11-18-2016

VDR-RIPK1 Interaction and its Implications in Cell Death and Cancer Intervention

Waise Quarni
University of South Florida, wquarni@health.usf.edu

VDR-RIPK1 Interaction and its Implications in Cell Death and Cancer Intervention

by

Waise Quarni

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Pathology and Cell Biology
Morsani College of Medicine
University of South Florida

Major Professor: Wenlong Bai, Ph.D.
Santo Nicosia, M.D.
Subhra Mohapatra, Ph.D.
Alvaro Monteiro, Ph.D.

Date of Approval:
November 18, 2016

Keywords: Vitamin D, Growth Suppression, Apoptosis, Necroptosis

ACKNOWLEDGMENTS

I would like to sincerely appreciate and thank my supervisor, Dr. Wenlong Bai for his relentless support and guidance throughout all these years. I will be indebted to him forever for everything I learned during my time under his mentorship. I would also like to thank my committee members Drs. Santo Nicosia, Subhra Mohapatra, Alvaro Monteiro and my former committee members Drs. Jerry Wu and Lori Hazlehurst for their wonderful guidance.

I would also like to thank my former and current lab members, Drs. Anfernee Tse, Yuefeng Sun, Panida Lungchukiet, Ravi Kasiappan and Pei Wang for their selfless help and guidance.

I also courteously thank Dr. Xiaohong Zhang for her kind help and suggestions and helping me with reagents. I am thankful to her current and former lab members Drs. Shengyan (Kevin) Xiang, Chen Hu, Jheng Yu-Wu, Kendra Williams for their kind help.

I also thank my wife for her mental support in this journey. Finally, I would like to thank my mom, for her love and for everything I have achieved in my life. Without her, I would not be here today. I also thank the Pathology and Cell Biology department and the Morsani College of Medicine at USF for the financial support throughout all these years.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AF-1 ..... Activation Function 1
AIF ..... Apoptosis Inducing Factor
ANK ..... Ankyrin
APAF ..... Apoptotic protease activating factor
AP ..... Alkaline Phosphatase
AR ..... Androgen Receptor
ATCC ..... American Type Culture Collection
cAMP ..... Cyclic Adenosine Monophosphate
CARD ..... Caspase Activation and Recruitment Domain
CBP ..... CREB Binding Protein
cDNA ..... Complementary DNA
CD95 ..... Cluster of Differentiation 95
cFLIP ..... Cellular FLICE Inhibitory Protein
cIAP ..... Cellular Inhibitor of Apoptosis Protein
CREB ..... cAMP Response Element-Binding protein
CYLD ..... Cylindromatosis
DAMP ..... Damage Associated Molecular Pattern
DBD ..... DNA Binding Domain
DD ..... Death Domain
DIK ..... Protein Kinase C-δ-interacting Protein Kinase
DMSO ..... Dimethylsulfoxide
DMEM ..... Dulbecco's Modified Eagle Medium
DNA ..... Deoxyribonucleic Acid
EcR ..... Ecdysone Receptor
EDTA ..... Ethylene Diamine Tetra Acetic acid
EGTA ..... Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER ..... Estrogen Receptor
EtOH ..... Ethanol
FasL ..... Fas Ligand
FasR ..... Fas Receptor
FBS ..... Fetal Bovine Serum
FXR ..... Fragile X mental retardation syndrome-related protein
GR ..... Glucocorticoid Receptor
HAT ..... Histone Acetyltransferase
HCL ..... Hydrochloric Acid
HDAC ..... Histone deacetylase
HEK293T ..... Human Embryonic Kidney 293T
HRE ..... Hormone Response Element
IKK ..... IκB Kinase

IL-1 ..... Interleukin 1
IL-2 ..... Interleukine 2
IL-6 ..... Interleukin 6
KD ..... Kinase Domain
KDa ..... KiloDalton
LBD ..... Ligand Binding Domain
LRR ..... Leucine Rich Repeat
LXRα/LXRβ ..... Liver X Receptor
Lys ..... Lysine
MAPK ..... Mitogen Activated Protein Kinase
MBP ..... Myelin Basic Protein
MEF ..... Mouse Embryonic Fibroblast
MLKL ..... Mixed Lineage Kinase domain-Like protein
MOMP ..... Mitochondrial outer membrane permeabilization
MR ..... Mineralocorticoid receptor
mRNA ..... Messenger RNA
MTT ..... 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl ..... Sodium Chloride
NcoR ..... Nuclear Receptor coreperssor
NCDD ..... Nomenclature Committee on Cell Death
Nec-1 ..... Necrostatin-1
NEMO ..... NF-kappa-B essential modulator
NF-κB ..... Nuclear Factor κ B
NGF-1β ..... Nerve Growth Factor-1 β
nM ..... Nanomolar
PCR ..... Polymerase Chain Reaction
PI ..... Propidium Iodide
PKA ..... Protein Kinase A
PKC ..... Protein Kinase C
PKK ..... Protein kinase C-associated Kinase
PPAR-α ..... Peroxisome proliferator-activated receptor- α
PPAR-β/δ ..... Peroxisome proliferator-activated receptor-β/δ
PR ..... Progesterone Receptor
RHIM ..... RIP Homotypic Interaction Motif
RIPK1 ..... Receptor Interacting Protein Kinase 1
RNA ..... Ribonucleic Acid
ROS ..... Reactive Oxygen Species
Roc ..... Ros of complex proteins
RXR ..... Retinoic Acid Receptor
SD ..... Standard Deviation
Ser ..... Serine
SIRT2 ..... Sirtuin 2
SMAC ..... Second Mitochondria-derived Activator of Caspases
SMRT ..... Silencing Mediator for Retinoid or Thyroid-hormone receptors
SRC-1 ..... Steroid Receptor Complex-1
TAK1 ..... TGFβ Activated Kinase 1

TAB2.....TAK Binding Protein
TCA.....Tricarboxylic Acid Cycle
TNFα..... Tumor Necrosis Factor α
TR ..... Thyroid Receptor
TRADD..... TRAF Associated Death Domain
TRAF .....TNF Receptor Associated Factor
UBD ..... Ubiquitin Binding Domain
VD3.....Vitamin D3
VDR ..... Vitamin D Receptor
VDR-KO ..... Vitamin D Receptor Knockout
VDR-WT.....Vitamin D Receptor Wild Type
VDRE-TK-luc..... Vitamin D Receptor-Thymidine Kinase-luciferase reporter plasmid
WD40..... Tryptophan and Aspartic acid 40 repeat
1,25D3.....1,25-dihydroxyvitamin D3
μM..... Micromolar

ABSTRACT

Receptor interacting protein kinase 1 (RIPK1) is an enzyme acting downstream of tumor necrosis factor alpha to control cell survival and death. RIPK1 expression has been reported to cause drug resistance in cancer cells; but so far, no published studies have investigated the role of RIPK1 in vitamin D action. In the present study, we investigated whether RIPK1 played any role in 1,25-dihydroxyvitamin D3 (1,25D3)-induced growth suppression. In our studies, RIPK1 decreased the transcriptional activity of vitamin D receptor (VDR) in luciferase reporter assays independently of its kinase activity, suggesting a negative role of RIPK1 in 1,25D3 action. RIPK1 also formed a complex with VDR and deletion analyses mapped the RIPK1 binding region to the C-terminal ligand-binding domain of VDR. Subcellular fractionation analyses indicated that RIPK1 increased VDR retention in the cytoplasm, which may account for the inhibition of VDR transcriptional activity. Consistent with the reporter analyses, 1,25D3-induced growth suppression was more pronounced in RIPK1-null mouse embryonic fibroblasts (MEF) and RIPK1 knockdown ovarian cancer cells than control cells. We have also shown that VDR was involved in RIPK1-mediated cell death pathway in a cell line specific manner. *In vivo* study showed that VDR deletion delayed the necroptotic response to tumor necrosis factor alpha in mice. Western blot analyses of platinum sensitive and resistant cell lines showed a correlation between RIPK1 expression and drug resistance, suggesting a possible role of RIPK1 in drug resistance. In conclusion, this study is the first to define RIPK1 as a VDR repressor, projecting

RIPK1 depletion as a potential strategy to increase the potency of 1,25D3 and its analogs for cancer intervention.

CHAPTER 1:

INTRODUCTION

1.1 Cell death

Cell death was formulated initially as a final outcome of inflammatory tissue damage. However, it was later found that this is a mechanism to eliminate pathogens and regulate inflammation that otherwise would cause further damage of the tissue or the whole organism [1]. Until the nineteenth century, forms of cell death had not been classified and differentiated from one another. In 1972, the term “apoptosis” was first coined to define the regulated form of cell death which is different from inflammatory necrotic cell death [2]. Before the advent of molecular diagnosis of cell death events, cell death pathways were characterized mainly based on morphological features for several reasons. Availability of light microscopy over fluorescence microscopes, easiness of observations and being unable to differentiate and characterize seemingly similar but mechanistically different cell death pathways have put forward morphological classification of cell death over molecular pathway based classification of cell death. Since then, major cell death pathways have been termed as: 1) Apoptosis, 2) Autophagy and 3) Necrosis [3].

Note to reader: Portion of this chapter has been previously published in Cellular & Molecular Immunology (2010) 7, 243–249, and has been reproduced with permission from Nature Publishing Group.

As it was eventually realized to be an overgeneralization to characterize cell death between apoptosis and necrosis on the basis of caspase dependency and independency, in 2012, the Nomenclature Committee on Cell Death (NCCD) has decided to classify major cell death pathways according to molecular mechanisms [4].

1.1.1 Apoptosis

Apoptosis or "programmed cell death" is a form of cell death where cells decide to die and follow a regulated molecular events leading to its death [5]. This type of cell death was first demonstrated during the normal development in the nematode *Caenorhabditis elegans* [6, 7]. It soon ignited an immense interest among scientists and until recently, this type of cell death is the most studied among all other cell death pathways. From the birth to the death of an organism, apoptosis works during the development, aging, DNA damage repair, immune defense etc. in response to a wide variety of physiological and pathological stimuli and conditions [8]. Morphologically, apoptosis is defined as cell shrinkage through dense cytoplasm and more tightly packed organelles. In addition to that, pyknotic nuclei due to chromatin condensation is another prominent characteristic of apoptosis visible through light microscope [9]. Cytoplasmic blebbing due to separation of cell fragments and the integrity of the plasma membrane are also important morphological hallmarks of apoptotic cell death [9]. Despite the same morphological features, apoptosis has been classifieds into extrinsic and intrinsic pathways based on the molecular pathways it follows. Extrinsic apoptosis is initiated through the binding of death ligands like tumor necrosis factor (TNF), TNF-related apoptosis inducing ligand (TRAIL) or Fas ligang (FasL/CD95L) to their cognate receptor TNFR, TRAILR, and FAS/CD95, respectively [10-12]. After tumor necrosis factor-α (TNFα) binds to TNFR, the activated receptor recruits

proteins including Fas-associated protein with death domain (FADD) protein, which has a death domain (DD) in it. The receptor also recruits TNFR associated death domain (TRADD), another death domain containing protein [13, 14]. The death effector domain (DED) of FADD binds to another DED domain of caspase-8. The protein complex in the death receptor is called death-inducing signaling complex (DISC) [14-16]. The autoactivation of pro-caspase-8 in the DISC cleaves downstream caspases like caspase-3, caspase-6 and caspase-7 to execute apoptosis [16, 17]. However, apoptosis can also be induced other than external stimuli. This type of apoptosis is called intrinsic apoptosis. As extrinsic apoptosis is caspase-mediated, intrinsic apoptosis can be either caspase dependent or caspase independent [18, 19]. In intrinsic pathway, the initiation of apoptosis does not rely on external ligand mediated signals rather than occurs by an abundant cellular stress like DNA damage, oxidative stress, hypoxia, hypothermia, toxins, over-accumulation of unfolded proteins in the endoplasmic reticulum etc. In all cases, cells can be either overwhelmed with the stress or fail to respond through the anti-apoptotic proteins. This leads to a common phenomenon called mitochondrial outer membrane permeabilization or MOMP [20]. The loss of mitochondrial membrane potential causes the release of cytochrome c along with its adaptor protein apoptotic protease activating factor, APAF1. Along with dATP and caspase-9, these proteins form apoptosome that initiates caspase-dependent intrinsic apoptotic cell death. On the other hand, release of apoptosis-inducing factor (AIF) causes DNA fragmentation [21]. In this caspase independent intrinsic pathway, energy depletion and respiratory chain inhibition are accompanied by production of reactive oxygen species (ROS). This also leads to apoptotic cell death [22, 23].

1.1.2 Autophagy

Morphologically, autophagy is termed as a form of cell death where the cell dies through cytoplasmic vacuolization and degradation by lysosome [24, 25]. Due to environmental factor such as stress, eukaryotic cells often form a double membrane vesicle or autophagosome where several organelles and part of the cytoplasm are trapped. This autophagosome is later delivered to lysosome to be degraded [26, 27].

1.1.3 Necrosis

Although necrosis has been studied much less than apoptosis, it is the oldest form of cell death that had been observed and recorded and considered as an outcome of accidental cell death due to cellular stress. Morphologically, necrotic cell death shows plasma membrane rupture and dissociation of organelles [28-30]. As the plasma membrane is no longer intact, necrotic cells become positive for both propidium iodide (PI) and annexin V (AV) staining, differentiating it from the apoptotic cell death pathway [31]. Until recently, necrosis was thought to follow no specific molecular pathways.

1.1.4 Other forms of cell death

NCDD also denotes other forms of atypical cell death. Mitotic catastrophic cell death is one such type. After a failed mitosis, for example, if the daughter cell's cytoplasm is unevenly distributed or has multinucleation, cell can die through apoptotic or necrotic cell death. This type of cell death is called mitotic catastrophe [32, 33]. On the other hand, anoikis occurs when a cell loses the attachment to the substrate or to another cell [34-36]. Pyroptosis is another form of cell death which involves the activation of caspase-1. This protein can form a protein complex called

"pyroptosome" which leads to interleukin-1β release. This type of cell death is observed in immune cells such as macrophage [37-39].

1.2 RIPK family

Receptor interacting protein 1 had been given the name since it was initially identified as a protein that interacts with the death domain of Fas (CD95) receptor through its C-terminal death domain region [40]. Eventually, it was found to be a serine/threonine kinase, hence alternatively called as RIP kinase 1 or RIPK1 [41]. The protein has three domains: the N-terminal kinase domain, the intermediate domain and the C-terminal death domain. Although initially thought as a single protein, other proteins were later found to be structurally related to this protein and RIPK was hence re-named as RIP1 or RIPK1 [42]. Proteins in the RIPK family share a common kinase domain. RIPK2 protein has a caspase activation and recruitment domain (CARD) [43]. It uses this domain to bind to another CARD domain containing protein. It can also bind to anti-apoptotic proteins like cellular inhibitor of apoptotic proteins (cIAPs) and induce Mitogen activated protein (MAP) kinase and NF-κB signaling pathways [42, 44]. Other functions of RIPK2 include T-cell receptor regulated proliferation and IL-1β maturation [45, 46]. Of all the members, RIPK1 and RIPK3 share a distinctive region, the RIP homotypic interaction motif (RHIM). This motif is special and thought to be evolutionary conserved since only a few proteins possess this motif. RIPK1 and RIPK3 interact with each other through this motif [47, 48].

In human, RIPK4 is known as protein kinase C-δ-interacting protein kinase (DIK) and in mouse; it is denoted as ROV (PKK). RIPK4 is known to activate c-Jun N-terminal kinase (JNK) and NF-κB signaling through interaction with TNF receptor associated factor (TRAF) protein

[49, 50]. RIPK4 knockout mice die after birth because of abnormal epidermal development suggesting its key role in developmental morphogenesis [51-53]. RIPK5 protein shares structural similarity with RIPK4 in kinase and ankyrin repeat domains. However, RIPK5 protein's function has not been fully understood yet. It has been observed that overexpression of RIPK5 induces apoptotic cell death through DNA fragmentation [54].

Compared to the other members of RIPK family, RIPK6 and RIPK7 are quite different. Although both proteins share the similar kinase domain, they also harbor leucine rich repeat (LRR) motif and Ros of complex proteins or C-terminal of Roc (Roc/COR) domain. RIPK6 has ankyrin repeat domain whereas RIPK7 protein has a unique tryptophan-aspartic acid repeat domain, also known as WD40 domain. The structural similarity of these two proteins suggests that they might have similar role in cell signaling. Indeed, mutations in these proteins lead to greater susceptibility to Parkinson's disease, although the mechanism of disease progression due to the mutation is still unclear [44, 55-57].

1.2.1 Domain structure of RIPK1

Among the members of RIPK family, RIPK1 has been the most-studied protein. In human, it is a 671 and in mouse, 656 amino acid residue long. Despite the length similarity, these two proteins are only 69% identical in sequence. However, they share similar domains, each with specific function. The N-terminal domain of human RIPK1 is about 300 residues long, known as the kinase domain with an ATP binding pocket. The kinase domain has auto- and trans-phosphorylation ability [58]. Enzyme digestion followed by mass spectrometry and *in vitro*

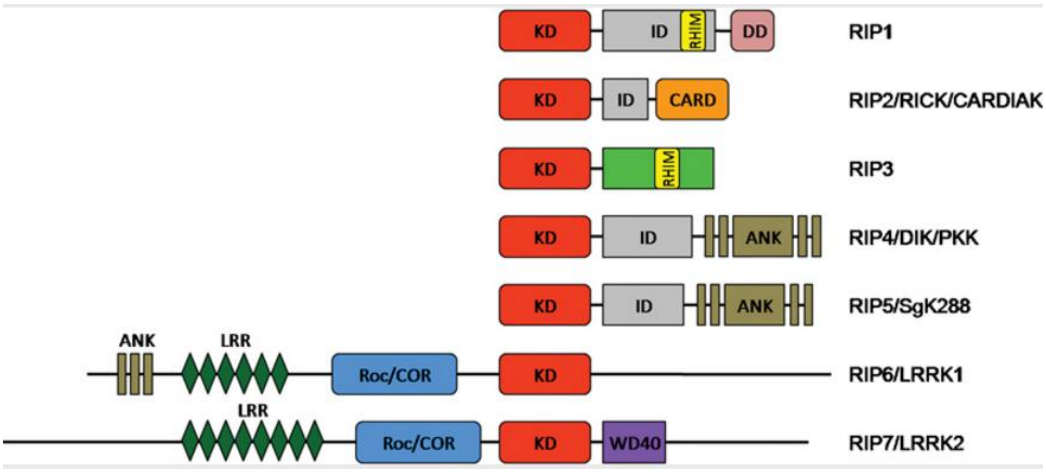

Fig. 1: RIPK family members and their domains

RIPK family proteins have a common kinase domain (KD). RIPK1 and RIPK3 share a unique RIP homotypic interaction motif (RHIM). RIPK2 has a caspase activation and recruitment domain. RIPK4, RIPK5 and RIPK6 share ankyrin repeat domain (ANK). RIPK6 and RIPK7 have unique leucine-rich repeat (LRR) and Ros of complex proteins/C-terminal of Roc (Roc/COR) domain which are not shared by other members of the family. RIPK7 also has WD40 motif.

Adapted from Zhang et.al. Receptor-interacting protein (RIP) kinase family; Cellular and Molecular Immunology (2010) 7, 243–249 with permission from Nature Publishing Group. Reprinted by permission from McMillan Publishers Limited: (Cellular and Molecular Immunology) [44] (http://www.nature.com/cmi/index.html). Copyright (2010).

kinase assays revealed that the autophosphorylation sites of RIPK1. Interestingly, all autophosphorylation sites are in the N-terminal kinase domain. Functionally, the kinase domain is responsible for phosphorylating the RIPK1 protein itself and other proteins including RIPK3 and initiating necroptotic cell death signaling. The intermediate domain is about 300 residues long. This domain contains a unique motif called RIP homotypic interaction motif (RHIM) spanning from 531 to 547 residues (Fig. 2). RIPK1 and RIPK3 bind to each other through this motif and hence, is important for necroptotic induction [59]. Intermediate domain is also crucial for ubiquitination of RIPK1 at Lysine 377 residue [60, 61]. There are other lysine residues in

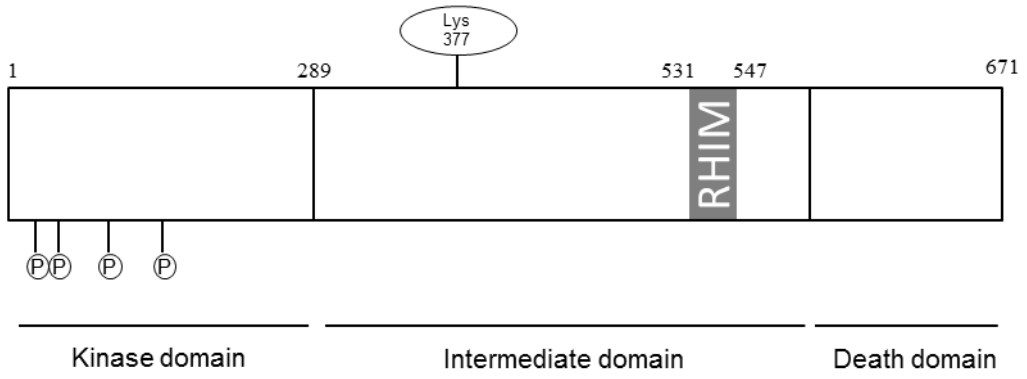

Fig. 2. Domain structure of the RIPK1 protein
RIPK1 protein is comprised of three domains. Each domain length has been indicated with the amino acid residues length shown in numbers. The kinase domain has autophosphorylation sites as indicated. The intermediate domain contains the RHIM motif and Lysine 377 ubiquitination site followed by death domain. RHIM: RIP homotypic interaction motif.

RIPK1 protein which can also be ubiquitinated [62]. These sites have not been extensively studied and should be explored in future. The death domain is the shortest domain of RIPK1 protein, ranging from 583 to 671 residues. Although small in size, this domain serves as a major docking site for other proteins to bind to RIPK1 to conduct ligand-mediated cell survival or death signaling [40, 63, 64].

1.2.2 Post-translational modifications of RIPK1

The three dimensional structure of proteins is known to be changed by post-translational modifications [65]. These modifications not only are important for making the protein suitable for binding to other proteins, but also accelerate the downstream signaling process mediated by the protein [66]. Important post-translational modifications are phosphorylation, acetylation, ubiquitination, sumoylation etc. As described above, RIPK1 proteins act as an important protein

in cell signaling pathway. The activity of RIPK1 protein can be regulated by post-translational modifications like phosphorylation and ubiquitination [62].

1.2.2.1 Phosphorylation of RIPK1

Soon after its discovery, RIPK1 has become known for both its kinase activity to phosphorylate itself and its role as an adaptor protein in NF-κB-mediated in cell signaling. The kinase activity of RIPK1 is needed for necrotic induction but dispensable for TNFR-induced NF-κB signaling [67]. The major phosphorylation sites of RIPK1 include Ser14/15, Ser20, Ser161 and Ser166, which are considered as autophosphorylation sites [68]. RIPK1 can also be phosphorylated on Ser166, Ser331 and Ser416 sites. However, these sites have not been found to be physiologically relevant and their role in RIPK1 functions should be further investigated [69].

1.2.2.1.1 Positive and negative phosphorylation of RIPK1

In search of potential phosphorylation sites of biological significance, various phosphorylation sites of RIPK1 have been mutated and the kinase or the necroptotic activities of the mutants have been measured. The mutation of the potential S161 site to alanine caused only a 20% reduction in kinase activity and did not inhibit the necroptotic potential of RIPK1 [70]. However, S161A-RIPK1 decreased the ability of necrostatin-1 (Nec-1) to inhibit RIPK1 kinase activity. Collectively, most of the phosphorylation sites show additive effect on the kinase activity of RIPK1, negating the possibility of a single site as the sole important phosphorylation site. Interestingly, although Ser89 site has not been defined as a phosphorylation site of RIPK1, mutational analyses suggest that this site serves as a potential inhibitor of the kinase activity of RIPK1. S89A-RIPK1 mutant showed increased kinase activity for downstream signaling and

necrosome formation, indicating that the phosphorylation at residue 89, if occurs, may negatively regulate RIPK1 kinase activity [69, 70].

1.2.2.1.2 Phosphorylation and necroptotic signaling of RIPK1

Being discovered in 1995, the initial assessment about RIPK1 considered this protein to be only effective in apoptotic cell death. However, RIPK1 was also an important mediator of caspase independent non-apoptotic cell death pathway [71]. As this form of cell death had necrotic morphology but showed regulated cell death like apoptosis, it was coined as "necroptosis" [72]. This form of cell death distinguishes itself from apoptotic cell death by its inflammatory characteristics of necrotic cell death. The kinase activity of RIPK1 is not important in NF-κB signaling [73] but indispensable for the downstream cell death signaling [74, 75].
To find out what other kinase proteins are also involved in necroptotic signaling, researchers from three individual laboratories performed siRNA-based screening against known kinase genes along with inducing cells by necroptotic signaling. The studies found out that RIPK3 was also a major player in this cell death pathway, which concurrently worked with RIPK1 as a binding partner [76-78]. It was observed that RIPK3 is an upstream regulator of RIPK1 since it can phosphorylate RIPK1 [77].

1.2.2.1.3 Downstream effectors of RIPK1 phosphorylation

Although it was known that the phosphorylation event of RIPK1 and RIPK3 initiates the necrosome formation; until recently, the downstream events of RIPK1 and RIPK3 mediated signaling pathway have not been defined. Sun et. al. performed immunoprecipitation with anti-

RIPK3 followed by mass spectrometry of the bands in silver staining and found the presence of mixed lineage kinase domain like protein (MLKL) in the complex [79]. Further studies also

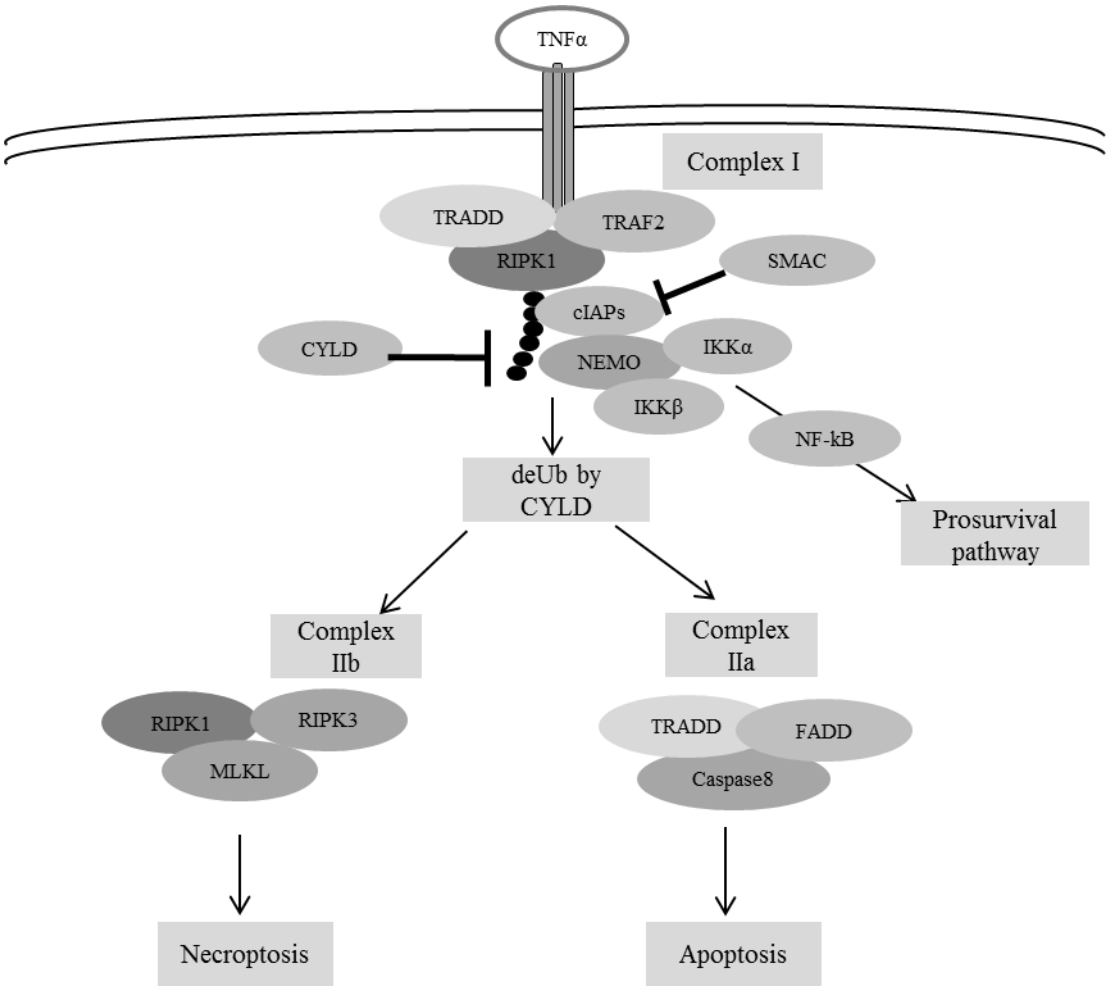

Figure 3: Apoptotic and necroptotic cell death induction by TNFα and RIPK1
Upon binding to its ligand-TNFα, TNFR recruits TRADD, TRAF2 and RIPK1 to form complex I. RIPK1 gets ubiquitinated by cIAPs through K63 linked ubiquitination. This can be inhibited by second mitochondria derived activator of caspases (SMAC) and can also be deubiquitinated by cylandromatosis (CYLD). This complex I further recruits inhibitor of nuclear factor kappa B kinase (IKK) subunits, IKKγ/NEMO (NF-κB essential modulator), to initiate NF-κB mediated cell survival signaling. After getting deubiquitinated by CYLD, RIPK1 binds to caspase-8 to induce apoptosis by forming complex IIa. If caspase-8 activity is inhibited, RIPK1 can bind to RIPK3 and initiate necroptotic cell death by forming complex IIb.

confirmed the presence of MLKL protein in RIPK1-RIPK3 complex [80, 81]. The fact that the kinase domain of RIPK3 was required for the binding to MLKL proteins emphasized the importance of MLKL as a potential target for phosphorylation by RIPK3 protein. *In vitro* phosphorylation assays also confirmed that MLKL got phosphorylated by RIPK3, but not by RIPK1 [82]. The major phosphorylation sites of murine MLKL are Ser345, Ser347 and Thr349, which correspond to human Thr357 and Ser358 sites [83, 84]. A potential inhibitor of necroptosis, necrosulfonamide, was found to bind to MLKL and inhibit necroptosis through the binding, further confirming that RIPK3-MLKL interaction drives necroptosis. After phosphorylation, RIPK3-MLKL complex is translocated to the membrane and causes membrane depolarization. The phosphorylation of MLKL is indispensable in membrane translocation since the mutant proteins were unable to translocate to the plasma membrane and induce necroptosis [80].

