian E3 ubiquitin ligases that serve as N-recognins (including UBR1, UBR2, UBR4 and UBR5), characterized by a ~70-residue zinc-finger domain (amino acids 113-194) labelled as Ubr box domain; in yeast, there is only one such N-recognin, Ubr1[109]. It was demonstrated that these N-recognins bear two distinct substrate recognition domains, the Ubr box domain required for type 1 primary destabilizing residues and the N-domain for type 2 primary destabilizing residues [20, 106, 108, 109]. In mammals, a set of pre–N-degrons (aspartate, glutamate, asparagine, glutamine, cysteine) can destabilize proteins via N-terminal arginylation via the action of arginyl-transferase ATE1. In addition, N-terminal Asn and Gln serve as tertiary destabilizing N-terminal residues through their enzymatic deamidation via N-terminal amidohydrolases into the secondary destabilizing N-terminal residues Asp and Glu, respectively.

As bacterial cells have no ubiquitin-proteasome system, the N-end rule degradation pathway mediates the destruction of target protein substrates via the function of ClpS, an adaptor protein that mediates the transfer of substrates to the proteolytic ClpAP complex[110]. Bulky hydrophobic (leucine, phenylalanine, tryptophan and tyrosine) and basic residues (arginine and lysine) serve as primary and secondary destabilizing residues, respectively (figure 3). It was revealed that there are similarities between ClpS and the eukaryotic N-domain, suggesting the bacterial origin of the type 2 branch of the eukaryotic N-end rule degradation pathway [108, 110]. In line with these studies, the crystal structures of ClpS [111, 112] and observations from other recent work [109–112] demonstrated that the N-domain and ClpS domain adopt similar substrate-binding pockets to mediate processive protein degradation.

Structural studies of the UBR box [113, 114], and of the ClpS adaptor protein (and, by homology, the N-domain of the UBR enzyme in eukaryotes) [111, 112], suggest that type 1 and type 2 domains adopt different ways to bind to a variety of N-terminal amino acid residues with various sizes and forms. For instance, it was demonstrated that UBR box domains interact with type 1

peptides via a shallow and acidic groove (113, 114), whereas ClpS encompass a deep hydrophobic pocket(111) that can accommodate the binding with the hydrophobic N-terminal side chain of type 2 N-terminal amino acid residues. Tellingly, the structural data on UBR box domain unveil that the substrate-interacting site of the UBR box is stabilized by via a fold that encompass two contiguous zinc fingers: a typical Cys_2His_2 zinc finger and a novel binuclear zinc finger where two zinc ions in tetrahedral coordination share a common cysteine ligand (113–115).

Despite the existence of basic structural differences and a significant evolutionary gap (20), the Ubr box and ClpS show some similarity with respect to fundamental molecular principles of substrate recognition. For instance, both the UBR box and ClpS recognize the free α-amino group of the N-terminal amino acid residue via three highly conserved hydrogen-bonds (20, 110, 114).Since the N-terminus α-amino group exist in all proteins, this weak and transient binding is not substrate selective yet can serve as a pivotal entry step in the context of the N-end rule, enabling N-recognin to rapidly scan a pool of protein N-termini. Once the N-recognin recognizes a good substrate, the interaction between the N-terminal amino acid and UBR box domain can be stabilized via selective interactions with the N-terminus side chain (114, 115),which in turn are further stabilized through hydrogen bonds with the first peptide bond and the side chain of the Penultimate amino acid residue. Taken together. It seems that the first two N-terminal acid residues impact the recognition by the UBR E3 ubiquitin ligases. Accordingly, N-recognins appear to minimize non–N-end rule specific interactions with the rest of the protein through limiting the major interactions to the first two N-terminal amino acids. This two-step N-end rule recognition provides the molecular basis for binding selectivity and affinity between the Ubr box and a peptide substrate (114, 115).

As the N-recognin binding to the target N-terminal amino acid proceeds, the N-recongin E3 ubiquitin activity mediates E2-dependent conjugation of ubiquitin to a properly-exposed lysine residue on the target substrate (114,115). Tellingly, significant evidence suggest that a genuine N-end rule substrate may encompasses an unstructured, flexible region between the N-terminal amino acid residue and the rest of protein fragment (114,115), which may be needed for search of a polyubiquitination site. Once ubiquitin chain begins to assemble, the N-recognin can be released from the substrate N-terminal residue to initiate a new round of N-end rule recognition. It is noteworthy to mention that recent biochemical studies suggest that UBR box domains have

relatively high dissociation rates, unveiling the reason behind the high processivity of N-end rule-mediated degradation found in eukaryotic cells (21, 96, 115, 116).

Although structural work reveals the crucial importance for the identity of the N-terminal amino acid residue, mounting lines of evidence suggest that the second amino acid residue also significantly contributes to N-end rule recognition and selectivity, yet much still unknown regarding a unifying principle for the importance of the second residue (117). Previous structural work demonstrated that human Ubr box domain favours acidic amino acid residues, which exist in the products of N-terminal arginylation of secondary N-terminal destabilizing residues(114); remarkably, bacterial ClpS also prefers arginine or lysine at the second position (110). It thus seems that the second N-terminal amino acid residue significantly impacts the affinity to the Ubr box in a a strategy specific to individual N-recognins. Indeed, more recent work unveiled that the mouse version of Ubr1 and Ubr2 can recognize proteins having the unacetylated N-terminal Methionine if it is followed by bulky hydrophobic amino acid residues (as Leu, Ile, Tyr, and Trp). Tellingly, Ubiquitination data and binding assays suggested that this N-degron can be recognized by type II binding site of UBR1 or UBR2 (118).

1.6.2 The N-end-rule pathway vis-à-vis caspase-generated proteolytic fragments: The beginning determines the end

Upon the activation of the proteolytic cascades in the context of apoptotic cell death, enormous number of various proteolytic cleavage events takes place. Tellingly, many of these cleavage events culminates in activation, inactivation, or alteration in cleaved proteins functions and localization. This proteolytic reprogramming of a protein can facilitate sculpting the apoptotic programme and is exemplified by the cleavage of crucial signalling kinases as PKC-Ө, BMX kinase and PKC-δ.

In contrast to the current model about the counteracting responses to activated caspases (119), our understanding about the counteracting molecular responses to caspases-generated proteolytic fragments (pro-apoptotic and anti-apoptotic fragments) still far from clear (20). Since It has been revealed, recently, that the destabilizing feature of the N-terminal residues of a cellular significant subset of caspase-generated proteolytic fragments is fully conserved in vertebrates (the metabolic

instability nature of Nt-residue is conserved, rather than the exact identity of the N-terminal residue of the proteolytic fragment) (9, 10, 20). This evolutionary conserved pattern of destabilizing N-terminal residues (P1' residue in the precursor protein), suggests that their metabolic instability is imperative for their functional attribute. Accordingly, it is possible that the N-end rule degradation pathway acts as one of the molecular mechanisms that counteract these caspase-generated proteolytic fragments (either pro- and anti-apoptotic fragments), via targeted protein degradation, that may be generated by basal and induced protease activity (10).

My broad research goal was to investigate how the N-end rule pathway regulates apoptosis progression and execution via selective protein degradation of specific key cleaved fragments. Implicit in this objective is the assumption that selective degradation by the Arg-N-end rule pathway is a control checkpoint after basal or treatment-induced caspase activation. My investigation focused on three cleaved protein fragments (Lyn kinase, BMX kinase and PKC-theta). The choice of these fragments over other possible proteins pertinent to apoptosis is because: First, the cleavage of these proteins have been reported more than once (6,9,10,20), so it is more likely to yield reproducible results in the laboratory. Second, the cleavage site (either caspase or calpain cleavage sites) and the destabilizing neo-N-terminal amino acids of these substrates are evolutionary conserved in vertebrates (6,9,10,20). Third, the presence of internal lysine residue (site for ubiquitination by the E3 ubiquitin ligases). Fourth, the diversity of functions (pro-and anti-apoptotic) of the substrates might reveal a broader role and mechanisms for the N-end rule pathway with respect to apoptosis.

In this work, I report the investigation of the role of the Arg-N-end rule pathway in regulating apoptotic cell death via selective protein degradation of specific key cleaved fragments in mammalian cell lines. Implicit in this objective is the assumption that selective degradation via the Arg-N-end rule pathway is a counteracting molecular response to basal or treatment-induced caspase activation. My investigation has focused on three cleaved protein fragments (Lyn kinase, BMX kinase and PKC-theta).

Selective degradation of the C-terminal fragments of these three diverse protein kinases implicates an expansive role for the N-end rule pathway in the complex network of apoptotic pathways. The degradation of the anti-apoptotic cleaved Lyn kinase by the Arg-N-end rule pathway in CML K562

revealed that Arg-N-end rule may target anti-apoptotic fragments and thus regulates the apoptotic threshold in certain cells (120). The pro-apoptotic cleaved BMX kinase degradation by the Arg-N-end rule pathway in cancer-derived cell lines provides an example for an N-end rule-mediated anti-apoptotic mechanism for cancer cell survival via destroying an endogenous pro-apoptotic protein fragment generated (121). Lastly, I have determined that the pro-apoptotic cleaved fragment of PKC-theta is unstable in cells as its N-terminal lysine targets it for proteasomal degradation via the N-end rule pathway and this degradation is inhibited by mutating the destabilizing N-Termini, knockdown of the UBR1 and UBR2 E3 ligases. Tellingly, I demonstrated that the metabolic stabilization of the cleaved fragment of PKC-theta or inhibition of the N-end rule augments the apoptosis-inducing effect of staurosporine in Jurkat cells. Collectively, this work expands the functional scope of N-end rule pathway and support the notion that targeting N-end rule machinery may have therapeutic implications.

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Chapter 2: The anti-apoptotic form of tyrosine kinase Lyn that is generated by proteolysis is degraded by the N-end rule pathway.

2.1 INTRODUCTION

Lyn is a member of the Src family of tyrosine kinases (SFKs) that is a pivotal signalling intermediary for signal transduction pathways involved in a broad range of cellular functions such as proliferation(1, 2), differentiation (3) , cell migration (4), autophagy(5) and apoptosis(6). The structure of the Lyn protein includes: (i) a unique N-terminal domain (SH4) that contains a myristoylation site and a palmitoylation site (7) required for membrane attachment, followed by (ii) two protein-protein interaction domains initially characterized in SFKs (SRC homology 2 and 3 domains, SH2 and SH3 respectively), and (iii) a kinase domain containing its catalytic activity.

The expression of Lyn has also been linked to cancer progression and drug resistance. While the development of imatinib (Gleevac), a tyrosine kinase inhibitor targeting the Bcr-Abl fusion protein for the treatment of chronic myeloid leukemia (CML), has greatly improved the control of CML a significant number of patient eventually develop imatinib resistance (8). While the most common resistance mechanism is the sporadic mutations to Bcr-Abl other mechanisms occurring at significant frequency include the overexpression of Src family kinases like Lyn (9,10).

During apoptosis in B cells, T cells and the CML cell line K562, Lyn is cleaved in its N-terminal SH4 domain by caspase-3 after aspartate 18, exposing a leucine as the new N-termini (11-13). This cleavage shown schematically in **Figure 1**, was demonstrated to result in almost full-length Lyn bearing an N-terminal leucine, but lacking the short N-terminal region containing the acylation sites necessary to maintain the association of Lyn to the plasma membrane. As a result, Lyn is free to diffuse from the plasma membrane into the cytosol of the cells (13). The caspase-cleaved form of Lyn (LynΔN) was shown to exert an anti-apoptotic function in B-cells and K562 cells (11, 12). In the case of the K562 cells, it was also demonstrated that LynΔN provided a significantly increased resistance in comparison to the full length un-cleaved Lyn protein (11). Additional investigation in mouse models have revealed that the expression of LynΔN results in a psoriasis-like inflammatory syndrome and impair TFNR1 signalling (14, 15).

The N-end rule relates the *in vivo* half-life of a protein to the identity of its N-terminal amino acid residue (16, 17). The components of the N-end rule pathway recognize proteins with specific N-termini and target these proteins for ubiquitin dependent degradation by 26 S proteasome. Similar but distinct versions of the N-end rule are present in all organisms from mammals to bacteria. In

eukaryotes, N-end rule pathway mediated protein degradation has been implicated in diverse biological processes such as: G-protein signalling (18), DNA repair (19), cardiovascular development (20) and apoptosis (21).

The activity of the N-end rule pathway has been linked to the regulation of programmed cell death via the targeted degradation of proteolytic products that promote or carry out apoptosis (21-24). In *D. melanogaster* it was demonstrated that the *Drosophila* inhibitor of apoptosis protein (DIAP1), which binds and inhibits active caspases, is cleaved by an active caspase. Caspase cleavage exposes an N-terminal asparagine which is an N-end rule destabilizing residue (21). As a result, the N-end rule degradation machinery targets DIAP1, and potentially the active caspase, for degradation via the proteasome. More recent investigations with mammalian cells have shown that the N-end rule pathway targets a variety of pro-apoptotic protein fragments, generated as a result of proteolysis by active proteases during apoptosis, for degradation (23-25). Furthermore, it was demonstrated that the partial ablation of the N-end rule pathway sensitizes mouse embryonic fibroblasts to apoptosis-inducing agents. Together, these results suggest a significant role for the N-end rule pathway on the suppression of the apoptotic program.

Here we investigate the role of N-end rule-mediated degradation of LynΔN, as caspase generated protein that counters cell death (11, 12). We present the first study on the stability of LynΔN and demonstrate that the N-terminal leucine of LynΔN targets it for degradation via the N-End rule pathway. With the exception of pathogen induced cell death (26), this is the first example of the N-End rule functioning in a pro-apoptotic role by the targeted degradation of an anti-apoptotic protein.

2.2 MATERIALS & METHODS:

2.2.1 Generation of ubiquitin fusion cleaved Lyn expression vector. To express the cleaved Lyn fragment we cloned the cleaved Lyn (isoform 1) as a fusion between an N-terminal ubiquitin and C-terminal triple FLAG tag (3x FLAG) that had been previously cloned in a pcDNA 3.1 hygro plasmid[24]. A cDNA clone (clone ID: MHS6278-211689273, Open Biosystems) was used for cloning the Lyn sequence, and the sequence used corresponds to amino acids 19 to 512. This sequence of Lyn corresponds to the sequence from the known caspase cleavage site (13) to the C-terminal end of the protein.

2.2.2 Site directed mutagenesis. Mutagenesis of the codon for the leucine corresponding to the N-termini of the cleaved Lyn protein were performed by site directed mutagenesis to change the codon to arginine (CGG), valine (GUG) and methionine (AUG). Similarly, the kinase dead form of cleaved Lyn was obtained through mutating the lysine residue at position 275 in the putative ATP-binding site to arginine (CGG) by site directed mutagenesis.

2.2.3 Cell culture. HEK293T cells were obtained from the ATCC and K562 cells were obtained from Dr. Chris Bleakly (University of Alberta, Canada). The HEK293T cells were cultured in DMEM supplemented with 10 % fetal bovine serum (FBS). K562 cells were cultured in RPMI1640 with 10% FBS.

2.2.4 Cell transfection. HEK293T were transfected using the Calcium phosphate-based method as previously described[31]. K562 cells were transfected using electroporation (Neon transfection system) according to manufacturer's procedures (Pulse voltage 1450 v, Pulse width (ms) 10 and 3 pulses).

2.2.5 Reagents and antibodies. DAPI was purchased from Sigma (Saint Louis, MO, USA). PARP antibody (cat#: 9542) was purchased from cell signalling technology (Beverly, MA, USA). Mouse anti-FLAG® M2 antibody (cat#: F1804) was purchased from Sigma. Rabbit anti-β-actin (I-19, cat#: sc-1616-R), anti-UBR1 (cat#: sc-100626) and anti-UBR2 (cat#: sc-135594) were purchased from Santa Cruz Biotechnology. Secondary antibodies for Western blot analysis (goat anti-mouse and goat anti-rabbit) coupled to IRDyes® were purchased from LI-COR. The Alexa Fluor 488-labeled secondary goat anti-mouse antibody for immunostaining was purchased from

Invitrogen. Rabbit Anti-GFP antibody was a gift from Luc Berthiaume (Department of Cell Biology, University of Alberta).

2.2.6 Western blotting. After SDS-PAGE (on 5 or 10% gels), proteins were transferred onto nitrocellulose membranes (LI-COR Biosciences). The membranes were blocked with 2.5% fish skin gelatin blocking buffer (0.5% of Cold Water Fish Skin Gelatin (Sigma) in $1\times$ phosphate buffered saline - pH 7.4 with 0.1% Triton X-100) and probed with primary and secondary antibodies and imaged with an Odyssey Infrared Imaging System using the manufacturer's recommended procedures (LI-COR).

2.2.7 CHX-chase assays and Western blot analysis. 24 hrs after transfection, 5×10^5 cells were treated with 100µg/ml of cycloheximide for the indicated amounts of time. Cells were harvested and then lysed in 150 µl of lysis buffer (50 mm Tris, pH 6.8, 8% glycerol (v/v), 0.016% SDS (w/v), 0.125% β-mercaptoethanol (v/v), 0.125% bromphenol blue (w/v), 1 mm PMSF, and 1 µg/ml of leupeptin). The samples were sonicated for 10 seconds with an amplitude of 30% (1 watt), then resolved by SDS-PAGE on 10% gels along with Precision Plus All Blue protein pre-stained standards (Bio-Rad) as molecular weight markers. After SDS-PAGE, proteins were transferred onto nitrocellulose membranes (LI-COR Biosciences). The membranes were blocked with fish skin gelatin blocking buffer (2.5% of Cold Water Fish Skin Gelatin in PBS and 0.1% Triton X-100) and probed with primary and secondary antibodies and imaged with an Odyssey® Infrared Imaging System using the manufacturer's recommended procedures (LI-COR).

2.2.8 Fluorescence immunostaining and imaging. Transiently transfected HEK 293T were seeded on coverslips in 12-well plates (2.5×10^3 cells/well) and grown for 48 hrs. Cells were then washed with PBS+ (PBS containing 1 mM $MgCl_2$ and $CaCl_2$) and fixed in 4% paraformaldehyde for 15 min at 22 °C. The cells were then permeabilized with 0.1% Triton X-100 for 2 min at 22 °C and then washed three times with PBS+. The cells were then blocked with 4% normal donkey serum at 22 °C for 1 hr and washed again with PBS+ prior to incubation with the 1° Antibody (1 in 150 dilution in 4% normal donkey serum) for 1 h at 22 °C. After a washing step (three washes with PBS+) the cells were then incubated with the 2° Antibody (1:200 dilution in 4% normal donkey serum) for 60 min at 22 °C in the dark. After washing, the cells were stained with DAPI nuclear counterstain (1:1000 dilution). After a single wash step the coverslips were mounted on

slides using Dako Fluorescence mounting medium and allowed to set overnight prior to imaging. Fluorescence images were obtained with an Axiocam on an Axio Observer microscope (Carl Zeiss, Jena, Germany) using a ×100 Plan Aprochromat Lens.

2.2.9 UBR1 and UBR2 shRNAs. Four UBR1 unique 29mer shRNA constructs in GFP-V-RS vectors (cat#: TG300681) were purchased from Origene. Four unique UBR2 29mer shRNA constructs in RFP-C-RS vectors (cat#: TF300680) were also purchased from Origene.

2.2.10 Cell viability assay. Cell counting and the trypan blue exclusion test were performed with a TC20™ Automated Cell Counter (BioRad).

2.2.11 Flow cytometry. Annexin V-FITC Apoptosis Detection Kit (eBioscience) was used for apoptosis analysis by flow cytometry. Cells (5×10^5 cells) were incubated for 10 minutes in 500 µl 1× binding buffer, 5 µl of Annexin V-FITC and 10ul of PI (20 µg /ml) prior to flow cytometry analysis on a LSR-Fortessa Instrument. Ten thousand events are acquired for statistical analysis.

2.3 RESULTS AND DISCUSION

The proteolytic cleavage of Lyn by caspase-3 after aspartate 18 sheds the N-terminal segment of the protein that contains both the sites of N-myristoylation and S-palmitoylation (13). The proteolytic release of the Lyn fragment has also been demonstrated to counter the apoptotic program and significantly increases the resistance of Ramos cells to IgM stimulation (12) and K562 cells to imatinib (11). **Figure 1a** shows the Western blot analysis after expressing a Lyn-GFP construct in K562 cells, a CML derived cell line, treated with imatinib. Imatinib treatments results in the appearance of a faster migrating species that corresponds to the cleaved Lyn protein. Cleavage of Lyn is concomitant with PARP cleavage, which is commonly utilized to detect caspase activity (27). The increased apparent amount of LynΔN over that of the full-length Lyn observed in the blot is attributed to poorer extraction of the full-length myristoylated and palmitoylated Lyn protein from the membrane insoluble fraction of the lysis buffer. The loss of the N-terminal domain of LynΔN with the fatty acid modifications results in the diffusion of LynΔN throughout the cytoplasm (12). To date there has been no investigations into the stability of LynΔN.

We utilized an expression system to investigate the stability and cellular effects of LynΔN that is independent of caspase cleavage. This was to circumvent the challenges of having ongoing formation of the cleaved protein during investigations of protein stability, as the continued cleavage by caspase-3 would convolute the analysis. To mimic the caspase-cleaved form of the Lyn kinase, a plasmid construct was created to express a recombinant ubiquitin-LynΔN fusion protein with a C-terminal 3× FLAG tag as we have previously described (24). The ubiquitin-fusion technique enables the expression of a protein with any desired N-terminal amino acid as the N-terminal ubiquitin is proteolytically removed by endogenous ubiquitin hydrolases, which releases LynΔN as generated by caspase-3 cleavage of the full length Lyn (13) (**Figure 1b**). This approach enables the expression of LynΔN with leucine as the N-terminal amino acid.
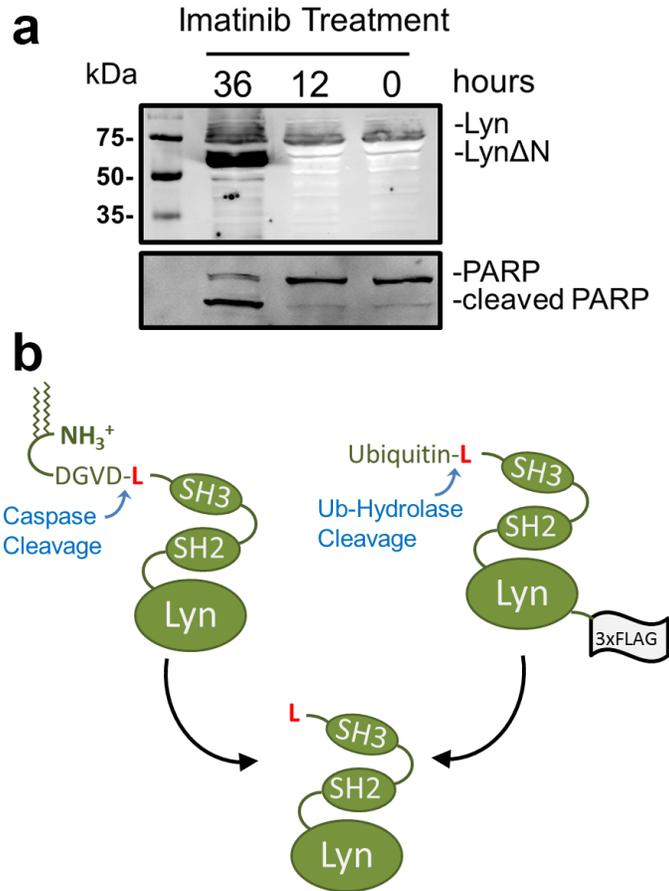
Figure 2.1 Protease dependent generation of LynΔN. (a) K562 cells were transfected with a Lyn-GFP expressing vector. 24 hours after transfection, the cells were treated with 1μM imatinib for the indicated times. The cells were then lysed and resolved by SDS-PAGE for Western blot (WB) analysis. Western blot analysis reveals a faster migrating species after 48 hours of imatinib treatment. For WB analysis the polyclonal rabbit primary antibody used was either anti-GFP or anti-PARP (b) Schematic depiction of the generation of LynΔN by either caspase cleavage after Asp18 or as a recombinant ubiquitin fusion. Both methods of generating LynΔN in cells results in an N-terminal leucine.