1.2.2.2 Acetylation of RIPK1

Acetylation of protein refers to the addition of an acetyl group to mostly lysine residues of the protein. It is a major post-translational modification for nuclear proteins like histones and transcriptional regulators [85]. Most of the proteins involved in energy metabolism pathways like glycolysis, TCA cycle, urea cycle, chromatin remodeling, cell cycle, splicing, nuclear transport, actin nucleation, and mitochondrial metabolism etc. are known to be acetylated, which renders this modification a major role in metabolic pathways [86]. Histone modifications by acetyation also change nuclear conformation, thereby influencing transcription. As acetylation changes the activity and stability of these enzymes, it is as important as other post-translational modifications like phosphorylation or ubiquitination. On the other hand, deacetylation of proteins is also

important as the event also changes protein's structure and shape and thereby, changes their functions. Sirtuin 2 (SIRT2) is a nicotinamide adenine dinucleotide (NAD+) dependent deacetylase that was suggested to play an important role in necroptotic signaling process. Narayan et.al. initially showed that SIRT2 binds to RIPK3 and deacetylates RIPK1, which was required for the formation of RIPK1-RIPK3 complex to induce necroptosis. They also showed that SIRT2 inhibitor blocked RIPK1-RIPK3 complex formation and necroptotic induction and that SIRT2-/- cells were not responsive to necroptotic cell death induction, suggesting that SIRT2 was required for necroptosis [87]. However, their work was challenged by multiple research groups who failed to reproduce the data. Additionally, SIRT2 -/- mice died of necroptotic cell death after tail-vein TNFα injection, suggesting that SIRT2 is dispensable in necroptotic cell death [88]. This controversial issue was addressed by a more recent paper showing that SIRT2 was indeed required for necroptosis. However, this group also introduced another member, SIRT5, of the same SIRT family as a potential protein to induce necroptosis redundantly with SIRT2. The reason that SIRT2 knockdown did not inhibit necroptosis in some cell lines was the functional redundancy played by SIRT5, which was initially ignored. This might also explain why SIRT2-/- died of necroptotic cell death after TNFα injection [89].

1.2.2.3 Ubiquitination of RIPK1

RIPK1 was first implicated in TNFα-stimulated NF-κB activation. Since RIPK1 itself is a kinase, its phosphorylation activity towards itself or other proteins was first assumed to be the activating factor. However, RIPK1 was also assumed to have further covalent modifications other than phosphorylations for several reasons. First, the phosphorylation of inhibitor of kappa B (IκB) kinase (IKK) was not efficient by RIPK1 itself. On the other hand, kinases present in the

whole cell complex were more capable of phosphorylating the IKK proteins. Therefore, RIPK1 was not involved in phosphorylating IκB kinase proteins. SDS-PAGE analyses of the TNFR complex also showed a ladder-like appearance of RIPK1 protein, which is a feature of ubiquitinated protein. Overexpression studies of RIPK1 in Jurkat cells stimulated with TNFα followed by immunoprecipitation of RIPK1 showed that RIPK1 gets ubiquitinated. Of the five consecutive lysine residues in the intermediate domain of RIPK1, each one was mutated to find out the specific ubiquitination site. The analyses showed that RIPK1 gets exclusively ubiquitinated at the lysine 377 site [60]. Mutational studies with of mutant ubiquitins showed that RIPK1 gets ubiquitinated through K63-linked ubiquitin chain. This polyubiquitination chain of RIPK1 recruits the member of IKK family, IKKγ or NEMO. Both the polyubiquitination of RIPK1 and the binding of NEMO are important for NF-κB activation. Polyubiquitination of RIPK1 by K63-linked chain also recruits and activates transforming growth factor beta (TGFβ) activated kinase (TAK1) protein through TAK1 binding protein (TAB) TAB2 and TAB3, which preferentially bind to K63-linked polyubiquitnated RIPK1 [71].

1.2.3 Ubiquitination in NF-κB signaling

Addition of ubiquitin to protein is called ubiquitination or ubiquitylation. Ubiquitin is a small protein of 76 amino acid residues and of 8.5kDa in molecular weight. It is a highly conserved protein found to be unique in eukaryotes [90-92]. The C-terminus of the ubiquitin covalently linked to the N-terminal end or the N-group of a lysine residue of the substrate. Of the ubiquitin's 76 residues, 7 are lysine residues that can be used to form polyubiquitin chains with other ubiquitin proteins. The ubiquitination process depends on three consecutive steps. 1) ubiquitin activation, 2) ubiquitin conjugation and 3) ubiquitin ligation. The ubiquitin activating

enzyme (E1) catalyzes the reaction of thioester linkage formation between ubiquitin. Ubiquitin conjugating enzyme (E2) catalyzes the binding of activated ubiquitin to itself. In the final step, ubiquitin ligase enzyme (E3) catalyzes the reaction of the binding between the lysine residue of the target protein and the C-terminal glycine residue of ubiquitin [90, 93, 94].

Different types of ubiquitination can occur in proteins. Monoubiquitination occurs when only one ubiquitin protein binds to the substrate protein. Multiple monoubiquitination occurs when ubiquitin binds to multiple sites of the protein. Polyubiquitination occurs when ubiquitin binds as a linear or branched pattern through its 7 lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 or Lys63). Different types of polyubiquitination are defined according to the lysine residues of the ubiquins used for the for4mation of polyubiquitin chains. For example, K48 ubiquitination is called if the connecting ubiquitin is attached to the Lys48 residue of the previously attached ubiquitin, and K63 ubiquitination is used if the incoming ubiquitin is attached to the Lys63 residue of the previous ubiquitin [90, 93]. Different types of ubiquitination are used in different molecular pathways. Monoubiquitination of proteins usually leads to protein trafficking, endocytosis and DNA repair [95]. K48 linked polyubiquitination signals mainly for proteolytic cleavage of the protein whereas K6, K28 and K63 polyubiquitination signals for non-proteolytic activation, such as kinase activation or the activation of NF-κB mediated cell survival pathway [94]. The length and types of the ubiquitin chain determine the outcome of the ubiquitinated protein.

One function of ubiquitination is to make the protein available for binding by other proteins. Proteins bind to the ubiquitinated protein through ubiquitin binding domain (UBD) [96]. UBD comprises of a diverse structure including zinc fingers and plekstrin homology motif,

which allows the flexibility and choice of binding proteins to target ubiquitinated proteins. A single protein can bind to multiple ubiquitination sites of another protein. Alternatively, two different proteins can bind to different ubiquitination sites of the same protein. In both cases, this binding results in the activation of a downstream signaling pathway to amplify the signal. Ubiquitinated proteins signal different outcomes through differently linked chains. Generally, K63-linked ubiquitination signals for cell survival whereas K48-linked ubiquitination targets proteins for proteasomal degradation [97].

1.2.4 RIPK1 ubiquitination is important for cell survival

As RIPK1 deficient mouse embryonic fibroblast (RIPK1-/- MEF) are highly sensitive to TNFα-induced cell death, it underscores the fact that the presence of RIPK1 is important for cell survival [98]. However, whether RIPK1 ubiquitination is also important or not has remained unknown for a while. To investigate this question, RIPK1 ubiquitin mutant construct (RIPK1 K377R) was generated and stably expressed in RIPK1-deficient cells. It was observed that RIPK1-K377R expressing cells were more susceptible to TNFα induced apoptotic cell death than cells expressing wild type RIPK1. Therefore, the ubiquitination of RIPK1 appeared important for cell survival [60].

1.2.5 RIPK1 as a survival protein

Besides being a kinase, RIPK1 can serve as an adaptor protein in cell signaling pathways. As discussed, diverse death ligands bind to their corresponding receptors and recruit adaptor proteins, including RIPK1. RIPK1 serves as the cross-road between the decision of life and death of the cells depending upon the interplay between the cell survival and cell death signaling

complexes [71]. RIPK1 mainly forms complexes with cell surface receptors after stimulation with death ligands. The initial assessment of RIPK1 protein in chemotherapeutic treatment was that, this protein can be used for killing of cancer cells by death ligands. Induction of pro-apoptotic or pro-necrotic complex like “Ripoptosome” or the RIPK1-RIPK3-caspase-8 complex seemed to be a promising target for chemotherapy [99, 100]. RIPK1-/- die shortly after birth through apoptotic cell death in major adipose and lymphoid tissues, indicating the role of RIPK1 in cell survival [98]. This also may be the case in cancer cell survival. Recent studies have shown that RIPK1 is upregulated in melanoma cells than normal skin cells [101]. This upregulation helped the melanoma proliferation since stable knockdown of RIPK1 in those melanoma cells inhibited the proliferation. The survival function is also supported by the fact that ectopic expression of RIPK1 increased melanoma cell proliferation and induced anchorage-independent melanocyte growth. The same group has also shown that RIPK1 is increased in endoplasmic reticulum stress by tunicamycin or thapsigargin treatments, which allows melanoma cells to survive and evade apoptosis by going through autophagy [102]. Knockdown of RIPK1 significantly decreased melanoma cell survival, which corroborated their earlier findings. Another study has also shown that RIPK1 serves as a chemoresistance inducing protein in lung cancer cells. Wang et.al. has shown that in RIPK1 knockdown lung cancer cells, the cytotoxic effect of cisplatin was significantly increased, suggesting that RIPK1 helps cancer cells to survive through chemotherapeutic treatment [103]. Current chemotherapy involves drugs that induce apoptosis in cancer cells by inducing reactive oxygen species (ROS) generation. Cancer cells have evolved mechanisms to subvert the effect of ROS by inducing antioxidant expression like catalase. It has also been observed that in RIPK1 knockdown cells, both the stability and synthesis rate of catalase were decreased. Further investigation in this pathway also led to the

finding that micro-RNA 16a expression was increased in RIPK1 knockdown cells, which targets catalase and degrades the protein. Therefore, this work summarizes a novel role of RIPK1 in cancer cell survival and shows how cancer cells can evade apoptosis through RIPK1 by inducing microRNA regulated catalase degradation.

1.2.6 Regulated necrosis or necroptosis

Apoptosis and necrosis had shown a distinct features in terms of initiation and morphological features. As mentioned earlier, while apoptotic pathway has already been well established to be regulated, necrosis was thought to be a totally unregulated event with physiologically catastrophic consequences. In addition to that, apoptosis and necrosis were thought to be initiated by different inducers until 1988 when Laster et.al. showed that both apoptosis and necrosis could be initiated by a single death ligand, TNF [104]. This group noticed that in response to TNF induction, F17 cells showed apoptotic cell death whereas L-M cells went through necrosis. This indicated that the type of cell death by TNF may depend on the types of cells. It was known that TNF induced necrotic cell death in L929 cells[105]. Interestingly, in the presence of caspase inhibitor, the TNF accelerated necrotic cell death to L929 cells[106]. This led to the notion that necrosis can also be triggered or regulated like apoptotic cell death pathway. However, to show a true regulation of necrotic cell death pathway, proteins that play roles in that event were yet to be identified. In 2000, Holler et. al. first showed that in caspase-8 inactivated T cells, FasL caused necrotic cell death. However, this type of FasL induced cell death was not observed in T cells lacking RIPK1 and FADD proteins [107]. This study first showed that RIPK1 and FADD are required for necrotic cell death pathway. Since this form of

cell death showed necrotic morphology yet showed regulated pathway like apoptosis, Degterev et.al. termed this type of cell death as “Necroptosis” [72].

1.2.6.1 Necroptosis is a kinase-mediated pathway

Myocardial infarction and cerebral ischemia are examples of acute physiological conditions where human tissues have necrotic cell death. As a subset of necrotic cell death is regulated event like necroptosis, a drug preventing necroptosis can be an acceptable treatment method for the patient. In search of a potential target for treating necrotic cell death, Degterev et.al. screened ~15,000 potential compounds and found necrostatin-1(Nec-1) to be a potent inhibitor of necroptotic cell death induced by death ligand [68]. Additionally, Nec-1 did not change the morphology of apoptotic cell death process, confirming that it exclusively works on necroptotic cell death [68, 108]. More importantly, Nec-1 significantly inhibited *in vivo* ischemic neuronal injury, suggesting it to be a potential drug candidate for inhibiting necrotic cell death related diseases [72]. Like other kinase inhibitors, Nec-1 has an indole ring that makes it an ideal kinase inhibitor. *In vitro* kinase assays showed that Nec-1 significantly decreased RIPK1 kinase activity. Structure-activity relationship analyses also concluded that Nec-1 specifically targets RIPK1[68]. Although RIPK1 was originally identified as the key protein in necroptosis, further research showed that necroptosis is indeed mediated by RIPK1 and RIPK3 complex [76-78].

1.2.6.2 RIPK3 is a necroptotic switch

As mentioned earlier, RIPK1 protein can act both in cell survival and cell death pathways. Although the kinase domain of RIPK1 is not required for NF-κB-mediated cell survival pathway, it is required for RIPK1 mediated cell death pathways. Although the kinase

activity of RIPK1 is generally required for necroptotic cell death, it is not the determinant of necroptosis in all cell lines [109]. Therefore, other proteins must be involved in necroptotic induction. It has already been known that z-VAD is a pan caspase inhibitor and, SMAC mimetic (SM) inhibits the anti-apoptotic functions of cIAP proteins [100, 110]. Therefore, treating cells with TNFα and SM induce apoptotic cell death in the absence of z-VAD. If z-VAD is added, caspase mediated apoptotic cell death is inhibited and accordingly, TNFα/SM co-treatment induces necroptotic cell death. A genome wide screening of siRNA pool in FADD-deficient Jurkat and human colorectal carcinoma HT-29 cells showed that the knockdown of RIPK3 also decreased necroptotic cell death induction along with RIPK1 knockdown as a positive control. On the other hand, the knockdown of the other members of RIPK family did not alter cell viability [76]. From previous studies, it was already known that RIPK1 and RIPK3 share common domains and bind to each other [47]. Furthermore, in RIPK3 stable knockdown cell line, TNFα/SM/z-VAD induced necroptotic death was abolished. Together, these data suggest that RIPK3 is the molecular activator of necroptotic cell death. RIPK3 is not expressed in all cell lines. A cellular expression profile of RIPK3 among mouse and human cell lines along with their necroptotic response showed that only those cells that express RIPK3 responded to necroptotic induction [76]. Compared to RIPK3, RIPK1 is ubiquitously expressed in different cell lines, further confirming that RIPK3, not RIPK1, is the molecular switch to necroptosis from apoptosis. The kinase activity of RIPK3 is also important in necroptosis. Although RIPK1 has autophosphorylation capability, no phosphorylation was observed in TNFα-treated RIPK3-/- MEF cell line, indicating that RIPK3 is required for RIPK1 phosphorylation and works upstream of its phosphorylation event [77].

1.2.7 Pathological consequences of necroptotic protein knockout in mice

Functions of proteins may vary in different developmental stages of an organism. This notion was more established when the physiological effect of the knockout mice of different important genes was generated. Kelliher et.al. showed that RIPK1-/- mice died perinatally within 3 days of birth [98]. This showed that RIPK1 is important for perinatal development of mice. This study is in the striking contrast with the established idea that RIPK1 is required for caspase-8 mediated apoptosis and RIPK3 mediated necroptosis [100]. However, further knockout models show that during development, RIPK1 works to inhibit both cell death pathways [111, 112]. As mentioned earlier, RIPK1-/- mice do not survive beyond 3 days of birth. On the other hand, knock-in mice carrying the kinase-dead mutant (D138N or K45A) of RIPK1 survive to adulthood [113, 114]. Therefore, it is not the kinase activity, rather than the structural role of RIPK1 that is required for the survival of the embryo and the development of mice. Tissues of RIPK1-/- mice show apoptotic cell death in histological assays [98]. It is already known that although RIPK3-/- mice are viable, RIPK1(-/-)/RIPK3(-/-) double knockout mice show perinatal death. As observed in earlier studies, caspase-8 null mice died at embryonic day 10.5. RIPK1 and caspase-8 double knockout mice survived further and died within 1 day of birth [115, 116]. This indicates that RIPK1 is not responsible for the death of caspase-8 null mice at E10.5. On the other hand, RIPK1/RIPK3/caspase-8 and RIPK1/RIPK3/FADD triple null mice survive. This supports that RIPK1 may be required to prevent the FADD and caspase-8 mediated apoptotic cell death at the time of birth [116].

Although RIPK1 and RIPK3 work together in necroptotic cell death pathway, the role of RIPK3 in developmental pathway seems different from RIPK1 [116]. Different from RIPK1-/- mice, RIPK3-/- mice survive to adulthood [114]. However, this type of mice is not responsive to

in vivo necroptotic induction, corroborating the role of RIPK3 in necroptosis [117]. In addition, whereas RIPK1 kinase dead mutant mice survived to adulthood, RIPK3 D161N (kinase dead mutant) mice did not. Interestingly, RIPK3 D161N (kinase dead mutant)/caspase-8-/- mice survived to adulthood [114, 118]. Since caspase-8-/- mice die at the same gestation period, the survival of RIPK3 D161N (kinase dead mutant)/caspase-8 -/- mice suggests that RIPK3 and its kinase activity are responsible for the death of caspase8 deficient embryos [114].

1.2.8 *In vitro* and *in vivo* induction of necroptosis

In cells, cIAP protein family acts as a checkpoint to control unintended apoptosis. cIAPs have ubiquitin ligase activity and keeps RIPK1 K63 ubiquitinated so that NFkB cell survival pathway is active. However, if cIAPs are inactivated by intracellular proteins or chemicals like SM, this checkpoint is compromised. Therefore, etoposide or SM can inhibit cIAPs and turn the TNFα-mediated cell survival pathway into cell death pathway. This cell death pathway can be both apoptotic or necrotic. In addition to TNFα, SM and z-VAD can inhibit caspase-8 mediated apoptotic cell death and direct the cell death to necroptosis. Therefore, the combination of TNFα, SM and z-VAD is used to induce necroptotic death *in vitro* in cells. However, in cell lines like L929 where TNFα is produced in an autocrine manner, TNFα alone is sufficient to induce necroptosis without z-VAD and SM. On the other hand, induction of necroptosis *in vivo* in mice is pretty straightforward. It has been observed that injecting TNFα in mice can provoke systemic inflammation as compared to systemic inflammatory respiratory syndrome (SIRS). However, addition of z-VAD sensitizes mice to TNFα more than TNFα alone [117, 119].

1.2.9 Necroptosis and inflammation

Inflammation is considered to have four hallmark signs as: color, calor (heat), rubor (redness), tumor (swelling), and dolor (pain) [120, 121]. Although inflammation was initially thought as a result of injury, now it is considered as a process of tissue repair. Rather than just mechanical insults to body, inflammation is now being considered as an event mediated by host cell itself [121]. Inflammation and cell death are integral to each other since the same receptor proteins involved in inflammation are responsible for cell death signaling as well. On the other hand, cell death processes lead to inflammation as well. The major host cell mediated inflammatory mechanisms include the rupture of blood and lymphatic vessels [122] and the release of host cell secreted molecules like hydrogen peroxide to induce inflammation on neighbor cells [1, 123]. Dying cells in tissues secrete cytokines including TNF, CD95L (FasL), TRAIL, interferon, etc., which bind to neighbor cells and decrease the cell survival signal to initiate inflammatory signaling, leading to cell death [120]. Of the known cell death pathways, apoptosis has more of a positive and resolving role in suppressing inflammation. It has been observed that neutrophils in inflammatory sites are engulfed by macrophages to reduce inflammation [124]. In addition to that, apoptotic cells expose phosphatidyl serine that helps phagocytes to be attracted to engulf the dying cells [125-127]. However, the role of apoptosis in inflammatory is not always suppressive. During late apoptosis, plasma membrane rupture is also evident. Therefore, the release of damage associated molecular patterns (DAMPs) also causes inflammation [128]. It is also known that apoptotic cancer cells after chemotherapeutic treatment can cause inflammation [129]. However, necroptosis has always been thought to be more inflammatory than apoptosis since the plasma membrane rupture causes massive DAMP to be released, which attracts inflammatory cells [130]. Both apoptotic and necroptotic cells are

eventually phagocytosed. However, given the amount of DAMPs secreted outside, it is clear that necroptotic cells trigger more inflammation. Recent study shows that necroptosis in keratinocytes is more inflammatory than apoptosis [111].

1.3 Nuclear receptor superfamily

From single cell bacteria to about 37 trillion cell-containing organism like human, gene expression regulation is an integral part of the central dogma of life [131]. Nuclear receptors have been an indispensable playmaker in this gene regulation having their ability to bind directly to DNA. Soon after the discovery of DNA structure, the concept and discoveries of gene regulators started gaining scientist's attention, and the field of research on nuclear receptors is growing with a fast pace since then [132]. It has been almost thirty years since the first isolation of the cDNA of glucocorticoid, thyroid and estrogen receptors [133-135]. Since then, significant discoveries have been accomplished in nuclear receptors. These receptors along with their ligand showed that chemically different ligands can bind to structurally related receptors. Specially, the first discovery of receptor for vitamin A gave a clue to scientists about the reality of a nuclear receptor superfamily [136, 137]. Along with the ligands for receptors, the receptor's ability to form homo or heterodimers gave the whole nuclear receptor superfamily an immense opportunity to play a role in diverse molecular pathways including homeostasis, reproduction, metabolism and development [132, 138].

Although initially thought merely functioning as transcription factors, nuclear receptors also function as signal transducers. Different organisms contain different number of phylogenetically related nuclear receptor proteins. The fly Drosophila melanogaster contains 21 genes, human contain 48 genes and surprisingly, Caenorhabditis elegans contains 270 genes

[139-141]. The high number of C. elegans is possibly due to the proliferation and diversification of the genes [141]. The ligands of the nuclear receptor are fat-soluble lipophilic steroid hormones having a high affinity to their receptors and originated exclusively from endocrine sources. Unlike their peptide hormone counterparts, these steroid hormones can get through the plasma membrane's lipid bilayer due to their lipophilic nature and bind to their respective receptors in the cell [142-144].

1.3.1 Nuclear receptor family classification

The nuclear receptor superfamily has been divided into 4 classes according to their DNA binding characteristics and the mode of dimerization.

Type I receptors are classical steroid hormone receptors such as glucocorticoid receptor (GR), mineralocorticoid receptor (MR), estrogen receptor (ER), androgen receptor (AR) and receptor progesterone (PR). These receptors form homodimer and bind to palindromic sequences with inverted repeats separated by 3 nucleotide base spacing. They control different metabolic and developmental pathways such as reproduction, carbohydrate metabolism, sexual differentiation and electrolyte balance [145-148]. ER has two subtypes- ERα and ERβ. Both proteins share similar regions with having a variable N-terminal region. Although they bind to similar DNA sequences, both proteins interact with different sets of proteins. Their tissue distributions are also different as well. Whereas ERα is more localized in major female organs like ovary, vagina, uterus, mammary gland etc. ERβ is found in ovary, prostate, spleen, testis, lung, thymus and hypothalamus [148]. PRs are expressed mainly in female reproductive tissues and central nervous system. Two isoforms of PR, named PR-A and PR-B are generated from two different promoters. PR-B is stronger as a transcription factor than PR-A. They differ only in the

N-terminal region; however, they have their exclusive as well as common target genes. For example, PR-B works on the mammary gland development whereas PR-A works on uterus [149]. Similar to ER and PR, GR also resides in the cytoplasm with chaperones. GR mainly works in liver, pancreas, muscle and adipose tissues. 5 different GR protein subtypes - GRα, GRβ, GRγ, GR-A and GR-P arise due to alternative splicing of the mRNA [150]. GRα increases cell's sensitivity to glucocorticoid hormone whereas GRβ decreases it. The MR is a 107KD protein which plays a major role in fluid transport and blood pressure control [151]. In addition to kidney, other organs also express MR and it is evident that MR acts on hippocampus, endothelium, heart as well. AR gene encodes a 110 KD protein and this protein binds to its cognate ligands, specially testosterone and 5-α-dihydrotestosterone. AR plays a major role in the development of male reproductive systems. AR presence has also been observed in female tissues including ovaries, uterus, skin, adipose and brain etc. [152].

Type II receptors are the group of nuclear receptors that bind to and form heterodimer with 9-cis retinoic acid receptor (RXR). The receptor heterodimers bind to DNA elements of direct repeat with different spacing sequences. The binding partners of RXR include trans retinoic acid receptor (RAR), thyroid hormone receptor (TR), vitamin D receptor (VDR) and ecdosyne receptor (EcR). These receptors work both on endocrine and lipid sensing receptor pathways. They also serve as the sensor for metabolites [153]. Type II receptors also have some previously known orphan receptors. These partner receptors of RXR heterodimers were put in the orphan receptor family since their ligands were unknown. However, they are now called adopted orphan receptors since their ligands have been discovered [154, 155]. These receptors include peroxisome proliferator-activated receptors (PPARs), oxysterols (LXR), constitutive androstane receptor (CAR), bile acids (FXR). PPARs mainly function in homeostasis in

development and inflammation as well as lipid sensing metabolism. Three major subtypes of PPAR have been identified: PPARα, PPARβ/δ and PPARγ [156, 157]. PPARα and γ are found in liver and adipose tissue, however, PPARβ/δ is found in almost all tissues. PPARα play a crucial role in cardiac lipid homeostasis by regulating the gene expression of fatty acid oxidation [156, 157]. PPARγ functions to regulate systemic lipid and glucose level [158-160]. It has been observed that the mutation in PPARγ gene results in diabetes, insulin resistance and hypertension [161]. On the other hand, PPARδ works as a sensor for very low density lipoprotein in macrophages [162]. Liver X receptors (LXRα and LXRβ) regulate the cholesterol homeostasis and lipid metabolism in skeletal muscle. These receptors are generally known as the cholesterol sensor [163].

Type III receptors include mainly the orphan receptors whose ligands are still unknown. Typically they form a homodimer with each other.. On the other hand, Type IV receptors bind to the core site as monomers. Rev-erbα , Rev-erbβ and NGF-1β are the examples of this receptor family. They also take part in lipid homeostasis and skeletal muscle function [164].