The N-terminal leucine of LynΔN renders it a potential N-End Rule substrate, as leucine is a type II primary destabilizing N-terminus (16). To investigate this possibility we evaluated whether LynΔN was degraded and whether degradation was dependent on the identity of the N-termini. A mutation to the ubiquitin-LynΔN fusion was made to change the N-terminal leucine of Lyn to valine, methionine and arginine, as valine and methionine are stabilizing N-termini and arginine is a type I destabilizing N-terminus (16). To investigate protein stability, the LynΔN constructs were transiently transfected into HEK293T. Twenty-four hours following transfection, cells were treated with 100 μg/ml of cycloheximide (CHX) to inhibit protein synthesis and the cells were then lysed at increasing time points after CHX addition. Lysates were then resolved by SDS-PAGE and the amount of LynΔN remaining was quantified by Western blot analysis with an anti-FLAG antibody using actin as a loading control. The data reveals that LynΔN with the wild type leucine N-termini is unstable (**Figure 2a**). In contrast, the N-terminal valine mutant (**Figure 2a**) and methionine mutant (**Figure 2b**) are stable. As predicted the mutant with the N-terminal arginine is rapidly degraded (**Figure 2b**), as protein is only observed at the initial time point. This N-terminal dependent degradation of Lyn suggests that LynΔN may be a *bona fide* N-end rule substrate.

As the N-end rule pathway is an upstream branch of the ubiquitin proteasome-system, we then investigated whether the degradation of LynΔN is proteasome-dependent. We studied the degradation of the wild type LynΔN using the CHX protocol described above using HEK293T cells in the presence or absence 10 μM of MG132 proteasome inhibitor. The data shown in **Figure 2c** reveals that the addition of MG132 results in stabilization of LynΔN, demonstrating a role for the proteasome for LynΔN degradation.

The targeted degradation of proteins via the N-end rule pathway includes the recognition of the destabilizing N-termini by specific E3 ubiquitin ligases. To identify the E3 ubiquitin ligases that mediate the degradation of LynΔN, we initially investigated the role of E3 ubiquitin ligases UBR1 and UBR2. UBR1 and UBR2 are redundant E3 ubiquitin ligase previously identified to be components of the N-end rule pathway which when deleted in combination result in embryonic lethality (28). To investigate whether UBR1 and UBR2 are required for degradation of LynΔN, the stability of LynΔN was investigated in co-transfection experiments with mixtures of plasmid vectors expressing shRNAs targeting UBR1 and UBR2. Both vector controls and plasmids

expressing scrambled shRNA sequences were used as controls. In all cases the total amount of plasmid DNA was used for each transfection was kept constant. The data in **Figure 2d** reveals that LynΔN is unstable in both control experiments (scrambled shRNAs and vector control). In contrast, when UBR1 and UBR2 targeting shRNAs are simultaneously expressed the degradation of LynΔN is nearly completely inhibited. The knock down of both UBR1 and UBR2 by shRNA expression were verified by Western blot analysis for endogenous UBR1 (**Figure 2e**) and UBR2 (**Figure 2f**). The dependence on UBR1 and UBR2 for LynΔN degradation is in agreement with the hypothesis that degradation is via the N-end rule pathway.

As Lyn is a kinase, we investigate whether its activity is required for degradation via the N-end rule. An inactivating mutant (K275R), as previously described (29), was introduced into the wild type ubiquitin-LynΔN fusion construct. The stability of the wild type and K275R mutant LynΔN were then investigated as described above. As seen in **Figure 2g**, no significant change to the stability of the protein was observed when the enzymatic activity of LynΔN blocked.
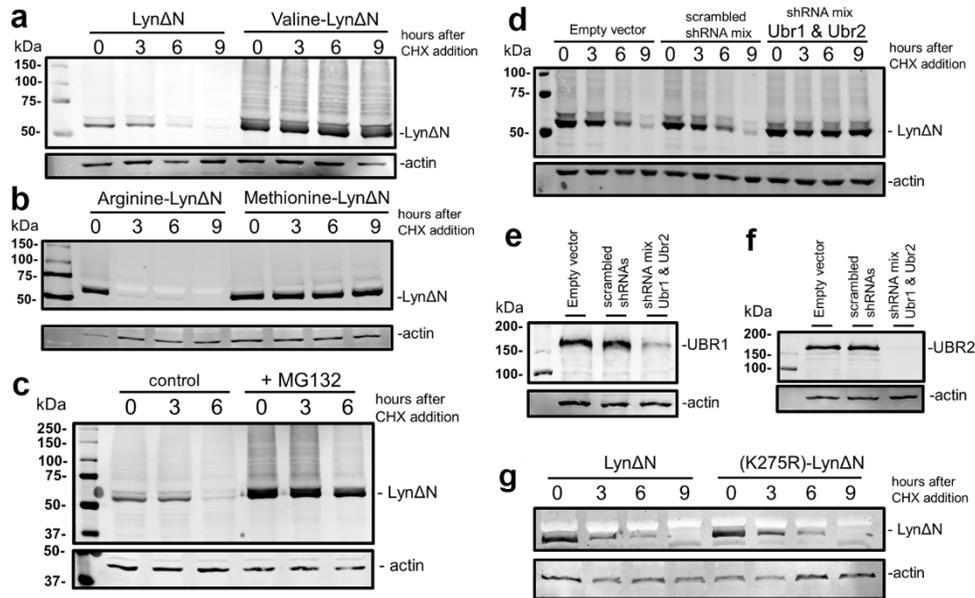
Figure 2.2 LynΔN is degraded by the N-end rule pathway. (a) Wild type LynΔN and valine-LynΔN were transfected into HEK293T. After transfection the cells were treated with 100 μg/ml cycloheximide (CHX) to block protein synthesis. At the indicated time points, cells were lysed and resolved by SDS-PAGE. The amount of LynΔN protein remaining was visualized by WB analysis with an anti-FLAG M2 antibody Loading control analysis was done by WB analysis using an anti-actin antibody. (b) LynΔN with N-terminal arginine (type I destabilizing N-termini) and methionine (stable N-termini) were transfected into HEK293T and analyzed as in (a). (c) Stability of LynΔN was visualized in HEK293T cells in the presence and absence of 10 μM MG132 and analyzed as in (a). (d) Stability of LynΔN was visualized in HEK293T cells that were also transfected with a plasmid mixture expressing shRNAs targeting UBR1 and UBR2 or controls. Both vector control and plasmids expressing scrambled shRNA sequences were used as controls. In all cases the total amount of plasmid DNA was used for each transfection was kept constant. (e) Knock down of UBR1 after shRNA treatment in (d) was verified by WB analysis for endogenous UBR1 after SDS-PAGE on a 5% gel. (f) Knock down of UBR2 after shRNA treatment in (d) was verified by WB analysis for endogenous UBR2 after SDS-PAGE on a 5% gel. (g) Stability of LynΔN was compared to the stability of the inactive K275R-LynΔN mutant.

Initial investigations on LynΔN reported that this fragment localizes to the cellular cytoplasm versus the plasma membrane for the intact Lyn kinase (13). We evaluated the localization of the recombinant LynΔN in HEK 293T cells to confirm its predicted cytoplasmic localization and that this localization is independent of the identity of the N-termini. Our results mimic the previously reported localization as the C-terminal fragment is localized to the cytoplasm (**Figure 3**). The cytoplasmic localization was independent of the identity of the N termini as identical results were obtained with fragments with N-terminal arginine or valine (**Figure 3**).

We then investigated how the N-end rule degradation of LynΔN influences the previously reported anti-apoptotic role of this protein fragment (11). Prior to investigating the cellular effects of LynΔN in K562 cells we first investigated that the LynΔN fragment was unstable in these cells as we observed for the HEK293T cells. The wild type LynΔN and the valine N-terminal mutant were both transfected into K562 cells and the stability of the proteins were investigated as described above with the utilization of cycloheximide. As observed in **Figure 4a**, the LynΔN with the wild type leucine N-termini was unstable while the valine N-terminal mutant was not degraded. We next investigated the sensitivity of K562 cells to imatinib that have been transfected with plasmids expressing either the wild type LynΔN, the stable valine N-terminal LynΔN mutant or a vector control. After transfection, 1 μM imatinib was added and the cells were evaluated by trypan blue exclusion staining to quantify cell viability after increasing times after imatinib addition. The data shown in **Figure 4b** reveal that the active degradation of LynΔN by the N-end rule significantly counters the imatinib resistance provided by LynΔN, this is demonstrated by the increased cell viability of K562 cells expressing the stable valine LynΔN mutant.

To verify that the increased viability during imatinib treatment as a result of LynΔN expression was a result of reduced apoptosis, we then proceed to analyze the cells for markers of apoptosis. The presence of phosphatidylserine on the outer leaflet of the plasma membrane[30] and caspase activity (27). The cells were stained with Annexin V and propidium iodide (PI) and then analyzed by FACS. Data from analyses are summarized in **Figure 4c**, where the percentage of cells that were Annexin V positive or both Annexin V and propidium iodide positive is plotted. In agreement with the trypan blue staining, the expression of the valine-LynΔN resulted in the fewest number of apoptotic cells as observed by Annexin V and PI staining upon treatment of the

cells with imatinib. FACS analysis was able to quantify a difference between the wild type LynΔN and the vector control, indicating that even with N-end rule degradation of LynΔN it can still function to counter the apoptotic stimuli to some extent. When the catalytically inactive form of LynΔN(K275R) is expressed, no significant change in the number of apoptotic cells was observed in comparison to the vector control. Western blot analysis to evaluate caspase activity by detecting caspase dependent PARP cleavage (27) is shown in **Figure 4d**. This data reveals that the expression of the stable valine-LynΔN protein results in a significant delay in the detection of the cleaved PARP fragment and thus caspase activation, where cleavage is only observed after 48 hours after the addition of imatinib. As a control for all the viability analysis we had also quantified the amount of expressed LynΔN in the cells (**Figure 4d**). As shown in the Western blot, all three forms were expressed but valine-LynΔN was detected in higher abundance. This increased amount is predicted as this is the stable form of LynΔN.

| | Anti-Flag | DAPI | Merge |

Leu-LynΔN

Val-LynΔN

Met-LynΔN

Arg-LynΔN
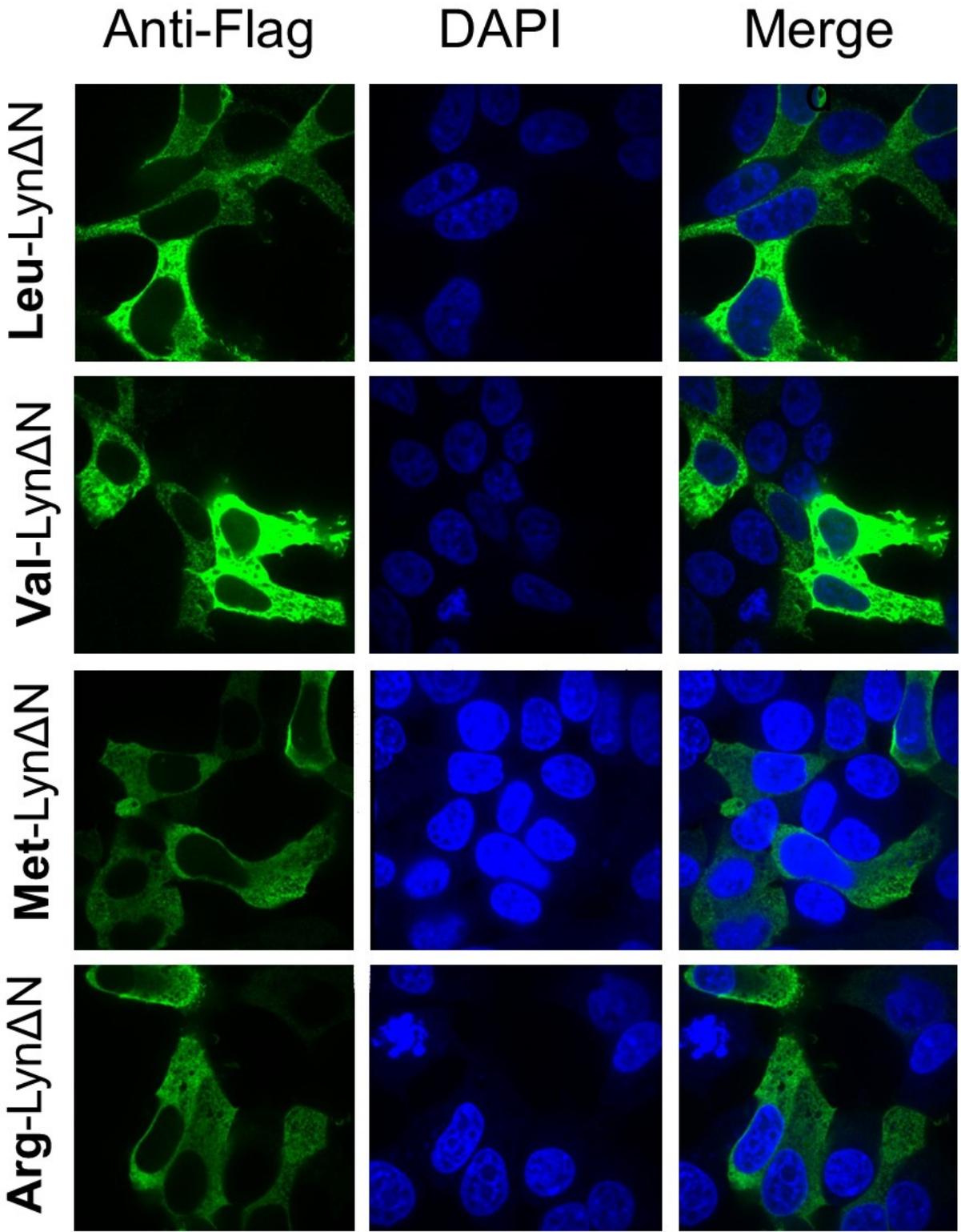
Figure 2.3 LynΔN is localized to the cytoplasm Immunofluorescent investigations of the cleaved fragment of Lyn (green) in transfected HEK293T cells in conjunction to nuclear staining with DAPI (blue). Localization of wild type LynΔN fragment N-terminal leucine (top) reveals a diffuse cytoplasmic localization. Localization of the mutant LynΔN fragments with the N-terminal valine, methionine and arginine all exhibit a similar cytoplasmic localization.

a

LynΔN | Valine-LynΔN | hours after CHX addition

kDa | 0 3 6 9 | 0 3 6 9

150-
100-
75-
50-

-LynΔN

-actin

b

p=0.011 p=0.021 p=0.053

Cell Viability (%)

No imatinib
Vector Control
LynΔN
Valine-LynΔN
(K275R)LynΔN

1 µM Imatinib

Time(hr): 0 24 48 72

c

Positive cells (%)

p=0.0006
p=0.0032

Annexin V
Annexin V & Propidium Iodide

Hours after Imatinib addition: 0 24 48 | 0 24 48 | 0 24 48 | 0 24 48 | 0 24 48

untreated | vector control | LynΔN | valine-LynΔN | (K275R)LynΔN

1 uM Imatinib

d

Hours after Imatinib Addition

0 | 24 | 48 | 72

No Imatinib
Vector
LynΔN
Val-LynΔN
(L275R)LynΔN

- LynΔN

-Cleaved PARP

57

Figure 2.4 LynΔN induced imatinib resistance. (a) Wild type LynΔN and valine-LynΔN were transfected into K562 cells, a CML derived cell line. After transfection the cells, CHX-chase experiment was done as previously described. The amount of LynΔN protein remaining was visualized by WB analysis with an anti-FLAG antibody and actin was analyzed as a loading control. (b) K562 cells were transfected to express the indicated proteins. Cells were then treated with imatinib for the indicated times then stained with trypan blue and analyzed on a TC20 automated cell counter (BioRad). The data represents the average and standard deviation from three independent experiments and p-values are derived from paired two tailed t-tests. (c) Quantified data from FACS analysis for K562 cells treated identically to those in (b). The percentage of cells that were stained with Annexin V (apoptotic cells) or both Annexin V and propidium iodide (dead cells) are shown. Cell staining was done with an Annexin V-FITC and propidium iodide. The data represents the average and standard deviation from three independent experiments. (d) Western Blot analysis of cell lysates from one set K562 cells used above. Blotting with an anti-FLAG antibody was used to detect LynΔN expression and an anti-PARP antibody was used to detect PARP cleavage.

Together our data demonstrate that LynΔN is degraded in cells and this degradation is a result of the proteolytically exposed N-terminal leucine being recognized by the N-end rule machinery. In agreement with our proposed model of N-end rule degradation of LynΔN (**Figure 5**), we have demonstrated that degradation can be prevented by changing the identity of the N-terminal amino acid to valine. It is somewhat remarkable that this relatively small change of leucine to valine make such a significant difference in protein stability. The importance of the role for this leucine may explain its complete conservation in vertebrates. Consistent with LynΔN being an N-End rule substrate is the proteasome, UBR1 and UBR2 dependent degradation.

The degradation of LynΔN by the N-end rule pathway is an additional example of how the N-End rule pathway can function in a pro-apoptotic function. This was demonstrated by how the valine-LynΔN stabilizing mutant provides significantly higher imatinib resistance to K562 cells. A previous example of N-End rule dependent cell death has been previously described with macrophages treated with anthrax lethal toxin (26). In conjunction with previous reports demonstrating anti-apoptotic functions of the N-End rule in other pathways (21, 23), it is becoming apparent that the role of the N-end Rule pathway cannot be specifically labeled as a pro- or anti-apoptotic pathway. The commonality of the reported examples of proteins being targeted for degradation is that the N-End rule counters the proteolytic activation of these proteins.
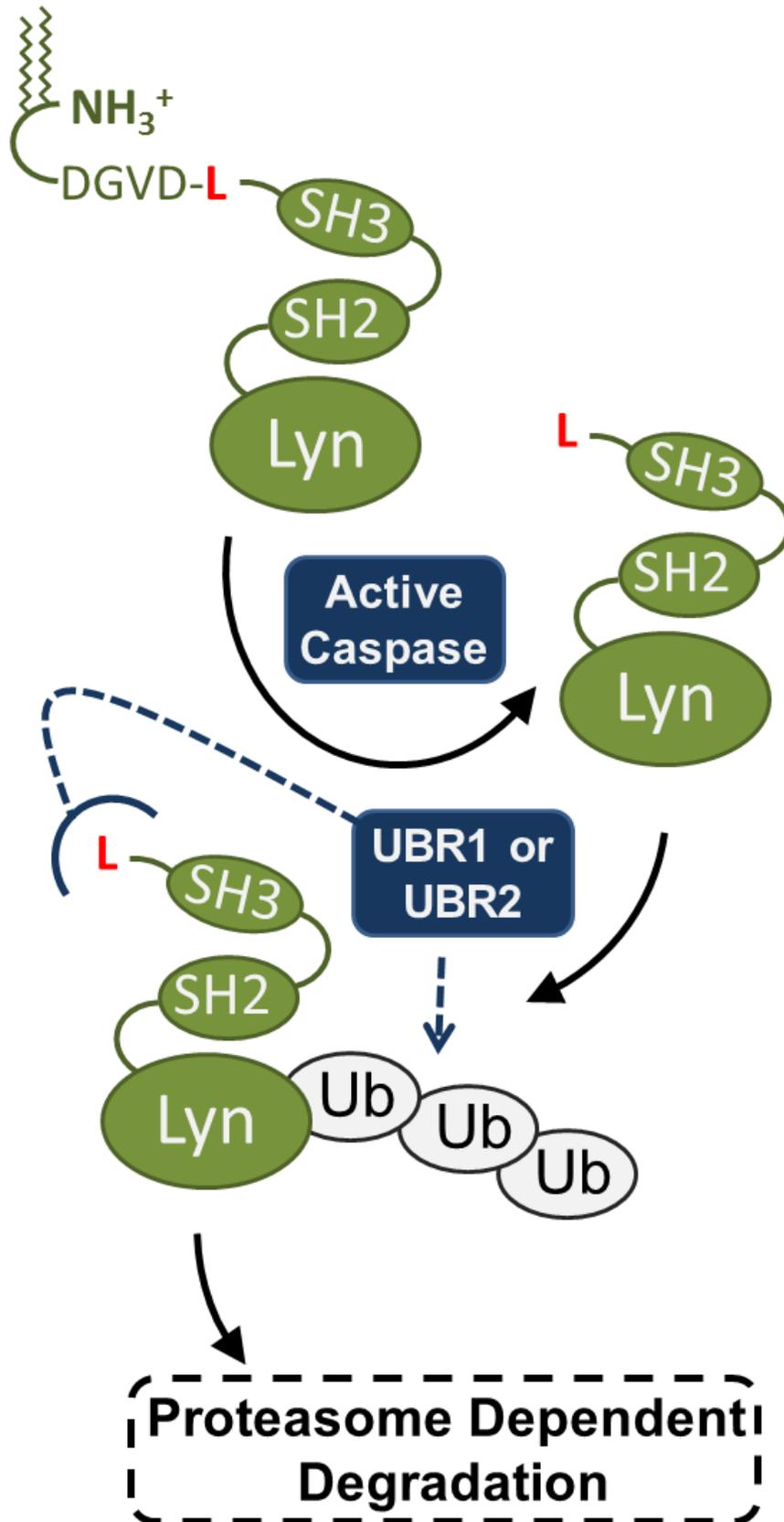
Figure 2.5 Model for LynΔN degradation. Our final model is that the caspase generated LynΔN is recognized by the degenerate UBR1 and UBR2 E3 ubiquitin ligases. UBR1/2 recognize the N-terminal leucine of LynΔN which then ubiquitinates the protein, targeting it to the proteasome for degradation.

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Chapter 3: Phosphorylation Impacts N-end Rule Degradation of the Proteolytically Activated Form of BMX Kinase.

3.1 INTRODUCTION

Bone marrow kinase on chromosome X (BMX) is a member of the Tec non-receptor tyrosine kinase family that also includes BTK, ITK, TEC and TXK(1-4). Members of this family share a modular structure, including an N-terminal pleckstrin homology (PH) domain, a Tec homology (TH) domain, a Src homology 2 and 3 (SH2 & SH3) domains and a C-terminal catalytic kinase domain(5). While there is tissue specific expression of several of the Tec family kinase, BMX is expressed in a variety of tissues and cell types, in contrast to what is implied by its name. BMX expression has been observed in epithelial cells, endothelial cells, cells of hematopoietic origins as well as various prostate cancer cell lines (5-7).

Investigations have revealed that BMX plays pivotal roles in signalling for diverse cellular processes such as cell differentiation, stem cell renewal, proliferation and transformation(1,5,8,9). BMX is involved in IL-6- induced neuroendocrine differentiation and neuropeptide-induced androgen-independent growth of prostate cancer cells (7). Additionally, BMX has been linked to Src-induced transformation of epithelial cells and fibroblasts via activation of STAT3 and to the transformation of human mammary epithelial cancer cells through activation of Pak1 (5,10).

BMX activity has been reported to have what appear to be contradictory roles in cell survival and apoptosis. The BMX kinase has been shown to play a protective role from radiation-induced apoptosis in prostate cancer cells, nasopharyngeal carcinoma and breast cancer cells (7,11-13). However, it has also been reported that the BMX kinase can mediate a pro-apoptotic function as well (14,15). How BMX kinase plays such a paradoxical role in cell survival has been attributed, at least in part, to the proteolytic activation of BMX kinase during apoptosis. For example, during apoptosis in PC3 prostate cancer cells, BMX kinase is cleaved by caspases after Asp 242, generating an active truncated BMXΔN kinase containing the complete SH2 and tyrosine kinase domains, but lacking the intact pleckstrin homology and SH3 domains(15). The truncated BMXΔN has been shown to exhibit increased kinase activity and sensitizes prostate cancer cells toward apoptosis in response to various apoptosis-inducing stimuli(15). While the function of the cleaved BMXΔN has been investigated, the stability of this protein fragment and the role of the neo-N-termini generated by proteolysis have never been investigated.

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Here we report the first study on the interplay and roles of N-End Rule-mediated degradation and phosphorylation on BMXΔN metabolic stability and drug-induced apoptosis in prostate cancer cells. The N-End rule pathway is one branch of N-termini dependent protein degradation (16-18). We have determined that the pro-apoptotic BMXΔN fragment is unstable in cells as its N-terminal tryptophan targets it for proteasomal degradation via the N-end rule pathway. Additionally, we have demonstrated that the metabolic stabilization of BMXΔN, either through the mutation of the destabilizing N-terminal amino acid to a stabilizing residue or via the inhibition of the N-end rule E3 ubiquitin ligases UBR1 and UBR2, augments the apoptosis-inducing effect of docetaxel in prostate cancer cells. Interestingly, we also determined that phosphorylation at tryrosine 566 of BMXΔN inhibits its degradation by the N-End rule pathway and this phosphorylation is also crucial for its pro-apoptotic function.

3.2 MATERIALS & METHODS

3.2.1 Generation of Ubiquitin fusion Cleaved Bmx Expression Vector: To express the cleaved BMX fragment we cloned the cleaved BMX as a fusion between an N-terminal ubiquitin and C-terminal triple FLAG tag (3× FLAG) to generate a Ub-BMX-FLAG pcDNA 3.1 vector as we have previously describe(45). A cDNA clone (cloneID: MHS1010-BC016652BE894841, Open Biosystems) was used to clone the BMX sequence corresponding to the caspase-3 cleavage site to the C-terminal end of the protein, amino acids 243-675. The final Ub-BMX-FLAG pcDNA 3.1 vector was verified by DNA sequencing. The full length BMX was similarly cloned with a C-terminal 3× FLAG in a pcDNA 3.1 plasmid.

3.2.2 Site directed Mutagenesis: Mutagenesis of the codon for the tryptophan corresponding to the N-termini of the cleaved BMX protein were performed by site directed mutagenesis to change the codon to Arg (CGG), Val (GTG), Tyr (TAC) or Lys (AAA). Similarly, the kinase dead form of cleaved BMX was obtained through mutating lysine 455 in the putative ATP-binding site to arginine (CGG) by site directed mutagenesis. To prevent phosphorylation at tyrosine 566 this residue was mutated to Phe (TTT). In Full length BMX-FLAG Tryptophan 243 was mutated to Valine (GTG) to change the neo-N-termini after caspase cleavage.

3.2.3 Cell culture: HEK293T, PC3 and LNCap cells were obtained from the ATCC. The HEK293T cells were cultured in DMEM supplemented with 10% FBS. PC3 cells were cultured in

DMEM/F12 with 10% FBS and LNCap cells were cultured in RPMI 1640 supplemented with 10 % FBS.

3.2.4 Cell transfection: HEK293T were transfected using the Calcium phosphate-based method. PC3 and LNCaP cells were transfected using electroporation (Neon transfection system) according to manufacturer's procedures. The transfection efficiencies for the PC3 cells ranged from 55-75% between experiments as determined by transfecting with a GFP expressing vector and FACS analysis. To ensure identical transfection of samples in each well of a culture plate for protein stability measurements and/or cell death measurements, a single batch of cells are transfected with a single Neon tip and then seeded into individual wells (equal number of cells per each well) in a 12 or 6 well plate.

3.2.5 Antibodies & Inhibitors: PARP antibody (9542) was purchased from Cell Signalling Technologies. Mouse anti-FLAG® M2 antibody (F1804) was purchased from Sigma. Rabbit anti-β-actin (I-19, sc-1616-R) anti-UBR1 (sc-100626), anti-UBR2 (sc-135594) and anti-BMX (sc-8874) were purchased from Santa Cruz Biotechnology. Secondary antibodies for Western blot analysis (goat anti-mouse and goat anti-rabbit) coupled to IRDyes® were purchased from LI-COR.

3.2.6 Protein Stability Assays and Western Blot Analysis: Twenty-four hours after transfection, 5×10^5 cells were treated with 100μg/ml of CHX for the indicated amounts of time. Cells were harvested and then lysed in 150 μl of lysis buffer (50 mm Tris, pH 6.8, 8% glycerol (v/v), 0.016% SDS (w/v), 0.125% β-mercaptoethanol (v/v), 0.125% bromphenol blue (w/v), 1 mm PMSF, and 1 μg/ml of leupeptin). The samples were sonicated and then resolved by SDS-PAGE on 10% gels along with Precision Plus All Blue protein pre-stained standards (Bio-Rad).

After SDS-PAGE, proteins were transferred onto nitrocellulose membranes (LI-COR Biosciences). The membranes were blocked with 2.5% fish skin gelatin (Truin Science) in 1×PBS with 0.1% Triton X-100) and probed with primary and secondary antibodies and imaged with an Odyssey® Infrared Imaging System using the manufacturer's recommended procedures (LI-COR).

3.2.7 UBR1 and UBR2 shRNAs*:* Four UBR1 unique 29mer shRNA constructs in GFP-V-RS vectors (cat#: TG300681) were purchased from Origene. Four unique UBR2 29mer shRNA constructs in RFP-C-RS vectors (cat#: TF300680) were also purchased from Origene. As individual shRNA resulted in only moderate knockdown of either UBR1 or UBR2, mixtures of the four shRNAs for each target were empirically screened to obtain the maximal knockdown of each protein. Specifically, the combinations of A, B & C (GFP-V-RS) and A, B, & D (RFP-C-RS)

gave the most effective knockdowns. These six shRNA expressing vectors were then transfected into PC3 cells and cultured in 1 μg/mL puromycin for four weeks to select for cells stably incorporating these plasmid vectors. FACS was then used to isolate the cells expressing both GFP and RFP (the reporters for each shRNA vector respectively). The PC3 cells expressing the shRNAs for UBR1 and UBR2 exhibited reduced proliferation rates when compared to either the wild type PC3 cells or PC3 cells similarly selected for using vector control plasmids. The doubling time for wild type PC cells was determined to be 27±3 hours and the vector control cells were determined to be 29±2 hours. In contrast, the UBR1 & UBR2 shRNA expressing cells was determined to be 55±9 hours. This impaired proliferation is not entirely surprising considering the previous well-documented role of the N-End rule in cell division and chromosome stability (46,47).

Flow cytometry: Annexin V-FITC Apoptosis detection Kit (eBioscience) was used for apoptosis analysis by flow cytometry using the manufacturers recommended procedures and analyzed on a LSR-Fortessa Instrument. Ten thousands events are acquired for statistical analysis.

3.2.8 Cell viability assay*:* Cell counting and the trypan blue exclusion test were performed with a TC20™ Automated Cell Counter (BioRad).

3.2.9 Protein dephosphorylation: 24 hrs after transfection, 5×10^5 cells, cells were harvested and then lysed in 150 μl of lysis buffer as described previously ((Wu *et al.,* 2001) and then incubation at 4 °C for 30 min. The sample was then incubated with 400 units of λ phosphatase (New England BioLabs) in λ phosphatase reaction buffer at 30 °C for 60 min.

3.2.10 Chemicals*:* The L-phenylalaninamide inhibitor, Cycloheximide, Staurosporine and sodium orthovanadate were purchased from Sigma.

3.2.11 Fluorescence immunostaining and imaging. Transiently transfected PC3 cells were seeded on coverslips in 12-well plates (2.5×10^3 cells/well) and grown for 48 hrs. Cells were then washed with PBS+ (PBS containing 1 mM MgCl$_2$ and CaCl$_2$) and fixed in 4% paraformaldehyde for 15 min at 22 °C. The cells were then permeabilized with 0.1% Triton X-100 for 2 min at 22 °C and then washed three times with PBS+. The cells were then blocked with 4% normal donkey serum at 22 °C for 1 hr and washed again with PBS+ prior to incubation with the 1° Antibody (1 in 150 dilution in 4% normal donkey serum) for 1 h at 22 °C. After a washing step (three washes with PBS+) the cells were then incubated with the 2° Antibody (1:200 dilution in 4% normal donkey serum) for 60 min at 22 °C in the dark. After washing, the cells were stained with DAPI nuclear counterstain (1:1000 dilution). After a single wash step the coverslips were mounted on

slides using Dako Fluorescence mounting medium and allowed to set overnight prior to imaging. Fluorescence images were obtained with an Axiocam on an Axio Observer microscope (Carl Zeiss, Jena, Germany) using a ×100 Plan Aprochromat Lens.

3.3 RESULTS & DISCUSSION

3.3.1 BMX is proteolytically cleaved in docetaxel treated PC3 cells to generate the pro-apoptotic BMXΔN fragment. Proteolytic generation of BMXΔN by caspase cleavage was reported over a decade ago(15) where cleavage releases the C-terminal BMXΔN fragment (amino acids 243-675) containing part of the SH3 domain, the SH2 domain and the kinase domain of the protein. To investigate this BMXΔN fragment we constructed plasmids to express either the full-length BMX kinase with a C-terminal 3×Flag tag or an ubiquitin (Ub)-BMXΔN 3×Flag tag fusion protein as schematically shown in **Figure 1A**. The Ub-BMXΔN fusion mirrors the caspase-cleaved form of the BMX kinase as endogenous ubiquitin hydrolases proteolytically remove the N-terminal ubiquitin to expose the N-terminal tryptophan. Both constructs were expressed in the androgen independent PC3 prostate cancer cell line via electroporation. The cells expressing the full length BMX were also treated with 10 nM docetaxel (or DMSO control), a chemotherapeutic that induces cell death in PC3 cancer cells by inducing mitotic catastrophe and caspase dependent apoptosis (19-21). As predicted, expression of the Ub-BMXΔN fusion construct results in a protein fragment with an electrophoretic mobility, of an approximate molecular weight of 50 kDa, that matches that of the docetaxel induced caspase cleaved form of the full-length BMX kinase (**Figure 1B**).

3.3.2 Increased apoptosis sensitivity in BMXΔN expressing cells. Previous investigations reported that the proteolytic removal of the N-terminal domain of the BMX kinase produce a catalytically active protein fragment, BMXΔN, which sensitizes PC3 cells toward Fas ligand induced apoptosis(15). To verify whether this enhanced sensitivity also applies to docetaxel treatment we investigated the effects of expressing full length BMX and BMXΔN on docetaxel induced cell death in PC3 cells. PC3 cells transfected to express either full length BMX, BMXΔN or a vector control were treated with 0 to 20 nM docetaxel for 48 hours, upon which the cells were evaluated for viability by a trypan blue exclusion staining assay and counted on an automated counter. As shown in **Figure 1B**, there is no difference in cell viability when expressing either BMX constructs when no docetaxel was present. In agreement with the previous investigation, reduced cell viability is observed in the BMXΔN expressing cells in the presence of 10 or 20 nM docetaxel (**Figure 1C**). An analogous experiment was also performed in the presence or absence of 10 nM docetaxel and was analyzed by FACS after Annexin V and propidium iodide (PI) staining. The data in Figure1D concurs with the data from trypan blue staining where the cells expressing BMXΔN exhibit increased Annexin V positive cells (dark grey) and double positive cells stained for both PI and

Annexin V (light grey) when treated with docetaxel. Again, the data reveals no observable cell death, versus vector control, when BMXΔN is expressed in the absence of docetaxel. Together our data indicates that the expression of BMXΔN enhances drug induced apoptosis in PC3 cells but is not toxic in untreated or unstressed cells as was previously observed for cells treated with Fas ligand(15). With the difference observed in sensitizing PC3 cells to apoptosis by full length BMX and BMXΔN, the cellular localization of the two different proteins were investigated. The loss of the PH domain and much of the SH3 domain may result in severe changes to cellular localization. Fluorescent imaging of both BMX and BMXΔN in **Figure 1E** reveal similar localization in foci located throughout the cytoplasm. Analysis of the images does not reveal evident differences in the cellular localization of the two proteins.

3.3.3 BMXΔN is unstable and is degraded by the proteasome. As the proteolytic generation of BMXΔN generates an N-terminal tryptophan. The reported specificity of the N-End Rule pathway(22) predicts that this protein fragment will be unstable in cells, but the stability of BMXΔN has never previously been investigated.

The stability of both full-length BMX and BMXΔN was investigated by expressing the proteins in PC3 cells and then adding cycloheximide (CHX) to the cells to block protein synthesis. Cells were then lysed at 0, 1 and 4 hours after CHX addition and the amount of protein present were relatively quantified by Western Blot analysis after resolving the samples by SDS-PAGE. The data in Figure 2A demonstrates that the full-length BMX protein is stable while BMXΔN was degraded over the four hour time course. Western Blot analysis for actin was performed to verify equal protein loading on the gel. This is the first demonstration that BMXΔN is unstable in cells.

To verify that BMXΔN degradation is via the proteasome, we investigated whether addition of a proteasome inhibitor, MG132, inhibits degradation. The stability of BMXΔN in PC3 cells was investigated in the presence or absence 10 μM MG132. The data in Figure 2B reveals that the addition of MG132 to the cells stabilized BMXΔN in contrast to the DMSO control. Together the data demonstrates that the BMXΔN protein fragment is unstable and is degraded by the proteasome.

A

Caspase Cleavage

Ub-Hydrolase Cleavage

Ubiquitin-**W**

B

	BMX	BMX	BMXΔN	
kDa	-	+	-	Docetaxel
100-				
75-				-BMX
50-				-BMXΔN
35-				

C

Viable Cells (%)

p=0.089
p=0.047

Docetaxel (nM)

D

Cell Labelling (%)

p = 0.0046
p = 0.016

Vector Control   BMX   BMXΔN   Docetaxel

E

Anti-FLAG   DAPI   merged

BMX

BMXΔN
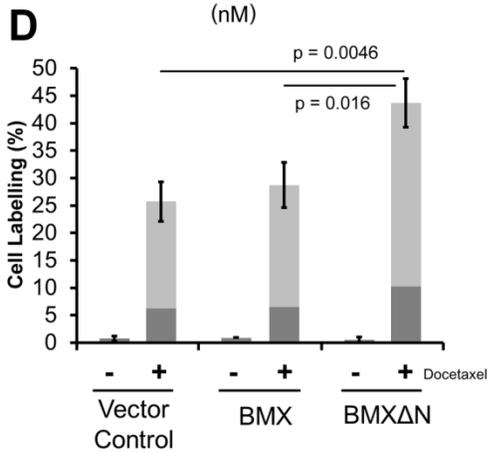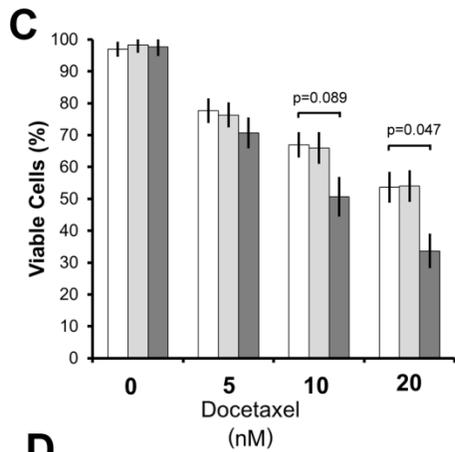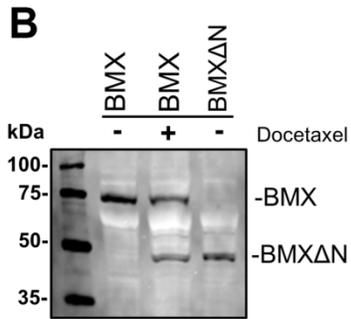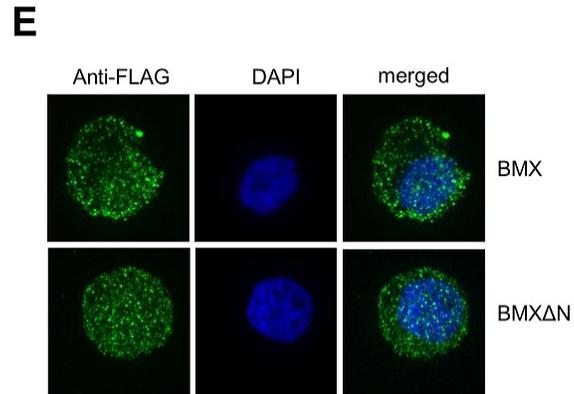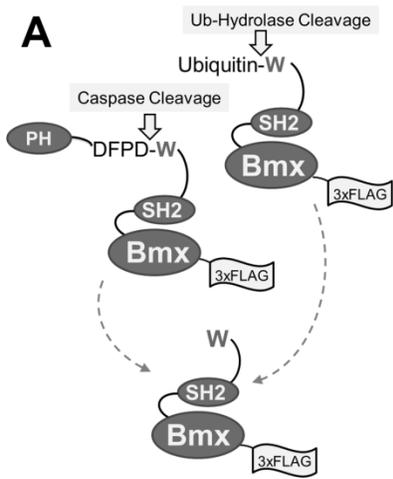
FIGURE 3.1. Protease dependent generation of BMXΔN. (A) Schematic depiction of the generation of BMXΔN by either caspase- cleavage after Asp 242 or as an ubiquitin fusion to generate BMXΔN with an N-terminal Tryptophan. (B) Transient transfection with BMX-Flag or Ub-BMXΔN-Flag in PC3 cells. After transfection the cells were treated with 10 nM docetaxel or left untreated prior to lysis and analysis by SDS-PAGE and Western Blot (WB) analysis with an anti-Flag antibody. WB analysis reveals that docetaxel treatment result in cleavage of full length BMX that results in a cleaved product with identical electrophoretic mobility as BMXΔN. (C) PC3 cells were transfected with plasmids to express full length BMX (light grey bars), BMXΔN (dark grey bars)or a vector control (white bars) . Cells were then treated with the indicated concentrations of docetaxel for 48 hours, then assayed for viability using trypan blue staining. The data represent the average and standard deviation from three independent experiments and p-values are derived from paired two-tailed t-tests. (D) FACS analysis of docetaxel treated PC3 cells. PC3 cells were transfected with indicated expression plasmids and then left untreated or were treated with 10 nM docetaxel for 48 hours. The percentage of cells that were only stained Annexin V (dark grey) or both Annexin V and propidium iodide (light grey) are shown. The data represent the average and standard deviation from three independent experiments and p-values are derived from paired two-tailed t-tests. (E) Immunofluroscent investigations of the full length BMX kinase and the cleaved fragment BMXΔN in transfected PC3 cells in conjunction with nuclear DAPI staining reveal similar cytoplasmic staining

3.3.4 The N-End Rule targets BMXΔN to the proteasome for degradation. As the proteolytic generation of BMXΔN exposes an N-terminal tryptophan residue, we hypothesized it is targeted to the proteasome for degradation by the components of the N-End rule pathway(16). The N-end rule pathway is a protein degradation pathway that recognizes proteins with a specific destabilizing N-terminal residues and then ubiquitinates these proteins for proteasome dependent degradation(16). In eukaryotes the primary N-terminal destabilizing residues are classified into positively charged amino acids (Type I), such as Arg and Lys, or bulky hydrophobic residues (Type II), such as Trp and Tyr. As BMXΔN has an N-terminal Tryptophan (a type II destabilizing residue), we investigated whether the N-end rule pathway targets BMXΔN for degradation.

To investigate the potential for N-End Rule degradation, we created a couple of BMXΔN N-terminal mutants where the Trp was mutated to either Val or Arg. If BMXΔN was degraded via the N-End Rule pathway, an N-terminal Val is predicted to stabilize BMXΔN as this is a stabilizing N-terminus(16). Mutating Trp to Arg, a type I destabilizing residue, is predicted to continue to render BMXΔN unstable(16). The wild type BMXΔN and the two N-terminal mutants were expressed in PC3 cells and the stability of the proteins was investigated after the addition of CHX to the cells. As predicted the N-terminal Val mutant was stabilized (**Figure 2C**) and the N-terminal Arg mutant remained unstable. This data is in agreement with the hypothesis that BMXΔN is degraded by the N-End Rule pathway.

To rule out the possibility that the BMXΔN degradation is specific to the androgen-independent prostate cancer cell line PC3, we also investigated the degradation of wild type BMXΔN, Val-BMXΔN and Arg-BMXΔN in the androgen-dependent prostate cancer cell line, LNCaP, and the unrelated HEK293T cell line. Despite the presence of some cell specific differences in the actual rates of degradation, the overall trends of N-termini dependent degradation were identical (**Figure 2C**).