1.3.2 Domain structure of nuclear receptors

During evolution, nuclear receptors have assumed different lengths due to sequence mutations. However, they follow a common pattern in terms of their domain structure [138, 165]. Typically, a nuclear receptor has six domains, they have been given the name of A to F domains from the N to C terminus. The A/B region is highly variable in receptors. The C domain is also called DNA binding domain (DBD). D domain is the hinge region that separates the E domain or the ligand binding domain (LBD) from the C domain. The LBD is followed by the variable F domain [166].

The N terminal A/B region is very weakly conserved among species. This region can function autonomously as an activation function that binds to the co-activators [138] and hence also called activation function-1 (AF-1). However, their activity may also be regulated by ligands bound to the receptor as well. The most conserved domain of nuclear receptor is the DBD. As the name implies, DBD binds to specific sequences of the DNA called hormone response element (HRE). It has a length of 66 highly conserved residues consisting of two zinc finger motifs, two α-helices and a COOH extension [166]. The first zinc finger motif contains a region called the proximal box or P-box that controls the high affinity recognition of the HRE. The second zinc finger contains a distal box or D-box that determines the receptor dimerization and half-site spacing [138, 167]. Following the DBD, the D region is a poorly conserved region of the nuclear receptor. It serves as a hinge region between the DBD and LBD, thus allowing the two domains to adopt different conformation. It also contains a nuclear localization signal and may contain protein-protein interaction sites. The LBD is also a conserved region of the nuclear receptors, though it is not as strictly conserved as the DBD. Whereas the DBD mainly controls the binding of the receptor to the DNA, the LBD has multiple functions with its different small regions in it. It contains a dimerization region that mediates the dimer formation with another nuclear receptor's LBD, followed by a ligand binding pocket for the lipophilic ligand. LBD also contains a co-regulator binding region with which it binds to either positive or negative coregulators, including the activation function-2 (AF-2). It is an α-helical domain which controls ligand dependent transcription and co-activator recruitment [166].

1.3.3 Mechanisms of nuclear receptor action

Nuclear receptors got activated in three major steps. When the receptors are unliganded, they bind to a co-repressor molecules that have histone deacetylase (HDAC) activity. After the ligand binding, the co-repressor is dissociated from the complex and the receptor binds to co-activators that have histone acetyltransferase activity (HAT) [167, 168]. The final step includes the interaction between co-activators and components of the RNA polymerase II complex to activate gene transcription [167].

1.3.4 Vitamin D and vitamin D receptor

Long before vitamin D was discovered, scientists understood with their experiences with diseases and diet that special organic micronutrients are required to prevent diseases like scurvy, rickets, beri-beri etc. [169]. It was Casimir Funk who first theorized that some food has "vital amine" in them which helps to maintain healthy life [170]. Later, it was discovered that a small water soluble molecule can prevent specific neurological diseases. Although not necessarily carrying amino group in them, scientists used the visionary idea of Funk and termed these factor as "Vitamin" [171, 172]. Soon after the first discovery, fat soluble molecules were also discovered and all of them were given the name of vitamin-A, B, C etc. Within a decade, another fat soluble molecule was discovered which could cure rickets in animal. This new vitamin was called, "vitamin D". Since then, several chemicals have been given the name vitamin D (VD) as vitamin D1, vitamin D2, vitamin D3 etc. However, vitamin D1 was found to be an artifact and was later removed from the list [172]. Soon, it was also found that sunlight can cure rickets [173]. However, it was soon realized that sunlight actually helps producing vitamin D3 (VD3) in animal skin [174]. Therefore, VD3 is actually a pro-hormone, not a vitamin

in true sense. VD3 is formed with the help of sunlight from its precursor, 7-dehydrocholesterol in the skin. Later on, VD3 is hydroxylated in the liver and kidney to become 1, 25 dihydroxyvitamin D3 (1,25D3), the active hormone. Although it was discovered that VD3 can improve bone mineralization and calcium absorption, directly adding VD3 to the VD3 deficient animal's serum did not improve the bone mineralization. Therefore, it was assumed that something else carried the signal of VD3 to induce the cell for VD's cellular activity [172]. Later it was discovered that VD3 acts through a receptor protein called vitamin D receptor (VDR) [175], a type II steroid nuclear receptor that forms a heterodimer with RXR for its function. As shown in Figure 4, VDR is comprised of four domains; the N-terminal DNA binding domain (DBD), a hinge region, the C-terminal ligand binding domain (LBD) and a small activation function-2 domain [176]. The DBD contains two zinc finger DNA binding motifs like other nuclear receptors. The hinge region allows VDR to adopt sufficiently different conformations through its flexibility [177].

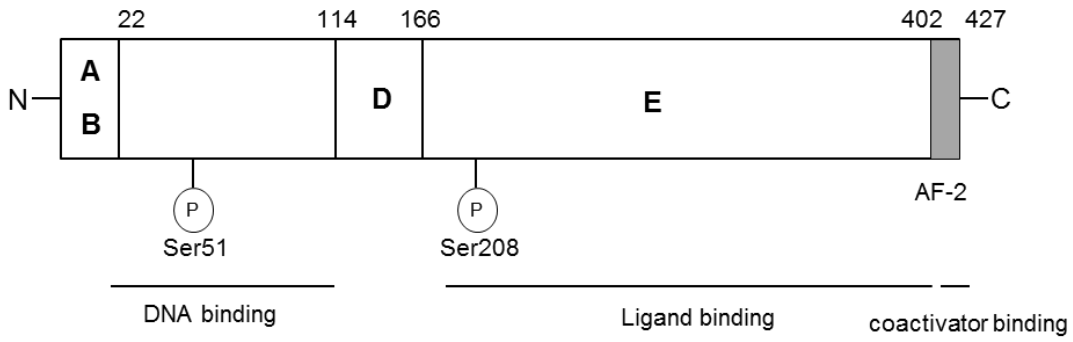

Figure 4: Domain structure of VDR

VDR contains four distinct domains. The DNA binding domain (DBD), the hinge region, the ligand binding domain (LBD) and the co-activator binding domain. DBD and LBD each has a major phosphorylation site.

1.3.4.1 Co-regulators of VDR

Previously, it was thought that nuclear receptors bind to their cognate ligands and execute their functions. However, it was soon revealed that the functional activity of these receptors rely much on the dimerization as well as other proteins. These proteins are called co-regulators [178]. As VDR has diverse functions in regulating gene expression, the capacity to work on regulating different genes relies on its ability to bind with diverse co-regulators. These co-regulators have VDR-interacting domains through which they bind to the DNA bound and ligand activated VDR protein to recruit other proteins and to stimulate or suppress transcription. They can also recruit other proteins with enzymatic activity, such as histone acetylase/deacetylase or RNA polymerase II. The first co-activator identified for VDR was steroid receptor co-activator-1(SRC-1) [179]. CREB binding protein (CBP) and p300 were also found as VDR co-activators to stimulate the transcription of VDR responsive genes. On the other hand, VDR also recruit co-repressor proteins such as silencing mediator of thyroid and retinoid receptors (SMRT) and nuclear receptor co-repressor (NcoR), which can inhibit the expression of genes [180]. Such co-repressors recruit histone deacetylase proteins that will repress the expression of target genes. However, there are also reports that these co-repressors do not associate with VDR as strongly as with other nuclear receptors [181].

1.3.4.2 Post-translational modifications of VDR

Like other proteins, VDR is also subjected to post-translational modifications and these modifications regulate VDR activity. *In vitro* and *in vivo* studies have shown that VDR gets phosphorylated at various sites. Whereas the phosphorylation at Ser-51 site by protein kinase C (PKC) negatively regulates VDR function [182], phosphorylation at Ser-208 position by casein

kinase II (CKII) enhances VDR transcriptional activity [183] . However, both positive and negative phosphorylations have been shown to be increased by stimuli given to cells. Therefore, the phosphorylations may be cell type specific. Other than phosphorylations, studies have also shown that plasma toxins from patients are able to bind to VDR and inhibit the VDR function by disrupting VDR-RXR heterodimer formation [184].

1.3.5 Mechanisms of VDR action

Subcellular localization analyses have shown that VDR is mostly distributed in the nucleus. A small portion of VDR resides in the cytoplasm [185-187]. Upon active ligand (1,25D3) binding, conformational changes occur in VDR, which makes the ligand binding domain more accessible to RXR. RXR has several isoforms and VDR is known to bind with all of them [188]. Unliganded VDR may also bind to the DNA, but this binding is loose. The VDR-RXR heterodimer is known to bind to the direct repeat 3 site where the recognition sequences or half sites are separated by three nucleotides. The VDR-RXR heterodimer then binds to transcription factor II protein as well as TATA binding protein to promote the formation of the preinitiation complex for transcription [189, 190]. To activate the gene expression, co-activators like SRC-1, CBP/p300 etc. are required. As previously discussed, these proteins have histone deacetylase activity which helps to unwind the DNA molecule and recruit RNA polymerase II to initiate transcription [189-191]. To repress gene expression, VDR-RXR can recruit co-repressors as previously described, which can deacetylate histones and stop the fromation of the preinitiation complex. Furthermore, the VDR-RXR heterodimer can also bind to the IL-2 promoter and hinder the binding of the nuclear factor of activated T-cells (NFAT) transcription factor, thereby, repressing the transcription [192].

1.3.6 Chemical analogs of vitamin D

1,25D3 plays a very important role in calcium homeostasis [193]. It has already been demonstrated that 1,25D3 can reduce the growth of cancer cells both *in vitro* and *in vivo* [194-196]. Therefore, it can be used as a potential drug for chemotherapy. However, studies have shown that it can also induce hypercalcemia if administered to the patient regularly with doses required for chemotherapy [197]. Therefore, it is important to design and synthesize analogs of 1,25D3 which will retain the therapeutic potential with less calcemic activity. Until now, thousands of chemical analogs of 1,25D3 have been developed [198]. However, only a handful of them seem to be usable as drug for human and animal use. The first set of analogs is alphacalcidol, doxercalciferol and dihydrotachysterol etc. [199, 200]. These drugs require further metabolic conversion to become fully active. A promising drug for hyperparathyroidism is maxacalcitol. It has much less side effect than 1,25D3 [201]. Seocalcitol or EB1089 has also become popular with *in vitro* experiments and have already been in the clinical trial to treat cancer patients [202]. This drug can inhibit growth and metastasis by inducing apoptosis in cancer cells [203]. Calcipotriol is another drug of the new generation which has been used to treat psoriasis [204]. It is also a promising drug for pancreatic cancer treatment [205].

1.3.7 Non-genomic action of 1,25D3

Due to the transcriptional and translational events exerted by 1,25D3 and VDR, the genomic response by the hormone is slow. However, 1,25D3 also induces nongenomic responses that occur quickly. Studies have shown that 1,25D3 can induce a fast formation of cAMP, calcium, and inositols. These secondary messengers molecules can activate Ca^{2+} channel. PKC, PKA, and MAPK pathways [206-210]. It can also induce rapid secretion of insulin by pancreatic

β cells by opening the Ca^{2+} and Cl^{-} channel [211]. A recent study has shown that 1,25D3 can also induce exocytosis by Sertoli cells in testis [212].

1.3.8 Vitamin D and cancer

1,25D3 or calcitriol is known to play a major role in bone mineralization through maintaining the calcium and phosphate metabolism. Different factors like living places (in terms of altitude), food habit, and exposure to sun determine the levels of circulating vitamin D in blood or in tissue and according to these factors, a person can have sufficient vitamin D or can be vitamin D deficient [213-215]. It is worth mentioning that the measuring standard of vitamin D is the circulating 25(OH) vitamin D3, 25(OH)D3, in the blood serum. The set point of deficiency of 25(OH)D3 is variable to different communities. The institute of medicine (IOM) sets the standard for deficiency as ≤20 ng per mL (50 nmol per L) and the endocrine society set the standard as 30 ng per mL (75 nmol per L) [216]. The deficiency determines the susceptibility of the person to bone related diseases like rickets. It is already known that a vast majority of people around the world, including those living in places with adequate sunlight, are vitamin D deficient [214]. Consequently, the prevalence of bone related diseases is also quite common. However, research works showed that vitamin D can also play an important role in preventing different diseases other than skeletal problems [217]. This is understandable because most cells in our body express VDR [218]. Therefore, vitamin D and VDR must have additional roles other than bone mineralization. 1, 25D3 has been reported to reduce the risk of diseases like hypertension, heart disease, neurological disease, diabetes, multiple sclerosis, asthma, autoimmune disease and more importantly, cancer [217].

The first *in vitro* study of the anticancer activity of 1,25D3 was shown by Colston et.al. This group showed that 1,25D3 can suppress the growth of malignant melanoma cells in culture [219]. Concurrently, another group also showed that 1,25D3 can differentiate mouse myeloid leukemia cells into macrophages [220]. Numerous studies have been done on the anticancer role of 1,25D3 and it is now established that it can suppress cancer growth both *in vitro* and *in vivo* [221-229]. Studies have shown that calcitriol or 1,25D3 can arrest breast cancer cell growth at G0/G1 stage by increasing the expression of p21, which can decrease the activity of cyclin dependent kinase activity. It has also been shown that EB1089 can decrease the expression of c-myc oncogene at the mRNA level and aromatase expression by directly inhibiting the mRNA synthesis [224]. Although more potent in estrogen sensitive cell lines, 1,25D3 can also decrease the growth of estrogen insensitive cancer cells as well [230]. EB1089 in combination with tamoxifen have exhibited a synergistic effect on reducing breast cancer cell growth [231]. Therefore, a combination therapy may be a better treatment for breast cancer.

1,25D3 is also known for its anti-inflammatory properties. Patients with acute or chronic inflammation have an increased propensity of developing prostate cancer [232]. Therefore, vitamin D might also play role in suppressing prostate cancer. Studies with prostate cancer cells have shown that 1,25D3 reduces the expression of proinflammatory cytokine like interleukine- 6 (IL-6). 1,25D3 induces the expression of MAP kinase phosphatase 5, which inhibits p38 activation by dephosphorylating p38 and ultimately decreases IL-6 production [233]. As IL-6 is directly involved in prostate cancer development, 1,25D3 treatment can hereby decrease prostate cancer progression. Furthermore, 1,25D3 analog BXL-628 inhibits prostate cancer growth and inflammation. This has already been tested positively in animal models as well [234]. 1,25D3 is known to modulate microRNA expression as well. In a recent study, combined treatments with

1,25D3 and testosterone of LNCaP prostate cancer cells, have shown that they can modulate the expression of fifteen microRNAs [235]. Upregulated micro-RNAs include miR-22, miR-29ab, miR-134, miR-1207-5p and miR-371-5p. These microRNAs target mRNAs with known fucntions in cell cycle progression, lipid synthesis and calcium homeostasis etc. In addition to the upregulation, this treatment also downregualted the expression of micro-RNAs like miR-17 and miR-20a, which are known as oncogenic micro-RNAs that promotes tumor progression. Therefore, combination of androgen and 1,25D3 can be an effective chemotherapy for prostate cancer patients. In addition, the expression of miR-100 and miR-125-b are downregualted. In previous studies, these two miRNAs are demonstrated to have a tumor suppressive role [236]. *In vivo* treatment of prostate cancer patients with 1,25D3 also changed the levels of these micro-RNA, thereby showing a promising role of 1,25D3 as an orally administrable drug to treat prostate cancer patients [237].

The role of vitamin D in suppressing ovarian cancer mortality rate has also been reported [238]. Further studies showed that vitamin D can play an important role in ovarian cancer prevention [239]. A randomized study to link single nuclear polymorphism and vitamin D deficiency related ovarian cancer susceptibility revealed that low circulating vitamin D is associated with increased risk of ovarian cancer [240]. Studies have also shown that daily intake of vitamin D could reduce the risk of incidence and death by ovarian cancer [214].

Our published studies in ovarian cancer have shown that 1,25D3 inhibits cancer growth by arresting cells at specific cell cycle checkpoints [194, 241, 242]. Further analyses have demonstrated that 1,25D3 induces microRNA target genes to decrease telomerase expression for cell death induction [196, 243] and to inhibit leptin and estrogen-induced tumor growth [195], suggesting that 1,25D3 has a particular role in lowering obesity-associated cancer risks in

women. More recent studies have shown that ovarian cancer invasion is suppressed by 1,25D3 through the cooperated actions of epithelial and stromal VDR [244]. Overall, these studies project VDR as a new therapeutic target for ovarian cancer intervention.

1.4 Central hypothesis

The main objective of this project is to investigate how 1,25D3 and TNFα crosstalk to control cell death pathways and how the cell death programs can be developed to benefit cancer intervention. Our central hypothesis is that, 1,25D3 and TNFα can regulate each other's cell death signaling pathway through a novel VDR-RIPK1 complex. This hypothesis has been formulated based on our preliminary data that 1,25D3 downregulated NF-κB regulated gene expression and that both TNFα and RIPK1 decreased VDR activity. Co-immunoprecipitation analyses showed that VDR and RIPK1 formed a protein complex, which was inducible by necroptotic stimuli. The rationale for the proposed research is, once it is known how 1,25D3 and TNFα regulate each other's signaling, new and innovative approaches can be developed to improve cancer therapy.

CHAPTER 2:

MATERIALS AND METHODS

2.1 Chemicals and antibodies

Anti-Flag antibody (F7425), anti-Flag M2 affinity gel (A2220), fetal bovine serum (FBS) (12306C), MG132 (C2211), and protease inhibitor cocktail (11836170001) were purchased from Sigma-Aldrich (St. Louis, MO). Anti-HA antibody (PRB-101P) was from Covance (BioLegend, San Diego, CA). Anti-VDR (C-20, D-6), anti-α-actinin (H-2, sc-17829) and anti-β-actin (AC-15, sc-69879) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-RIPK1 antibody (BD 610458) was from BD Bioscience (San Jose, CA). Luciferase substrates were from Promega (Madison, WI). The ECL Western blotting substrates were from Thermo Scientific (Waltham, MA). Penicillin and streptomycin (30-002-CI) solution was from Corning **(**Tewksbury, MA**).** Lipofectamine 2000 (11668-019) and trypsin (25200-056) were from Life Technologies (Grand Island, NY). Etoposide was from Sigma, Smac Mimetic (SM) was from Chemietek. (Indianapolis, IN). 1,25D3 (calcitriol) and necrostatin-1 (Nec-1) was purchased from Calbiochem (La Jolla, CA). 1,25D3 was reconstituted in 100% ethanol (EtOH) and Nec-1 in DMSO. Both stock solutions were stored at −20 °C. Handling of 1,25D3 and Nec-1 was performed under indirect lighting. Concentrations of 1,25D3 varied in each experiment and have been stated in the figure legends.

2.2 Plasmids

Control and RIPK1 shRNA plasmids (pRS-MIG-HRS and pRS-MIG-RIP193, respectively) were kind gifts from Dr. Martin Leverkus of University of Heidelberg [245]. The RIPK1 K45M mutant vector was kindly provided by Dr. Junying Yuan of Harvard University[68]. The p23 VDR reporter containing rat 24-hydroxylase promoter in pMAMMneoLuc was provided by Dr. H. F. Deluca of the University of Wisconsin-Madison [246]. RIPK1 K377R mutant was kindly provided by Dr. Zhijian Chen of the University of Texas Southwestern Medical Center at Dallas [60]. HA-RIPK1, Flag-RIPK1, HA-VDR and Flag-VDR plasmids were generated by cloning full length RIPK1 and VDR cDNA into HA- and Flag-tagged pcDNA3.1 vectors, respectively. Deletion constructs of VDR and RIPK1 were generated by PCR and cloned into HA-tagged pcDNA3.1 and Flag-tagged pCMV vectors, respectively. All primer sequences have been shown in tables 1, 2 and 3.

2.3 Cell lines

293T and L929 cells were purchased from American type culture collection (ATCC) (Manassas, VA). RIPK1 wild type and null mouse embryonic fibroblasts (MEFs) were kindly provided by Dr. Junying Yuan of Harvard University [247]. PE01 and PE04 cells were kindly provided by Dr. Toshiyasu Taniguchi of Fred Hutchinson Cancer Research Center [248]. Mouse mammary tumor VDR wild type and null cells were kindly provided by Dr. JoEllen Welsh from State University of New York, University at Albany [249]. VDR+/- and VDR-/- MEFs were from Dr. Jun Sun from University of Illinois at Chicago [250]. PE01, PE04, TykNu, TykNuR, M41 and M41R cells were maintained in RPMI medium containing 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS. OVCAR3 cells were maintained in

RPMI medium containing 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 15% FBS. All other cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/ml streptomycin, and 10% FBS.

2.4 Stable knockdown of RIPK1

For stable knockdown of RIPK1, PE01 and OVCAR3 cells were plated in a 60mm dish. At 70-80% confluency, cells were transfected with 5μg of pRS-MIG-HRS (HRS: hyper random sequences) control or pRS-MIG-RIPK193 plasmid. Forty-eight hours post-transfections, stable clones were selected in media containing 2 μg/mL puromycin. Cells were maintained in puromycin containing medium for two to three weeks. Single colonies were picked knockdown clones. RIPK1 knockdown in stable clones was verified by Western blot analyses. All cells were maintained in a 37°C humidified incubator with 5% CO_2. Both pRS-MIG-HRS control and pRS-MIG-RIPK193 constructs were a kind gift of Dr. Martin Leverkus, University of Heidelberg as mentioned previously.

2.5 Immunological analyses

For co-immunoprecipitations, 293T cells were plated in 60mm dishes. For simplicity, one co-immunoprecipitation experiment detail is given here. At 60%-70% confluency, 293T cells were transfected with 1.5 μg of Flag-tagged VDR and HA-tagged RIPK1 (for example). 48 hours post-transfection, cells were washed with ice-cold phosphate buffered saline (PBS) and were lysed in cell lysis buffer containing 20 mM Tris-HCL (pH 7.5), 150 mM NaCl, 1% (v/v) NP-40, 1 mM PMSF and protease inhibitor cocktail. After two consecutive 6-second sonications

separated by brief cooling, cells were kept on ice for 10 minutes before centrifugation. Cellular extracts were incubated overnight at 4°C with M2 antibodies conjugated with beads. For co-precipitations of endogenous VDR and RIPK1, L929 cells were cultured in 100mm dishes in DMEM medium. Cells were lysed with lysis buffer containing 25 mM Tris•HCl pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 5% glycerol. Protein concentration was measured through Bradford assay and 2μg of cellular protein was incubated with 1μg of anti-VDR antibody (Santa Cruz; C-20 antibody) overnight at 4°C, followed by a 4-hour incubation with protein G beads. Rabbit IgG antibody was used as a negative control. After incubations, the beads were washed five times with lysis buffer and precipitated proteins were detected by Western blot analyses.

2.6 Transfection and reporter assays

2×10^5 293T cells were plated in 12-well plates and transfected with plasmids the next day. For example, to perform luciferase assay with p23 VDR reporter plasmid, 293T cells were transfected with 200 ng of p23 VDR reporter plasmid, 20 ng of pCMVβgal, 50 ng of Flag-VDR and indicated amounts of Flag-RIPK1. Twenty-four hours post-transfections, cells were treated with either EtOH or 1,25D3 for an additional 24 hours. After the incubation, cells were washed with ice-cold PBS and 200μl of 1X lysis buffer was added to each well to lyse the cell. The 5X lysis buffer contained: 125mM Tris-phosphate (pH 7.8), 10mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 50% glycerol and 5% Triton X-100. 5mM of DTT was added to the final lysis buffer solution. Cells were sonicated twice for 6 seconds and were centrifuged at 13,000 rpm for 15 minutes. 25μl of the lysate was used to measure the luciferase activity and 100μl of the lysate was used to measure β-galactosidase activity. Luciferase activity was

measured by adding luciferase substrate from Promega (E1501) and normalized with cognate β-galactosidase activity. Each data point was analyzed in triplicates (n=3).

2.7 Cytoplasmic and nuclear fractionation

For cytoplasmic protein extraction, cells were washed and scraped with ice-cold PBS. After pelleting, cells were re-suspended in lysis buffer (10 mM Hepes, pH7.9; 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) and kept on ice for 15 minutes. 10% NP-40 was added and followed by centrifugation for 1 minute at 14,000 rpm. The supernatant was collected as cytoplasmic protein. The pellet was washed with ice-cold PBS, re-suspended in nuclear extraction buffer (20 mM Hepes, pH 7.9; 400 mM NaCl, 1 mM EDTA, 1 mM EGTA), and kept on ice for 30 minutes with 3- to 5-second vortexing at 5-minute intervals. After centrifugation at 14,000 rpm for 5 minutes, the supernatant was collected as nuclear extracts.

2.8 Necroptotic cell death induction

Cells were plated in 96-well plates and left overnight for attachment. Next day, day 0 reading value was measured after MTT treatment and rest of the cells were treated with TNFα (10ng/ml), Etoposide (100μM), SM (1μM), z-VAD (5μM), Nec-1(20μM) alone or in combination for 16 hours in respective experiments unless otherwise stated. Cell growth suppression was measured by MTT assays.

2.9 Methylthiazol tetrazolium (MTT) assays

Cell growth was quantified using MTT assays. Cells were plated in 96-well plates and treated with either EtOH or 1,25D3 for 6 days. MTT assays were performed as described [251].

In brief, MTT was added to wells at a final concentration of 0.5 mg/mL and incubated for 3 hours. The media were removed after the incubation and 200 μl of DMSO was added to the wells. The absorption at 595 nm was measured in a MRX microplate reader (DYNEX Technologies, Chantilly, VA). . Six samples were analyzed in parallel for each data point (n=6).

2.10 Necroptotic study in mice

VDR+/− mice of C57B16 background (The Jackson Laboratory, Bar Harbor, ME) were mated to produce VDR+/+ (WT) and VDR−/− (KO) mice [244]. Only VDR-WT and VDR-KO male mice were used for this study. 8-14 weeks old mice were injected intravenously through tail-vein with murine TNFα (Peprotech) 450μg/kg per mouse. Core body temperature of mice was measured before and after injection by a lubricated rectal thermometer probe purchased from Kent Scientific. Mice with temperature below 23.6°C was sacrificed due to ethical reason. All procedures were reviewed and approved by the Institutional Animal Care and Use Committees at the University of South Florida.

2.11 Statistical analyses

Significant analyses were performed with the Student *t*-test. All values were presented as mean ± standard deviation (SD) with the number of observations (*n*) indicated in the figure legends. $p < 0.05$ was considered to be statistically significant.

Table 1: Primers used for cloning

Primer name	Sequence
VDR-F	GAGGGATCCATGGAGGCAATGGCGGCCAG
VDR-R	GAGCTCGAGTCACTGGAAGGAGAGGCAGCGGT
RIPK1-F	GAGAGATCTATGCAACCAGACATGTCCTT
RIPK1-R	GAGGTCGACTTAGTTCTGGCTGACGTAAA

Table 2: Primers used for VDR deletion analyses

Primers	Domain	Sequence
VDR-full length- F	Full length	GAGGGATCCATGGAGGCAATGGCGGCCAG
VDR-full length- R	Full length	GAGGTCGACGGAGATCTCATTGCCAAACA
N-VDR- F:	DNA binding domain	GAGGGATCCATGGAGGCAATGGCGGCCAG
N-VDR R:	DNA binding domain	GAACTCGAGTCACATCATGTCTGAAGAGGTGA
C-VDR F:	Ligand binding domain	GAGGGATCCATGGACTCGTCCAGCTTCTCCAA
C-VDR R:	Ligand binding domain	GAGGTCGACGGAGATCTCATTGCCAAACA
ΔAF2-F	GAGGGATCCATGGAGGCAATGGCGGCCAG	
ΔAF2-R	GAGGTCGACCTGGAAGGAGAGGCAGCGGT	

Table 3: Primers used for RIPK1 deletion analyses

Primers name	Domain	Sequence
RIPK1-FL F:	FL	GAGGAATTCATGCAACCAGACATGTCCTT
RIPK1-FL R:	FL	GAGGTCGACTTAGTTCTGGCTGACGTAAATC
KD-RIPK1 F:	KD	GAGGAATTCATGCAACCAGACATGTCCTT
KD-RIPK1 R:	KD	GAGGTCGACTCAATAAAAAGGCCTAAATTTTTCT
KD+IDs-RIPK1 F:	KD+IDs	GAGGAATTCATGCAACCAGACATGTCCTT
KD+IDs-RIPK1 R:	KD+IDs	GAGGTCGACTCACATATAATTGTAGGCTCCAATC
KD+ID-RIPK1 F:	KD+ID	GAGGAATTCATGCAACCAGACATGTCCTT
KD+ID-RIPK1 R:	KD+ID	GAGGTCGACTCACGTCAGACTAGTGGTATTATC
ID-RIPK1 F:	ID	GAGGAATTCTATTTAAGTCAATTAGAAGA
ID-RIPK1 R:	ID	GAGGTCGACTCACGTTCACGTCAGACTAGTGGTATTATC
ΔKD-RIPK1 F:	ΔKD	GAGGAATTCTATTTAAGTCAATTAGAAGA
ΔKD-RIPK1 R:	ΔKD	GAGGTCGACTTAGTTCTGGCTGACGTAAATC

CHAPTER 3:

RESULTS

As aforementioned in the introduction section, several cancers respond to 1,25D3 for growth suppression, including ovarian cancer. However, not all ovarian cancer cells responded to 1,25D3 for growth suppression in our studies. This suggests the existence of additional molecular determinants which can suppress 1,25D3 action. We have examined the role of RIPK1 in 1,25D3 action and report that RIPK1 is a transcriptional inhibitor of VDR and decreases 1,25D3-induced growth suppression. Interestingly, VDR inhibition occurred independently of RIPK1 kinase activity and was likely mediated through a RIPK1-VDR protein interaction that increased the cytoplasmic retention of VDR.