Selective degradation via the N-End Rule pathway involves the recognition of the N-terminal destabilizing residue on the substrate protein by the UBR containing E3 ubiquitin ligases. The mammalian UBR E3 ubiquitin ligases have two key recognition domains, the UBR box domain and the N-domain, which function independently as recognition domains for either type I and type II N-terminal destabilizing residues respectively(22). To verify that BMXΔN is a *bona fide* N-End rule substrate we verified that its degradation was dependent on these E3 ubiquitin ligases. We

investigated the two functionally redundant E3 ubiquitin ligases UBR1 and UBR2, as they play an important role in recognition of type II N-terminal destabilizing residues in mammals(22). BMXΔN was expressed in PC3 cells stably expressing control shRNAs, a mixture of shRNAs to target both UBR1 and UBR2 or vector control cells. The simultaneous knock down of both UBR1 and UBR2 by shRNA expression was verified by Western Blot analysis for endogenous UBR1 (**Figure 2D**) and UBR2 (**Figure 2E**). The stability of BMXΔN was then investigated in these cells after treatment with CHX. **Figure 2F** reveals that the simultaneous knockdown of UBR1 and UBR2 inhibits the degradation of BMXΔN while the protein is still rapidly degraded in either the shRNA control expressing cells or the vector control cells. This result parallels what we have observed for the caspase cleaved form of the Lyn kinase which is targeted for degradation by the N-End Rule pathway(23).

This data along with the stability of the N-terminal mutants above is strong evidence that BMXΔN is an N-End rule substrate.

3.3.5 Inhibition of BMXΔN by the UBR Inhibitor. The N-domains of UBR1 and UBR2, which are structurally and functionally homologous to the bacterial ClpS protein(24), recognize N-termini with hydrophobic destabilizing residues such as tryptophan. A recent investigation has reported that some phenylalanine derivatives can inhibit the recognition and degradation of an N-End Rule model substrate, nsP4, with an N-terminal Tyr in rabbit reticulocyte lysates(25). We investigated whether one of the compounds identified in this study, L-phenylalaninamide (Phe-NH_3), can inhibit the degradation of BMXΔN in living cells. The stability of BMXΔN and four different N-terminal mutants, which included Tyr (type I), Lys (type II), Arg (type II) and Val (stabilizing) were investigated in PC3 cells in the presence and absence of Phe-NH_3. The data in **Figure 2G** reveals that when cells are pre-treated with Phe-NH_3 only the degradation of BMXΔN with type II destabilizing N-termini (Try and Tyr) are inhibited. Degradation of BMXΔN mutants with type I N-termini (Arg and Lys) are unaffected by Phe-NH_3 and the N-terminal Val mutant remains stable. While not a potent inhibitor this is the first data that verifies the use of Phe-NH_3 to selectively inhibit the degradation of proteins with type II N-termini in cultured cells.
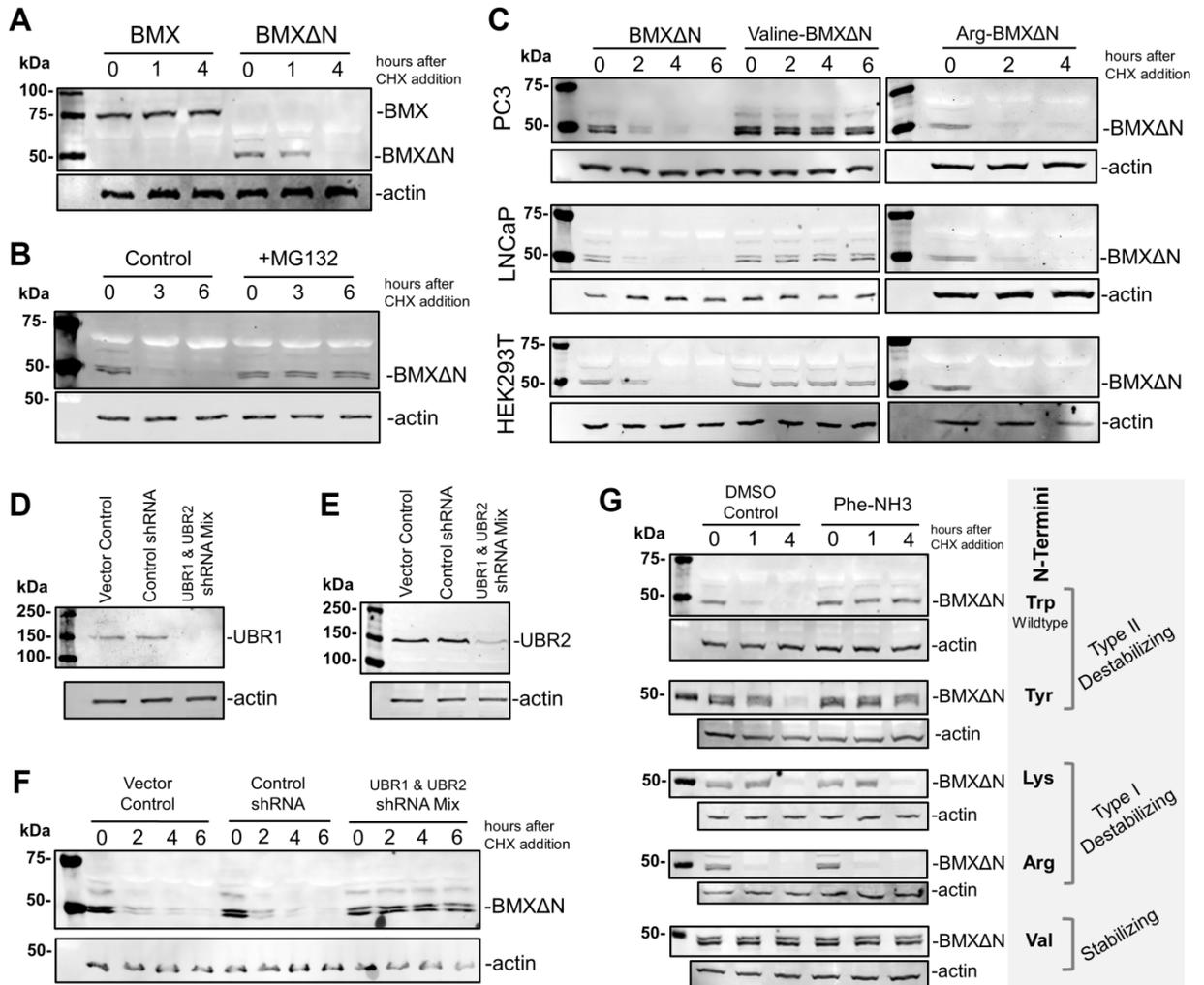
FIGURE 3.2. BMXΔN is degraded by the N-end rule pathway. (A) The stability of full-length BMX and BMXΔN were determined in transfected PC3 cells by treating the cells with 100 ug/mL cycloheximide (CHX), to block protein synthesis, and then the cell cell lysates were analyzed by WB analysis at the indicated times. An anti-Flag antibody was used to detect BMX and an anti-actin antibody was used as a loading control. (B) Stability of BMXΔN was investigated in PC3 cells in the presence and absence of MG132 (10 μM) and analyzed as in (A). (C) Wild type-BMXΔN with N-terminal tryptophan (type II destabilizing N-termini) and N-terminal mutants of arginine (type I destabilizing N-termini) and valine (stablizing N-termini) were transfected into the cell lines indicated and BMXΔN stability was determined as described in (A). (D) Verification of shRNA knock down of UBR1 in (F) by WB analysis with an anti-UBR1 antibody. (E) Verification of shRNA knock down of UBR2 in (F) by WB analysis with an anti-UBR2 antibody. (F) Stability of BMXΔN was visualized in PC3 cells that also expressed shRNAs targeting UBR1 and UBR2, control shRNAs or a pcDNA3.1 vector control.(G) Stability of wild type BMXΔN and the listed N-terminal mutants in the presence and absence of 200 μM Phe-NH3 (pre-incubated for 4 hours prior to the addition of CHX), an inhibitor reported to block the degradation of type II N-termini while not affecting the degradation of proteins with type I N-termini.

The results for the different methods to inhibit the N-End Rule: N-termini mutations, shRNA knockdown of UBR1 and UBR2 or pharmacological inhibition with Phe-NH₃, are all in agreement with a model where BMXΔN is targeted for N-Rule degradation by the UBR1 and UBR2 E3 ligases.

3.3.6 The Role of the N-End Rule on BMXΔN induced sensitivity to apoptosis. Given the degradation of BMXΔN by the N-End Rule we investigated the impact of degradation on its pro-apoptotic function. PC3 cells stably expressing shRNAs targeting UBR1 and UBR2 or shRNA controls were transfected with either a vector control or plasmids to express either wild-type BMXΔN or the stable N-terminal Val-BMXΔN mutant. After the transfection, the cells were treated with 10 nM docetaxel for 48 hours and then investigated by trypan blue staining to quantify cell viability. The data in **Figure 3A** reveals that stabilizing BMXΔN by the N-terminal valine mutation increases the cells sensitivity to docetaxel. When BMXΔN and Val-BMXΔN are expressed in UBR1/2 knock down cells, the sensitivity to docetaxel are essentially the same. Together the data suggests that BMXΔN results in lower docetaxel sensitivity than the Val-BMXΔN mutant as a result of its degradation.

A more detailed investigation of the effect of stabilizing BMXΔN on apoptosis was done by cell staining and FACS analysis. PC3 cells were transfected with wild-type BMXΔN, the stable N-terminal Val-BMXΔN mutant or a vector control. The cells were then treated with 5 or 10 nM docetaxel for 48 hours and then stained with PI and Annexin V prior to FACS analysis. The data in **Figure 3B** reveals that stabilizing BMXΔN with the valine N-terminal mutation results in increased numbers of Annexin V (dark grey) and PI – Annexin V doubly labeled (light grey) cells at both docetaxel concentrations when compared to wild type BMXΔN. The presence of the increased amounts of both early and late apoptotic cells upon docetaxel treatment when BMXΔN is stabilized indicates that the N-End rule is attenuating the pro-apoptotic activity of BMXΔN. As already mentioned above, despite its instability, wild type BMXΔN expression also leads to increased docetaxel sensitivity in **Figure 3B** when compared to the vector control. The difference between the wild-type BMXΔN and vector control indicates that even in presence of active N-End rule degradation of BMXΔN, it can still function as a pro-apoptotic molecule.

3.3.7 The N-End Rule Impacts Apoptosis. Our data on cell viability also reveals that UBR1 and UBR2 exhibit a BMXΔN independent influence on apoptosis. The data in **Figure 3A** reveals a

difference in docetaxel sensitivity between the UBR1/2 knock down cells versus the shRNA control cells, even when BMXΔN is not expressed. While we have not further investigated this observation, this finding is in agreement with previous reports. Specifically, UBR1/UBR2 knock out mouse embryonic fibroblasts were shown to be hypersensitive to apoptosis-inducing agents such as UV or staurosporine, a kinase inhibitor known to induce apoptotic cell death (26). Second, mice spermatocytes that lack UBR2 ligase undergo apoptosis during meiosis which eventually leads to male infertility (27).
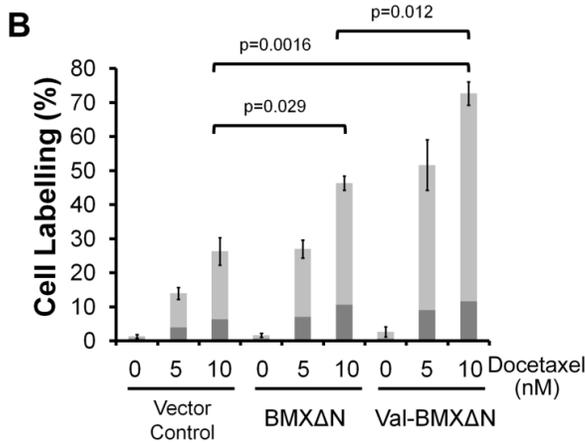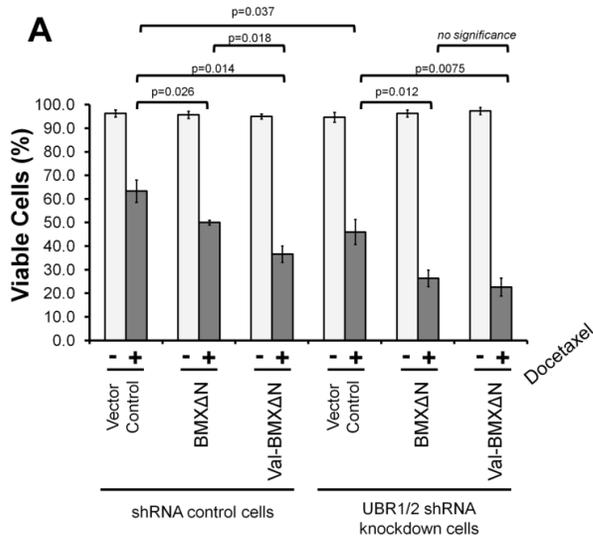
FIGURE 3.3. BMXΔN stability and the N-End Rule influencing apoptosis. (A) PC3 cells expressing control or UBR1/2 targetting shRNAs were transfected to express either wild type BMXΔN, the valine N-terminal mutant or a vector control. 24 hours after transfection cells were treated with 10 nM docetaxel for 48 hours. The cells were then analyzed by trypan blue staining. The data represent the average and standard deviation from three independent experiments and p-values are determined from paired two-tailed t-tests. (B) FACS analysis of docetaxel treated PC3 cells. PC3 cells were transfected to express either wild type BMXΔN, the valine N-terminal mutant or a vector control. Cells were then treated with the indicated concentrations of docetaxel for 48 hours. Cells were then analyzed by FACS for Anexin V and propidium iodine staining. The percentage of cells that were stained with either Annexin V (dark grey) or doubly labelled with both Annexin V and propidium iodide (grey) are indicated. The data represents the average and standard deviation from three independent experiments and p-values are derived from paired two –tailed t-tests.

3.3.8 N-End Rule Degradation of Caspase generated BMXΔN. In addition to studying the degradation of BMXΔN using the recombinant ubiquitin fusion constructs, we investigated whether caspase-mediated generation of BMXΔN from full-length BMX kinase in PC3 cells is accompanied by degradation by the N-End Rule pathway. PC3 cells were transfected to express full-length BMX or a W243V mutant. Caspase cleavage of the W243V mutant will generate the stable Val-BMXΔN variant. After 24 hours the cells were treated with 10 nM doctaxel for 48 hours, upon which cleavage of BMX is observed (**Figure 4A**). When Z-VAD-FMK, a pan caspase inhibitor(28), was added for two hours to halt the ongoing production of BMXΔN by caspase cleavage of full length BMX, a decrease in BMXΔN is observed (**Figure 4A**). This Z-VAD-FMK dependant reduction in BMXΔN is hypothesized to be a result of N-End Rule degradation and as predicted, is inhibited by the addition of MG132 or UBR1/2 shRNA knock down. The Z-VAD-FMK dependent drop in BMXΔN is also not observed with the W243V mutant, which is in agreement with N-End Rule degradation as the resulting BMXΔN with a valine N-terminal residue is predicted to be stable.

To investigate whether N-End rule degradation of caspase generated BMXΔN impacts cell viability, PC3 cells expressing either full length wild type or W243V BMX were treated with docetaxel for 24 hours and assayed for cell viability by trypan blue staining (**Figure 4B**). As shown, the expression of full length wild type BMX had no measurable impact on cell viability, similar to what was also demonstrated above by FACS analysis (**Figure 1D**). In contrast, when the W243V BMX mutant was expressed, a statistically significant decrease in cell viability was observed upon docetaxel treatment (**Figure 4B**). A parsimonious interpretation for this data is that while BMXΔN generated by caspase cleavage has the potential to be pro-apoptotic, the active degradation of this fragment by the N-End Rule-pathway effectively counters this function.

3.3.9 N-End Rule Degradation of endogenous BMXΔN Generated by Caspase cleavage. To investigate whether BMXΔN generated from caspase cleavage of endogenous BMX was similarly targeted for degradation by the N-End Rule, the following experiment was performed. PC3 cells expressing the UBR1/2 shRNAs (or control shRNAs) were treated with 10 nM docetaxel for 48 hours to induce apoptosis and BMX cleavage (**Figure 4C**). As described above, Z-VAD-FMK was utilized to prevent the ongoing formation of BMXΔN after docetaxel treatment. Three hours after the addition of Z-VAD-FMK the cells were then also

harvested for Western Blot analysis (**Figure 4C**). The data in **Figure 4C** reveals that treatment of the cells with docetaxel results in the formation of BMXΔN from endogenous BMX in both the control and UBR1/2 knock down cells. When the cells were then treated with Z-VAD-FMK, the BMXΔN fragment disappears in the control shRNA expressing cells, as was observed for recombinant full-length BMX (**Figure 4A**). In contrast, when the UBR1/2 knock down cells are similarly treated, BMXΔN remains present after the addition of Z-VAD-FMK. This is in agreement with our model that BMXΔN is stabilized in the absence of UBR1 and UBR2. In sum, the data supports a model were the caspase-mediated cleavage of endogenous BMX kinase generates a cleaved fragment that is a *bona fide* N-end rule substrate in cells.
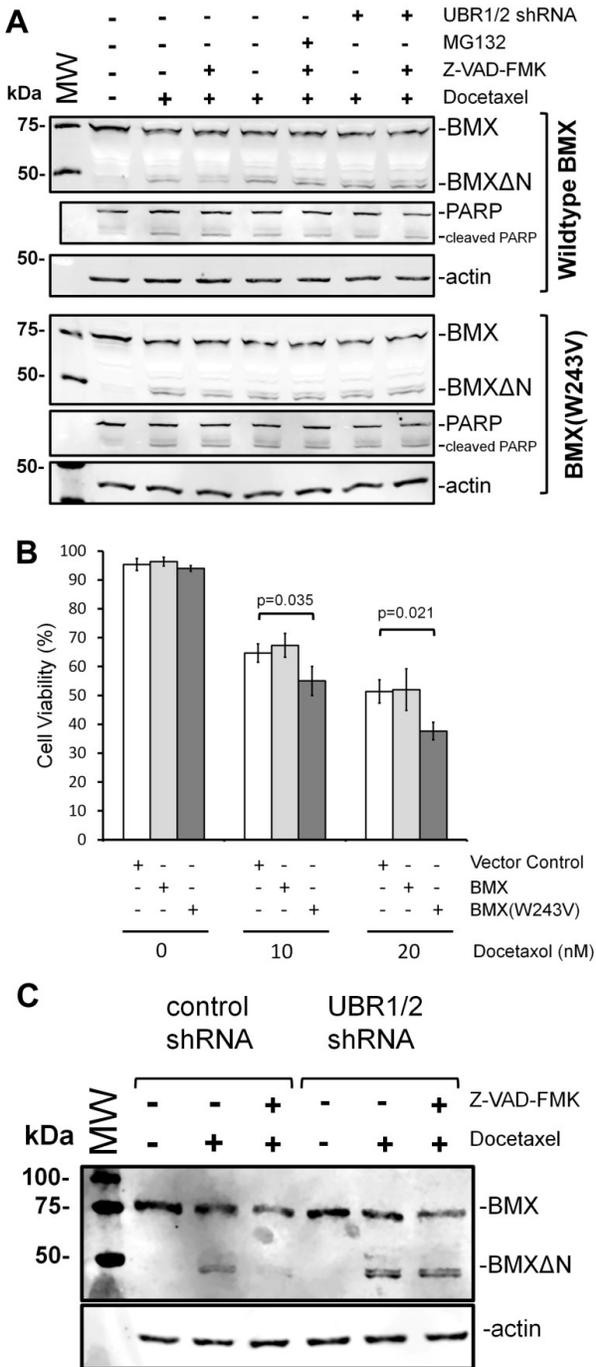
FIGURE 3.4 Cleavage of full length recombinant or endogenous BMX and C-terminal fragment degradation. (A) PC3 cells that either stably express shRNAs targeting UBR1 and UBR2 or shRNA controls were transfected to express full-length BMX (upper panel) or the W243V mutant BMX. 24 hours after transfection the cells were either untreated or treated with 10 nM docetaxel for 48 hours. The indicated samples were also treated with MG132 or Z-VAD-fmk. The pan-caspase inhibitor, Z-VAD-fmk was added, for two hours, to the indicated samples to prevent ongoing formation of BMXΔN by continued casapse activity. Western Blot analysis of cell lysates from the resulting experiments was performed to detect BMX and BMXΔN amounts, PARP or an actin loading control. When Z-VAD-fmk was added to prevent the ongoing formation of BMXΔN, the disappearance of the C-terminal is inhibited by knockdown of UBR1/2 with shRNAs or the W243V mutation which results in a BMXΔN fragment with a stabilizing valine N-termini. (B) PC3 cells expressing either recombinant full-length BMX or the W243V mutant were treated with the indicated amounts of docetaxel for 48 hours and then analyzed by trypan blue staining. The data represent the average and standard deviation from three independent experiments and p-values are determined from paired two-tailed t-tests. (C) PC3 cells that either stably express shRNAs targeting UBR1 and UBR2 or shRNA controls were either untreated or treated with 10 nM docetaxel for 48 hours to induce apoptosis. The pan-caspase inhibitor, Z-VAD-fmk was added, for three hours, to the indicated samples after docetaxel to prevent ongoing formation of BMXΔN by continued casapse activity. Western Blot analysis of cell lysates from the resulting experiments was performed to detect endogenous BMX and BMXΔN resulting from caspase cleavage

3.3.10 Catalytic Activity of BMXΔN is Dispensable for Degradation. With the pro- apoptotic role for BMXΔN, we next investigated roles for its catalytic activity on both protein degradation and docetaxel sensitivity. An inactivating mutation (K455R), as previously described(15), was introduced into the wild type ubiquitin-BMXΔN construct. The stability of the wild type and K455R mutant BMXΔN were then investigated. The data in **Figure 5A and 6E** does not reveal an observable difference in the stabilities of the proteins, leading to the conclusion that BMXΔN activity was not required for its degradation. Concomitantly, further analysis reveals that the abrogation of the catalytic activity of BMXΔN doesn't impact the stability of long-lived mutant Val-BMXΔN (**Figure 5A**).

In contrast to protein degradation, the catalytic activity of BMXΔN is essential for its pro-apoptotic function. This is demonstrated in **Figure 5B** where expression of the kinase dead BMXΔN (K455R) mutant in PC3 cells results in the same cell viability after docetaxel treatment as the vector control. In contrast to this is the decreased viability upon docetaxel treatment when cell are expressing either the wild type or the stable Val-BMXΔN mutant. Noteworthy, the expression of double mutant Val-BMXΔN (K455R) in PC3 cells also doesn't result in significant difference in cell death after docetaxel treatment with respect to cells expressing vector control (**Figure 5B**). This finding has two-fold consequences, first it precludes the possibility that the lack of pro-apoptotic activity by the (K455R) mutant being a simple result of its instability, as the (K455R) mutant does not exhibit pro-apoptotic activity when it is stabilized with an N-terminal valine. Second it further confirms the pivotal role of catalytic activity of BMXΔN in mediating its pro-apoptotic function in PC3 cells upon docetaxel treatment.