3.1 RIPK1 inhibits VDR transcriptional activity independent of its kinase activity

To test the effect of RIPK1 on VDR, HEK293T cells were transfected with a luciferase reporter construct containing rat 24-hydroxylase promoter in pMAMMneoLuc [246] together with plasmids encoding VDR and RIPK1 and treated with a vehicle (Ethanol) or 1,25D3. The transcriptional activity of VDR was measured by luciferase assays. 1,25D3 simulated luciferase activity and the co-expression of RIPK1 substantially reduced its capacity to do so in a dose dependent manner (Fig. 5A, bar graphs). RIPK1 did not decrease VDR protein abundance (Fig. 5A, lower panels) and thus apparently decreased the specific activity of VDR. To validate the data, we repeated the same experiment with another reporter construct containing VDRE-TK-luc

promoter. RIPK1 also decreased VDRE-TK-luc promoter activity (Fig.5B, bar graphs). In this case too, RIPK1 did not decrease VDR expression (Fig.5B, lower panel). Therefore, RIPK1 decreased that activity of VDR irrespective of the VDR promoter type.

Since RIPK1 is a protein kinase, we next sought to determine whether the enzymatic activity of RIPK1 is required for the inhibition of VDR. As shown in Fig. 6 (bar graphs), the kinase-dead K45M RIPK1 mutant inhibited 1,25D3-induced VDR activity to the same extent as wild-type RIPK. The addition of RIPK1 kinase inhibitor Nec-1 did not relieve the inhibition. In the absence of ectopic RIPK1, Nec-1 slightly elevated the activity of VDR, accompanied by a similar increase in the levels of VDR protein expression (Fig 6, lower panels). In our studies, Nec-1 increased the expression levels of both wild-type RIPK1 and the K45M mutant and both wild-type RIPK1 and the kinase-dead mutant increased the levels of VDR protein expression. The increased expressions of RIPK1 and VDR by Nec-1 and RIPK1, respectively, were consistently observed in our studies. To confirm that VDR is not a target of RIPK1 kinase activity, *in vitro* kinase assay was performed whereas VDR was used as a substrate of RIPK1 protein. Although RIPK1 phosphorylated myelin basic protein (MBP) as a positive control, it did not phosphorylate VDR (Fig. 7A). In addition to the RIPK1 mediated VDR activity decrease, we also wanted to determine the effect of TNFα on VDR activity. As shown in Fig. 8, TNFα alone also decreased the activity. Since HEK293T cells have endogenous RIPK1, TNFα may have worked through the RIPK1 that was already in the cell. However, addition of RIPK1 also decreased the VDR luciferase activity and RIPK1 combined with TNFα treatment had an additive effect to decrease the activity. Both TNFα and RIPK1 increased VDR level (Fig. 8, lower panel), confirming that the decrease of VDR activity was not due to the loss of VDR protein.

Overall, the studies suggest that RIPK1 inhibits the transcriptional activity of VDR independent of its kinase activity.

3.2 RIPK1 forms a protein complex with VDR

As aforementioned, RIPK1 can perform biological functions through its enzymatic activities or as an adaptor. The evidence of RIPK1's ability to inhibit VDR activity is independent of its kinase activity prompted us to ask whether RIPK1 forms a complex with VDR. HEK293T cells were transfected with plasmids encoding tagged RIPK1, VDR or both and co-immunoprecipitation experiments were performed. M2 anti-Flag beads co-precipitated HA-RIPK1 with Flag-VDR (Fig. 9A) and HA-VDR with Flag-RIPK1 (Fig. 9B) from cells expressing both proteins but not either protein alone. Thus, RIPK1 associates with VDR in overexpression studies. The binding of RIPK1 to VDR was detected both in the absence and presence of 1,25D3 but its amount was reduced by the hormone treatment (Fig. 10). Moreover, the complex formation also occurred between endogenous VDR and RIPK1 in L929 mouse fibroblast cells (Fig. 11), ruling out the possibility that the complex formation might be an artifact of ectopic overexpression.

3.3 The complex formation between RIPK1 and VDR involves the kinase domain of RIPK1 and the ligand-binding domain of VDR

To define the site in VDR that mediates RIPK1 binding, VDR deletion constructs (Fig. 12) were generated and their ability to bind to RIPK1 was assessed by co-immunoprecipitation analyses. As shown in Fig. 13, the VDR fragment deleted of the N-terminal DNA binding domain and hinge region bound to RIPK1 whereas the fragment deleted of the C-terminal ligand

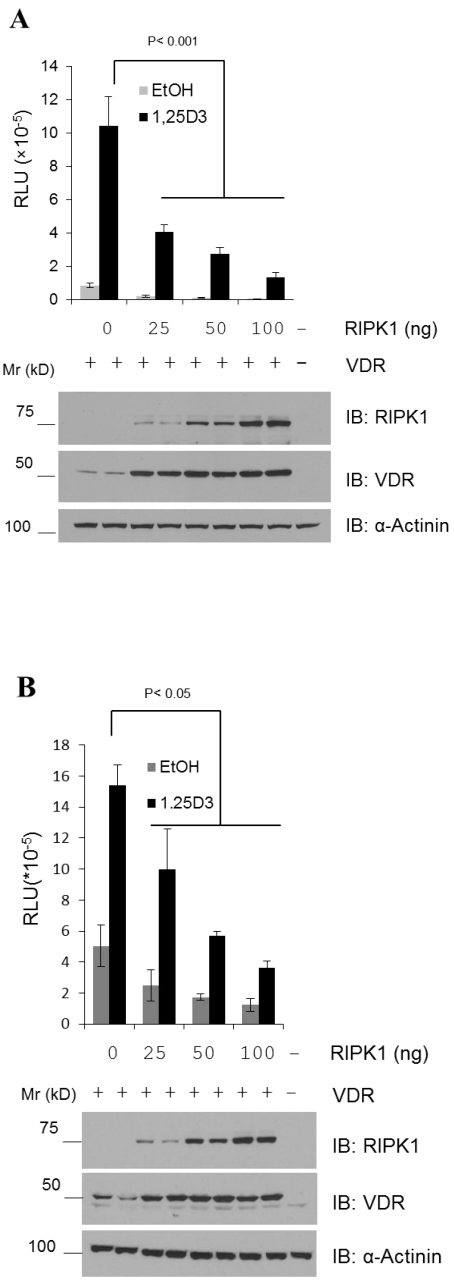

Figure 5: RIPK1 decreases VDR activity

293T cells were transfected with 20 ng of pCMVβgal, 50 ng of Flag-VDR and indicated amounts of Flag-RIPK1 and **A)** 200 ng of p23 VDR reporter plasmid or **B)** 200 ng of VDRE-Tk-Luc VDR reporter plasmid,. The next day, cells were treated with either EtOH or 1,25D3 for 24 hours. Luciferase activity was determined and normalized with cognate β-gal activity. Each data point was analyzed in triplicates (n-3) and reproduced two times. Western blot analyses were performed with antibodies as indicated.

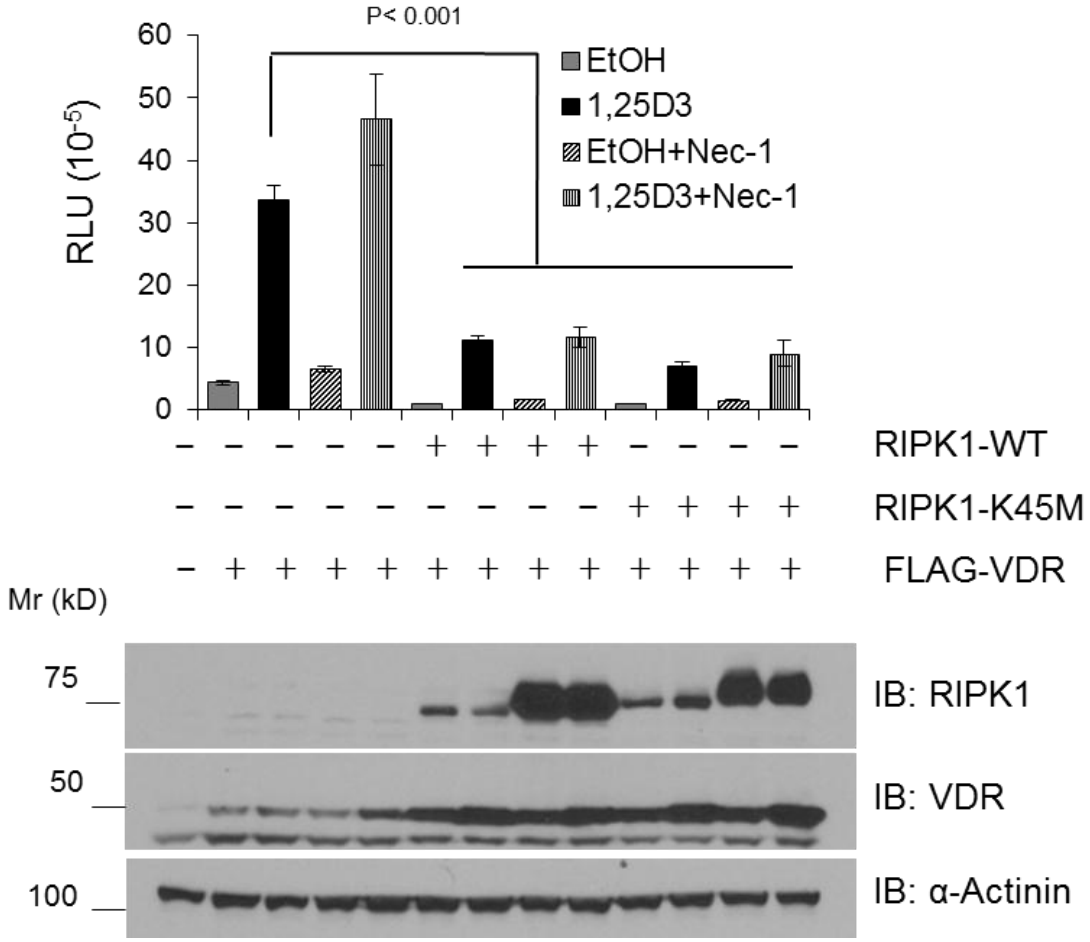

Figure 6: Suppression of VDR activity by RIPK1 is kinase-independent
293T cells were transfected with 200 ng of p23 VDR reporter plasmid, 20 ng of pCMVβgal, 50 ng of Flag-VDR and either with wild-type (RIPK1-WT) or kinase-dead mutant (RIPK1-K45M) RIPK1 and treated with 1,25D3 and/or RIPK1 inhibitor Nec-1 (20 μM). Luciferase activity was determined and normalized with cognate β-gal activity. Each data point was analyzed in triplicates (n-3) and reproduced two times. Western blot analyses were performed with antibodies as indicated.

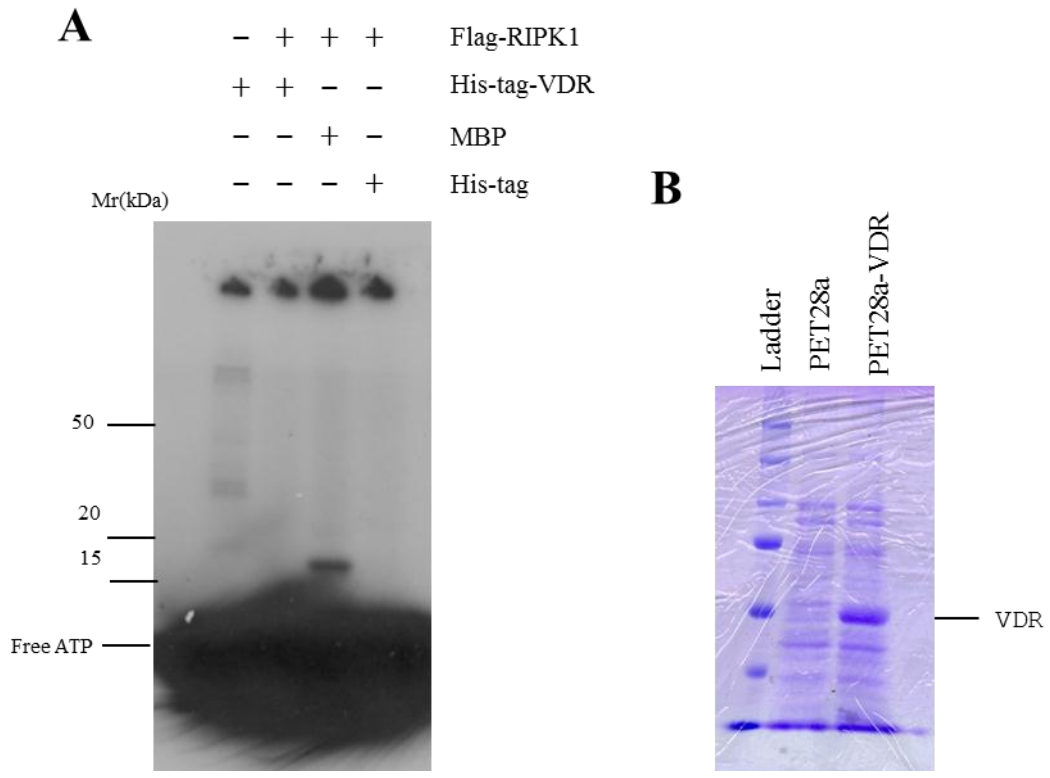

Figure 7: RIPK1 does not phosphorylate VDR
A. Flag-RIPK1 was transfected in H2K293T cells and expressed RIPK1 was immunoprecipitated with anti-Flag antibodies. In vitro kinase assay was performed with His-tag-VDR as a substrate. Myelin basic protein (MBP) was used as a positive control. **B.** Coomasie blue imgae to show VDR expression.

binding domain failed to bind, showing LBD as the site for RIPK1 binding. The VDR fragment deleted of the activation function 2 (AF2) region retained the ability to associate with RIPK1 (Fig. 13), revealing expendability of the AF2 region for binding. Using a similar approach, the regions in RIPK1 required for VDR interaction were also defined by deletion analyses. A set of RIPK1 deletion constructs were generated (Fig. 14) and their ability to form complexes with VDR was determined in co-precipitation analyses. As shown in Fig. 15, neither the kinase nor the intermediate domain alone was co- precipitated with VDR whereas the fragment containing both regions was co-precipitated.

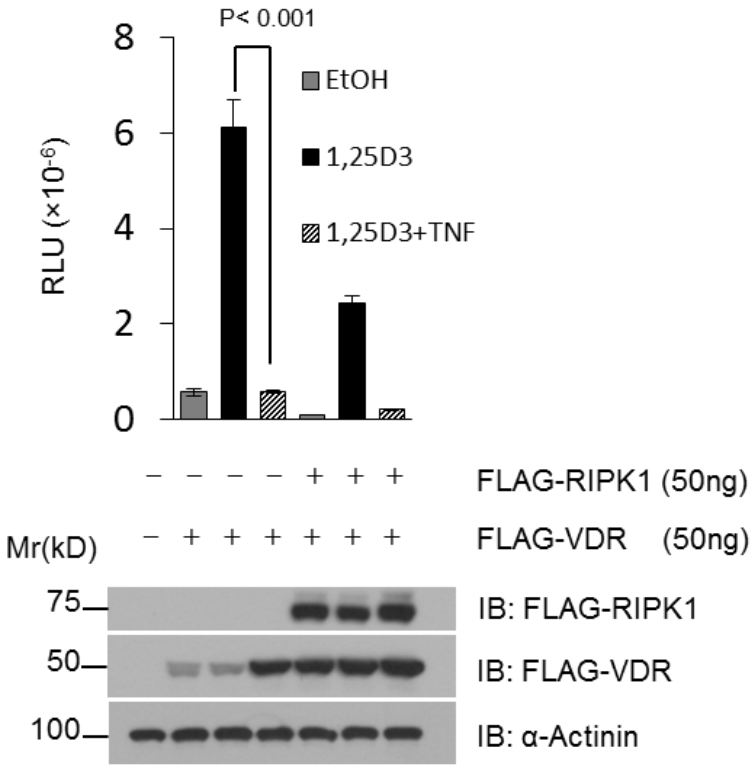

Figure 8: TNFα also suppresses VDR activity
293T cells were transfected with 200 ng of p23 VDR reporter plasmid, 20 ng of pCMVβgal and indicated amounts of Flag-VDR and Flag-RIPK1. 24 hours post-transfection, cells were treated with either EtOH or 1,25D3 with or without TNFα for another 24 hours. Luciferase activity was determined and normalized with cognate β-gal activity. Each data point was analyzed in triplicates (n-3) and reproduced two times. Western blot analyses were performed with antibodies as indicated.

The analyses suggest that, although the kinase or intermediate domain alone is not sufficient, they both are required for binding to VDR. The deletion of C-terminal death domain and the region between RHIM and the death domain did not abolish the VDR binding, showing that these regions are expendable.

Overall, the binding analyses tell us that RIPK1 employs multiple domains to interact with the LBD of VDR. Although the ability of RIPK1 to repress the transcriptional activity of

VDR is independent of its enzymatic activity, the kinase domain is physically involved in the complex formation.

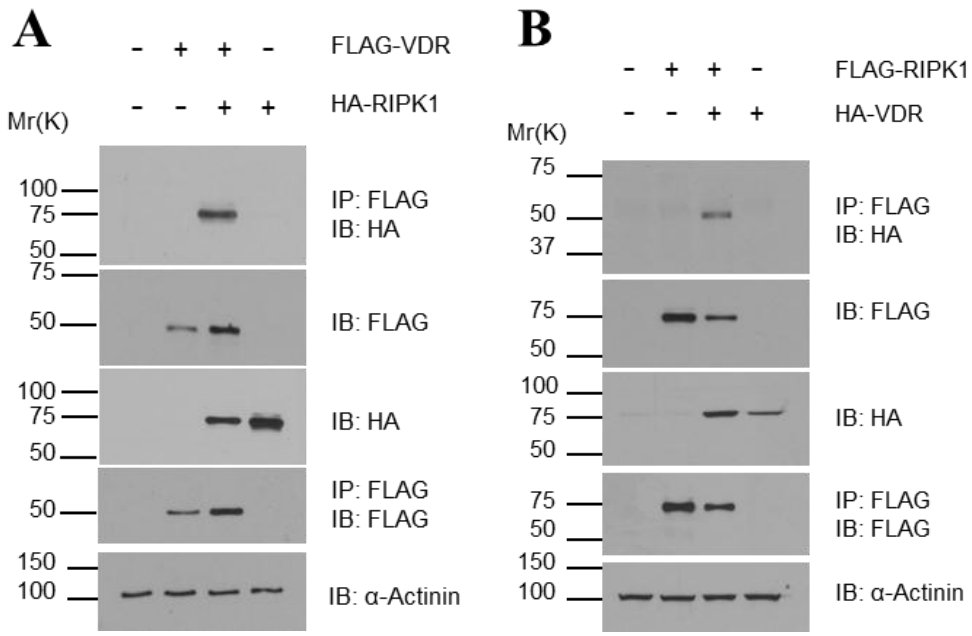

Figure 9: RIPK1 forms a protein complex with VDR
In reciprocal immunoprecipitation, 293T cells were transfected with **A)** 1.5 μg of Flag-tagged VDR and HA-tagged RIPK1 and **B)** 1.5 μg of Flag-tagged RIPK1 and HA-tagged VDR as indicated. 48 hours posttransfection, cell extract were immunoprecipitated with Flag antibodies followed by immunoblotting with indicated antibodies.

3.4 VDR-RXRα complex was not affected by RIPK1

Since VDR forms a heterodimer with RXRα, we sought to determine whether RIPK1 binding with VDR influenced the VDR-RXRα complex formation or not. Co-precipitation of VDR along with RXRα in absence or presence of RIPK1 showed that RIPK1 did not change the VDR-RXRα complex formation (Fig. 16). In both cases, 1,25D3 increased the binding, showing that RIPK1 did not decrease the binding even in presence of the hormone, either.

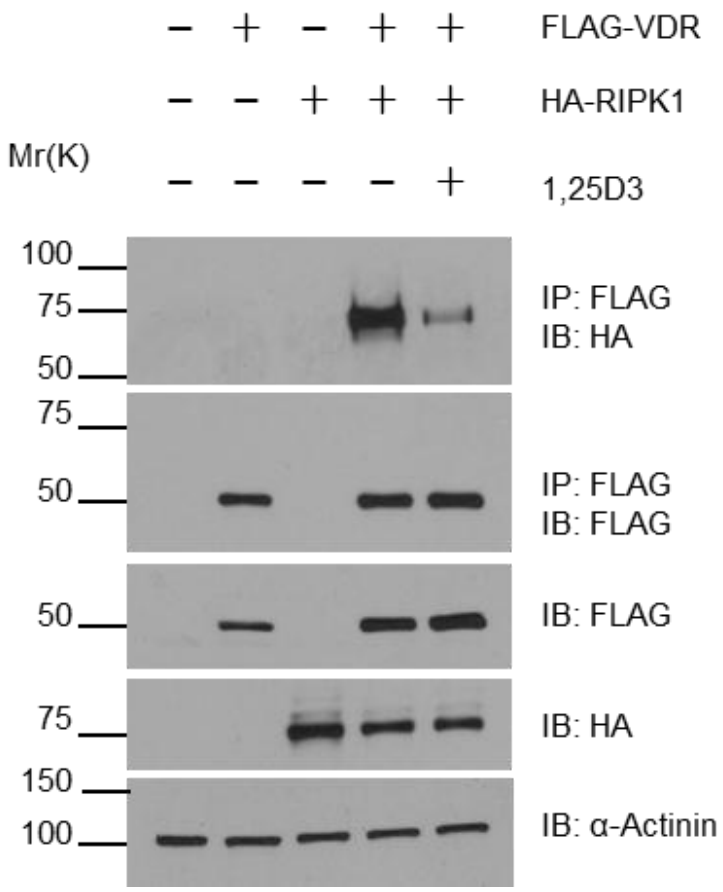

Figure 10: RIPK1-VDR complex formation is decreased by 1,25D3 treatment
293T cells were transfected with 1.5 μg of Flag-tagged VDR and HA-tagged RIPK1 as indicated. 24 hours posttransfection, cells were treated either with EtOH or 10^{-7} nM 1,25D3 for 6 hours. Cellular lysates were immunoprecipitated with anti-Flag antibody conjugated beads. Western blot analyses were performed with indicated antibodies.

3.5 RIPK1 increases VDR retention in the cytoplasm

In an effort to understand the mechanisms underlying VDR repression by RIPK1, we next assessed the effect of RIPK1 on VDR cellular localization. As shown in Fig. 17A, the VDR protein ectopically expressed in 293T cells was mainly localized to the nucleus but the co-transfection of RIPK1 increased the cytoplasmic VDR signal. Due to the positive effect of ectopic RIPK1 on the overall levels of VDR expression, the impact of ectopic RIPK1 on the

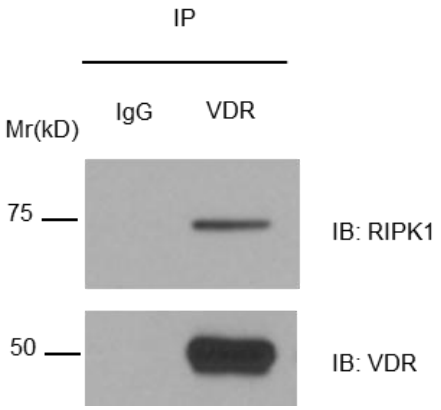

Figure 11: Interaction of endogenous VDR and RIPK1 in L929 cells
Whole cell lysates of L929 cells were immunoprecipitated with anti-VDR antibody followed by Western blot analyses with anti-VDR and RIPK1 antibodies as indicated.

nuclear VDR was not obvious from the Western blots (Fig. 17A). However, after quantification with ImageJ and normalization with cognate whole cell signals, it became clear that the increased VDR retention in the cytoplasm was accompanied by a decrease in the nuclear VDR (Fig. 17B, bar graphs). The data argue that the increased cytoplasmic retention is the possible mechanism underlying the VDR repression by RIPK1. Next, we sought to determine how the hormone influenced the retention of VDR into the cytoplasm by RIPK1. We performed the same experiment but treated with or without 1,25D3 for 24 hours post-transfection. The cytoplasmic and nuclear localization protein expression of VDR and RIPK1 (Fig. 18B) compared with the whole cell expression (Fig. 18A) showed there was more VDR retention in the cytoplasm than the nucleus in presence of RIPK1 and this was not relieved by 1,25D3 treatment (normalized in Fig. 18C, bar graphs). This data further proved the previous finding that RIPK1 indeed retained VDR into the cytoplasm.

Figure 12: Schematic representation of different VDR deletion constructs
The different deletion constructs of Flag tagged-human VDR used in this study. Amino acid residue numbers are shown at the top of the graphs. ΔC: C-terminal LBD deleted (aa.1-203); ΔN: N-terminal DNA binding domain and hinge region deleted (aa.204-427); ΔAF-2: Helix-12 deleted (aa.1-408).

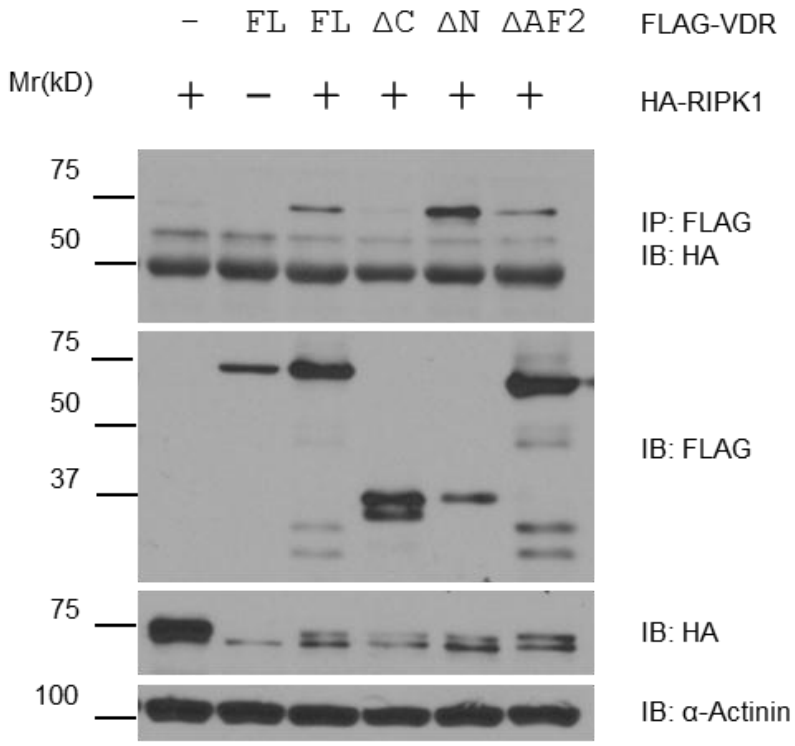

Figure 13: RIPK1 binds to the C-terminal LBD of VDR
293T cells were transfected with 1.5 μg of HA-RIPK1 together with 1.5 μg of Flag-tagged full-length (FL) VDR or its deletion constructs as indicated. Cellular extracts were subjected to co-immunoprecipitation analyses with antibodies as indicated.