After the induction of apoptotic-cell death in mammalian cells, proteases, such as caspases and calpains, cleave a large number of proteins(29). A number of cleaved fragments have pro-apoptotic activity; that is they can amplify apoptosis signalling, presumably via participating in positive feedback loops. For instance, cleaved RIPK1 enhances caspases activation (30,31), and cleaved BAX augments the mitochondrial outer membrane permeability (32). Our findings with BMXΔN reveal that while the catalytic activity of the kinase is required for its function in a positive feedback loop for apoptotic signalling, this activity is being attenuated by the N-End rule as has been observed for proteolytic products of RIPK1 and LIMK1(26).
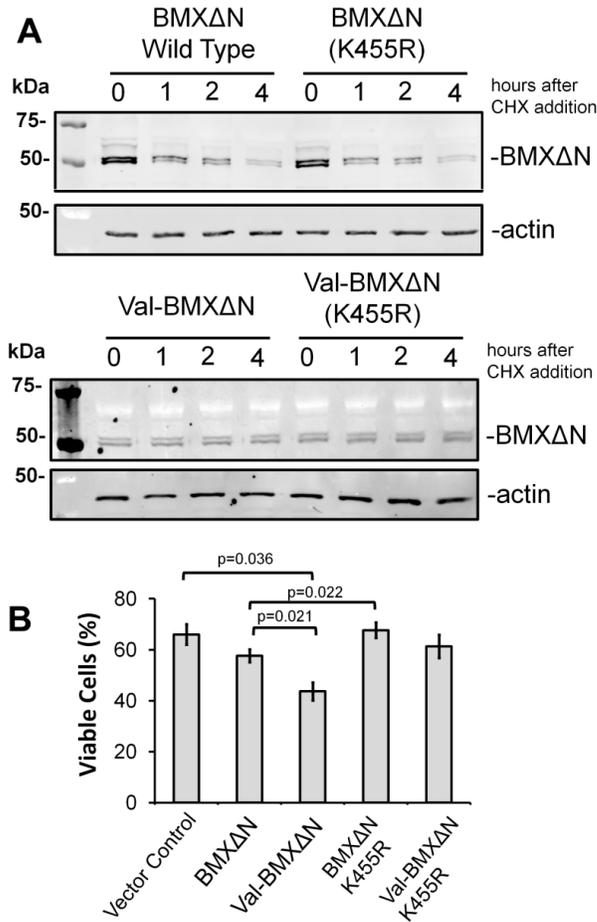
FIGURE 3.5 BMXΔN kinase activity is required for its pro-apoptotic activity. (A) Wild type (*upper panel*) and the N-terminal valine mutant (*lower panel*) BMXΔN along with their corresponding kinase inactive mutants (K455R) were expressed in PC3 cells. The stability of the proteins was investigated by treating the cells with 100 ug/mL CHX and collecting lysates at the indicated times. The lysates were resolved by SDS-PAGE and the amount of protein remaining was determined by WB analysis with either an anti-Flag or anti-actin antibodies. (B) PC3 cells were transfected to express the indicated BMX variants. Cells were then treated with 10 nM of docetaxel for 48 hours and were evaluated for viability by staining with trypan blue. The data represent the average and standard deviation from three independent experiments and p-values are derived from paired two-tailed t-tests

3.3.11 Phosphorylation of BMXΔN. Given the observation that BMXΔN fragment migrates as a 50-kDa doublet of bands in our Western Blot analysis of both recombinant and endogenous BMXΔN (**Figure 4A** and **4C**), we hypothesized that this doublet is a result of a posttranslational modification such as phosphorylation. The observation of a doublet also suggests that the modification is incomplete and that BMXΔN exists in both modified and unmodified populations.

To address this possibility of phosphorylation, λ-phosphatase treatment was performed with cell lysate from PC3 cells where the wild type BMXΔN was expressed. As opposed to the untreated control, the data in **Figure 6A** reveals a single band upon phosphatase treatment. To further investigate BMXΔN phosphorylation, wild type BMXΔN was expressed in PC3 cells treated with 1μM staurosporine, a non-specific kinase inhibitor (33), for 30 minutes. As seen in **Figure 6B**, staurosporine treatment culminated in a relative accumulation of the lower band, presumably the non-phosphorylated band, of the doublet as well as a reduction in the net level of BMXΔN which may be presumably attributed to its phosphorylation inhibitory effect and the consequences on BMXΔN metabolic stability. Crucially, to further demonstrate the role of phosphorylation in the regulation of BMXΔN doublet band turnover, BMXΔN was again expressed in PC3 cells which were either untreated or treated with 1 μM sodium orthovanadate, a tyrosine phosphatase inhibitor (34), for 1 hour. After the sodium orthovanadate pre-incubation, CHX was added to the cells in order to monitor BMXΔN stability. **Figure 6C** reveals that pre-treatment with the phosphatase inhibitor, when compared to the untreated control, leads to the accumulation of the upper band of the doublet, presumably the phosphorylated form of BMXΔN. Additionally, the data in **Figure 6C** reveals that sodium orthovanadate treatment stabilizes BMXΔN where the upper, presumably phosphorylated, band persists even at the 3-hour time point after CHX treatment. From this data it appears that the phosphorylated form of BMXΔN is degraded more slowly but at this point cannot rule out secondary indirect cellular effects of the phosphatase inhibitor. Nonetheless the data with λ-phosphatase, staurosporine and sodium orthovandate strongly suggest that the BMXΔN doublet observed by Western Blotting is a result of phosphorylation.

The presence of the doublet with the kinase dead mutant (**Figure 5A**) indicates that this phosphorylation is not a result of auto-phosphorylation by the proteolytically activated BMXΔN kinase.

3.3.12 BMXΔN Phosphorylation at tyrosine 566. Previous reports identified that BMX phosphorylation at Y566, within the activation loop, is essential for full length BMX kinase activity (1,5,35,36). This highly conserved tyrosine residue has been shown to be activated by Src family kinases and is required for subsequent full length BMX auto-phosphorylation (5,37). To investigate whether potential Y566 phosphorylation plays a role in BMXΔN degradation and docetaxel sensitization, a non-phosphorylatable mutation (Y566F) was introduced into the ubiquitin-BMXΔN fusion constructs. The wild type and Y566F BMXΔN constructs were transfected into PC3 cells investigated for protein stability. The data in **Figure 5D** reveals two essential points, the first is that the Y566F mutant is no longer observed as a complete doublet band and second, the Y566F mutant is more rapidly degraded than wild type BMXΔN. The quantified data from at least three replicate experiments is shown in **Figure 6E**. Our interpretation of this data is that phosphorylation at Y566 is crucial for the formation of a complete doublet band observed by Western Blot analysis. There may be additional phosphorylations that occur after Y566 phosphorylation but our current data cannot address this possibility. Autophosphorylation events are known to occur with the Tec family of kinases after src kinase phosphorylation of Y566(37), but in BMX both of these reported sites, Y216 and Y224, are located in the N-terminal fragment that is removed by proteolysis at D242 so the presence of additional phosphorylation sites in BMXΔN remains undetermined.

Taken together, our data demonstrating increased degradation of Y566F BMXΔN (**Figure 6D & 6E**) along with the reduced degradation of wild type BMXΔN in the presence of sodium orthovanadate (**Figure 6C**) are consistent with a model that phosphorylation of Y566 inhibits BMXΔN degradation.

The stability of a valine N-terminal mutant of the Y566F construct was also investigated to ensure that the degradation of the Y566F mutant was still a result of N-End rule degradation and not by an alternative mechanism (**Figure 6D & 6E**).

To additionally verify that the inhibition of protein degradation by phosphorylation is up- or downstream on N-End rule recognition we evaluated the stability of wild type and the Y566F BMXΔN mutant in the presence of MG132 or upon UBR1/2 shRNA knockdown. **Figure 6F** reveals that both MG132 and shRNA knockdown of UBR1/2 inhibit degradation of both wild type and the Y566F mutant BMXΔN. Inhibition of degradation upon UBR1/2 knockdown suggests that

Y566F phosphorylation prevents degradation via the N-End Rule. Inhibition may be at the point of substrate recognition or ubiquitination by UBR1 and UBR2. This is the first demonstrated example of phosphorylation regulating the recognition of a caspase proteolytic product by the N-End Rule pathway.

Given the data for BMXΔN generated by caspase cleavage of endogenous BMX also reveals the presence of the doublet band (**Figure 4C**), we believe this phosphorylation is not an experimental artifact as a result of expressing BMXΔN as an ubiquitin fusion. What we cannot determine from our data is whether the phosphorylation was already present in the full-length protein or whether it occurs subsequent to cleavage.

With the Y566F being more rapidly degraded than wild type, with half-lives of 0.55 and 1.5 hours respectively (**Figure 6E**), we then investigated its expression on docetaxel sensitivity. Wild type, the Y566F mutant BMXΔN and a vector control were transfected into PC3 cells which were then evaluated for viability by trypan blue staining after a 48 hour treatment with 10 nM docetaxel. **Figure 6G** reveals that the expression of the Y566F mutant does not alter docetaxel sensitivity, in comparison of a vector control. Wild type BMXΔN expression again results in increased docetaxel sensitivity. Moreover, the data in **Figure 6G** rules out the possibility that the lack of pro-apoptotic activity by the Y566F mutant being a simple result of its instability, as the Y566F mutant does not exhibit pro-apoptotic activity when it is stabilized with an N-terminal valine (**Figure 6G**). So like full length BMX, it appears BMXΔN requires Y566 phosphorylation on the activation loop for activity. The specific mechanism on how phosphorylation of Y566 inhibits N-End Rule degradation remains to be determined. In addition, the more rapid turn-over of the Y566F mutant and the apparent similar turnover of the BMXΔN band doublets in all our experiments suggests that the cellular equilibrium of BMXΔN phosphorylation and dephosphorylation is likely rapid in comparison to the timescale of BMXΔN degradation.
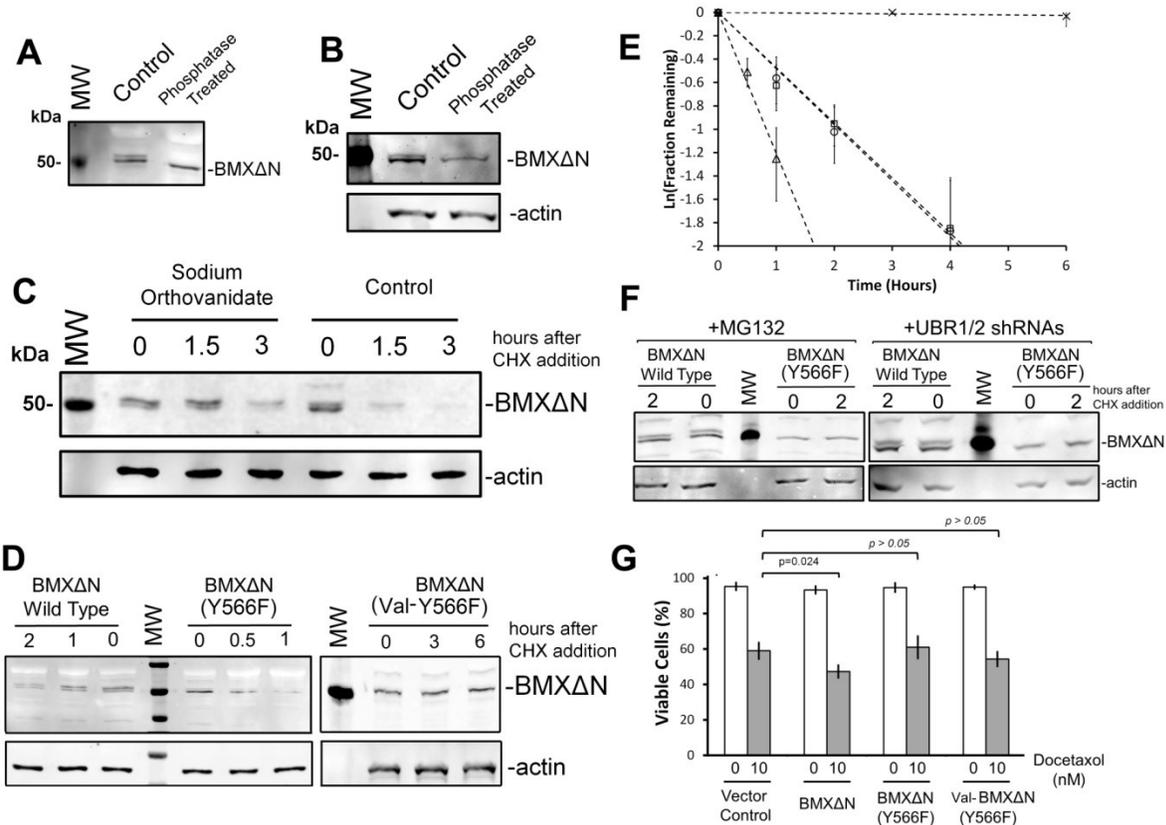
FIGURE 3.6 BMXΔN phosphorylation regulates its degradation and pro-apoptotic function. (A) Cell lysates from wild type BMXΔN expressing cells were treated (or untreated control) with λ phosphatase prior to SDS-PAGE and WB analysis with an anti-Flag antibody to detect BMXΔN. Phosphatase treatment resulted in the loss of the band doublet. (B) PC3 cells expressing BMXΔN cells were treated with the kinase inhibitor staurosporin (1μM) for 30 minutes prior to lysis and WB analysis with an anti-Flag antibody. (C) The stability of wildtype BMXΔN was investigated in PC3 cells after a 60 minutes pre-treatment with the phosphatase inhibitor sodium orthovanadate (or untreated control). The stability of the proteins was investigated by treating the cells with 100 ug/mL CHX and collecting lysates at the indicated times. The lysates were resolved by SDS-PAGE and the amount of protein remaining was determined by WB analysis with either an anti-Flag or anti-actin antibodies. (D) Wild type BMXΔN,Y566F and the valine N-terminal Y566F double mutant BMXΔN were expressed in PC3 cells. The stability of the proteins was determined as described for (C). (E) Quanification of the degradation of wild type, K455R, Y566F and valine N-terminal Y566F double mutant BMXΔN from a minimum of three individual experiments. The data was plotted to fit an apparent first order reaction to determine apparent rates and half-life. The amounts remaining of each of the different mutants (wild type, K455R, Y566F and valine N-terminal Y566F double mutant BMXΔN) are quantified relative to an actin loading control where the rate of disappearance are depicted as follows: the Y566F BMXΔN mutant (Δ), the valine N-terminal Y566F double mutant BMXΔN (×), the wild-type BMXΔN (○) mutant and the K455R mutant (□). (F)Stability of wild type BMXΔN and the Y566F mutant were determined in the presence and absence of 10 μM MG132 (left panel) or in UBR1 and UBR2 shRNA expressing cells (right panel). In all cases for the Y566F mutant the doublet band is no longer observed. (G) PC3 cells were transfected to express either BMXΔN, the Y566F mutant, the valine N-terminal Y566F double mutant or a vector control. 24 hours after transfection, the cells were treated with docetaxel for 48 hours. The cells were then stained with trypan blue to quantify cell viability. The data represent the average and standard deviation from three independent experiments and p-values are derived from paired two-tailed t-tests.

3.3.13 Conclusions. Cross-talk between phosphorylation and ubiquitin-dependent protein degradation plays a prominent role in cellular signalling and may take many forms(38). Notably, phosphorylation can regulate protein ubiquitination and degradation machinery via regulating the activity of E3-ubiquitin ligases as previously reported (39). Alternatively, the coordinated targeting of a protein substrate by phosphorylation and ubiquitin-dependent degradation provides another avenue of cross-regulation (40). While there are a number of examples of how phosphorylation impacts, either positively or negatively, target protein substrate degradation by ubiquitin-proteasome system (40,41), Herein we describe, to the best of our knowledge, the first clear example of phosphorylation regulating recognition of a caspase proteolytic product by the N-End Rule degradation pathway which was discovered about three decades ago(42). While the impact on degradation is clear, the mechanism of how phosphorylation at this internal residue inhibits recognition and proteasome targeting by UBR1 and UBR2 has yet to be determined. It remains unclear whether degradation of the phosphorylated form of BMXΔN is slower or whether it must first be dephosphorylated prior to degradation (**Figure 7**). Tellingly, it remains also to be determined whether there are other phosphorylation sites that can play role in the regulation of stability of BMXΔN. Future investigations may identify potential mechanisms, such as the inhibition of recognition of an essential lysine by the N-domain preventing ubiquitination.

Overall our findings have revealed an unforeseen interplay between phosphorylation and N-End rule degradation of a caspase product. Our observations are suggestive of increased complexity of signalling networks. While proteomics techniques have identified examples of how phosphorylation can regulate caspase cleavage(43) our data now demonstrates that phosphorylation can also regulate the outcome of some caspase products.

The degradation of the caspase generated BMXΔN is also a novel example of a protease product generated upon the activation of apoptotic program, which is actively degraded by the N-End Rule pathway. This emerging role for the N-End Rule pathway in attenuating the apoptotic program by degrading proteolytically activated proteins(26,44,45) is revealing this pathway to be a potential target to sensitize cells to cell-death promoting therapies.
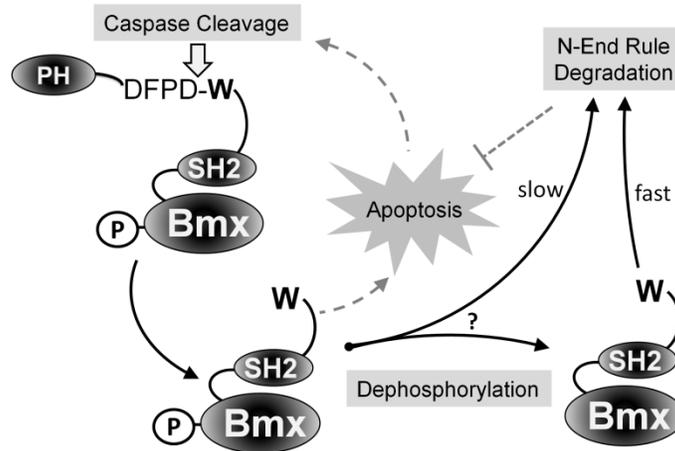
FIGURE 3.7 Model for BMXΔN degradation. Our proposed model is that the caspase generated BMXΔN is recognized by the degenerate UBR1 and UBR2 E3 ubiquitin ligases. UBR1/2 recognize the N-terminal tryptophan of BMXΔN which then ubiquitinate the protein, targeting it to the proteasome for degradation and thus attentuate its pro-apoptotic function. Phosphorylation of BMXΔN at tryrosine 566 is required its pro-apoptotic function and realtively inhibits its degradation by the N-End rule pathway.

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Chapter 4: N-end-rule-mediated Degradation of the Proteolytically Activated Form of PKC-theta Attenuates its Pro-Apoptotic Function.

A version of this chapter has been prepared for submission as Eldeeb MA & Fahlman RP (2017). N-end-rule-mediated Degradation of the Proteolytically Activated Form of PKC-theta Kinase attenuates its Pro-Apoptotic Function.

4.1 INTRODUCTION

PKC-θ is a member of the ubiquitously expressed PKC serine/threonine kinase family that encompasses several known isoforms that are classified into 3 subfamilies, classical (cPKC; α, β, γ), novel ((nPKC; δ, ϵ , η , θ , μ) and atypical (aPKC; ζ , λ) (1-3). Although these groups vary in their activation requirements based on their second messengers need. All members of PKC family share a modular structure, including C-terminal catalytic domain containing an ATP-binding site (C3), and a substrate-binding domain (C4) yet differ in the regulatory domains C1 and C2 (2-3). Members of the cPKC subfamily require calcium and DAG/phorbol esters for activation. Members of the nPKC subfamily lack the typical C2 homology domain and do not require calcium for activation, whereas members of the aPKC subfamily lack the calcium-binding C2 domain and part of the DAG/phorbol ester-binding C1 homologous domains and consequently, are insensitive to DAG/phorbol esters and calcium (2-3). Tellingly, PKC-θ has a relatively restricted expression pattern in mammalian tissues; this is exemplified by the relatively augmented expression of PKC-θ in skeletal muscle and lymphoid tissues including lymph nodes and thymus (1-3). In addition, PKC-θ exhibits some distinct features, which differentiate it from other T cell-expressed PKCs, noticeable among which, is its distinct intracellular localization in relatively well-defined locations of the T cell. Notably, PKC-θ is distinct in its translocation to the plasma membrane of the T-cell at the site of attachment between antigen (Ag)-specific T cells and Ag-presenting cells (APCs) (4), the so-called immunological synapse (IS) (5). The IS, which includes membrane receptors, signalling proteins, cytoskeletal elements and lipids microdomains (lipid rafts), acts as an activation platform for transducing the activation signals needed for sustained T cell activation.

Previous Investigations have established PKC-theta as a crucial player in T-cell signalling for regulating diverse cellular processes such as T-cell activation, proliferation and apoptosis (6-9). Data from PKC-θ$^{-/-}$ T cells demonstrated that PKC-theta is pivotal for the activation of two crucial transcription factors, *i.e.*, nuclear factor κB (NF-κB) and activator protein-1 (AP-1) in TCR/CD28-costimulated T-cell activation and proliferation (10,11). Both transcription factors (NF-κB and AP-1) are essential for IL-2- induced T-cell growth, activation and proliferation.

The activity of PKC-θ has been reported to have what appears to be contradictory roles cell survival and apoptotic cell death. The PKC-θ kinase has been demonstrated to mediate a protective role from Fas-L-induced apoptosis in Jurkat Cells (7,12). Furthermore, it was unveiled that this PKC-θ-dependent anti-apoptotic function is mediated, to large extent, by phosphorylation and

inactivation of BAD. However, intriguingly, it has also been reported that the PKC-θ kinase can mediate a pro-apoptotic function as well (6,13). How PKC-θ kinase plays such a paradoxical role in cell survival has been attributed, at least in part, to the proteolytic activation of PKC-θ kinase during apoptotic cell death induction. For example, upon apoptosis in Jurkat T-cells, PKC-θ kinase is cleaved at the hinge region by caspases generating an active truncated PKC-θ ΔN kinase containing almost the intact C-terminal catalytic kinase domain (6). The truncated PKC-θ ΔN has been demonstrated to exhibit an augmented kinase activity and sensitizes different cancer-derived cell lines, including Jurkat cells and neuroblastoma-derived cell line SK-N-BE, toward apoptosis in response to various apoptosis-inducing stimuli (6,13). Although the pro-apoptotic function of the cleaved PKC-θ ΔN has been investigated, the role of the neo-N-termini generated by proteolysis and its potential impact on the metabolic stability of this potent pro-apoptotic fragment have never been investigated (6,14).