3.6 Depletion of RIPK1 expression increases sensitivity of cells to 1,25D3-induced growth suppression

So far, we have shown that ectopic RIPK1 repressed VDR by binding to and holding the receptor in the cytoplasm. If this is true, depletion of RIPK1 expression should potentiate 1,25D3 action and increase the sensitivity of cells to the growth suppressive effect of the hormone. Indeed, in comparison to wild-type MEFs, RIPK1-null MEFs exhibited a dramatic and significant increase in their sensitivity to 1,25D3-induced growth suppression at all concentrations tested (Figs.19A and 19B).

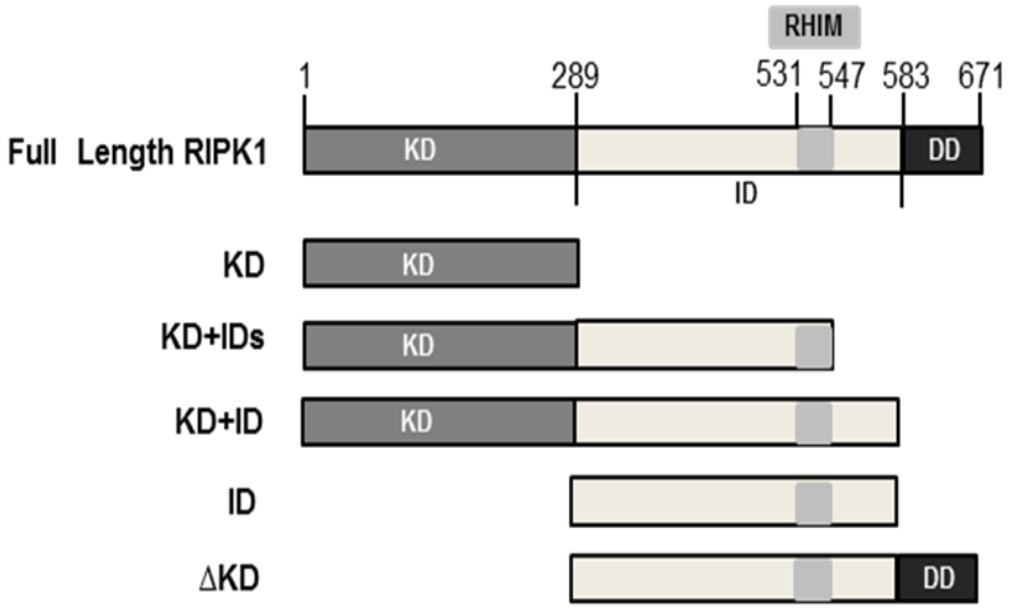

Figure 14: Schematic representation of different RIPK1 deletion constructs
Schematic representation of the domain structure of HA-RIPK1 and its deletion constructs. Amino acid residue numbers are shown at the top of the graphs. KD: kinase domain (aa.1-289); ID: Intermediate domain (aa.290-583); IDs: Smaller ID (aa.290-547); ΔKD: Kinase deleted (aa.290-583).

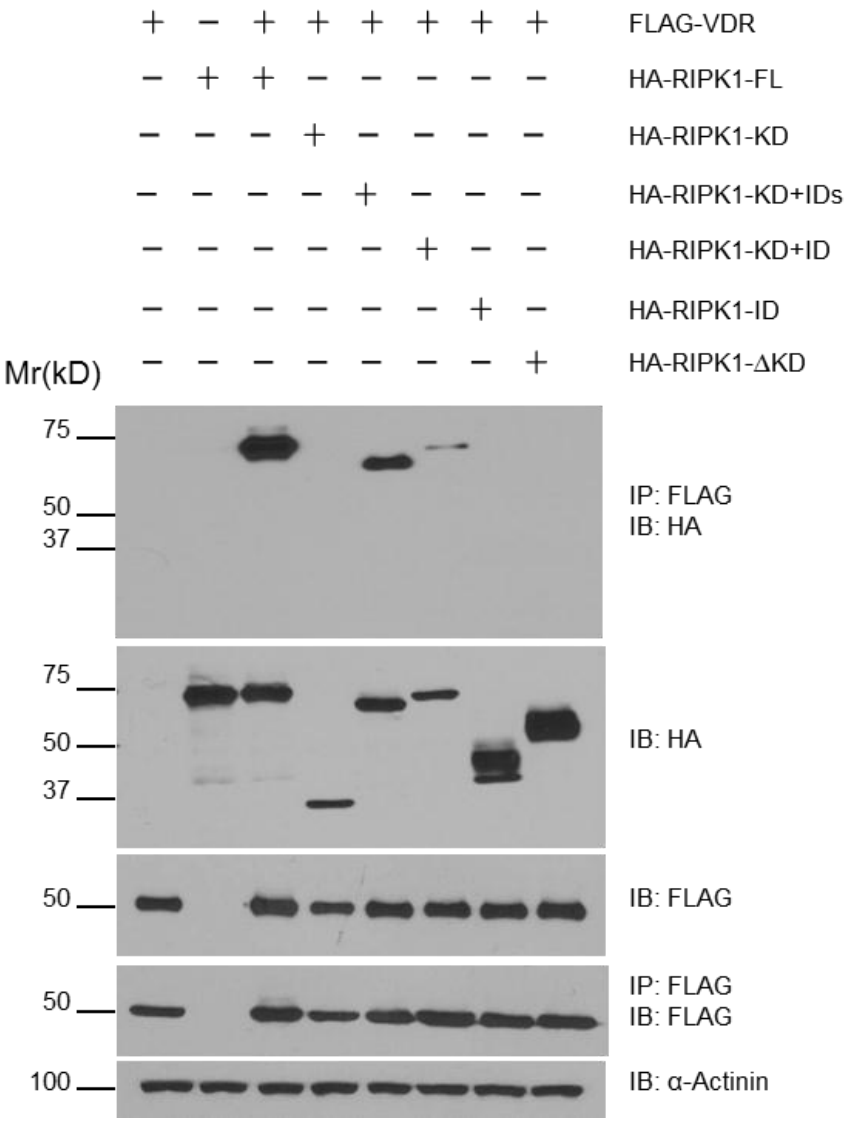

Figure 15: Multiple regions of RIPK1 mediate its binding to VDR
293T cells were transfected with 1.5 μg of Flag-VDR together with 1.5 μg of full-length (FL) or deletion constructs of HA-RIPK1 as indicated. Cellular extracts were subjected to co-immunoprecipitation analyses with antibodies as indicated.

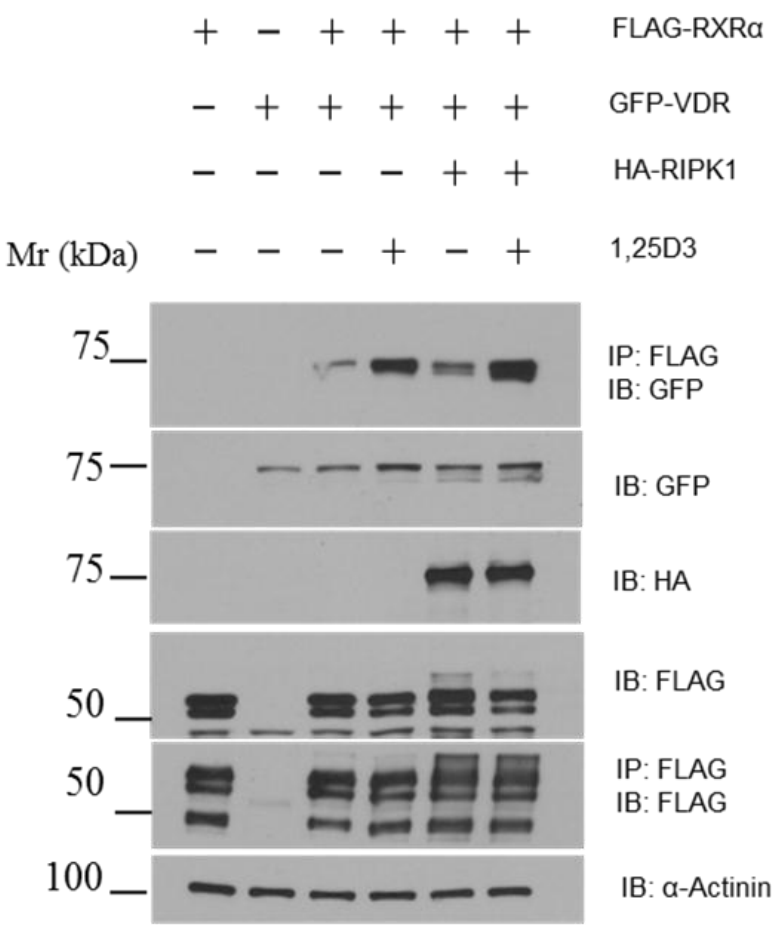

Figure 16: VDR-RXRα binding is not decreased by RIPK1
293T cells were transfected with Flag-tagged RXRα, GFP-tagged VDR and HA-tagged RIPK1 as indicated. 24 hours posttransfection, cells were treated with EtOH or 1,25D3 and after 24 hours of the treatment, cell extracts were immunoprecipitated with Flag antibodies followed by immunoblotting with indicated antibodies.

Consistent with the data from RIPK1-null MEFs, RIPK1 knockdown by stable shRNA expression in OVCAR3 (Figs. 20A and 20B) and PE01 (Figs. 21A and 21B) ovarian cancer cells also significantly increased their sensitivity to 1,25D3-induced growth suppression. The ability of RIPK1 depletion to potentiate the growth response to 1,25D3 was observed in multiple cell types with a stable shRNA pool (Fig. 20B) and two independent clones (Fig. 21B), ruling out the potential artifact of clonal effects and stable selection with antibiotics.

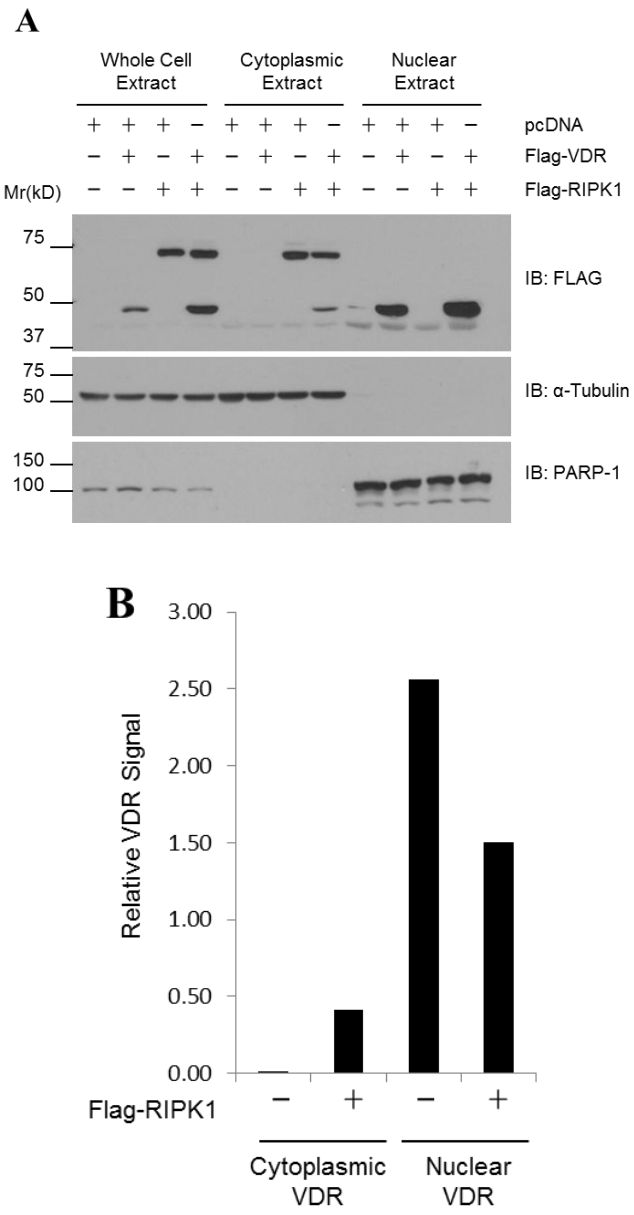

Figure 17: RIPK1 increases VDR cytoplasmic localization
A) 293T cells were transfected with Flag-VDR and Flag-RIPK1 constructs. Thirty-six hours post-transfections, cells were treated with EtOH or 10^{-7} nM 1,25D3 for 6 hours. Whole cell, cytosolic, and nuclear extracts were prepared and subjected to Western blot analyses with indicated afanantibodies. Anti-α-tubulin and PARP-1 Western blots were included as loading controls for cytoplasmic and nuclear proteins, respectively. B) The intensity of Western blot bands in figure 17 A was quantified by ImageJ (NIH). VDR signals were first normalized with cognate loading controls. Cytoplasmic and nuclear VDR were further normalized with corresponding whole-cell VDR and plotted as relative VDR signals.

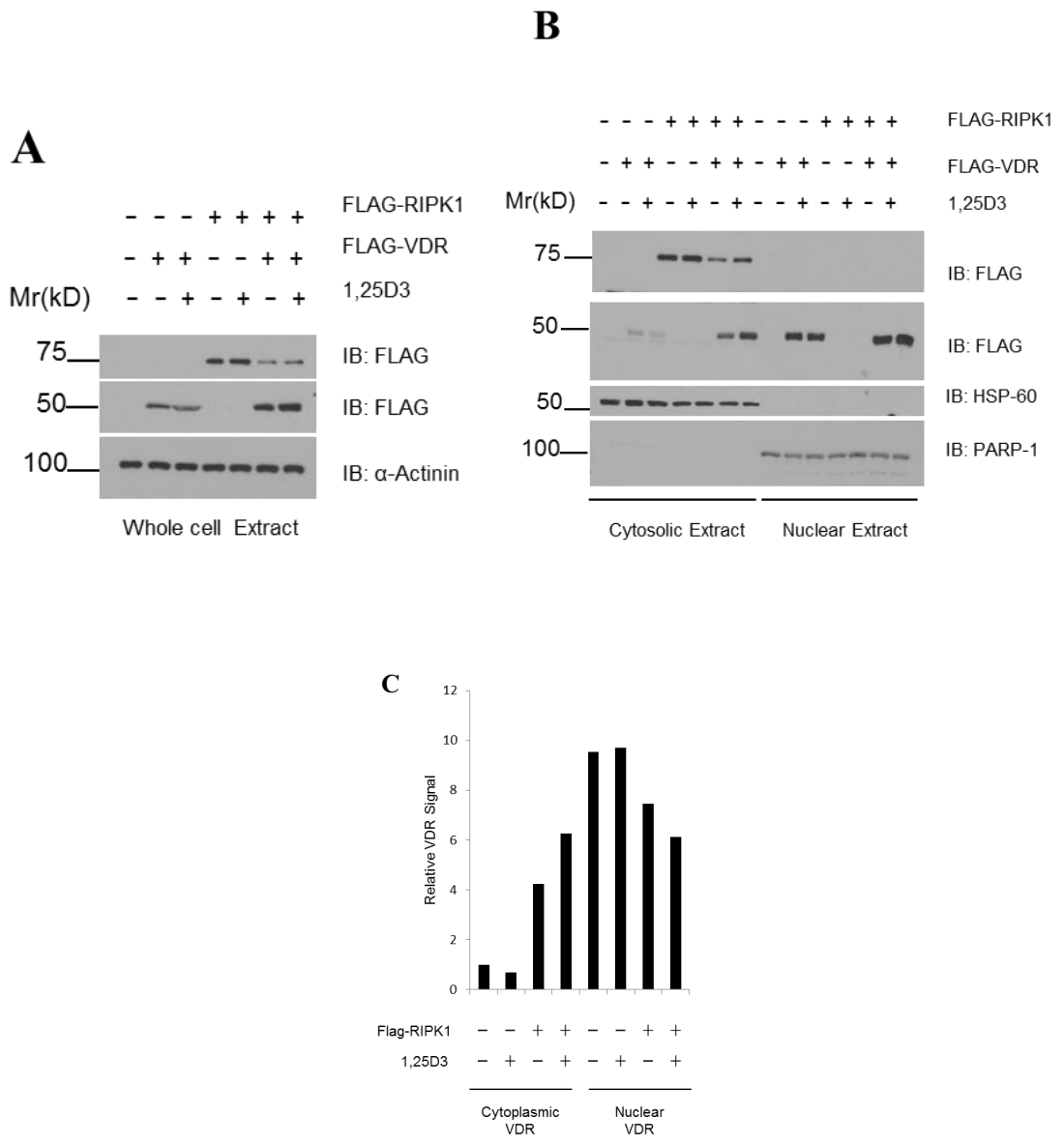

Figure 18: RIPK1 increases VDR cytoplasmic localization in the presence of 1,25D3
1,25D3 treatment increases VDR localization in presence of RIPK1. 293T cells were transfected with Flag-VDR and Flag-RIPK1 constructs. Thirty-six hours post-transfections, cells were treated with EtOH or 10^{-7} nM 1,25D3 for 6 hours. A) Whole cell, B) cytosolic and nuclear extracts were prepared and subjected to Western blot analyses with indicated antibodies. Anti-HSP60 and PARP-1 Western blots were included as loading controls for cytoplasmic and nuclear proteins, respectively. C) The intensity of Western blot bands in figure 18B was quantified by ImageJ (NIH). VDR signals were first normalized with cognate loading controls. Cytoplasmic and nuclear VDR were further normalized with corresponding whole-cell VDR and plotted as relative VDR signals.

Overall, the growth analyses with RIPK1-depleted cells support the conclusion of transfection studies that RIPK1 is a VDR repressor. The studies argue that RIPK1 depletion may represent an effective strategy for increasing the potency of 1,25D3 and its analogs in cancer intervention.

3.7 VDR-RIPK1 form endogenous complex in response to necrotic cell death induction

Overexpression data in HEK293T cell lines showed the VDR-RIPK1 complex formation. However, this complex may not be observed at the endogenous level in this cell line since HEK293T has very low level of VDR expression. Therefore, we sought to induce L929 cells as our model cell line for endogenous VDR-RIPK1 binding (Fig.11). This cell line is routinely used to show complex formation by RIPK1 protein with cell death induction [76] . We first treated L929 cells with TNFα, SM or MG132 (proteasome inhibitor) individually or in combination for 4 hours. After the treatment, cell lysates were immunoprecipitated with VDR antibody followed by western blot. Neither of the drugs could induce VDR-RIPK1 complex alone, whereas the combined treatment of all three drugs could induce the binding (Fig. 22A). This finding also led us to think using etoposide as alternative drug of SM. We performed the same cell death induction experiment combining TNFα and etoposide with or without MG132. Here, a time based treatment showed that the complex formation was induced by TNFα, etoposide and MG132 combined (Fig. 22B). The complex formation was stronger with time and formed only when MG132 was used. This suggests that VDR-RIPK1 complex is also formed by cell death induction at the endogenous level and may be proteolytically degraded.

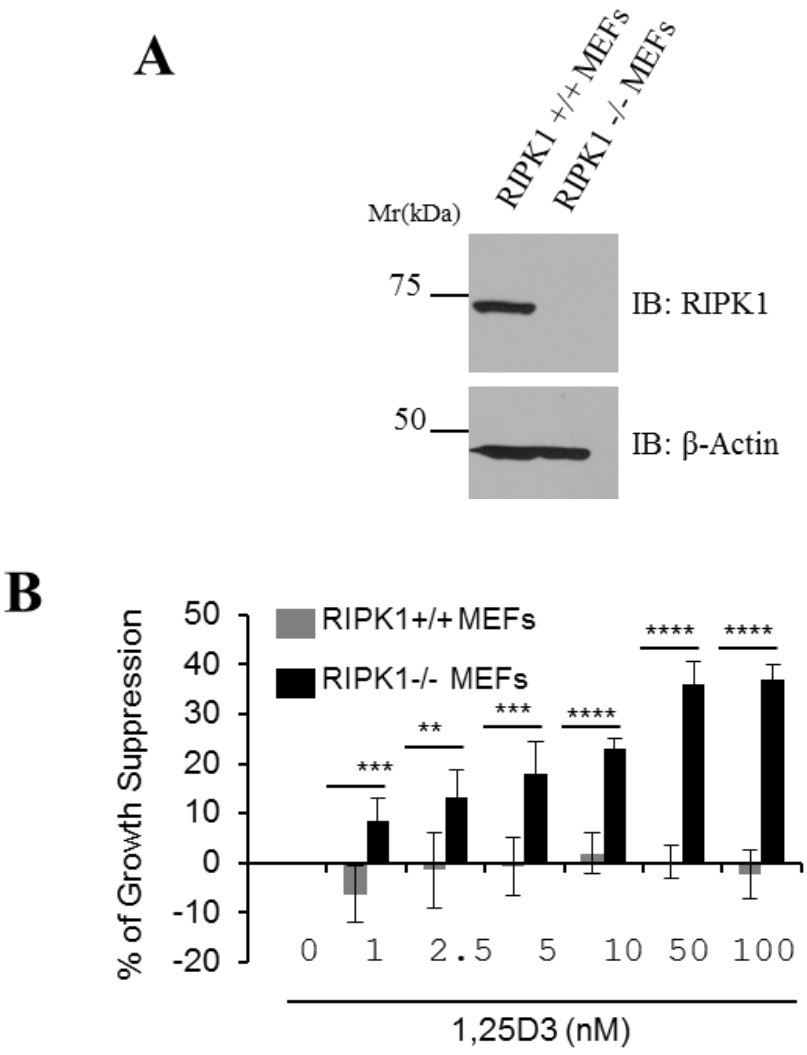

Figure 19: RIPK1 depletion sensitizes MEFs to 1,25D3
A) RIPK1+/+ and RIPK1-/- MEF cells were plated into 100mm dishes. Cell lysates were subjected to Western blot to detect the RIPK1 in both cells with anti-RIPK1 antibody. **B)** MEFs were plated in 96-well plates and treated with either EtOH or indicated concentrations of 1,25D3 for 6 days. MTT assay was performed. Six samples were analyzed in parallel for each data point (n=6) and the experiment was repeated twice. Percentages of cell growth were first calculated by subtracting MTT values at day zero from those in day 6 followed by dividing with day zero values. Percentages of growth suppression by 1,25D3 were calculated by dividing percentages of cell growth in 1,25D3-treated groups with those of the EtOH controls. ** $p<0.005$; *** $p<0.001$, **** $p<0.0001$.

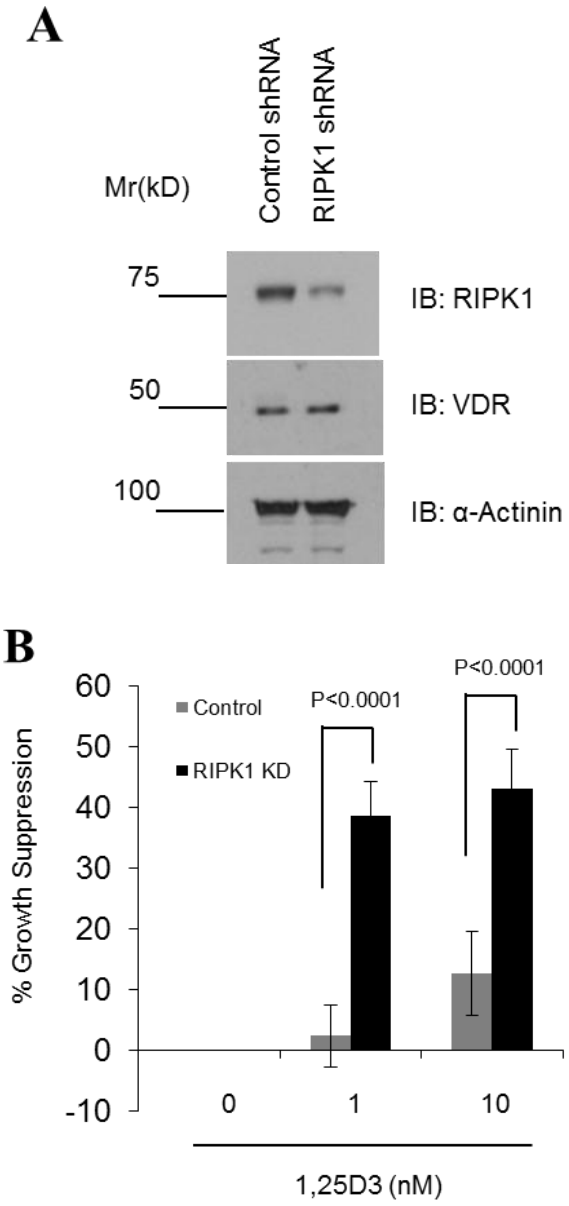

Figure 20: Knockdown of RIPK1 increases 1,25D3 induced growth suppression in OVCAR3 cells

A) RIPK1 and VDR expression was determined by Western blot analyses in an OVCAR3 cell pool stably expressing control or RIPK1 shRNA. **B)** The OVCAR3 control and RIPK1 KD cells were treated with EtOH or 1,25D3 for 6 days and the percentage of growth suppression was measured in MTT assays as in figure 19B.

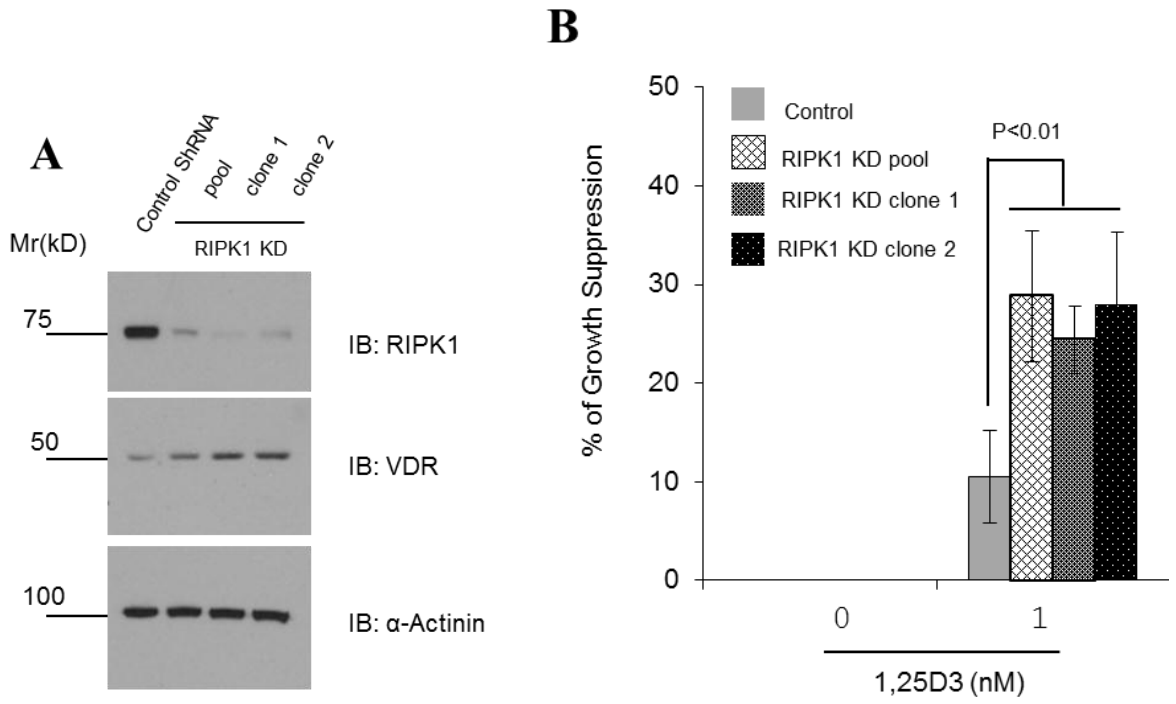

Figure 21: Knockdown of RIPK1 increases 1,25D3 induced growth suppression in PE01 cells
A) RIPK1 and VDR expression was determined by Western blot analyses in PE01 cell clones stably expressing control or RIPK1 shRNA. **B)** PE01 clones were treated with EtOH or 1,25D3 for 6 days and the percentage of growth suppression was determined in MTT assays as in figure 19B.

3.8 VDR-RIPK1 complex may be degraded by proteasome pathways

As discussed in the introduction section, RIPK1 protein has Lysine 377 residue in the intermediate domain (ID) which is a major ubiquitination site. The K48 ubiquitination targets the protein for proteasomal degradation. Since the ID is also important for binding to VDR along with its kinase domain (KD); it may pose an important question whether the ubiquitination influences the binding or not. For this kind of study, ubiquitin mutant has been used where the lysine residue has been modified to other residue such as arginine so that it can no longer be ubiquitinated [60]. If RIPK1-VDR complex is ubiquitinated at the K377 site and further targeted

for proteasome degradation, the K377R ubiquitin mutant of RIPK1 should show a stronger binding with VDR than the wild type. This study can alternatively supported by the use of a proteasome inhibitor MG132. MG132 targets the proteasome inhibitor complex and inhibits its activity. Addition of MG132 should rescue the wild type RIPK1-VDR complex formation from degradation and will not change mutant RIPK1-K377R-VDR complex formation. Since our endogenous binding between VDR and RIPK1 suggested that VDR-RIPK1 complex may be subjected to proteasomal degradation, we sought to determine whether mutation in K377 site of RIPK1 also supports this finding. To determine whether RIPK1–VDR complex gets ubiquitinated followed by degradation, we transfected 293T cells with HA-VDR along with Flag tagged wild type RIPK1 or flag tagged mutant RIPK1-K377R constructs. As shown in Fig. 23, RIPK1 ubiquitin mutant construct showed more binding with VDR. In addition to that, although MG132 substantially increased the complex formation of VDR with wild type RIPK1, it did not change as much in case of mutant RIPK1. This suggests that VDR-RIPK1 complex may get ubiquitinated and degraded by proteasome pathway.

3.9 VDR may potentiate necroptosis in mammary tumor cells

It is already known that cytokines can stimulate both prosurvival and prodeath signals [100]. Their selection on stimulating either pathway depends upon cell type or other stimulants. For example, TNFα treatment on L929 cells induces necroptotic cell death [252]. On the other hand, etoposide or SM can change the prosurvival signaling of TNFα into prodeath signaling [100]. Both of these drugs inhibit or deplete cIAPs from cells, rendering RIPK1 mediated apoptotic or necroptotic cell death pathway [100]. As caspase mediated apoptotic cell death can be blocked by pan caspase inhibitor z-VAD, the RIPK1 mediated apoptotic or necroptotic cell

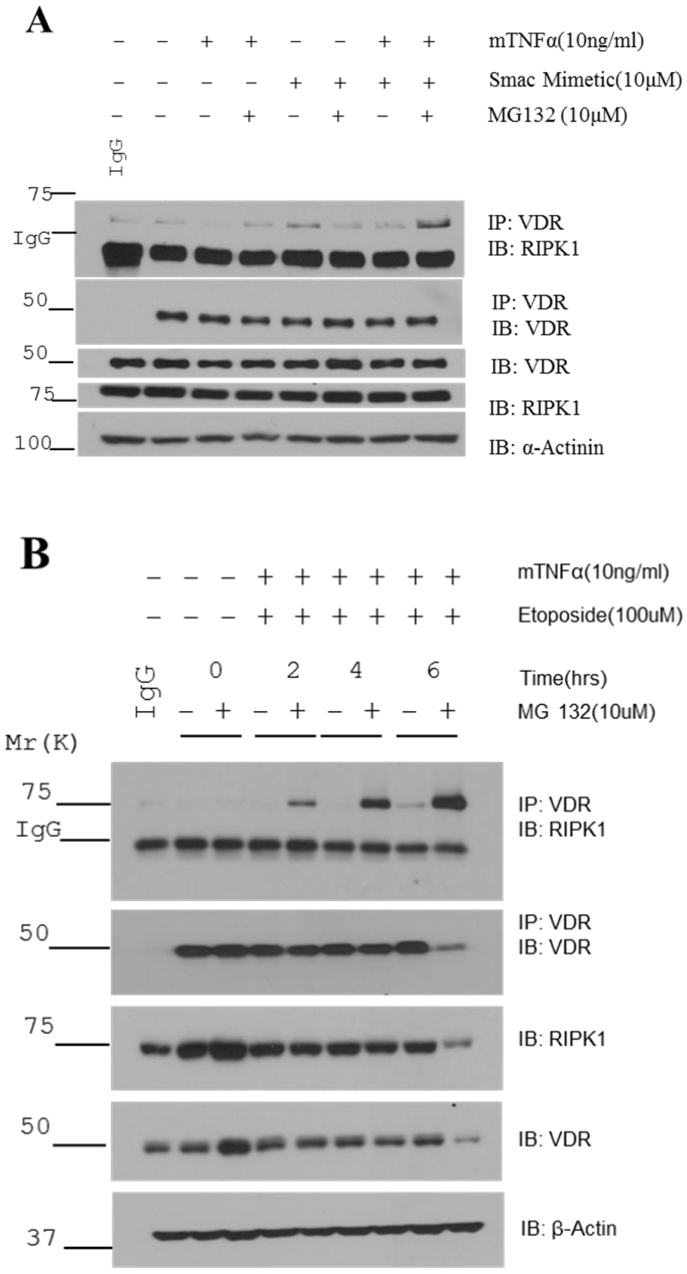

Figure 22: Endogenous binding of VDR-RIPK1 forms upon cell death induction
A) L929 cells were treated with murine TNFα, SM and MG132 alone or in combination as indicated for 6 hours. B) L929 cells were treated with murine TNFα(10ng/ml), Etoposide(100μM) and MG132(10μM) in combination for different time period as indicated. Aliquots of 1.5mg of cell lysates were immunoprecipitated with anti-VDR (D-6) antibody. The levels of RIPK1 and VDR in the immunocomplex and in the cell extracts were determined by Western blot analysis.

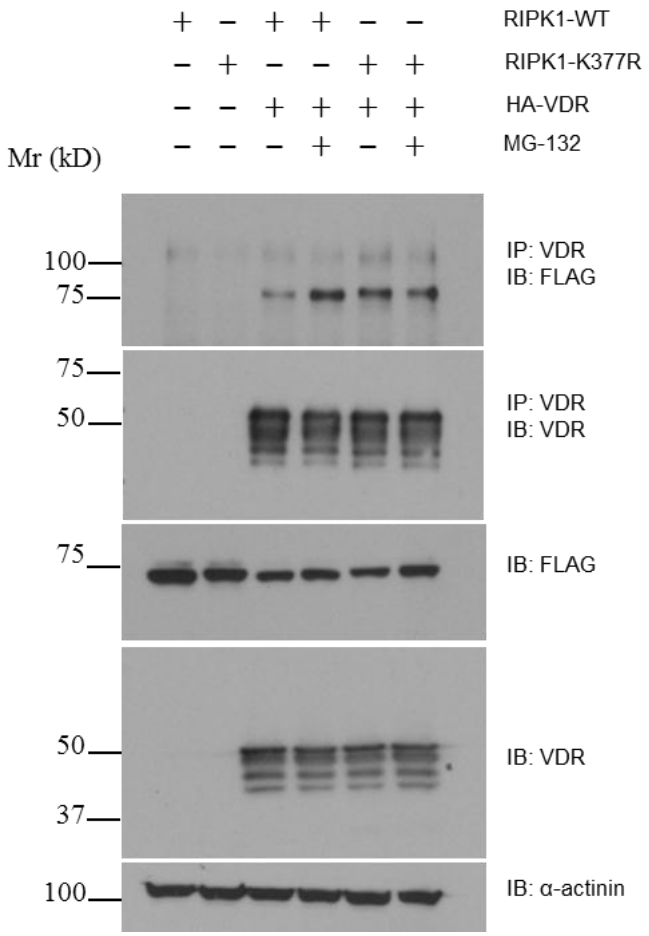

Figure 23: RIPK1-VDR complex may get degraded by proteasome
293T cells were transfected with 1.5 μg of HA-tagged VDR and either RIPK1-WT or ubiquitin mutant RIPK1-K377R (Flag-tagged). 44 hours posttransfection, cells were treated with either DMSO or 10μM MG132 for 4 hours. Cell extracts were immunoprecipitated with Flag antibodies followed by immunoblotting with indicated antibodies.

death can still occur through RIPK1 mediated pathway. To understand the role of VDR in apoptotic or necroptotic cell death pathway, we induced VDR wild type and VDR null mouse mammary tumor cells (MMT-VDR-WT and MMT-VDR-KO, respectively) with murine TNFα and etoposide alone or in combination for 16 hours to induce cell death. As shown in Fig. 24A, TNFα or etoposide alone did not suppress cell growth; however, combination of those two drugs

substantially suppressed growth in MMT-VDR-WT cells. On the other hand, MMT-VDR-KO cells did not show much growth suppression. z-VAD along with TNFα and etoposide could not relieve the WT cells from growth suppression, indicating that the suppression was not occurring through caspase-8 mediated pathway. Interestingly, Nec-1 almost prevented the cell death in WT cells. This indicates that, VDR is required for TNFα and etoposide mediated cell death and it may occur through necroptotic pathway. As SM also depletes IAP level, we performed the same experiment using SM instead of etoposide. Fig. 24B shows that MMT-VDR-WT cells responded the same way as Fig. 24A. Therefore, it might be concluded that VDR may potentiate RIPK1 mediated necroptotic cell death in mouse mammary tumor cells.

3.10 VDR role in necroptosis is cell line specific

Mouse embryonic fibroblast (MEF) cell line is an important candidate in cell death processes. As this cell line contains endogenous RIPK3 [76], it can be used as an ideal candidate for necroptosis study. To validate our findings in an epithelial cell line like the mammary tumor cells, we sought to determine how the presence or absence of VDR responded to necroptotic cell death. We have used VDR heterozygous (VDR+/-) MEF cell line as an alternative to VDR-WT MEF. As shown in Fig. 25, MEF cell line responded to the opposite manner of MMT cell line. Here, VDR null MEF cell line responded to necroptotic cell induction much more than VDR +/- MEF cell line. One explanation might be mammary tumor cells are epithelial cells, whereas MEF cells are mesenchymal cells. Therefore, the response of VDR to necroptotic induction was cell line specific.

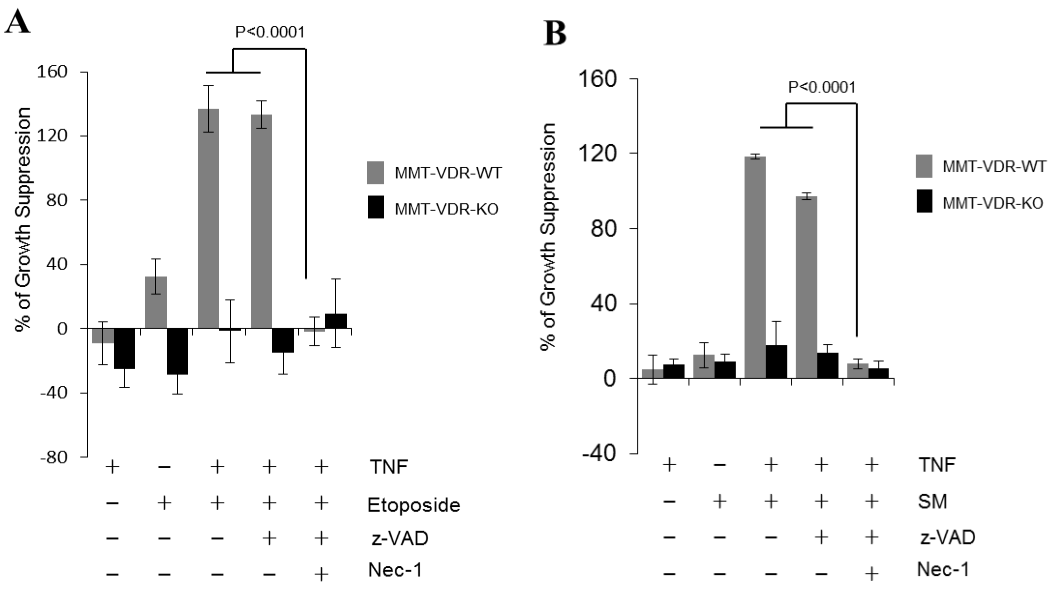

Figure 24: MMT-VDR null cells are not responsive to necroptosis
Mouse mammary tumor VDR wild type and VDR null cell lines, MMT-VDR-WT and MMT-VDR-KO, respectively cells were plated onto 96 well plate. 24 hours later, cells were treated with TNFα (10ng/ml), etoposide (100μM) (Fig. **A**), Smac mimetic (SM) (1μM) (Fig. **B**), z-VAD(10μM), Nec-1(10μM) alone or in combination as indicated, for 16 hours. Cell growth suppression was measured by MTT assay. Six samples were analyzed in parallel for each data point (n=6) and the experiment was repeated twice.

3.11 VDR does not modulate RIPK1-RIPK3 complex formation

As RIPK1 and RIPK3 form a complex that is important in necroptotic cell death, we sought to determine whether VDR decreases or increases the RIPK1-RIPK3 complex formation by binding to it. HEK293T cells were transfected with RIPK1, RIPK3 and VDR containing respective tags. Immunoprecipitaiton followed by western blot showed that the RIPK1-RIPK3 complex formation did not change due to the presence of VDR (Fig. 26). However, with our experience on coimmunoprecipitation analyses, RIPK1-RIPK3 complex seemed much stronger than RIPK1-VDR complex. Therefore, ectopic expression data might not reflect the endogenous influence of

VDR on RIPK1-RIPK3 complex formation. Hence, it may be required to repeat the experiment in endogenous protein level.

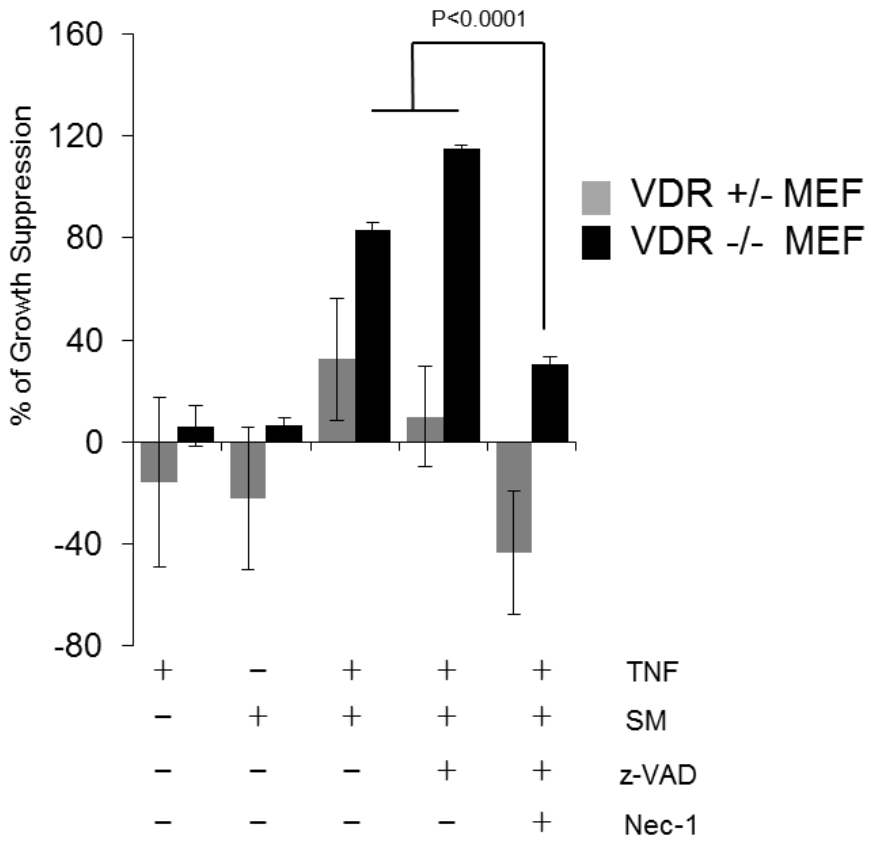

Figure 25: The involvement of VDR in necroptosis appears cell line specific
VDR heterozygous and VDR null mouse embryonic fibroblast (VDR+/-MEF and VDR-/-MEF, respectively) cells were plated onto 96 well plate. 24 hours later, cells were treated with TNFα (10ng/ml), SM (1μM), z-VAD (10μM), Nec-1(10μM) alone or in combination as indicated, for 16 hours. Cell growth suppression was measured by MTT assay. Six samples were analyzed in parallel for each data point (n=6) and the experiment was repeated twice.

3.12 RIPK1 expression varies between platinum sensitive and resistant cell lines

The problem with chemotherapeutic drugs even after first successful chemotherapy is that, the patient becomes chemoresistant within months. It is more prevalent in ovarian cancer [253]. To determine the effect of RIPK1 expression in platinum sensitive and resistant cancer cell lines, we utilized three pairs of ovarian cancer cisplatin sensitive and resistant cancer cell lines. Sensitive cell lines were TykNu, PE01, M41 and their resistant counterpart cell lines were TykNuR, PE04, M41R, respectively. We first performed a western blot to see whether there is any differential pattern of expression of RIPK1 among the sensitive and resistant cancer cell lines. Interestingly, cisplatin resistant cell lines showed either slight (TykNuR, M41R) or significantly (PE04) increased expression of RIPK1 compared to their cisplatin sensitive counterpart cell line (Fig 27). This suggests that during the process of gaining resistance to the drug, these cell lines also had more RIPK1 expression. This also potentiates the fact that RIPK1 may work in cancer cell survival.

3.13 VDR deletion defer mice death by necroptotic shock *in vivo*

As the role of VDR in necroptotic induction is cell line specific, it is important to determine the role of VDR in necroptosis *in vivo*. A well-established test for necroptotic induction in mice is injecting mTNFα through tail-vein injection [88, 117] (Fig. 28). This induces systemic inflammatory respiratory syndrome (SIRS) and septic shock in mice. SIRS happens due to systemic inflammation. The final outcome of the treatment is severe hypothermia and death [117]. To know whether VDR plays a role in inducing necroptosis *in vivo* or rather protects mice from it, we injected mTNFα in C57BL6 VDR wild type and null mice through tail-vein injection. VDR wild type mice suffered necroptotic shock and started dying within 2 hours of mTNFα

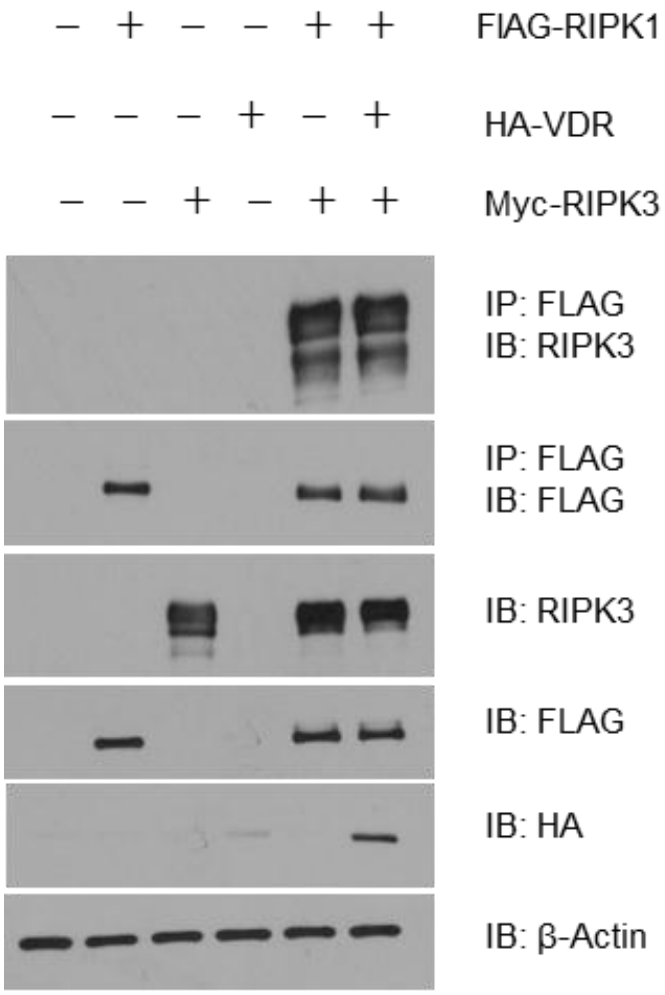

Figure 26: RIPK1-RIPK3 *in vitro* binding does not get affected by VDR

293T cells were transfected with 1.5 μg of Flag-tagged RIPK1, HA-tagged VDR and Myc-tagged RIPK3 as indicated. 48 hours posttransfection, cell extract were immunoprecipitated with Flag antibodies followed by immunoblotting with indicated antibodies.

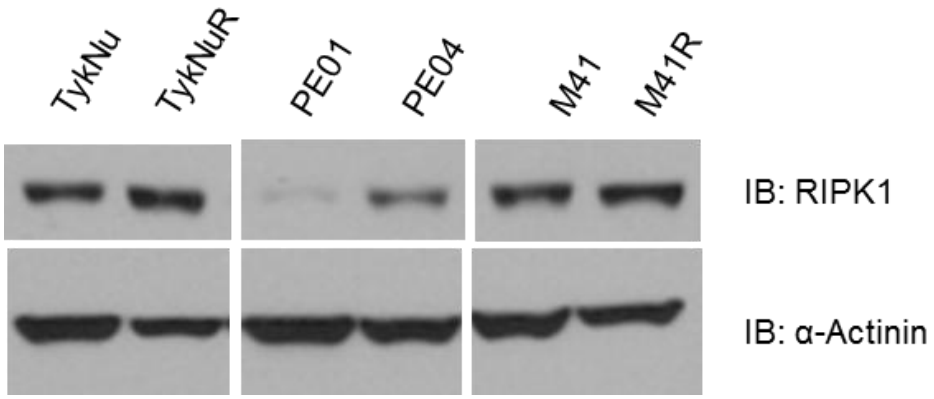

Figure 27: RIPK1 level is increased in platinum resistant ovarian cancer cell lines
Cisplatin sensitive ovarian cancer cell lines, TykNu, PE01, M41 and their resistant counterpart cell lines TykNuR, PE04, M41, respectively were cultured in 100mm dish. Cell extracts were immunoblotted to measure the expression level of RIPK1.

injection and continued to show the response afterwards. On the other hand, VDR null mice showed delayed response to mTNFα (Fig. 29). Although VDR deletion could not fully rescue the mice from necroptotic shock, it could delay the death more than VDR wild type mice (at 8 hours, p=0.0148).

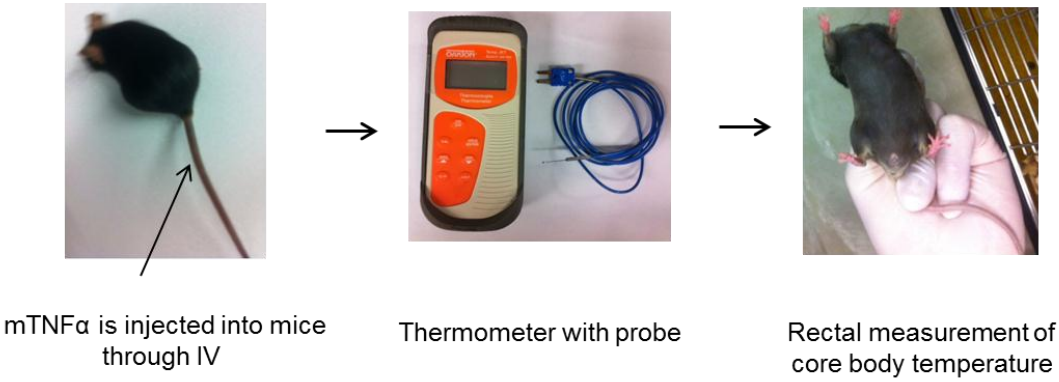

Figure 28: Procedure of necroptotic induction in mice and temperature measurement
Mice are injected mTNFα intravenously through tail-vein injection. A rectal probe attached with a thermometer is used to measure the core-body temperature.

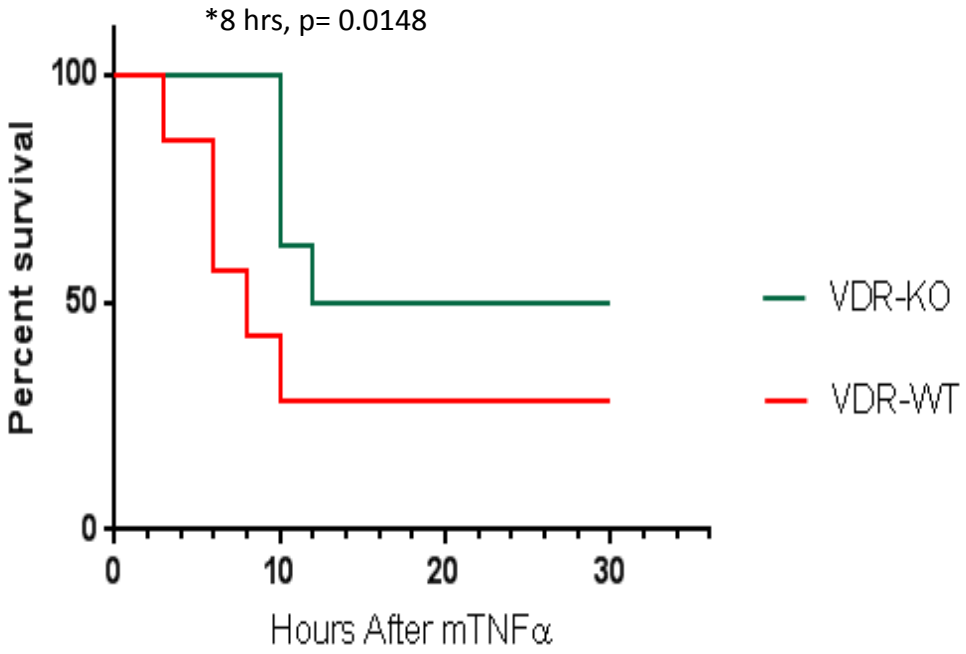

Figure 29: VDR deletion delays necroptotic induction in mice
Kaplan-Meier survival plot of C57BL/6 male VDR wild type (n=7) or null mice (n=8). Mice were injected with mTNFα (450µg/kg, Peprotech) intravenously through tail-vein injection. Core body temperature was measured through indicated time period for the next 48 hours. Mice with temperature below 23.6° were euthanized for ethical reasons.

CHAPTER 4
DISCUSSION

Our lab has previously shown that 1,25D3 regulates signaling pathways mediated by cell death ligands [254]. Therefore, it is perceivable that the downstream effector proteins of 1,25D3 and cell death ligands may cross-talk with each other. RIPK1 is well established as an essential molecule involved in mediating cell survival and death mechanisms. In this study, we have defined a novel function for RIPK1 in nuclear receptor action by identifying it as a VDR repressor. Reporter analyses on two different VDR promoter constructs have first shown that RIPK1 inhibits the transcriptional activity of VDR. Further studies also showed that this inhibition was independent of its kinase activity. Subsequent co-precipitation analyses have revealed that RIPK1 forms a complex with VDR, and deletion analyses defined the interaction interface to the LBD of the receptor. Interestingly, the kinase domain of RIPK1 appears to be required for its binding to VDR, even though its kinase activity is expendable for VDR repression. While the molecular mechanisms underlying VDR repression by RIPK1 remains largely to be defined, our studies suggest that increased cytoplasmic retention of VDR may be part of the picture. More importantly, RIPK1-knockout or knockdown increased the sensitivity of cells to 1,25D3-induced growth suppression, demonstrating the significance and clinical relevance of the knowledge gained in our mechanistic studies. Overall, our studies suggest that the depletion of RIPK1 may be an effective way to increase the sensitivity of cancers to 1,25D3 and its analogs.

Our studies have clearly demonstrated that VDR repression by RIPK1 occurs independently of its kinase activity and is mostly likely mediated through its adaptor function as a VDR-interacting protein. Although our data suggest that the increased cytoplasmic retention of VDR by RIPK1 binding as part of the mechanisms underlying VDR suppression, it should be noted that the nuclear localization signals have been identified in the DNA-binding domain and hinge region of VDR, whereas RIPK1 binding was mapped to the LBD (Fig.13). On the other hand, nuclear exporting signals have been located to the LBD of VDR. It is thus perceivable that, RIPK1 might promote VDR nuclear export. The issue is that RIPK1 is a cytoplasmic protein and we have not detected nuclear RIPK1 in our studies, making it less likely for RIPK1 binding to directly promote the nuclear export of VDR. Nevertheless, our mapping analyses suggest that multiple regions of RIPK1 are involved in its binding to VDR. It is possible that, after the primary contact with the LBD is made, RIPK1 may interrupt the interaction between VDR and nuclear pore structures through steric hindrance by secondary contacts with other regions in VDR. Besides hormone binding, the LBD is also known to be involved in mediating interactions between VDR and cofactors. Thus, RIPK1 binding may cause VDR repression not only by increasing VDR retention in the cytoplasm, but also by interfering with the recruitment of transcriptional cofactor. One possible explanation of VDR downregulation by RIPK1 is through the inhibition of the formation of VDR-RXR heterodimer, since RXR binds to VDR through the C-terminal LBD. However, our data showed that RIPK1 did not interfere with the VDR-RXR binding. Hence, other transcriptional activator or repressor might have played a role in VDR activity suppression by RIPK1.