The N-end rule pathway is a protein degradation pathway that relates the identity of the N-termini of a protein to its *in vivo* half-life (14). The N-end rule pathway degradation machinery recognizes proteins with specific N-termini and targets these proteins for ubiquitin- dependent degradation by the proteasome. Similar yet distinct formats of the N-end rule are present in all organisms from mammals to bacteria (14). In eukaryotes, N-end rule pathway mediated protein degradation has been implicated in strikingly diverse biological processes such as: G-protein signalling (15), cardiovascular development (16), mitophagy (17) and apoptosis (18-22).

The activity of the N-end rule pathway has been linked to the regulation of programmed cell death via the targeted degradation of proteolytic products that promote or carry out apoptosis (18-23). Investigations with mammalian cells have revealed that the N-end rule pathway targets several pro-apoptotic and anti-apoptotic protein fragments, generated as a result of proteolysis by active proteases during apoptosis, for degradation (18-23). Furthermore, it was demonstrated that the partial ablation of the N-end rule pathway sensitizes mouse embryonic fibroblasts to apoptosis-inducing agents (19). Together, these results suggest a significant role for the N-end rule pathway on the regulation of the apoptotic programme in various mammalian cells.

Here we report the first study on the role of N-End Rule mediated degradation on PKC-θ ΔN metabolic stability and drug-induced apoptosis in Jurkat T cell line. We have determined that the pro-apoptotic PKC-θ ΔN is unstable in cells as its N-terminal lysine targets it for proteasomal

degradation via the N-end rule pathway. Additionally, we have demonstrated that the metabolic stabilization of PKC-θ ΔN, either through the mutation of the destabilizing N-terminal amino acid to a stabilizing residue or via the inhibition of the N-end rule E3 ubiquitin ligases UBR1and UBR2, augments the apoptosis-inducing effect of Fas-ligand (Fas-L) in Jurkat cells. Taken together, our study expands the range of substrates of the Arg-N-end rule pathway and further supports the notion that UBR1/UBR2 of the N-end rule pathway could be a potential therapeutic target.

4.2 MATERIALS &METHODS

4.2.1 Generation of Ubiquitin fusion Cleaved PKC-θ Expression Vector: To express the cleaved pkc theta fragment we cloned the cleaved PKC theta as a fusion between an N-terminal ubiquitin and C-terminal triple FLAG tag (3× FLAG) to generate Ub- PKC-θ **ΔN** -FLAG pcDNA 3.1 vector as we have previously described (20). A cDNA clone (cloneID: MHS1010-BC016652BE894841, Open Biosystems) was used to clone the PKC-theta sequence corresponding to the caspase-3 cleavage site to the end of the protein, amino acids 355-706. The final Ub- PKC-θ ΔN -FLAG pcDNA 3.1 vector was verified by DNA sequencing. The full-length PKC theta was similarly cloned to generate to generate PKC-θ-FLAG pcDNA 3.1 plasmid.

4.2.2 Site directed Mutagenesis: Mutagenesis of the codon for the lysine corresponding to the N-termini of the cleaved pkc-theta protein was performed by site directed mutagenesis to change the codon to Arg (CGG) and Val (GUG). Similarly, the kinase dead form of cleaved PKC theta was obtained through mutating lysine 409 in the putative ATP-binding site to arginine (CGG) by site directed mutagenesis as previously described (6). In Full length PKC-theta-FLAG Lysine 365 was mutated to Valine (GUG) to change the neo-N-termini after caspase cleavage.

4.2.3 Cell culture: HEK 293T cells, Jurkat cells and Hela cells were obtained from the ATCC. The HEK293T and Hela cells were cultured in DMEM supplemented with 10% FBS. Jurkat cells were cultured in RPMI 1640 supplemented with 10 % FBS.

4.2.4 Cell Transfection: HEK293T were transfected using the Calcium phosphate-based method as previously described (31). Hela cells were transfected using the lipofectamine-based method as according to manufacturer's procedures and Jurkat cells were transfected using electroporation (Neon transfection system) according to manufacturer's procedures.

4.2.5 Antibodies: Mouse anti-FLAG® M2 antibody (cat#: F1804) was purchased from Sigma. Rabbit anti-β-actin (I-19, cat#: sc-1616-R) anti UBR1 (cat#: sc-100626) and anti-UBR2 (cat#: sc-135594) were purchased from Santa Cruz Biotechnology. Secondary antibodies for Western blot analysis (goat anti-mouse and goat anti-rabbit) coupled to IRDyes® were purchased from LI-COR. The rabbit poly-clonal PKC θ Antibody (C-18) was purchased from santa-cruz.

4.2.6 Protein Stability Assays and Western Blot Analysis: Twenty-four hrs after transfection, 5×10^5 cells were treated with 100µg/ml of cycloheximide (Chx) for the indicated amounts of time. Cells were harvested and then lysed in 150 µl of lysis buffer (50 mm Tris, pH 6.8, 8% glycerol (v/v), 0.016% SDS (w/v), 0.125% β-mercaptoethanol (v/v), 0.125% bromphenol blue (w/v), 1 mm PMSF, and 1 µg/ml of leupeptin). The samples were sonicated and then resolved by SDS-PAGE on 10% gels along with Precision Plus All Blue protein prestained standards (Bio-Rad). After SDS-PAGE, proteins were transferred onto nitrocellulose membranes (LI-COR Biosciences). The membranes were blocked with 2.5% fish skin gelatin blocking buffer in 1× PBS with 0.1% Triton X-100) and probed with primary and secondary antibodies and imaged with an Odyssey® Infrared Imaging System using the manufacturer's recommended procedures (LI-COR). Western blots for PARP and cleaved PARP were done using the reagents as previously described (12).

4.2.7 UBR1 and UBR2 shRNAs: Four UBR1 unique 29mer shRNA constructs in GFP-V-RS vectors (cat#: TG300681) were purchased from Origene. Four unique UBR2 29mer shRNA constructs in RFP-C-RS vectors (cat#: TF300680) were also purchased from Origene. Jurkat cells stably expressing a mixture of UBR1 shRNA's & UBR2 shRNA's were established as previously described (25)

4.2.8 Flow cytometry: Annexin V-FITC Apoptosis Detection Kit (eBioscience) was used for apoptosis analysis by flow cytometry using the manufacturers recommended procedures and analyzed on a LSR-Fortessa Instrument. Ten thousand events are acquired for statistical analysis.

4.2.9 Cell viability assay: Cell counting and the trypan blue exclusion test were performed with a TC20™ Automated Cell Counter (BioRad).

4.2.10 DNA fragmentation assay: To investigate whether cells underwent apoptotic DNA fragmentation, DNA was isolated and its oligonucleosomal fragmentation pattern was determined as previously described (26).

4.2.11 Reagents: Cycloheximide, Staurosporine were purchased from sigma. Purified human Fas-L (hbA 175) (175 amino acid protein fragment corresponding to an extracellular domain of FAS-L of human origin) were purchased from Santa-Cruz.

4.3 Results and Discussion

4.3.1 PKC-theta is proteolytically cleaved in Jurkat cells undergoing apoptosis to generate the pro-apoptotic PKC-θ ΔN fragment. Proteolytic generation of PKC-θ ΔN by caspase cleavage was reported around two decades ago where cleavage releases the C-terminal PKC-θ ΔN fragment (amino acids 355-706) containing almost the intact C-terminal catalytic kinase domain of the protein(6). We wished to investigate the PKC-θ ΔN fragment and so we constructed plasmids to express both the full-length PKC-θ kinase with a C-terminal 3×Flag tag and an ubiquitin (Ub)-PKCθΔN 3×Flag tag fusion protein as schematically shown in **Figure 4.1**. The Ub- PKC-θ-ΔN fusion mirrors the caspase-cleaved form of the PKC-θ kinase as endogenous ubiquitin hydrolases will proteolytically remove the N-terminal ubiquitin to expose the N-terminal lysine. Both constructs were expressed in the human T-cell leukemia Jukat cells via electroporation. The cells expressing the full length PKC-θ were also treated with DMSO or 200nM staurosporine (STS), a kinase inhibitor and an apoptosis-inducing agent in Jurkat T cells (27). As predicted, expression of the Ub-PKC-θ ΔN fusion construct results in a protein species with an electrophoretic mobility, of an approximate molecular weight of about 40 kDa, that matches that of the staurosporine-induced caspase cleaved form of the full-length PKC-θ kinase (**Figure 4.2c**). In addition, to further confirm the proteolytic generation of PKC-θ ΔN from the full-length counterpart, Jurkat cells expressing the full-length PKC-θ were also treated with either DMSO, a Fas-L (an extrinsic-apoptosis inducing agent) or staurosporine for different time points as indicated in figures 4.**2A** and **4.2B**. Indeed, Jurkat cells that were treated with either Fas-L or staurosporine result in production of PKC-θ ΔN at different time points of treatment.

4.3.2 Increased cell death sensitivity in PKC-θ ΔN expressing cells. Previous investigations reported that the proteolytic removal of the N-terminal domain of the PKC-θ kinase produces a catalytically active protein fragment, PKC-θ ΔN, which sensitizes a number of cancer cells toward apoptosis-inducing agents (6,13). To verify whether this enhanced sensitivity also applies to staurosporine and Fas-L treatments we investigated the effects of expressing full length PKC-θ and PKC-θ ΔN on staurosporine-induced cell death and Fas-L-induced cell death in Jurkat cells. Jurkat cells transfected to express full length PKC-θ, PKC-θ ΔN or a vector control were treated with staurosporine for different time points (Figure **4.2D**), upon which the cells were evaluated for viability by a trypan blue exclusion staining assay and counting on an automated counter. As shown in **Figure 4.2D**, there is some reduction in cell viability when expressing PKC-θ ΔN

constructs when no staurosporine was present. In agreement with the previous investigations (6,13), reduced cell viability is observed in the PKC-θ ΔN expressing cells in the presence of 200 nM staurosporine **Figure 4.2D.** An analogous experiment was also performed in the presence or absence of 50ng/ml of Fas-L and was analyzed by a trypan blue exclusion staining assay and counting on an automated counter. The data in **Figure 4.2E** concurs with the data from **Figure 4.2D** where the cells expressing PKC-θ ΔN exhibit increased cell death in absence and presence of Fas-L. Of note, the data with Fas-L reveals a significant protective role for PKC-θ-expression against Fas-L- induced-cell death in Jurkat cells. Together our data indicates that the expression of PKC-θ ΔN enhances drug induced apoptosis in Jurkat cells as was previously observed for cells treated with different apoptosis inducing agents (6,13). Conversely, the expression of the full-length PKC-θ confers Jurkat cells some resistance against Fas-L-induced-cell death as was previously reported (7).
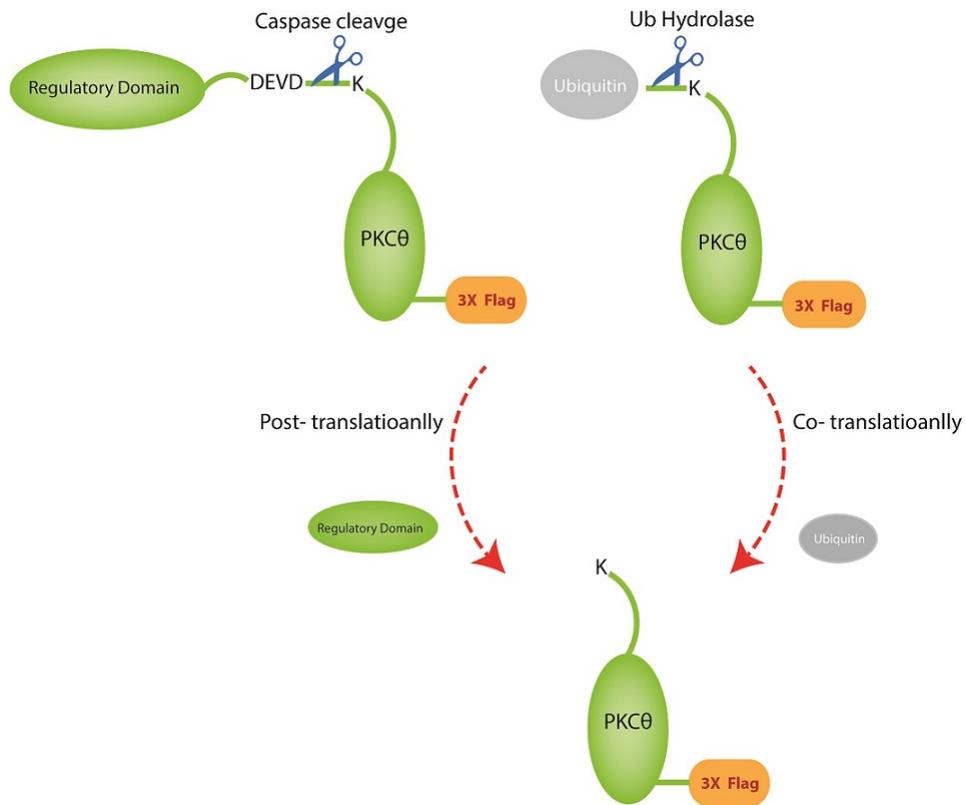
Figure 4.1 Schematic depiction of the generation of PKCθ ΔN by either caspase- cleavage after Asp 354 or as an ubiquitin fusion to generate PKCθ ΔN with an N-terminal lysine.
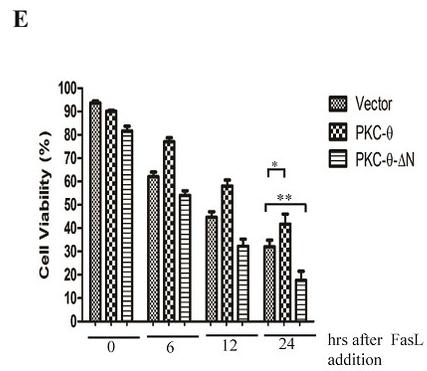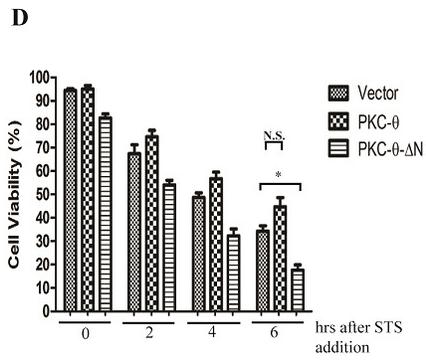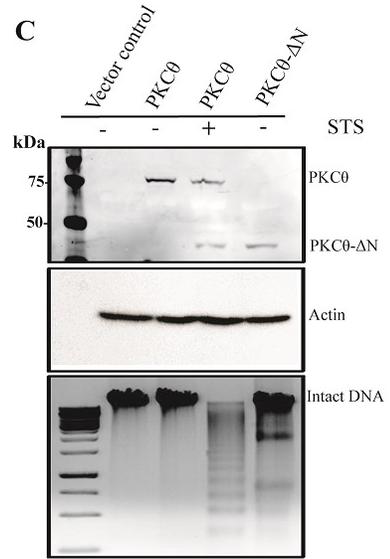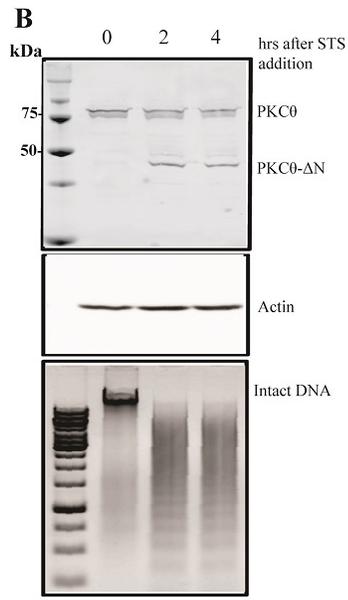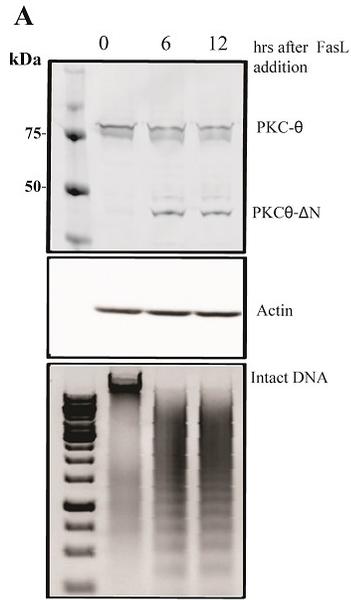
Figure 4.2 Protease dependent generation of pro-apoptotic PKC-θ ΔN. *A,* Jurkat cells were transiently transfected with PKC-θ-Flag. After transfection, the cells were treated with 200 nM staurosporine for the indicated time points (2 and 4 hours) or left untreated prior to lysis and analysis by SDS-PAGE and Western Blot (WB) analysis with an anti-Flag antibody. WB analysis reveals that staurosporine treatment results in cleavage of full length PKC-θ that results in a cleaved product with molecular mass of around 40 kDa, and an anti-actin antibody was used as a loading control. DNA fragmentation assay were done on the corresponding DNA samples as a positive indication for caspase activation. *B*, as in *(A)* but the cells were treated with a Fas-L (50 ng/ml) for the indicated time points (6 and 12 hours). *C*, Transient transfection with PKC-θ-Flag or Ub-PKC-θ ΔN-Flag in Jurkat cells. After transfection, the cells were treated with 200 nM staurosporine or left untreated prior to lysis and analysis by SDS-PAGE and Western Blot (WB) analysis with an anti-Flag antibody. WB analysis reveals that staurosporine treatment results in cleavage of full length PKC-θ that results in a cleaved product with identical electrophoretic mobility as PKC-θ-ΔN. *D,* Jurkat cells were transfected to express the indicated proteins. Cells were then treated with 200 nM of staurosporine for the indicated time points, then assayed for viability using trypan blue staining. The data represent the average and standard deviation from three independent experiments and p-values are derived from paired two-tailed t-tests. *E,* as in *D* but the cells were treated with a Fas-L (50 ng/ml) for the indicated time points (6, 12, 24 hours). Data sets that are deemed not significantly different (N.S.; >0.05) and data sets that are significant: * $p<0.05$,

4.3.3 PKC-θ ΔN is unstable and is degraded by the proteasome.
Since the cleaved fragment PKC-θ ΔN bears a destabilizing residue (N-terminal lysine), we predict that PKC-θ ΔN would be targeted for degradation in cells via the N-End rule degradation machinery yet the metabolic stability of this protein fragment has never been investigated. The stability of both full-length PKC-θ and PKC-θ ΔN were evaluated by expressing the proteins in Jurkat cells and then cycloheximide (CHX) was added to the cells to halt protein synthesis globally. Cells were then lysed at 0, 1 and 4 hours after CHX addition and the amount of protein remaining was determined by Western Blot analysis after resolving the samples by SDS-PAGE. The data in **Figure 4.3A** demonstrates that the full length PKC-θ protein is stable while PKC-θ ΔN is degraded over the four-hour time course. Western Blot analysis for actin was performed to verify equal protein loading on the gel. This is the first demonstration that PKC-θ ΔN is unstable in cells.

To verify that PKC-θ ΔN degradation is via the proteasome, we investigated whether addition of a proteasome inhibitor, MG132, inhibits degradation. The stability of PKC-θ ΔN in Jurkat cells was investigated in the presence or absence 10 μM MG132. The data in **Figure 4.3B** reveals that the addition of MG132 to the cells stabilized PKC-θ ΔN in contrast to the DMSO control. Together the data demonstrates that the PKC-θ ΔN protein fragment is unstable and is degraded by the proteasome.

4.3.4 The N-End Rule targets PKC-θ ΔN to the proteasome for degradation.
 As the proteolytic generation of PKC-θ ΔN exposes an N-terminal lysine residue, we hypothesized it is targeted to the proteasome for degradation by the components of the N-End rule pathway. The N-end rule pathway is a protein degradation pathway that recognizes proteins with specific destabilizing N-terminal residues and ubiquitinates these proteins for targeting to the proteasome for degradation (14). In eukaryotes, the primary N-terminal destabilizing residues are classified into positively charged amino acids (Type I), such as Arg as Lys, or bulky hydrophobic residues (Type II), such as Trp and Tyr. As PKC-θ ΔN has an N-terminal lysine (a type I destabilizing residue), we investigated whether the N-end rule pathway targets PKC-θ ΔN for degradation.

To investigate the potential for N-End Rule degradation, we created a couple of PKC-θ ΔN N-terminal mutants where the Lys was mutated to either Val or Arg. If PKC-θ ΔN was degraded via the N-End Rule, an N-terminal Val is predicted to stabilize PKC-θ ΔN as this is a stabilizing N-terminus (V. Mutating Lys to Arg, a type I destabilizing residue, is predicted to continue to render BMXΔN unstable (14). The wild type PKC-θ ΔN and the two N-terminal mutants were expressed

in Jurkat cells and the stability of the proteins was investigated after the addition of CHX to the cells. As predicted the N-terminal Val mutant was stabilized (**Figure 4.3C**) and the N-terminal Arg mutant remained unstable. This data agrees with the hypothesis that PKCθ ΔN is degraded by the N-End Rule pathway.

To rule out the possibility that the PKC-θ ΔN degradation is specific to the Jurkat cells, we also investigated the degradation of wild type PKC-θ ΔN, Val- PKC-θ ΔN and Arg- PKC-θ ΔN in the unrelated HEK293T and Hela cell lines. As shown in **Figure 4.3C**, the data reveals that, despite the presence of some cell specific differences in the actual rates of degradation, the overall trends of N-termini dependent degradation are similar.

Selective degradation via the N-end rule degradation machinery pathway involves the recognition of N-terminal destabilizing residue on the target protein by the UBR-box containing E3 ubiquitin ligases (14,24, 28-31). The mammalian UBR E3 ubiquitin ligases have two key recognition domains, the UBR box domain and the N-domain, which function independently as recognition domains for type I and type II N-terminal destabilizing residues respectively. To validate that PKC-θ ΔN is a *bona fide* N-End rule substrate we verified that its degradation was dependent on these E3 ubiquitin ligases. We investigated the two functionally overlapping E3 ubiquitin ligases UBR1 and UBR2, as they play a crucial role in recognition of type I N-terminal destabilizing residues in mammals (28-31). PKC-θ ΔN was expressed in Jurkat cells stably expressing control shRNAs, a mixture of shRNAs to target both UBR1 and UBR 2 or vector control cells. The stability of PKC-θ ΔN was then investigated in these cells after treatment with CHX. **Figure 4.4D** reveals that the knockdown of UBR1 and UBR inhibit the degradation of PKC-θ ΔN while the protein is still rapidly degraded in both the shRNA control expressing cells and the vector control cells. This result is analogous to what we have observed for the caspase cleaved forms of the Lyn kinase and BMX-kinase which are targeted for degradation by the N-End Rule pathway (20,25). An analogous experiment was also performed in cells stably expressing control shRNAs, a mixture of shRNAs to target either UBR1 or UBR 2 or vector control cells. The data in **Figure 4.4 E&4.4F** reveal that the knockdown of either UBR1 or UBRs doesn't effectively halt the degradation of PKCθ ΔN with respect to the PKC-θ ΔN degradation rate occurring in shRNA control expressing cells and the vector control cells. These data further confirm the notion that UBR1 and UBR2 are redundant E3 ubiquitin ligases.

The knock down of both UBR1 and UBR2 by shRNA expression was verified by Western Blot analysis for endogenous UBR1 (**Figures 4.4A &4.4 C**) and UBR2 (**Figures 4.4B &4.4C**). This data along with the stability of the N-terminal mutants above is strong evidence that PKC-θ ΔN is an N-End rule substrate.

The above results for the different methods to inhibit the N-End Rule pathway, shRNA knockdown of UBR1/2 or the general inhibition of proteasome via MG132, are in agreement with a model where PKC-θ ΔN is targeted for N-Rule degradation by the UBR1 and UBR2 E3 ligases. Nonetheless, our data doesn't preclude the possibility of involvement of other N-end rule pathway E3 ubiquitin ligases such as UBR4 as recent work demonstrated that UBR4 is involved in targeting basic destabilizing N-terminal residues for N-end rule mediated protein degradation (17,32).
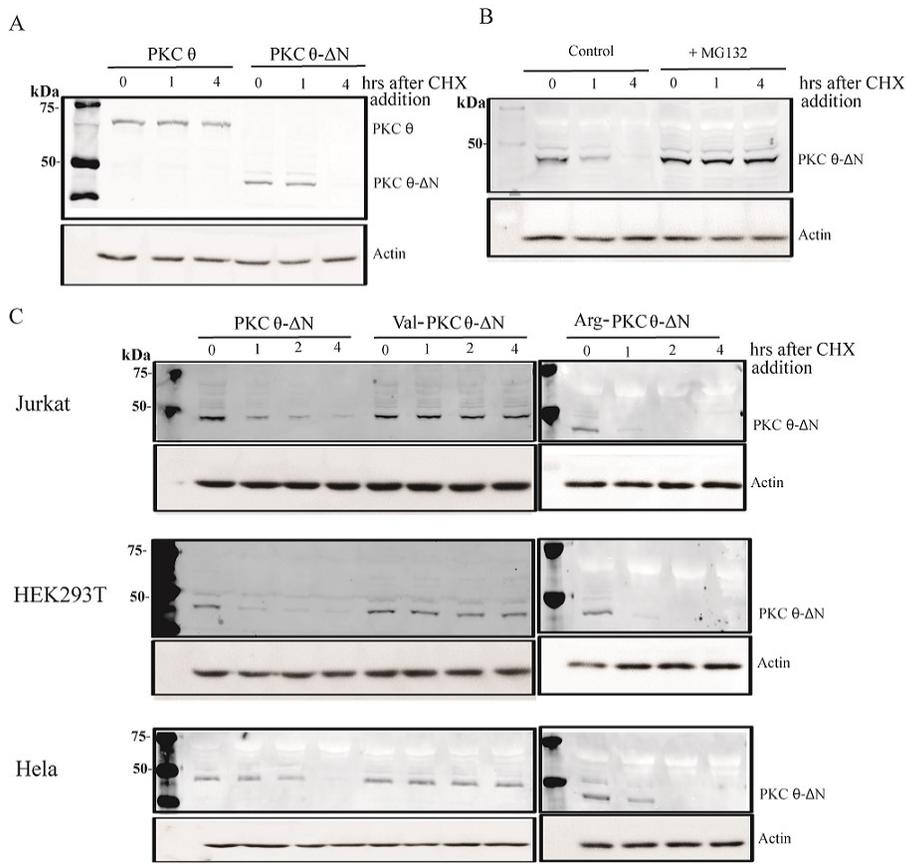
Figure 4.3 N-terminal dependent degradation of PKC-θ *A*, the stability of full-length PKC-θ and PKCθ ΔN was determined in transfected Jurkat cells by treating the cells with 100 μg/ml CHX, to block protein synthesis, and then the cell lysates were analyzed by WB analysis at the indicated times. An anti-FLAG antibody was used to detect PKC-θ, and an anti-actin antibody was used as a loading control to ensure equal loading. *B*, stability of BMXΔN was investigated in Jurkat cells in the presence and absence of MG132 (10 μM) and analyzed as in *A*. *C*, wild type PKC-θ ΔN with N-terminal lysine (type I destabilizing N termini) and N-terminal mutants of arginine (type I destabilizing N termini) and valine (stabilizing N termini) were transfected into the cell lines indicated, and PKC-θ ΔN stability was determined as described in *A*.
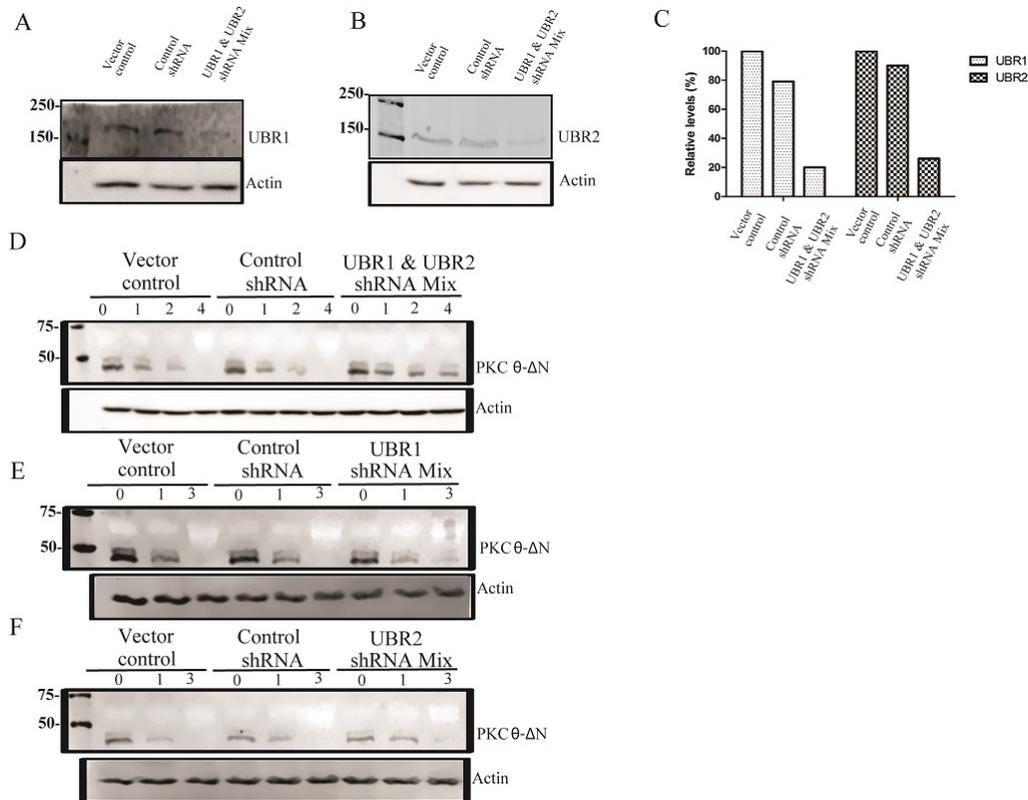
Figure 4.4 PKC-θ is degraded by the N-end rule UBR1/2 E3 ubiqutiin ligases pathway *A*, verification of shRNA knockdown of UBR1 in *D & E* by WB analysis with an anti-UBR1 antibody. *B*, verification of shRNA knockdown of UBR2 in *D & F* by WB analysis with an anti-UBR2 antibody. *C*, quantification of the relative levels of UBR1 and UBR2 in *A & B*. *D*, stability of PKC-θ ΔN was visualized in Jurkat cells that also expressed shRNAs targeting UBR1 and UBR2, control shRNAs, or a pcDNA 3.1 vector control. *E*, stability of wild type PKC-θ ΔN was visualized in Jurkat cells that also expressed shRNAs targeting UBR1, control shRNAs, or a pcDNA 3.1 vector control. *F*, stability of wild type PKC-θ ΔN was visualized in Jurkat cells that also expressed shRNAs targeting UBR2, control shRNAs, or a pcDNA 3.1 vector control.

4.3.5 The role of the N-End Rule on PKC-θ-induced sensitivity to apoptotic cell death. Given the degradation of PKC-θ ΔN by the N-End Rule we investigated the impact of degradation on its pro-apoptotic function. Jurkat cells stably expressing shRNAs targeting UBR1 and UBR2 or shRNA controls were transfected with either a vector control or plasmids to express either wild-type PKCθ ΔN or the stable N-terminal Val-PKC-θ ΔN mutant. After 24 hours, the cells were treated with 50 ng/ml Fas-L for 6 hours and then evaluated by trypan blue staining to quantify cell viability. The data in **Figure 4.5A** reveals that stabilizing PKC-θ ΔN by the N-terminal valine mutant increases the cells sensitivity to Fas-L. When PKC-θ ΔN and Val- PKC-θ ΔN are expressed in UBR1/2 knock down cells, the sensitivity to Fas-L are approximately the same. Together the data suggests that PKC-θ ΔN results in lower Fas-L sensitivity than the Val- PKCθ ΔN mutant because of its instability.

A more detailed investigation of the effect of stabilizing PKCθ ΔN on apoptosis was done by cell staining and FACS analysis. Jurkat cells were transfected with wild-type PKC-θ ΔN, the stable N-terminal Val- PKC-θ ΔN mutant or a vector control. The cells were then treated with 50 ng/ml Fas-L for 3 hours and then stained with PI and Annexin V prior to FACS analysis. The data in **Figure 4.5B** reveals that stabilizing PKC-θ ΔN with the valine N-terminal mutation results in increased numbers of Annexin V labeled cells when compared to wild type PKC-θ ΔN. The presence of the increased amounts of apoptotic cells upon Fas-L treatment when PKC-θ ΔN is stabilized indicates that the N-End rule is attenuating the pro-apoptotic activity of PKC-θ ΔN. Although we mentioned previously in **Figures 4.1D &4.1E** that despite its instability, wild type PKC-θ ΔN expression leads to increased Fas-L sensitivity as assayed by trypan blue staining assay when compared to the vector control. In this experiment in **Figure 4.5B**, we didn't observe a statistically significant effect of wild type expression on Jurkat cells sensitivity to Fas-L-induced apoptosis with respect to vector control. One possible reason for this observation could be the relative short-time of apoptosis-induction in our apoptosis experiment (**Figure 4.5B).**

4.3.6 The N-End Rule Directly Influences Apoptotic Stimulation. Our data also reveals that UBR1/2 exhibits a PKC-θ ΔN independent influence on apoptosis. The data in **Figure 4.5A** reveals a difference in Fas-L sensitivity between the UBR1/2 knock down cells versus the shRNA control cells, even when PKC-θ ΔN is not expressed. Although we have not further investigated this observation, this finding agrees with previous reports. Specifically, UBR1/UBR2 knock out mouse

embryonic fibroblasts was shown to be hypersensitive to apoptosis-inducing agents such as UV or staurosporine, a kinase inhibitor known to induce apoptotic cell death (19). Second, mice spermatocytes that lack UBR2 ligase undergo apoptotic cell death during meiosis and this eventually leads to male infertility (33).

A

B
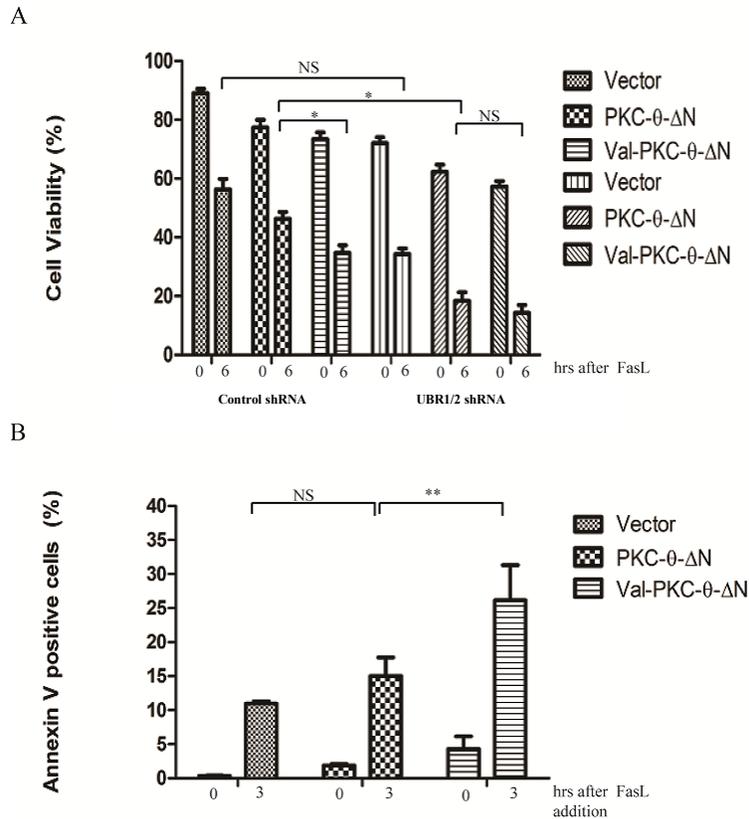
Figure 4.5 PKC-θ ΔN stability and the N-end rule influencing apoptosis. *A*, Jurkat cells expressing control or UBR1/2 targeting shRNAs were transfected to express either wild type PKC-θ ΔN, the valine N-terminal mutant, or a vector control. 24 h after transfection, cells were treated with 50 ng/ml Fas-L for 6 h. The cells were then analyzed by trypan blue staining. The data represent the average and S.D. (*error bars*) from three independent experiments, and *p* values were determined from paired two-tailed *t* tests. *B*, FACS analysis of Fas-L treated Jurkat cells. Jurkat cells were transfected to express either wild type PKCθ ΔN, the valine N-terminal mutant, or a vector control. Cells were then treated with Fas-L for 3 h. Cells were then analyzed by FACS for annexin V and propidium iodine staining. The percentage of cells that were doubly labeled with both annexin V and propidium iodide are indicated. The data represent the average and S.D. from three independent experiments and *p* values were derived from paired two-tailed *t* tests. Data sets that are deemed not significantly different (N.S.; >0.05) and data sets that are significant: * p<0.05, ** p<0.01.

4.3.7 N-End Rule Degradation of Caspase Generated PKC-θ ΔN. In addition to studying the degradation of PKC-θ ΔN using the recombinant ubiquitin fusion constructs, we investigated whether caspase-mediated generation of PKC-θ ΔN from full-length PKC-θ kinase in jurkat cells is accompanied by degradation via the N-end rule pathway. Jurkat cells were transfected to express full-length PKC-θ, K355V mutant, or the non-cleavable mutant (D354A). Cleavage of the K355V mutant by caspases will generate the stable Val-PKCθ ΔN variant. After 24 hours, the cells were treated with 200 nM staurosporine for 6 hours, upon which cleavage of PKCθ is observed (**Figure 4.6 A**) except in the non-cleavable mutant (D354A) and is concomitant with DNA fragmentation, which is used to detect caspase activation (34-36). When Q-VD-OPH, a broad spectrum caspase inhibitor (37), was added to halt the production of PKC-θ ΔN by ongoing caspase cleavage of full length PKC-θ, a mitigation in PKCθ ΔN levels is observed (**Figure 4.6A**). This Q-VD-OPH dependant reduction in PKCθ-ΔN is hypothesized to be a result of N-End rule degradation and as predicted is inhibited by the addition of MG132 or UBR1/2 shRNA knock down. The Q-VD-OPH dependent drop in PKCθ-ΔN is also not observed with the K355V mutant which is also predicted as the resultant Val- PKCθ-ΔN is stable. In sum, the data supports a model were the caspase-mediated cleavage of full length PKC-θ kinase generates a cleaved fragment that is a *bona fide* N-end rule substrate.

To investigate whether N-End rule degradation of caspase generated PKC-θ ΔN impacts cell viability, Jurkat cells expressing full length wild type PKC-θ, K355V PKC-θ or the non-cleavable mutant (D354A PKC-θ), were treated with staurosporine for 6 hours and assayed for cell viability by trypan blue staining (**Figure 4.6B**). As shown, the expression of full length wild type PKC-θ had no measurable impact on cell viability. In contrast, when the K355V PKC-θ mutant was expressed, a statistically significant decrease in cell viability was observed upon staurosporine treatment (Figure 4.6B), indicating the potent pro-apoptotic role of the caspase generated stable Val-PKCθ ΔN that outweighs the anti-apoptotic function of full-length PKC-θ. This is further demonstrated by the results from the significant anti-apoptotic effect of expression of non-cleavable mutant (D354A PKCθ) that mitigates the staurosporine-induced cell death in Jurkat cells with respect to vector control cells. Tellingly, a parsimonious interpretation for the non-observed pro-apoptotic activity of the caspase generated PKCθ ΔN is that while PKCθ ΔN generated by caspase cleavage has the potential to be pro-apoptotic, the active degradation of this fragment by the N-End Rule-pathway effectively counters this function. An analogous cell death experiment

was done (with Fas-L as an apoptosis-inducing agent) and similar results were obtained upon treatement with Fas-L as shown in **Figure 4.6C**.

4.3.8 N-End Rule Degradation of endogenous PKCθ ΔN Generated by Caspase cleavage.
To investigate whether PKC-θ ΔN generated from caspase cleavage of endogenous PKC-θ was similarly targeted for degradation via the N-End Rule pathway, the following experiment was performed. Jukrat cells stably expressing the UBR1/2 shRNAs (or control shRNAs) were treated with 200 nM staurosporine for 6 hours to induce apoptosis and PKC-θ cleavage (**Figure 4.7A**). As described previously, Q-VD-OPH was utilized to prevent the ongoing production of PKC-θ ΔN after staurosporine treatment. Two hours after the addition of Q-VD-OPH the cells were then also harvested for Western Blot analysis (**Figure 4.7A**). The data in Figure 4.7A reveals that treatment of the cells with staurosporine results in the formation of PKCθ ΔN from endogenous PKCθ in both the control and UBR1/2 knock down cells. When the cells were then treated with Q-VD-OPH, the PKCθ ΔN fragment disappears in the control shRNA expressing cells, as was observed for recombinant full length PKCθ (**Figure 4.7A**). In contrast, when the UBR1/2 knock down cells are similarly treated, PKCθ ΔN persists after the addition of Q-VD-OPH. This is in agreement with our model that PKCθ ΔN is stabilized in the absence of UBR1 and UBR2. Furthermore, treatment of the Jurkat cells with Fas-L culminates in the formation of PKC-θ ΔN from endogenous PKCθ in both the control and UBR1/2 knock down cells. Nevertheless, in contrast to the low steady-state levels of PKC-θ ΔN that appears in control cells, UBR1/2 knock down cells exhibit more intense steady-state levels of PKC-θ ΔN which may be attributed to the inhibition of degradation of PKC-θ ΔN resulted from the partial ablation of UBR1/UBR2 enzymes in the UBR1/2 knock down cells (**Figure 4.7B**). In sum, the data supports a model where the caspase-mediated cleavage of endogenous PKC-θ kinase generates a cleaved fragment that is a *bona fide* N-end rule substrate in Jurkat cells.
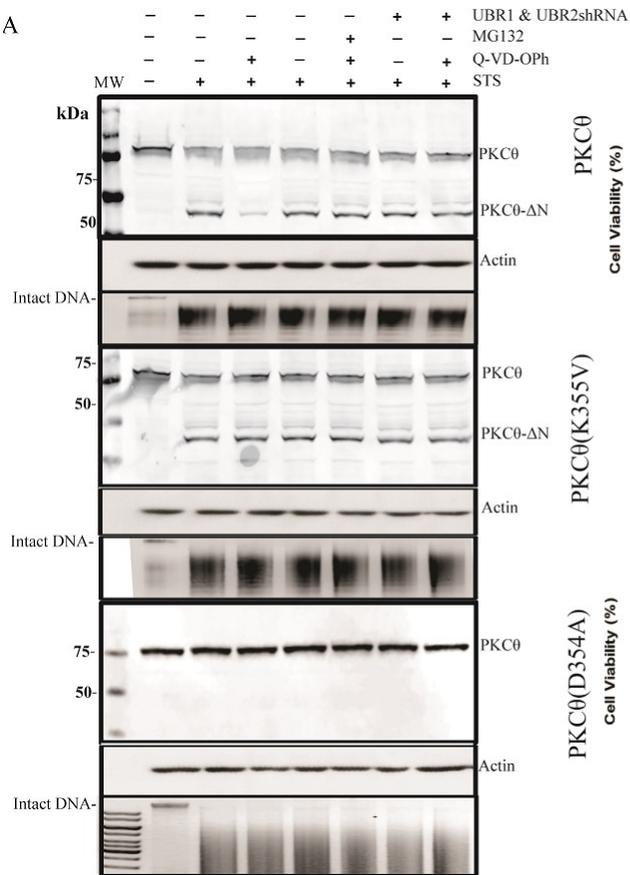
A

								UBR1 & UBR2shRNA
	−	−	−	−	−	+	+	
	−	−	−	−	+	−	+	MG132
	−	−	+	−	+	−	+	Q-VD-OPh
MW	−	+	+	+	+	+	+	STS

kDa

PKCθ
PKCθ-ΔN

75-
50-

Actin

Intact DNA-

PKCθ
PKCθ-ΔN

75-
50-

Actin

Intact DNA-

PKCθ

75-
50-

Actin

Intact DNA-

PKCθ (vertical)
PKCθ(K355V) (vertical)
PKCθ(D354A) (vertical)

B

Cell Viability (%)

hrs after STS addition

Vector
PKC-θ (D354A)
PKC-θ
PKC-θ(K355V)

C

Cell Viability (%)

hrs after FasL addition

Vector
PKC-θ (D354A)
PKC-θ
PKC-θ(K355V)