A large amount of epidemiological and molecular studies have shown that vitamin D reduces cancer risk and that 1,25D3 analogs are promising agents for cancer treatment. However, the potential of 1,25D3 and its analogs in cancer intervention has yet to be realized in clinics. A majority of the published studies about vitamin D and cancer have been dedicated to pathways through which the hormone suppresses cancer growth and invasion; very little is known about the molecules and pathways that suppress VDR action and cause resistance of cancer cells to 1,25D3 and its analogs. The presented studies suggest that the expression of RIPK1 may cause resistance to 1,25D3-induced growth suppression and identify RIPK1 as a molecular target for increasing the therapeutic potential of 1,25D3 analogs for cancer. The fact that VDR suppression is not mediated by the kinase activity of RIPK1 makes it more challenging to target RIPK1 for cancer intervention. However, technological advancements in small interference RNA and gene editing tools could make it possible to develop strategies for targeted depletion of RIPK1 in cancer.

Studies have also shown that RIPK1 is subjected to ubiquitination [60, 255]. Our data showed that the complex formation between VDR and the ubiquitin mutant of RIPK1 (RIPK1-K377R) was stronger than VDR and wild type RIPK1 (Fig. 23). This was also supported by our endogenous binding data in L929 cells. Cell death induction by TNFα and etoposide/SM showed increased endogenous binding in presence of proteasome complex inhibitor MG132 (Fig. 22A and 22B). Therefore, our date clearly indicated that VDR-RIPK1 complex is subjected to be degraded after formation. This might be postulated that proteasome complex can recognize this complex and degrade it by ubiquitination to keep the cell alive.

The VDR-RIPK1 complex appeared to be regulated by necroptotic stimuli and we have detected a cell line specific effect of VDR on necroptosis (Figs.24A and B, 25). However, more extensive studies are needed to explain the results from different cell lines that appear to be conflicting. It should be mentioned that necroptotic stimuli can also lead to RIPK1 mediated apoptotic cell death, in case if RIPK3 is not expressed in the cell line [100]. Therefore, to confirm the role of VDR in necroptosis in both MMT-VDR and VDR-MEF cell lines, further experiments are required. Downstream effect of necroptotic induction such as phosphorylation of RIPK3 and MLKL as well as time lapse video microscopy through staining of RIPK1-RIPK3 complex have not been done in our study. Therefore, these experiments might be done in future to confirm the role of VDR in necroptotic cell death in these cell lines.

While the presented studies have focused on the effect of RIPK1 on VDR action and 1,25D3-induced growth suppression, the complex formation between RIPK1 and VDR suggest that VDR and its ligand may regulate RIPK1 functions. We first assumed that VDR interaction with RIPK1 may interfere with RIPK1-RIPK3 complex formation. However, our overexpression data does not indicate that VDR disrupts RIPK1-RIPK3 binding (Fig. 26). This should be noted that RIPK1-RIPK3 binding may be stronger than VDR-RIPK1 complex and hence, is not visibly getting affected. This experiment was done in overexpression system and should also be done in endogenous system to see whether VDR can disrupt the RIPK1-RIPK3 binding or not. MEF cell line is a good candidate to show RIPK1-RIPK3 complex formation through necroptotic induction. Therefore, VDR+/- and VDR-/- MEF cell lines can be used to see whether VDR interferes with RIPK1-RIPK3 complex formation at the endogenous level. Stable expression of VDR in VDR-/- MEF cell line can confirm the finding in this experiment.

In the current study, we have also shown the *in vivo* role of VDR role in necroptosis. Established *in vivo* studies of TNFα induced necroptosis employs tail-vein injection of TNFα. This causes a rapid core body temperature due to SIRS, which shows necrotic cell death in mice. Our study showed that although VDR in cell death assays showed differential role depending upon the cell line, VDR is actually required to induce necroptosis *in vivo*. Despite the fact that VDR deletion could not completely inhibit TNFα induced necroptotic death in mice, the VDR-KO mice survived longer than VDR-WT mice. This underscores the role of VDR in inducing necroptotic induction *in vivo*.

Our comparative expression study in cisplatin sensitive and resistant cells showed that RIPK1 is differentially expressed in cells. We also observed that RIPK1 level in those platinum resistant cell lines was slightly increased. Whether RIPK1 level also increased during the process of drug resistance can also be worth exploring. Ovarian cancer in most of the patients is detected at the advanced stage when the tumor has already metastasized to other organs. The role of RIPK1 in both cell survival and cell death pathway makes it an ideal candidate for testing this protein to play any role in cancer cell survival in chemotherapy. Recent studies show that RIPK1 is required in cancer cell survival and downregulation of RIPK1 in those cells increases chemotherapeutic response [101, 103]. Our present study suggests that knockdown of RIPK1 increases 1,25D3 induced cancer cell growth suppression. While we did not perform any cell death assay after cisplatin or carboplatin treatment on these RIPK1 knockdown cancer cells, our work can also be extended to chemotherapeutic efficacy on these cells. It may be also studied whether the combined treatment of 1,25D3 and platinum drugs have any additive or synergistic effect on RIPK1 knockdown ovarian cancer cells.

4.1 Summary and perspective

Our data allow us to draw the following conclusions. 1) RIPK1 decreases VDR activity and the suppressive action does not depend on its kinase activity of RIPK1; 2) RIPK1 binds to VDR, specifically the binding is mediated by the kinase and intermediate domain of RIPK1 and the C-terminal ligand binding domain of VDR; 3) RIPK1 retains VDR into the cytosol and controls the nuclear translocation of VDR both in absence and in presence of 1,25D3; 4) Depletion of RIPK1 in cancer cell increases 1,25D3 mediated cell growth suppression; 5) The role of VDR in necroptotic cell death is cell line specific. 6) VDR deletion decreases necropototic inflammation *in vivo* in mice.

Based on the above conclusions, future studies should focus on the investigation of the following observations for their potential significance in understanding 1,25D3 actions in cancer and potential clinical relevance.

First, although it has been shown that RIPK1 decreased VDR activity by inhibiting nuclear translocation, the mechanism is not fully understood yet. It has not been studied yet whether RIPK1 also cross-talks with transcriptional coactivators or corepressors of VDR. Detailed mechanistic study is required to answer questions such as whether RIPK1 can also squelch SRC-1 to the cytoplasm to contribute to the inhibition of VDR activity.

Secondly, the cross-talk between VDR and RIPK3 may be possible in cell survival and death signaling through RIPK1. Whereas RIPK1 was present in all cell lines that we have studied, RIPK3 expression has not been tested (Fig 27). RIPK3 expression study can also be done in these cell lines to find out potential relationship between platinum resistance and RIPK3 expression. Our overexpression data showed that VDR did not interfere with RIPK1-RIPK3 binding (Fig 26). However, this study was done in overexpression system where abundant

proteins were present to bind with each other and in HEK293T cell line, which is nonresponsive to necroptotic induction. Therefore, studies with endogenous proteins in cell lines and in VDR-WT and null MEFs need to be performed to see whether VDR affects RIPK1-RIPK3 interaction during necroptotic cell death. Restoring VDR expression in VDR-null MEF can be used to confirm the role of VDR in necroptosis.

Lastly, our studies appear to corroborate with the previous findings that RIPK1 is an oncogenic protein and that downregulating RIPK1 increases the chemosensitivity of cancer cells. In our studies, we have shown that RIPK1 knockdown also increased 1,25D3 mediated cell growth suppression in ovarian cancer cell lines. This can be further tested in other vitamin D sensitive cancer cell lines. Along with this, the sensitivity of ovarian cancer cells to therapeutic drugs can be tested in our RIPK1 knockdown cells. As for the 1,25D3 sensitivity, it is worth to find out whether combined treatments of 1,25D3 and other chemotherapeutic drugs have more profound effects, additive or synergistic, on suppressing cancer cell growth in RIPK1 knockdown cells.

In summary, my thesis work is the first to demonstrate an interaction between RIPK1 and VDR. The studies suggest that inhibiting RIPK1 expression can be an effective strategy to potentiate the effects of 1,25D3 in suppressing tumor growth. Together with RIPK1 depletion, 1,25D3 treatments may become an important component of standard cancer therapy in clinics.

References:

1. Wallach, D., T.B. Kang, and A. Kovalenko, Concepts of tissue injury and cell death in inflammation: a historical perspective. Nat Rev Immunol, 2014. **14**(1): p. 51-9.
2. Kerr, J.F., A.H. Wyllie, and A.R. Currie, Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer, 1972. **26**(4): p. 239-57.
3. Kroemer, G., et al., Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ, 2009. **16**(1): p. 3-11.
4. Galluzzi, L., et al., Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death Differ, 2012. **19**(1): p. 107-20.
5. Kerr, J.F., History of the events leading to the formulation of the apoptosis concept. Toxicology, 2002. **181-182**: p. 471-4.
6. Ellis, H.M. and H.R. Horvitz, Genetic control of programmed cell death in the nematode C. elegans. Cell, 1986. **44**(6): p. 817-29.
7. Horvitz, H.R., Genetic control of programmed cell death in the nematode Caenorhabditis elegans. Cancer Res, 1999. **59**(7 Suppl): p. 1701s-1706s.
8. Renehan, A.G., C. Booth, and C.S. Potten, What is apoptosis, and why is it important? BMJ, 2001. **322**(7301): p. 1536-8.
9. Elmore, S., Apoptosis: a review of programmed cell death. Toxicol Pathol, 2007. **35**(4): p. 495-516.
10. Wajant, H., The Fas signaling pathway: more than a paradigm. Science, 2002. **296**(5573): p. 1635-6.
11. Schutze, S., V. Tchikov, and W. Schneider-Brachert, Regulation of TNFR1 and CD95 signalling by receptor compartmentalization. Nat Rev Mol Cell Biol, 2008. **9**(8): p. 655-62.
12. Mehlen, P. and D.E. Bredesen, Dependence receptors: from basic research to drug development. Sci Signal, 2011. **4**(157): p. mr2.
13. Nagata, S., Apoptosis by death factor. Cell, 1997. **88**(3): p. 355-65.
14. Kim, J.W., E.J. Choi, and C.O. Joe, Activation of death-inducing signaling complex (DISC) by pro-apoptotic C-terminal fragment of RIP. Oncogene, 2000. **19**(39): p. 4491-9.
15. Hughes, M.A., et al., Reconstitution of the death-inducing signaling complex reveals a substrate switch that determines CD95-mediated death or survival. Mol Cell, 2009. **35**(3): p. 265-79.
16. Kischkel, F.C., et al., Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J, 1995. **14**(22): p. 5579-88.
17. Muzio, M., et al., FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. Cell, 1996. **85**(6): p. 817-27.

18. Kiechle, F.L. and X. Zhang, Apoptosis: biochemical aspects and clinical implications. Clin Chim Acta, 2002. **326**(1-2): p. 27-45.
19. Fulda, S., et al., Cellular stress responses: cell survival and cell death. Int J Cell Biol, 2010. **2010**: p. 214074.
20. Tait, S.W. and D.R. Green, Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol, 2010. **11**(9): p. 621-32.
21. Cande, C., et al., Apoptosis-inducing factor (AIF): caspase-independent after all. Cell Death Differ, 2004. **11**(6): p. 591-5.
22. Simon, H.U., A. Haj-Yehia, and F. Levi-Schaffer, Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis, 2000. **5**(5): p. 415-8.
23. Um, H.D., J.M. Orenstein, and S.M. Wahl, Fas mediates apoptosis in human monocytes by a reactive oxygen intermediate dependent pathway. J Immunol, 1996. **156**(9): p. 3469-77.
24. Mizushima, N., Autophagy: process and function. Genes Dev, 2007. **21**(22): p. 2861-73.
25. Glick, D., S. Barth, and K.F. Macleod, Autophagy: cellular and molecular mechanisms. J Pathol, 2010. **221**(1): p. 3-12.
26. Levine, B. and D.J. Klionsky, Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell, 2004. **6**(4): p. 463-77.
27. Tasdemir, E., et al., Methods for assessing autophagy and autophagic cell death. Methods Mol Biol, 2008. **445**: p. 29-76.
28. Golstein, P. and G. Kroemer, Cell death by necrosis: towards a molecular definition. Trends Biochem Sci, 2007. **32**(1): p. 37-43.
29. Kanduc, D., et al., Cell death: apoptosis versus necrosis (review). Int J Oncol, 2002. **21**(1): p. 165-70.
30. Nikoletopoulou, V., et al., Crosstalk between apoptosis, necrosis and autophagy. Biochim Biophys Acta, 2013. **1833**(12): p. 3448-59.
31. Brauchle, E., et al., Cell death stages in single apoptotic and necrotic cells monitored by Raman microspectroscopy. Sci Rep, 2014. **4**: p. 4698.
32. Castedo, M., et al., Cell death by mitotic catastrophe: a molecular definition. Oncogene, 2004. **23**(16): p. 2825-37.
33. Vakifahmetoglu, H., M. Olsson, and B. Zhivotovsky, Death through a tragedy: mitotic catastrophe. Cell Death Differ, 2008. **15**(7): p. 1153-62.
34. Grossmann, J., Molecular mechanisms of "detachment-induced apoptosis--Anoikis". Apoptosis, 2002. **7**(3): p. 247-60.
35. Gilmore, A.P., Anoikis. Cell Death Differ, 2005. **12 Suppl 2**: p. 1473-7.
36. Taddei, M.L., et al., Anoikis: an emerging hallmark in health and diseases. J Pathol, 2012. **226**(2): p. 380-93.
37. Brennan, M.A. and B.T. Cookson, Salmonella induces macrophage death by caspase-1-dependent necrosis. Mol Microbiol, 2000. **38**(1): p. 31-40.
38. Fernandes-Alnemri, T., et al., The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. Cell Death Differ, 2007. **14**(9): p. 1590-604.
39. Bergsbaken, T., S.L. Fink, and B.T. Cookson, Pyroptosis: host cell death and inflammation. Nat Rev Microbiol, 2009. **7**(2): p. 99-109.
40. Stanger, B.Z., et al., RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. Cell, 1995. **81**(4): p. 513-23.

41. Hsu, H., et al., TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. Immunity, 1996. **4**(4): p. 387-96.
42. Meylan, E. and J. Tschopp, The RIP kinases: crucial integrators of cellular stress. Trends Biochem Sci, 2005. **30**(3): p. 151-9.
43. Chou, J.J., et al., Solution structure of the RAIDD CARD and model for CARD/CARD interaction in caspase-2 and caspase-9 recruitment. Cell, 1998. **94**(2): p. 171-80.
44. Zhang, D., J. Lin, and J. Han, Receptor-interacting protein (RIP) kinase family. Cell Mol Immunol, 2010. **7**(4): p. 243-9.
45. Kobayashi, K., et al., RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. Nature, 2002. **416**(6877): p. 194-9.
46. Martinon, F. and J. Tschopp, Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. Cell, 2004. **117**(5): p. 561-74.
47. Sun, X., et al., Identification of a novel homotypic interaction motif required for the phosphorylation of receptor-interacting protein (RIP) by RIP3. J Biol Chem, 2002. **277**(11): p. 9505-11.
48. Upton, J.W., W.J. Kaiser, and E.S. Mocarski, Cytomegalovirus M45 cell death suppression requires receptor-interacting protein (RIP) homotypic interaction motif (RHIM)-dependent interaction with RIP1. J Biol Chem, 2008. **283**(25): p. 16966-70.
49. Holland, P., et al., RIP4 is an ankyrin repeat-containing kinase essential for keratinocyte differentiation. Curr Biol, 2002. **12**(16): p. 1424-8.
50. Meylan, E., et al., RIP4 (DIK/PKK), a novel member of the RIP kinase family, activates NF-kappa B and is processed during apoptosis. EMBO Rep, 2002. **3**(12): p. 1201-8.
51. Hu, Y., et al., Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. Science, 1999. **284**(5412): p. 316-20.
52. Li, Q., et al., IKK1-deficient mice exhibit abnormal development of skin and skeleton. Genes Dev, 1999. **13**(10): p. 1322-8.
53. Takeda, K., et al., Limb and skin abnormalities in mice lacking IKKalpha. Science, 1999. **284**(5412): p. 313-6.
54. Zha, J., et al., RIP5 is a RIP-homologous inducer of cell death. Biochem Biophys Res Commun, 2004. **319**(2): p. 298-303.
55. Paisan-Ruiz, C., et al., Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. Neuron, 2004. **44**(4): p. 595-600.
56. Greggio, E., et al., Mutations in LRRK2/dardarin associated with Parkinson disease are more toxic than equivalent mutations in the homologous kinase LRRK1. J Neurochem, 2007. **102**(1): p. 93-102.
57. Braithwaite, S.P., LRRK2 in Parkinson's disease: building an understanding of disease etiology. FEBS J, 2009. **276**(22): p. 6427.
58. Shalini, S., et al., Old, new and emerging functions of caspases. Cell Death Differ, 2015. **22**(4): p. 526-39.
59. Li, J., et al., The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. Cell, 2012. **150**(2): p. 339-50.
60. Ea, C.K., et al., Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol Cell, 2006. **22**(2): p. 245-57.
61. Li, H., et al., Ubiquitination of RIP is required for tumor necrosis factor alpha-induced NF-kappaB activation. J Biol Chem, 2006. **281**(19): p. 13636-43.

62. Ofengeim, D. and J. Yuan, Regulation of RIP1 kinase signalling at the crossroads of inflammation and cell death. Nat Rev Mol Cell Biol, 2013. **14**(11): p. 727-36.
63. Lin, Y., et al., Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. Genes Dev, 1999. **13**(19): p. 2514-26.
64. Park, Y.H., et al., Formation of the death domain complex between FADD and RIP1 proteins in vitro. Biochim Biophys Acta, 2013. **1834**(1): p. 292-300.
65. Xin, F. and P. Radivojac, Post-translational modifications induce significant yet not extreme changes to protein structure. Bioinformatics, 2012. **28**(22): p. 2905-13.
66. Duan, G. and D. Walther, The roles of post-translational modifications in the context of protein interaction networks. PLoS Comput Biol, 2015. **11**(2): p. e1004049.
67. Berger, S.B., et al., Cutting Edge: RIP1 kinase activity is dispensable for normal development but is a key regulator of inflammation in SHARPIN-deficient mice. J Immunol, 2014. **192**(12): p. 5476-80.
68. Degterev, A., et al., Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol, 2008. **4**(5): p. 313-21.
69. Dondelinger, Y., P. Vandenabeele, and M.J. Bertrand, Regulation of RIPK1's cell death function by phosphorylation. Cell Cycle, 2016. **15**(1): p. 5-6.
70. McQuade, T., Y. Cho, and F.K. Chan, Positive and negative phosphorylation regulates RIP1- and RIP3-induced programmed necrosis. Biochem J, 2013. **456**(3): p. 409-15.
71. Festjens, N., et al., RIP1, a kinase on the crossroads of a cell's decision to live or die. Cell Death Differ, 2007. **14**(3): p. 400-10.
72. Degterev, A., et al., Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol, 2005. **1**(2): p. 112-9.
73. Lee, T.H., et al., The kinase activity of Rip1 is not required for tumor necrosis factor-alpha-induced IkappaB kinase or p38 MAP kinase activation or for the ubiquitination of Rip1 by Traf2. J Biol Chem, 2004. **279**(32): p. 33185-91.
74. Wang, L., F. Du, and X. Wang, TNF-alpha induces two distinct caspase-8 activation pathways. Cell, 2008. **133**(4): p. 693-703.
75. Humphries, F., et al., RIP kinases: key decision makers in cell death and innate immunity. Cell Death Differ, 2015. **22**(2): p. 225-36.
76. He, S., et al., Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell, 2009. **137**(6): p. 1100-11.
77. Cho, Y.S., et al., Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell, 2009. **137**(6): p. 1112-23.
78. Zhang, D.W., et al., RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science, 2009. **325**(5938): p. 332-6.
79. Sun, L., et al., Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell, 2012. **148**(1-2): p. 213-27.
80. Cai, Z., et al., Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis. Nat Cell Biol, 2014. **16**(1): p. 55-65.
81. Zhao, J., et al., Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. Proc Natl Acad Sci U S A, 2012. **109**(14): p. 5322-7.
82. Nogusa, S., et al., RIPK3 Activates Parallel Pathways of MLKL-Driven Necroptosis and FADD-Mediated Apoptosis to Protect against Influenza A Virus. Cell Host Microbe, 2016. **20**(1): p. 13-24.

83. Murphy, J.M., et al., The pseudokinase MLKL mediates necroptosis via a molecular switch mechanism. Immunity, 2013. **39**(3): p. 443-53.
84. Wang, H., et al., Mixed lineage kinase domain-like protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP3. Mol Cell, 2014. **54**(1): p. 133-46.
85. Drazic, A., et al., The world of protein acetylation. Biochim Biophys Acta, 2016. **1864**(10): p. 1372-401.
86. Zhao, S., et al., Regulation of cellular metabolism by protein lysine acetylation. Science, 2010. **327**(5968): p. 1000-4.
87. Narayan, N., et al., The NAD-dependent deacetylase SIRT2 is required for programmed necrosis. Nature, 2012. **492**(7428): p. 199-204.
88. Newton, K., et al., Is SIRT2 required for necroptosis? Nature, 2014. **506**(7489): p. E4-6.
89. Preyat, N., et al., Intracellular nicotinamide adenine dinucleotide promotes TNF-induced necroptosis in a sirtuin-dependent manner. Cell Death Differ, 2016. **23**(1): p. 29-40.
90. Hershko, A. and A. Ciechanover, The ubiquitin system. Annu Rev Biochem, 1998. **67**: p. 425-79.
91. Pickart, C.M., Mechanisms underlying ubiquitination. Annu Rev Biochem, 2001. **70**: p. 503-33.
92. Ulrich, H.D., Degradation or maintenance: actions of the ubiquitin system on eukaryotic chromatin. Eukaryot Cell, 2002. **1**(1): p. 1-10.
93. Hochstrasser, M., Ubiquitin-dependent protein degradation. Annu Rev Genet, 1996. **30**: p. 405-39.
94. Pickart, C.M. and M.J. Eddins, Ubiquitin: structures, functions, mechanisms. Biochim Biophys Acta, 2004. **1695**(1-3): p. 55-72.
95. Woelk, T., et al., The ubiquitination code: a signalling problem. Cell Div, 2007. **2**: p. 11.
96. Hicke, L., H.L. Schubert, and C.P. Hill, Ubiquitin-binding domains. Nat Rev Mol Cell Biol, 2005. **6**(8): p. 610-21.
97. Chen, Z.J. and L.J. Sun, Nonproteolytic functions of ubiquitin in cell signaling. Mol Cell, 2009. **33**(3): p. 275-86.
98. Kelliher, M.A., et al., The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. Immunity, 1998. **8**(3): p. 297-303.
99. Feoktistova, M., et al., cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. Mol Cell, 2011. **43**(3): p. 449-63.
100. Tenev, T., et al., The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. Mol Cell, 2011. **43**(3): p. 432-48.
101. Liu, X.Y., et al., RIP1 Kinase Is an Oncogenic Driver in Melanoma. Cancer Res, 2015. **75**(8): p. 1736-48.
102. Luan, Q., et al., RIPK1 regulates survival of human melanoma cells upon endoplasmic reticulum stress through autophagy. Autophagy, 2015. **11**(7): p. 975-94.
103. Wang, Q., et al., Receptor-interacting protein 1 increases chemoresistance by maintaining inhibitor of apoptosis protein levels and reducing reactive oxygen species through a microRNA-146a-mediated catalase pathway. J Biol Chem, 2014. **289**(9): p. 5654-63.
104. Laster, S.M., J.G. Wood, and L.R. Gooding, Tumor necrosis factor can induce both apoptic and necrotic forms of cell lysis. J Immunol, 1988. **141**(8): p. 2629-34.

105. Grooten, J., et al., Cell membrane permeabilization and cellular collapse, followed by loss of dehydrogenase activity: early events in tumour necrosis factor-induced cytotoxicity. Cytokine, 1993. **5**(6): p. 546-55.
106. Vercammen, D., et al., Tumour necrosis factor-induced necrosis versus anti-Fas-induced apoptosis in L929 cells. Cytokine, 1997. **9**(11): p. 801-8.
107. Holler, N., et al., Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol, 2000. **1**(6): p. 489-95.
108. Smith, C.C. and D.M. Yellon, Necroptosis, necrostatins and tissue injury. J Cell Mol Med, 2011. **15**(9): p. 1797-806.
109. Cook, W.D., et al., RIPK1- and RIPK3-induced cell death mode is determined by target availability. Cell Death Differ, 2014. **21**(10): p. 1600-12.
110. Sun, H., et al., Design of small-molecule peptidic and nonpeptidic Smac mimetics. Acc Chem Res, 2008. **41**(10): p. 1264-77.
111. Dannappel, M., et al., RIPK1 maintains epithelial homeostasis by inhibiting apoptosis and necroptosis. Nature, 2014. **513**(7516): p. 90-4.
112. Takahashi, N., et al., RIPK1 ensures intestinal homeostasis by protecting the epithelium against apoptosis. Nature, 2014. **513**(7516): p. 95-9.
113. Kaiser, W.J., et al., RIP1 suppresses innate immune necrotic as well as apoptotic cell death during mammalian parturition. Proc Natl Acad Sci U S A, 2014. **111**(21): p. 7753-8.
114. Newton, K., et al., Activity of protein kinase RIPK3 determines whether cells die by necroptosis or apoptosis. Science, 2014. **343**(6177): p. 1357-60.
115. Dillon, C.P., et al., RIPK1 blocks early postnatal lethality mediated by caspase-8 and RIPK3. Cell, 2014. **157**(5): p. 1189-202.
116. Silke, J., J.A. Rickard, and M. Gerlic, The diverse role of RIP kinases in necroptosis and inflammation. Nat Immunol, 2015. **16**(7): p. 689-97.
117. Duprez, L., et al., RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome. Immunity, 2011. **35**(6): p. 908-18.
118. Kaiser, W.J., et al., RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature, 2011. **471**(7338): p. 368-72.
119. Cauwels, A., et al., Caspase inhibition causes hyperacute tumor necrosis factor-induced shock via oxidative stress and phospholipase A2. Nat Immunol, 2003. **4**(4): p. 387-93.
120. Rock, K.L. and H. Kono, The inflammatory response to cell death. Annu Rev Pathol, 2008. **3**: p. 99-126.
121. White, E.S. and A.R. Mantovani, Inflammation, wound repair, and fibrosis: reassessing the spectrum of tissue injury and resolution. J Pathol, 2013. **229**(2): p. 141-4.
122. Mori, W., The Shwartzman reaction: a review including clinical manifestations and proposal for a univisceral or single organ third type. Histopathology, 1981. **5**(2): p. 113-26.
123. Henson, P.M. and R.B. Johnston, Jr., Tissue injury in inflammation. Oxidants, proteinases, and cationic proteins. J Clin Invest, 1987. **79**(3): p. 669-74.
124. Savill, J.S., et al., Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. J Clin Invest, 1989. **83**(3): p. 865-75.
125. Voll, R.E., et al., Immunosuppressive effects of apoptotic cells. Nature, 1997. **390**(6658): p. 350-1.

126. Fadok, V.A., et al., Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J Clin Invest, 1998. **101**(4): p. 890-8.
127. Venegas, V. and Z. Zhou, Two alternative mechanisms that regulate the presentation of apoptotic cell engulfment signal in Caenorhabditis elegans. Mol Biol Cell, 2007. **18**(8): p. 3180-92.
128. Silva, M.T., A. do Vale, and N.M. dos Santos, Secondary necrosis in multicellular animals: an outcome of apoptosis with pathogenic implications. Apoptosis, 2008. **13**(4): p. 463-82.
129. Krysko, D.V., et al., Immunogenic cell death and DAMPs in cancer therapy. Nat Rev Cancer, 2012. **12**(12): p. 860-75.
130. Kaczmarek, A., P. Vandenabeele, and D.V. Krysko, Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. Immunity, 2013. **38**(2): p. 209-23.
131. Bianconi, E., et al., An estimation of the number of cells in the human body. Ann Hum Biol, 2013. **40**(6): p. 463-71.
132. Evans, R.M. and D.J. Mangelsdorf, Nuclear Receptors, RXR, and the Big Bang. Cell, 2014. **157**(1): p. 255-66.
133. Green, S., et al., Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature, 1986. **320**(6058): p. 134-9.
134. Hollenberg, S.M., et al., Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature, 1985. **318**(6047): p. 635-41.
135. Weinberger, C., et al., The c-erb-A gene encodes a thyroid hormone receptor. Nature, 1986. **324**(6098): p. 641-6.
136. Giguere, V., et al., Identification of a receptor for the morphogen retinoic acid. Nature, 1987. **330**(6149): p. 624-9.
137. Petkovich, M., et al., A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature, 1987. **330**(6147): p. 444-50.
138. Robinson-Rechavi, M., H. Escriva Garcia, and V. Laudet, The nuclear receptor superfamily. J Cell Sci, 2003. **116**(Pt 4): p. 585-6.
139. Adams, M.D., et al., The genome sequence of Drosophila melanogaster. Science, 2000. **287**(5461): p. 2185-95.
140. Robinson-Rechavi, M. and V. Laudet, Bioinformatics of nuclear receptors. Methods Enzymol, 2003. **364**: p. 95-118.
141. Sluder, A.E., et al., The nuclear receptor superfamily has undergone extensive proliferation and diversification in nematodes. Genome Res, 1999. **9**(2): p. 103-20.
142. McEwan, I.J., Nuclear receptors: one big family. Methods Mol Biol, 2009. **505**: p. 3-18.
143. Nagy, L. and J.W. Schwabe, Mechanism of the nuclear receptor molecular switch. Trends Biochem Sci, 2004. **29**(6): p. 317-24.
144. Mangelsdorf, D.J., et al., The nuclear receptor superfamily: the second decade. Cell, 1995. **83**(6): p. 835-9.
145. Beato, M., P. Herrlich, and G. Schutz, Steroid hormone receptors: many actors in search of a plot. Cell, 1995. **83**(6): p. 851-7.
146. Cole, T.J., et al., Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Genes Dev, 1995. **9**(13): p. 1608-21.

147. Couse, J.F. and K.S. Korach, Exploring the role of sex steroids through studies of receptor deficient mice. J Mol Med (Berl), 1998. **76**(7): p. 497-511.
148. Couse, J.F. and K.S. Korach, Estrogen receptor null mice: what have we learned and where will they lead us? Endocr Rev, 1999. **20**(3): p. 358-417.
149. Obr, A.E. and D.P. Edwards, The biology of progesterone receptor in the normal mammary gland and in breast cancer. Mol Cell Endocrinol, 2012. **357**(1-2): p. 4-17.
150. Moraitis, A.G., et al., The role of glucocorticoid receptors in metabolic syndrome and psychiatric illness. J Steroid Biochem Mol Biol, 2016.
151. Shibata, S., et al., Mineralocorticoid receptor phosphorylation regulates ligand binding and renal response to volume depletion and hyperkalemia. Cell Metab, 2013. **18**(5): p. 660-71.
152. Kimura, N., et al., Immunocytochemical localization of androgen receptor with polyclonal antibody in paraffin-embedded human tissues. J Histochem Cytochem, 1993. **41**(5): p. 671-8.
153. Moore, J.T., J.L. Collins, and K.H. Pearce, The nuclear receptor superfamily and drug discovery. ChemMedChem, 2006. **1**(5): p. 504-23.
154. Chawla, A., et al., Nuclear receptors and lipid physiology: opening the X-files. Science, 2001. **294**(5548): p. 1866-70.
155. Giguere, V., Orphan nuclear receptors: from gene to function. Endocr Rev, 1999. **20**(5): p. 689-725.
156. Barish, G.D. and R.M. Evans, PPARs and LXRs: atherosclerosis goes nuclear. Trends Endocrinol Metab, 2004. **15**(4): p. 158-65.
157. Evans, R.M., G.D. Barish, and Y.X. Wang, PPARs and the complex journey to obesity. Nat Med, 2004. **10**(4): p. 355-61.
158. Gurnell, M., et al., The metabolic syndrome: peroxisome proliferator-activated receptor gamma and its therapeutic modulation. J Clin Endocrinol Metab, 2003. **88**(6): p. 2412-21.
159. Rangwala, S.M. and M.A. Lazar, Peroxisome proliferator-activated receptor gamma in diabetes and metabolism. Trends Pharmacol Sci, 2004. **25**(6): p. 331-6.
160. Rosen, E.D. and B.M. Spiegelman, PPARgamma : a nuclear regulator of metabolism, differentiation, and cell growth. J Biol Chem, 2001. **276**(41): p. 37731-4.
161. Barroso, I., et al., Dominant negative mutations in human PPARgamma associated with severe insulin resistance, diabetes mellitus and hypertension. Nature, 1999. **402**(6764): p. 880-3.
162. Chawla, A., et al., PPARdelta is a very low-density lipoprotein sensor in macrophages. Proc Natl Acad Sci U S A, 2003. **100**(3): p. 1268-73.
163. Muscat, G.E., et al., Regulation of cholesterol homeostasis and lipid metabolism in skeletal muscle by liver X receptors. J Biol Chem, 2002. **277**(43): p. 40722-8.
164. Smith, A.G. and G.E. Muscat, Orphan nuclear receptors: therapeutic opportunities in skeletal muscle. Am J Physiol Cell Physiol, 2006. **291**(2): p. C203-17.
165. Huang, P., V. Chandra, and F. Rastinejad, Structural overview of the nuclear receptor superfamily: insights into physiology and therapeutics. Annu Rev Physiol, 2010. **72**: p. 247-72.
166. Germain, P., et al., Overview of nomenclature of nuclear receptors. Pharmacol Rev, 2006. **58**(4): p. 685-704.
167. Aranda, A. and A. Pascual, Nuclear hormone receptors and gene expression. Physiol Rev, 2001. **81**(3): p. 1269-304.

168. Pawlak, M., P. Lefebvre, and B. Staels, General molecular biology and architecture of nuclear receptors. Curr Top Med Chem, 2012. **12**(6): p. 486-504.
169. Carr, A.C. and M.C. Vissers, Synthetic or food-derived vitamin C--are they equally bioavailable? Nutrients, 2013. **5**(11): p. 4284-304.
170. Funk, C., On the chemical nature of the substance which cures polyneuritis in birds induced by a diet of polished rice. J Physiol, 1911. **43**(5): p. 395-400.
171. Drummond, J.C., The Nomenclature of the so-called Accessory Food Factors (Vitamins). Biochem J, 1920. **14**(5): p. 660.
172. Deluca, H.F., History of the discovery of vitamin D and its active metabolites. Bonekey Rep, 2014. **3**: p. 479.
173. Chick, D.H., Study of rickets in Vienna 1919-1922. Med Hist, 1976. **20**(1): p. 41-51.
174. Holick, M.F., et al., Photometabolism of 7-dehydrocholesterol to previtamin D3 in skin. Biochem Biophys Res Commun, 1977. **76**(1): p. 107-14.
175. Brumbaugh, P.F. and M.R. Haussler, 1 Alpha,25-dihydroxycholecalciferol receptors in intestine. I. Association of 1 alpha,25-dihydroxycholecalciferol with intestinal mucosa chromatin. J Biol Chem, 1974. **249**(4): p. 1251-7.
176. Pike, J.W. and M.B. Meyer, The vitamin D receptor: new paradigms for the regulation of gene expression by 1,25-dihydroxyvitamin D(3). Endocrinol Metab Clin North Am, 2010. **39**(2): p. 255-69, table of contents.
177. Nwachukwu, J.C. and K.W. Nettles, The nuclear receptor signalling scaffold: insights from full-length structures. EMBO J, 2012. **31**(2): p. 251-3.
178. Smith, C.L. and B.W. O'Malley, Coregulator function: a key to understanding tissue specificity of selective receptor modulators. Endocr Rev, 2004. **25**(1): p. 45-71.
179. Onate, S.A., et al., Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science, 1995. **270**(5240): p. 1354-7.
180. Sanchez-Martinez, R., et al., Vitamin D-dependent recruitment of corepressors to vitamin D/retinoid X receptor heterodimers. Mol Cell Biol, 2008. **28**(11): p. 3817-29.
181. Tagami, T., et al., The interaction of the vitamin D receptor with nuclear receptor corepressors and coactivators. Biochem Biophys Res Commun, 1998. **253**(2): p. 358-63.
182. Hsieh, J.C., et al., Human vitamin D receptor is selectively phosphorylated by protein kinase C on serine 51, a residue crucial to its trans-activation function. Proc Natl Acad Sci U S A, 1991. **88**(20): p. 9315-9.
183. Jurutka, P.W., et al., Human vitamin D receptor phosphorylation by casein kinase II at Ser-208 potentiates transcriptional activation. Proc Natl Acad Sci U S A, 1996. **93**(8): p. 3519-24.
184. Patel, S.R., et al., Inhibition of calcitriol receptor binding to vitamin D response elements by uremic toxins. J Clin Invest, 1995. **96**(1): p. 50-9.
185. Sher, E., et al., Whole-cell uptake and nuclear localization of 1,25-dihydroxycholecalciferol by breast cancer cells (T47 D) in culture. Biochem J, 1981. **200**(2): p. 315-20.
186. Walters, M.R., et al., Factors affecting the distribution and stability of unoccupied 1,25-dihydroxyvitamin D3 receptors. J Recept Res, 1981. **2**(4): p. 331-46.
187. Walters, S.N., et al., Intracellular location of unoccupied 1,25-dihydroxyvitamin D receptors: a nuclear-cytoplasmic equilibrium. Arch Biochem Biophys, 1986. **246**(1): p. 366-73.

188. Wang, X., et al., Induction of kinase suppressor of RAS-1(KSR-1) gene by 1, alpha25-dihydroxyvitamin D3 in human leukemia HL60 cells through a vitamin D response element in the 5'-flanking region. Oncogene, 2006. **25**(53): p. 7078-85.
189. Christakos, S., et al., Vitamin D: Metabolism, Molecular Mechanism of Action, and Pleiotropic Effects. Physiol Rev, 2016. **96**(1): p. 365-408.
190. Haussler, M.R., et al., The vitamin D hormone and its nuclear receptor: molecular actions and disease states. J Endocrinol, 1997. **154 Suppl**: p. S57-73.
191. Issa, L.L., G.M. Leong, and J.A. Eisman, Molecular mechanism of vitamin D receptor action. Inflamm Res, 1998. **47**(12): p. 451-75.
192. Alroy, I., T.L. Towers, and L.P. Freedman, Transcriptional repression of the interleukin-2 gene by vitamin D3: direct inhibition of NFATp/AP-1 complex formation by a nuclear hormone receptor. Mol Cell Biol, 1995. **15**(10): p. 5789-99.
193. Horst, R.L., J.P. Goff, and T.A. Reinhardt, Role of vitamin D in calcium homeostasis and its use in prevention of bovine periparturient paresis. Acta Vet Scand Suppl, 2003. **97**: p. 35-50.
194. Li, P., et al., p27(Kip1) stabilization and G(1) arrest by 1,25-dihydroxyvitamin D(3) in ovarian cancer cells mediated through down-regulation of cyclin E/cyclin-dependent kinase 2 and Skp1-Cullin-F-box protein/Skp2 ubiquitin ligase. J Biol Chem, 2004. **279**(24): p. 25260-7.
195. Kasiappan, R., et al., Vitamin D suppresses leptin stimulation of cancer growth through microRNA. Cancer Res, 2014. **74**(21): p. 6194-204.
196. Jiang, F., et al., Induction of ovarian cancer cell apoptosis by 1,25-dihydroxyvitamin D3 through the down-regulation of telomerase. J Biol Chem, 2004. **279**(51): p. 53213-21.
197. Gulliford, T., et al., A phase I study of the vitamin D analogue EB 1089 in patients with advanced breast and colorectal cancer. Br J Cancer, 1998. **78**(1): p. 6-13.
198. Brown, A.J. and E. Slatopolsky, Vitamin D analogs: therapeutic applications and mechanisms for selectivity. Mol Aspects Med, 2008. **29**(6): p. 433-52.
199. Lind, L., B. Wengle, and S. Ljunghall, Blood pressure is lowered by vitamin D (alphacalcidol) during long-term treatment of patients with intermittent hypercalcaemia. A double-blind, placebo-controlled study. Acta Med Scand, 1987. **222**(5): p. 423-7.
200. Dennis, V.C. and G.L. Albertson, Doxercalciferol treatment of secondary hyperparathyroidism. Ann Pharmacother, 2006. **40**(11): p. 1955-65.
201. Kazama, J.J., et al., Maxacalcitol is a possible less phosphatemic vitamin D analog. Ther Apher Dial, 2005. **9**(4): p. 352-4.
202. Evans, T.R., et al., A phase II trial of the vitamin D analogue Seocalcitol (EB1089) in patients with inoperable pancreatic cancer. Br J Cancer, 2002. **86**(5): p. 680-5.
203. Pepper, C., et al., The vitamin D3 analog EB1089 induces apoptosis via a p53-independent mechanism involving p38 MAP kinase activation and suppression of ERK activity in B-cell chronic lymphocytic leukemia cells in vitro. Blood, 2003. **101**(7): p. 2454-60.
204. Scott, L.J., C.J. Dunn, and K.L. Goa, Calcipotriol ointment. A review of its use in the management of psoriasis. Am J Clin Dermatol, 2001. **2**(2): p. 95-120.
205. Sherman, M.H., et al., Vitamin D receptor-mediated stromal reprogramming suppresses pancreatitis and enhances pancreatic cancer therapy. Cell, 2014. **159**(1): p. 80-93.

206. Caffrey, J.M. and M.C. Farach-Carson, Vitamin D3 metabolites modulate dihydropyridine-sensitive calcium currents in clonal rat osteosarcoma cells. J Biol Chem, 1989. **264**(34): p. 20265-74.
207. Bissonnette, M., et al., 1,25(OH)2 vitamin D3 activates PKC-alpha in Caco-2 cells: a mechanism to limit secosteroid-induced rise in [Ca2+]i. Am J Physiol, 1994. **267**(3 Pt 1): p. G465-75.
208. Vazquez, G., R. Boland, and A.R. de Boland, Modulation by 1,25(OH)2-vitamin D3 of the adenylyl cyclase/cyclic AMP pathway in rat and chick myoblasts. Biochim Biophys Acta, 1995. **1269**(1): p. 91-7.
209. de Boland, A.R. and A.W. Norman, 1alpha,25(OH)2-vitamin D3 signaling in chick enterocytes: enhancement of tyrosine phosphorylation and rapid stimulation of mitogen-activated protein (MAP) kinase. J Cell Biochem, 1998. **69**(4): p. 470-82.
210. Song, X., et al., Stimulation of phosphorylation of mitogen-activated protein kinase by 1alpha,25-dihydroxyvitamin D3 in promyelocytic NB4 leukemia cells: a structure-function study. Endocrinology, 1998. **139**(2): p. 457-65.
211. Norman, A.W., M.T. Mizwicki, and D.P. Norman, Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. Nat Rev Drug Discov, 2004. **3**(1): p. 27-41.
212. Menegaz, D., et al., 1alpha,25(OH)2-Vitamin D3 stimulation of secretion via chloride channel activation in Sertoli cells. J Steroid Biochem Mol Biol, 2010. **119**(3-5): p. 127-34.
213. Garland, C.F. and F.C. Garland, Do sunlight and vitamin D reduce the likelihood of colon cancer? Int J Epidemiol, 1980. **9**(3): p. 227-31.
214. Garland, C.F., et al., The role of vitamin D in cancer prevention. Am J Public Health, 2006. **96**(2): p. 252-61.
215. Webb, A.R., L. Kline, and M.F. Holick, Influence of season and latitude on the cutaneous synthesis of vitamin D3: exposure to winter sunlight in Boston and Edmonton will not promote vitamin D3 synthesis in human skin. J Clin Endocrinol Metab, 1988. **67**(2): p. 373-8.
216. Feldman, D., et al., The role of vitamin D in reducing cancer risk and progression. Nat Rev Cancer, 2014. **14**(5): p. 342-57.
217. Holick, M.F., Vitamin D: importance in the prevention of cancers, type 1 diabetes, heart disease, and osteoporosis. Am J Clin Nutr, 2004. **79**(3): p. 362-71.
218. Wang, Y., J. Zhu, and H.F. DeLuca, Where is the vitamin D receptor? Arch Biochem Biophys, 2012. **523**(1): p. 123-33.
219. Colston, K., M.J. Colston, and D. Feldman, 1,25-dihydroxyvitamin D3 and malignant melanoma: the presence of receptors and inhibition of cell growth in culture. Endocrinology, 1981. **108**(3): p. 1083-6.
220. Abe, E., et al., Differentiation of mouse myeloid leukemia cells induced by 1 alpha,25-dihydroxyvitamin D3. Proc Natl Acad Sci U S A, 1981. **78**(8): p. 4990-4.
221. Cheung, F.S., F.J. Lovicu, and J.K. Reichardt, Current progress in using vitamin D and its analogs for cancer prevention and treatment. Expert Rev Anticancer Ther, 2012. **12**(6): p. 811-37.
222. Deeb, K.K., D.L. Trump, and C.S. Johnson, Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. Nat Rev Cancer, 2007. **7**(9): p. 684-700.

223. Gocek, E. and G.P. Studzinski, Vitamin D and differentiation in cancer. Crit Rev Clin Lab Sci, 2009. **46**(4): p. 190-209.
224. Krishnan, A.V., S. Swami, and D. Feldman, Vitamin D and breast cancer: inhibition of estrogen synthesis and signaling. J Steroid Biochem Mol Biol, 2010. **121**(1-2): p. 343-8.
225. Krishnan, A.V. and D. Feldman, Mechanisms of the anti-cancer and anti-inflammatory actions of vitamin D. Annu Rev Pharmacol Toxicol, 2011. **51**: p. 311-36.
226. Leyssens, C., L. Verlinden, and A. Verstuyf, Antineoplastic effects of 1,25(OH)2D3 and its analogs in breast, prostate and colorectal cancer. Endocr Relat Cancer, 2013. **20**(2): p. R31-47.
227. Mehta, R.G., et al., Vitamin D and breast cancer: emerging concepts. Cancer Lett, 2013. **334**(1): p. 95-100.
228. Pereira, F., M.J. Larriba, and A. Munoz, Vitamin D and colon cancer. Endocr Relat Cancer, 2012. **19**(3): p. R51-71.
229. Tang, J.Y., et al., Vitamin D in cutaneous carcinogenesis: part II. J Am Acad Dermatol, 2012. **67**(5): p. 817 e1-11; quiz 827-8.
230. Colston, K.W., et al., Growth inhibition of both MCF-7 and Hs578T human breast cancer cell lines by vitamin D analogues is associated with increased expression of insulin-like growth factor binding protein-3. J Mol Endocrinol, 1998. **20**(1): p. 157-62.
231. Vink-van Wijngaarden, T., et al., Inhibition of breast cancer cell growth by combined treatment with vitamin D3 analogues and tamoxifen. Cancer Res, 1994. **54**(21): p. 5711-7.
232. Nelson, W.G., et al., The role of inflammation in the pathogenesis of prostate cancer. J Urol, 2004. **172**(5 Pt 2): p. S6-11; discussion S11-2.
233. Nonn, L., et al., Inhibition of p38 by vitamin D reduces interleukin-6 production in normal prostate cells via mitogen-activated protein kinase phosphatase 5: implications for prostate cancer prevention by vitamin D. Cancer Res, 2006. **66**(8): p. 4516-24.
234. Adorini, L., et al., Inhibition of prostate growth and inflammation by the vitamin D receptor agonist BXL-628 (elocalcitol). J Steroid Biochem Mol Biol, 2007. **103**(3-5): p. 689-93.
235. Wang, W.L., et al., Effects of 1alpha,25 dihydroxyvitamin D3 and testosterone on miRNA and mRNA expression in LNCaP cells. Mol Cancer, 2011. **10**: p. 58.
236. Sun, D., et al., miR-99 family of MicroRNAs suppresses the expression of prostate-specific antigen and prostate cancer cell proliferation. Cancer Res, 2011. **71**(4): p. 1313-24.
237. Giangreco, A.A., et al., Tumor suppressor microRNAs, miR-100 and -125b, are regulated by 1,25-dihydroxyvitamin D in primary prostate cells and in patient tissue. Cancer Prev Res (Phila), 2013. **6**(5): p. 483-94.
238. Lefkowitz, E.S. and C.F. Garland, Sunlight, vitamin D, and ovarian cancer mortality rates in US women. Int J Epidemiol, 1994. **23**(6): p. 1133-6.
239. Garland, C.F., et al., Role of ultraviolet B irradiance and vitamin D in prevention of ovarian cancer. Am J Prev Med, 2006. **31**(6): p. 512-4.
240. Ong, J.S., et al., Association of vitamin D levels and risk of ovarian cancer: a Mendelian randomization study. Int J Epidemiol, 2016.
241. Jiang, F., et al., G2/M arrest by 1,25-dihydroxyvitamin D3 in ovarian cancer cells mediated through the induction of GADD45 via an exonic enhancer. J Biol Chem, 2003. **278**(48): p. 48030-40.

242. Shen, Z., et al., The coupling of epidermal growth factor receptor down regulation by 1alpha,25-dihydroxyvitamin D3 to the hormone-induced cell cycle arrest at the G1-S checkpoint in ovarian cancer cells. Mol Cell Endocrinol, 2011. **338**(1-2): p. 58-67.
243. Kasiappan, R., et al., 1,25-Dihydroxyvitamin D3 suppresses telomerase expression and human cancer growth through microRNA-498. J Biol Chem, 2012. **287**(49): p. 41297-309.
244. Lungchukiet, P., et al., Suppression of epithelial ovarian cancer invasion into the omentum by 1alpha,25-dihydroxyvitamin D3 and its receptor. J Steroid Biochem Mol Biol, 2015. **148**: p. 138-47.
245. Geserick, P., et al., Cellular IAPs inhibit a cryptic CD95-induced cell death by limiting RIP1 kinase recruitment. J Cell Biol, 2009. **187**(7): p. 1037-54.
246. Arbour, N.C., et al., A highly sensitive method for large-scale measurements of 1,25-dihydroxyvitamin D. Anal Biochem, 1998. **255**(1): p. 148-54.
247. Christofferson, D.E., et al., A novel role for RIP1 kinase in mediating TNFalpha production. Cell Death Dis, 2012. **3**: p. e320.
248. Sakai, W., et al., Functional restoration of BRCA2 protein by secondary BRCA2 mutations in BRCA2-mutated ovarian carcinoma. Cancer Res, 2009. **69**(16): p. 6381-6.
249. Zinser, G.M., K. McEleney, and J. Welsh, Characterization of mammary tumor cell lines from wild type and vitamin D3 receptor knockout mice. Mol Cell Endocrinol, 2003. **200**(1-2): p. 67-80.
250. Sun, J., et al., Increased NF-kappaB activity in fibroblasts lacking the vitamin D receptor. Am J Physiol Endocrinol Metab, 2006. **291**(2): p. E315-22.
251. Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods, 1983. **65**(1-2): p. 55-63.
252. Vanlangenakker, N., et al., TNF-induced necroptosis in L929 cells is tightly regulated by multiple TNFR1 complex I and II members. Cell Death Dis, 2011. **2**: p. e230.
253. Armstrong, D.K., Relapsed ovarian cancer: challenges and management strategies for a chronic disease. Oncologist, 2002. **7 Suppl 5**: p. 20-8.
254. Zhang, X., et al., Suppression of death receptor-mediated apoptosis by 1,25-dihydroxyvitamin D3 revealed by microarray analysis. J Biol Chem, 2005. **280**(42): p. 35458-68.
255. O'Donnell, M.A., et al., Ubiquitination of RIP1 regulates an NF-kappaB-independent cell-death switch in TNF signaling. Curr Biol, 2007. **17**(5): p. 418-24.

APPENDIX A: Copyright permission

NATURE PUBLISHING GROUP LICENSE TERMS AND CONDITIONS

Oct 13, 2016

This Agreement between Waise Quarni ("You") and Nature Publishing Group ("Nature Publishing Group") consists of your license details and the terms and conditions provided by Nature Publishing Group and Copyright Clearance Center.

License Number	3967091389327
License date	Oct 13, 2016
Licensed Content Publisher	Nature Publishing Group
Licensed Content Publication	Cellular & Molecular Immunology
Licensed Content Title	Receptor-interacting protein (RIP) kinase family
Licensed Content Author	Duanwu Zhang, Juan Lin, Jiahuai Han
Licensed Content Date	Apr 12, 2010
Licensed Content Volume Number	7
Licensed Content Issue Number	4
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 1: Domain organization of the RIP kinase family
Author of this NPG article	no
Your reference number	
Title of your thesis / dissertation	VDR-RIPK1 Interaction and its Implications in Cell Death and Cancer Intervention
Expected completion date	Nov 2016
Estimated size (number of pages)	150

United States
Attn: Waise Quarni

Total 0.00 USD

Terms and Conditions

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use,subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.

2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run).NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).

4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.

5. The credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)
   For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

   Note: For republication from the *British Journal of Cancer*, the following credit lines apply.
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

   Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

   Note: For adaptation from the *British Journal of Cancer*, the following credit line applies.
   Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visithttp://www.macmillanmedicalcommunications.com for more information.Translations of

Ethics topics to consider when publishing:

- **Authorship of the paper:** Authorship should be limited to those who have made a significant contribution to the conception, design, execution, or interpretation of the reported study.
- **Originality and plagiarism:** The authors should ensure that they have written entirely original works, and if the authors have used the work and/or words of others, that this has been appropriately cited or quoted.
- **Data access and retention:** Authors may be asked to provide the raw data in connection with a paper for editorial review, and should be prepared to provide public access to such data.
- **Multiple, redundant or concurrent publication:** An author should not in general publish manuscripts describing essentially the same research in more than one journal or primary publication. Elsevier does not view the following uses of a work as prior publication: publication in the form of an abstract; publication as an academic thesis; publication as an electronic preprint. Information on prior publication is included within each Elsevier journal's Guide for Authors. Note: *Cell Press* ↗ , *The Lancet* ↗ , and some society-owned titles have different policies on prior publication. Information on these is available on the journal homepage.
- **Acknowledgement of sources:** Proper acknowledgment.
- **Disclosure and conflicts of interest:** All submissions must include disclosure of all relationships that could be viewed as presenting a potential conflict of interest.
- **Fundamental errors in published works:** When an author discovers a significant error or inaccuracy in his/her own published work, it is the author's obligation to promptly notify the journal editor or publisher and cooperate with the editor to retract or correct the paper.
- **Reporting standards:** Authors of reports of original research should present an accurate account of the work performed as well as an objective discussion of its significance.
- **Hazards and human or animal subjects:** Statements of compliance are required if the work involves chemicals, procedures or equipment that have any unusual hazards inherent in their use, or if it involves the use of animal or human subjects.

APPENDIX B: IACUC approval

USF UNIVERSITY OF SOUTH FLORIDA

RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

MEMORANDUM

TO:	Wenlong Bai,
FROM:	Farah Moulvi, MSPH, IACUC Coordinator Institutional Animal Care & Use Committee Research Integrity & Compliance
DATE:	1/4/2016
PROJECT TITLE:	Vitamin D and Its Analog in Cancer Prevention and Treatment
FUNDING SOURCE:	USF department, institute, center, etc.
IACUC PROTOCOL #:	R IS00000491
PROTOCOL STATUS:	**APPROVED**

Your request for continuation of this study was received and will be reported to the Institutional Animal Care and Use Committee (IACUC). The IACUC acknowledges that this study is currently on going as previously approved. Please be advised that **continuation of this study is in effect for a one-year period beginning 3/4/2016:**

Please take note of the following:

• **IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system.** After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

• **All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification.** Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.

RESEARCH & INNOVATION • RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
PHS No. A4100-01, AAALAC No. 000434, USDA No. 58-R-0015
University of South Florida • 12901 Bruce B. Downs Blvd., MDC35 • Tampa, FL 33612-4799
(813) 974-7106 • FAX (813) 974-7091

ABOUT THE AUTHOR

Waise Quarni received his Bachelor and Masters degree from the University of Dhaka. In 2009, he moved to the United States at the University of Central Florida to finish his Masters in Molecular Biology and Microbiology. In 2011, he joined the PhD Integrated Biomedical Sciences at the University of South Florida. He joined Dr. Wenlong Bai's lab at the department of Pathology and Cell Biology in 2012. During his thesis, Waise has been very active in Dr. Bai's lab. He has co-authored publications in several high impact peer reviewed scientific journals. Waise has received Vitamin D workshop trainee travel award and gave oral presentation at that international workshop. He has also received AMSGS travel award from the Morsani College of Medicine.