118
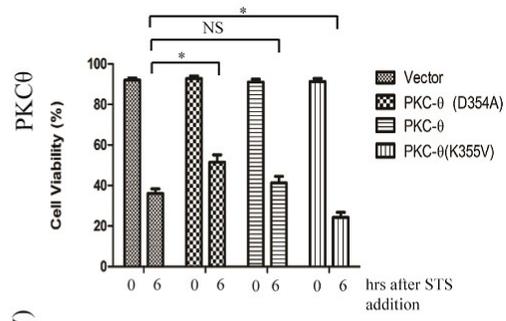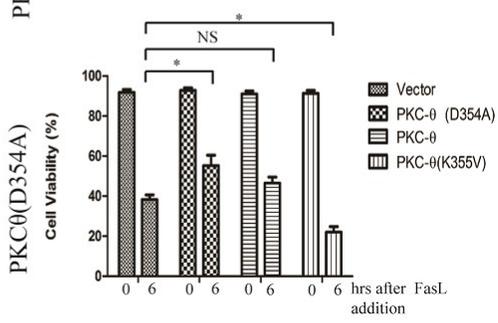
Figure 4.6 Cleavage of full-length recombinant PKCθ and C-terminal fragment degradation. *A*, Jurkat cells that stably express either shRNAs targeting UBR1 and UBR2 or shRNA controls were transfected to express full-length BMX (*top*), the K355V mutant PKCθ, or the non-cleavable mutant (D354A). 24 h after transfection, the cells were either untreated or treated with 200 nM staurosporine for 4 h. The indicated samples were also treated with MG132 or Q-VD-OPH. The pan-caspase inhibitor Q-VD-OPH was added, for 2 h, to the indicated samples to prevent ongoing formation of PKCθ ΔN by continued caspase activity. Western blotting analysis of cell lysates from the resulting experiments was performed to detect PKCθ and PKCθ ΔN amounts, DNA fragmentation pattern, or an actin loading control. When Q-VD-OPH was added to prevent the ongoing formation of PKCθ ΔN, the disappearance of the C-terminal fragment is inhibited by knockdown of UBR1/2 with shRNAs or the K355V mutation, which results in a PKCθ ΔN fragment with a stabilizing valine N terminus. *B*, Jurkat cells expressing either recombinant full-length PKCθ or the K355V mutant were treated with 100 nM of staurosporine for 6 h and then analyzed by trypan blue staining. The data represent the average and S.D. (*error bars*) from three independent experiments, and *p*values were determined from paired two-tailed *t* tests. *C, as in B* but the cells were treated with Fas-L (50 ng/ml) for the indicated time. The data represent the average and S.D. (*error bars*) from three independent experiments, and *p*values were determined from paired two-tailed *t* tests. Data sets that are deemed not significantly different (N.S.; >0.05) and data sets that are significant: * p<0.05, ** p<0.01.
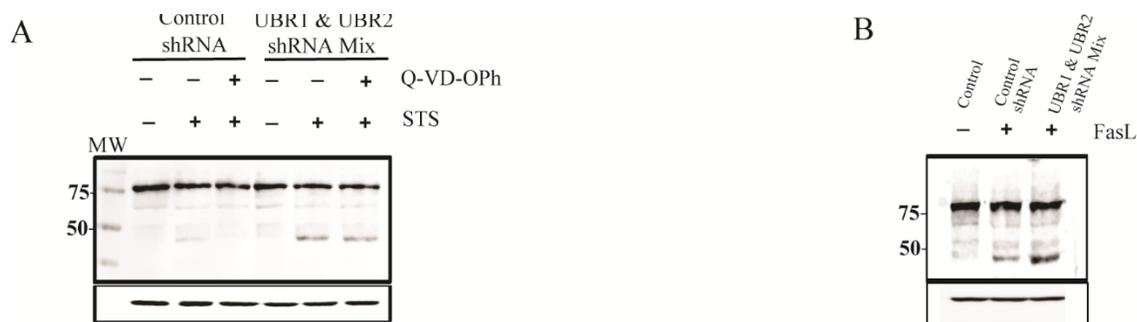
Figure 4.7 N-End Rule Degradation of endogenous PKC-θ ΔN Generated by Caspase Cleavage. *A*, Jurkat cells that stably express either shRNAs targeting UBR1 and UBR2 or shRNA controls were either untreated or treated with 200 nM staurosproine for 4 h to induce apoptosis. The pan-caspase inhibitor Q-VD-OPH was added, for 2 h, to the indicated samples after staurosporine to prevent ongoing formation of PKCθ ΔN by continued caspase activity. Western blotting analysis of cell lysates from the resulting experiments was performed to detect endogenous PKCθ and PKCθ ΔN resulting from caspase cleavage. *B,* Jurkat cells that stably express either shRNAs targeting UBR1 and UBR2 or shRNA controls were treated with 50 ng/ml Fas-L for 8 h to induce apoptosis. Jurkat cells that stably express shRNA controls were left treated to serve as control.

4.3.9 Catalytic Activity of PKC-θ ΔN is Dispensable for Degradation. With the pro-apoptotic role for PKC-θ ΔN we next investigated functions for its catalytic activity on both protein degradation and Fas-L sensitivity. An inactivating mutation (K409W), as previously described (6), was introduced into the wild type ubiquitin- PKC-θ ΔN construct. The stability of the wild type and K409W mutant PKC-θ ΔN were then investigated. The data in **Figure 4.8 B** does not reveal significant difference in the stabilities of the proteins; leading to the conclusion that PKC-θ ΔN activity was not a prerequisite for its degradation. Concomitantly, further analysis reveals that the abrogation of the catalytic activity of PKC-θ ΔN doesn't influence the stability of long-lived mutant Val- PKC-θ ΔN (**Figure 4.8A**).

Beside the N-end rule-mediated degradation of PKC-θ ΔN, the catalytic activity of PKC-θ ΔN is an important regulator for its pro-apoptotic function. This is demonstrated in **Figure 4.8C** where expression of the kinase dead PKC-θ ΔN (K409W) mutant in Jurkat cells results in the same cell viability after Fas-L treatment as the vector control. In contrast to this is the increased viability upon Fas-L treatment when cells are expressing the stable Val- PKC-θ ΔN mutant. Noteworthy, the expression of double mutant Val- PKC-θ ΔN (K409W) in Jurkat cells also doesn't result in a significant difference in cell death after Fas-L treatment with respect to cells expressing vector control (**Figure 4.8C**). This finding has two-fold consequences, first it precludes the possibility that the lack of pro-apoptotic activity by the (K409W) mutant being a simple result of its instability, as the (K409W) mutant does not exhibit pro-apoptotic activity when it is stabilized with an N-terminal valine. Second it further confirms the crucial role of catalytic activity of PKC-θ ΔN in mediating its pro-apoptotic function in Jurkat cells upon treatment.

4.3.10 Conclusions.
After induction of apoptotic-cell death in mammalian cells, proteases, such as caspases and calpains, cleave a large number of proteins (38). A number of cleaved fragments have pro-apoptotic activity; that is, they can amplify apoptosis signalling, presumably via participating in positive feedback loops. For instance, cleaved RIPK1 enhances caspases activation (39). Our findings with PKC-θ ΔN reveal that while the catalytic activity of the kinase is required for its pro-apoptotic function, this activity is attenuated by the N-End rule-mediated degradation as has been observed for catalytic fragment of RIPK1(19). With the expanding role of the N-End Rule pathway for the attenuation of proteolytically activated apoptotic factors (19, 20, 23–25), it may be a novel target to augment pro-apoptotic therapies.
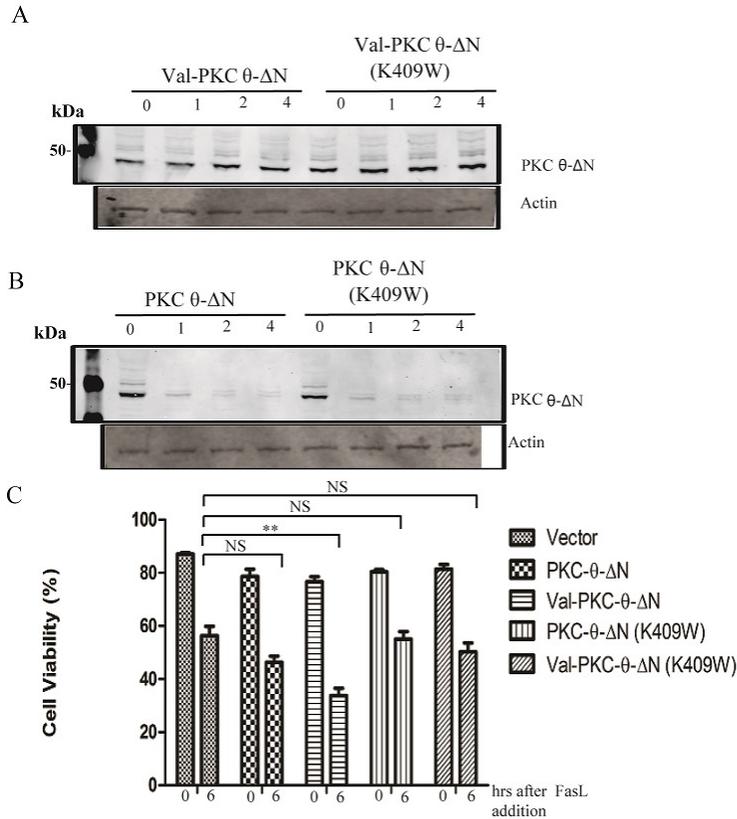
Figure 4.8 PKC-θ ΔN kinase activity is required for its pro-apoptotic activity. *A & B*, wild type (*bottom*) and the N-terminal valine mutant (*Top*) PKC-θ ΔN along with their corresponding kinase inactive mutants (K409W) were expressed in Jurkat cells. The stability of the proteins was investigated by treating the cells with 100 μg/ml CHX and collecting lysates at the indicated times. The lysates were resolved by SDS-PAGE, and the amount of protein remaining was determined by WB analysis with an anti-FLAG and an anti-actin antibody was used to determine actin levels as loading control. *B*, Jurkat cells were transfected to express the indicated PKCθ variants. Cells were then left untreated or treated with 50ng/ml Fas-L for 6 h and were evaluated for viability by staining with trypan blue. Data sets that are deemed not significantly different (N.S.; >0.05) and data sets that are significant: * p<0.05, ** p<0.01.
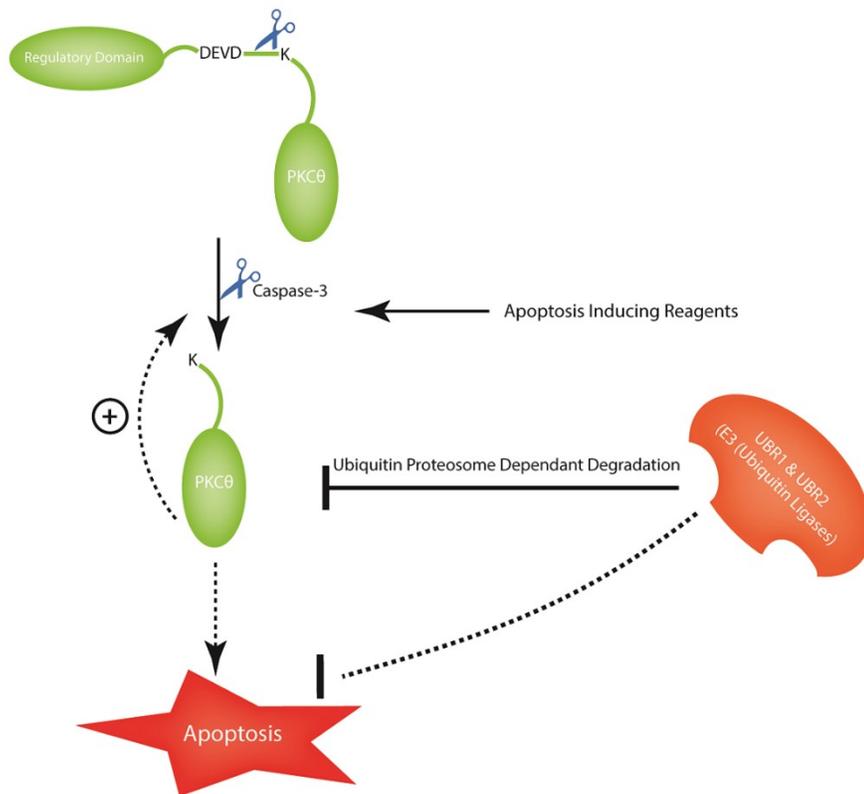
Figure 4.9 Model for PKC-θ ΔN degradation. Our proposed model is that the caspase-generated PKC-θ ΔN is recognized by the degenerate UBR1 and UBR2 E3 ubiquitin ligases. UBR1/2 recognize the N-terminal lysine of PKC-θ ΔN, which then ubiquitinates the protein, targeting it to the proteasome for degradation and thus attenuating its pro-apoptotic function.

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Chapter 5: Conclusion and Future Directions

5.1 Concluding remarks

The elucidation of the functions and roles of the Arg-N-end rule degradation machinery with respect to apoptotic cell death is confounded, at least in part, by the strikingly diverse potential N-end rule target protein substrates in the mammalian cellular system and the complexities of apoptotic cell death signalling cascades during different apoptotic phases (initiation and execution phases) (1-7). My work demonstrated that the previously identified proteolytic protein fragments (caspase activated fragments of Lyn kinase, BMX kinase, and PKC-θ) are short-lived substrates of the Arg/N-end rule pathway in mammalian-derived cell lines. Although the entire set of caspases-generated proteolytic fragments that are potentially short-lived Arg/N-end rule substrates is certainly more than the 3 substrates validated and studied in the present work (7), and several other previously identified proteolytic fragments remain to be examined for their degradation via the N-end rule degradation machinery. Furthermore, more of such active proteolytic fragments are likely to be identified in future studies. By targeting these fragments for proteasomal degradation, the Arg/N-end rule pathway counteracts their caspases-mediated activation and thereby dampens their pro-apoptotic or anti-apoptotic reactions that regulate apoptotic signalling (3).

Although recent work revealed that, potentially, the N-terminal dependent protein degradation (the Arg-N-end rule pathway and other branches of the N-end rule) may target significant number of caspase-generated C-terminal proteolytic fragments (estimated to be around 20 % of total caspase-generated protein fragments) (7,8), Nonetheless, many of these proteolytic products have other N termini that are not recognized by the N-end rule degradation machinery (7,8). In addition, there are also some functional N-terminal proteolytic fragments of the protein substrates to be considered as well (5,9). It is likely that N-end rule degradation machinery acts as one of the components of the signalling networks occurring during these proteolytically active cascades.

5.2 The N-end rule degradation machinery counteracts apoptotic cell death

Protease-generated proapoptotic fragments likely act as components of positive feedback loops that amplify specific aspects of apoptotic cell death, including caspase activation(10). Tellingly, several kinases exhibit elevated kinase activity once their N-terminal regulatory domain removed by the proteolytic cleavage events as part of positive amplification loop during the context of apoptosis (11-13). One example of such protein kinase fragments is the C-terminal fragment of PKC-θ. Our work reveals that the pro-apoptotic cleaved fragment of PKC-θ ΔN is unstable in Jurkat T-cells as its N-

terminal lysine targets it for proteasomal degradation via the N-end rule pathway and this degradation is inhibited by mutating the destabilizing N-Termini, inhibiting the proteasome (MG132 treatment), or knocking down the UBR1 and UBR2 E3 ligases. Tellingly, we demonstrated that the metabolic stabilization of the cleaved fragment of PKC-theta or inhibition of the N-end rule machinery augments the apoptosis-inducing effect of diverse apoptosis-inducing agents in Jurkat T-cell.

We also demonstrated, for the first time, that the caspase cleaved form of the BMX tyrosine kinase is actively degraded by the N-end Rule pathway in prostate-derived cancer cell lines. Interestingly, we have also discovered, that the N-end rule-mediated degradation of the cleaved pro-apoptotic BMX kinase dampens its pro-apoptotic function. Collectively, our data on the emerging role of the N-end-Rule pathway in attenuating the apoptotic programme via destroying active pro-apoptotic kinase fragments is revealing this pathway to be a potential target to sensitize cancer cells to cell-death promoting therapies.

5.3 The N-end rule pathway can mediate pro-apoptotic signalling

Intriguingly, our work unveils that the-N-end rule pathway can target the anti-apoptotic LynΔN for proteasomal-degradation and thus dampens its anti-apoptotic function, suggesting that the N-end rule degradation machinery can mediate a pro-apoptotic role. The proteasomal-mediated degradation of LynΔN is a result of the proteolytically exposed N-terminal leucine being recognized by the N-end rule degradation machinery. Tellingly, we have demonstrated that degradation can be prevented by changing the identity of the N-terminal amino acid to valine. The complete conservation of N-terminal leucine in vertebrates suggests the importance of this destabilizing residue as a major functional attribute of this active anti-apoptotic kinase fragment.

The degradation of LynΔN by the N-end rule pathway provides an example of how the N-End rule degradation pathway can function in a pro-apoptotic manner. This was demonstrated by how the valine-LynΔN stabilizing mutant provides significantly higher imatinib resistance to K562 cells. A previous example of N-End rule dependent cell death has been previously described with macrophages treated with anthrax lethal toxin (14). In addition, recent work revealed, interestingly, that that Arg-N-end rule pathway enzyme ATE1 can promote cell death and/or growth arrest, depending on the context, type and level of stress stimulus (15).

5.4 The N-end rule-mediated degradation of a caspase-generated fragment can be modulated by phosphorylation

Although some pro-apoptotic fragments have been validated as short-lived substrates for the N-end rule pathway, we have found that the N-end rule-mediated degradation of the cleaved BMX kinase is inhibited by its phosphorylation (2). This is an entirely novel mechanism of regulation as phosphorylation of a protease-generated substrate has never been reported to alter N-End-Rule degradation pathway which was discovered three decades ago (16). Our work also addresses some of duality reported regarding the pro/anti- survival function of the BMX kinase (17,18). Overall our findings have revealed an unforeseen interplay between phosphorylation and N-end- Rule-protein degradation of a caspase product, revealing an increasing complex regulatory network of apoptotic signalling cascades.

It is yet to be explored if there are other N-end rule substrates that can be regulated by phosphorylation. Although there are a number of examples of how phosphorylation impacts, either positively or negatively, target protein substrate degradation by the ubiquitin-proteasome system (19), our work presented for the first time the first clear example of phosphorylation regulating recognition of a caspase proteolytic product via the N-end rule degradation pathway, which was discovered about 3 decades ago. The mechanism of how internal phosphorylation inhibits recognition and proteasome targeting by UBR1 and UBR2 has yet to be explored. It remains unclear whether degradation of the phosphorylated form of BMXΔN is slower or whether it must first be dephosphorylated before degradation. Tellingly, it also remains to be determined whether there are other phosphorylation sites that can play a role in the regulation of stability of BMXΔN. Future investigations may identify potential mechanisms, such as the inhibition of recognition of an essential lysine by the N-domain preventing ubiquitination.

5.5 Mutual repression between the N-end rule pathway and apoptotic activated proteolytic machinery

Given the significant counteracting response of the Arg/N-end rule pathway to caspase-generated pro-apoptotic fragments (2,4,10,20), might there be a mutual repression between this degradation machinery and critical proapoptotic effectors such as activated caspases? Recent work demonstrated that key recognition components of the Arg-N-end rule degradation machinery are abrogated upon

apoptotic cell death progression (10). Tellingly, it was found that ATE1 arginyl-transferase is degraded in apoptotic cells, that activated caspases can mediate the cleavage of ATE1 arginyl-transferase and the UBR1 E3 Ubiquitin ligase, and that these cleavage events can functionally inactivate ATE1 arginyl-transferase, indicating a dynamic inhibitory cross-talk between the Arg/N-end rule degradation machinery and the potent proapoptotic activated caspases (10). A tempting interpretation for this mutual repression dictates that N-end rule degradation of caspase-generated proteolytic fragments would be relatively high at the early stages of apoptotic cell death signalling. However, upon progression of apoptotic cell death signalling, caspase-mediated suppression of the N-end rule degradation machinery would increase, specifically at later apoptotic phases, when a potent proapoptotic signaling begins to outplay the antiapoptotic response of the Arg/N-end rule pathway and other antiapoptotic networks in a manner that the proapoptotic signalling reach the point of commitment to cellular demise or the point of no return.

My recent preliminary data (data not shown) have demonstrated that UBR1 E3 ubiquitin ligases is cleaved completely in Jurkat cells following staurosporine treatment (after 8 hours of 200 nM STS) and as a result the caspase-generated PKC-θ ΔN is not degraded anymore. Despite being preliminary in nature, this data further supports the disposition that at later stages of apoptotic cell death the N-end rule degradation machinery components are proteolyzed and might functionally be inactivate. Meanwhile, the proteolytic pro-apoptotic fragments, which were degraded via the N-end rule at earlier stages of apoptosis, are not degraded anymore promoting the commitment of cellular demise. It is yet to be investigated whether, at later stages of apoptosis, other related ubiquitin-proteasome degradation machinery components are affected.

5.6 Future direction: Beyond the N-end rule: The N-end-code beyond the mere identity of the N-terminal amino acid residue.

Although the N-end rule pathway has been postulated as a rule that dictates the relationship between the nature of the N-terminal amino acid residue and the half-life of a given protein. Mounting lines of evidences unveil that the actual disposition is much more complex than currently appreciated (21,22). For instance, N-terminal acetylation of the N-terminal amino residue has been demonstrated to trigger protein degradation via creating specialized degrons called (Ac-N-degron) that can be recognized by the so-called Ac-N-end rule pathway (22). Notably, it has been demonstrated recently

that the generation of Ac/N-degrons contributes to protein quality control networks and fine-tuning of intracellular protein homeostasis (23).

Another emerging pivotal factor in the regulation of the N-terminal dependent protein degradation is the identity of second amino acid residue. Recent lines of evidences support a crucial role for the identity of amino acid residue in the penultimate position in recognition and subsequent degradation via specialized N-end rule degradation components (21). For example, recent work in yeast demonstrated that unacetylated N-terminal methionine can trigger the degradation of a given protein if the second amino acid residue is bulky hydrophobic residue and that this degradation depends on the yeast UBR1 N-recognin (24).Furthermore, more recent work unveiled that Pck1, a gluconeogenic enzyme found in yeast, which contains Pro at position 2 can be targeted for degradation by a new branch of the N-end rule pathway called Pro-N-end rule pathway. Gid4 was identified as the N-recognin of the Pro-N-end rule pathway. While the structural basis of this work is far from clear, this work revealed that the recognition depends on the identity of the first four N-terminal amino acid residues (21). It is tempting to speculate that future work may unfold some of the complexity of N-degron, which could encompass a combination of features " code" like the identity of N-terminal residue, specific PTM, specific structural features, and certain localizations features.

My most recent preliminary investigation (data not shown) on N-terminal-dependent protein degradation resulted in the unforeseen discovery of a novel specificity for N-end rule degradation machinery in some cancer cell types by a potential novel pathway (The selective degradation of MK-bearing protein(s) in K562 cell line). This discovery is anticipated to have broad implications as it recognizes significantly common N-termini and is predicted to affect large number of proteins (more than 5% of the cellular proteome). With the predicted significant impact to cellular proteome, we are beginning to elucidate the spectrum of these alterations with respect to cancer cell proteome. Although my initial observation of this novel N-termini dependent protein degradation was done with recombinant reporter proteins. We have since verified the degradation of an endogenous protein with matching N-terminal residue. The endogenous protein is Dicer, a ribonuclease and crucial regulator of microRNA biogenesis. Future work may involve the biochemical characterization of this altered specificity of this novel N-termini protein degradation pathway by investigating the stability of a complete set of reporter proteins that exhibit a complete range of mutations in first and second N-

terminal amino-acid residue positions. In addition, of crucial importance is the identification of the recognition component of this N-terminal degradation pathway that target proteins with matching N-termini for destruction. Given the recent developments in methods to study protein-protein interactions (mass-spectrometry-based methods or AP-based methods coupled to Mass-spectrometry), it is expected that future studies may identify novel N-end rule degradation components that are relevant to distinct cellular physiological inputs and further unfold the role of N-terminal dependent-protein turnover on cellular biology with potentially far-reaching implications.

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