The Inhibitor of Apoptosis (IAP) Protein Ubiquitome

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Abstract

The Inhibitor of Apoptosis (IAP) proteins are a highly conserved group of anti-apoptotic proteins. Cellular IAP 1 and 2 (cIAP1 and 2) are two members of the IAP protein family that regulate the activity of the Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor family. The role and mechanism of the IAPs in ubiquitination are not yet completely understood due to the complexity of this posttranslational modification process. Additionally, The IAPs are involved in a myriad of cellular processes, and many of the process-specific mechanisms by which the IAPs are involved is unknown. I aim to delve deeper into the signalling pathways that are controlled by cIAP1 and cIAP2 by discovering currently unknown protein-protein interactions. In doing so, I will determine which proteins interact with the cIAPs and what signalling pathways these proteins are involved in. Using a BioID approach, I sought to characterize the cIAP1 interactors involved in the canonical and non-canonical NF-κB pathways. I generated a stable cell line expressing TurboID-cIAP1 fusion protein in HEK 293T cells that expressTurboID-cIAP1 at levels comparable to endogenous cIAP1. I identified multiple potential cIAP1 interactors that have ties to the NF-κB pathway. These proteins regulate NF-κB signalling in multiple ways including influencing acetylation and nuclear retention of the NF-κB transcription factors, phosphorylation of NF-κB transcription factors, and RNA splicing of genes involved in the TNFR1 complex I. Further work needs to be done to confirm these interactions and to discover the mechanisms by which these interactions occur. NF-κB signalling is known to have widespread function within the cell and within diseases such as cancer; it will be beneficial to study these interactions to better understand how cancer develops and how to treat it best, especially in patients with a poor prognosis.

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List of Abbreviations

APEX	Ascorbate Peroxidase
ATCC	American Tissue Culture Collection
BAFFR	B Cell Activating Factor Receptor
CAD	Caspase-activated recruitment Domain
CARD	Caspase Recruitment Domain
CD40	Cluster of Differentiation 40
CD40L	Cluster of Differentiation 40 Ligand
cFLIP	cellular FLICE-like inhibitory protein
cIAP1	cellular IAP 1
cIAP2	cellular IAP 2
Co-IP	Co-immunoprecipitation
DBI	Diazepam Binding Inhibitor
DD	Death Domain
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic Reticulum
ERK	Extracellular-signal Regulated Kinase
FADD	Fas-associated Death Domain
FBS	Fetal Bovine Serum
FN14	Fibroblast Growth Factor-inducible Factor 14
FRT	Flippase Recognition Target
GARIN1A	Golgi-associated RAB 2 Interacting Protein 1A
HCC	Hepatocellular Carcinoma
HEK	Human Embryonic Kidney
HN1	Haematologically and Neurologically expressed 1
HRP	Horseradish Peroxidase
IAA	Indanyloxyacetic acid
IAP	Inhibitor of Apoptosis
IBM	IAP Binding Motif
ICAD	Caspase-activated recruitment Domain Inhibitor
IKBKAP	IκB kinase associated protein gene
ILP-1	IAP-like Protein 1
ILP-2	IAP-like Protein 2
IPO13	Importin 13
IκB	Inhibitor of Kappa B
IκK	Inhibitor of Kappa B Kinase
JPT1	Jupiter Microtubule Associated Homolog 1
KAP1	KRAB-associated Protein 1
KRAB	Krüppel-associated Box

LC/MS	Liquid Chromatography Mass Spectrometry
MKP3	Mitogen Activated Protein Kinase Phosphatase 3
MLKL	Mixed Lineage Kinase Domain-like
MYMK	Myomaker
NAIP	Neuronal Apoptosis Inhibiting Protein
NEA	N-ethylmaleimide
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells
NIK	NF-κB Inducing Kinase
NLR	Nod-like Receptor
NSCLC	Non-small Cell Lung Cancer
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PL	Proximity Labelling
PLA	Proximity Ligation Assay
POTEE	POTE ankyrin domain family, member E
POTE	Prostate, Ovary, Testes, and Embryo
PTBP1	Polypyrimidine Tract Binding Protein 1
RAB2A	Ras-related protein 2A
RAB2B	Ras-related protein 2B
RANK	Receptor Activator for NF-κB
RING	Really Interesting New Gene
RIPK1	Receptor Inducing Protein Kinase 1
S1P	Sphingosine 1 Phosphate
SASP	Senescence-associated Secretory Phenotype
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SLC38A11	Solute carrier family 38 member 11
SMAC	Secondary Mitochondrial Activator of Caspases
SPHK1	Sphingosine Kinase 1
STAT3	Signal Transducer and Activator of Transcription 3
TBS/T	Tris-buffered Saline/Tween-20
Tc1	TurboID-cIAP1
Tc2	TurboID-cIAP2
TMEM8C	Transmembrane Protein 8C
TNF-R1	Tumour Necrosis Factor Receptor 1
TNF-α	Tumour Necrosis Factor Alpha
TNFRSF	Tumour Necrosis Factor Receptor Super Family
TO	Tetracycline Operator
TRAF2	TNFR-associated Factor 2
TRIM28	Tripartite Motif Containing 28

TsIAP	Testis-specific IAP
UBA	Ubiquitin Associated Domain
UBC	Ubiquitin Conjugating domain
URGCP	Upregulator of Cell Proliferation
XIAP	X-linked IAP

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Chapter 1: Introduction

Apoptosis

Apoptosis is a form of non-inflammatory programmed cell death that is characterized by its unique morphological characteristics, including cell shrinkage "budding" of apoptotic bodies from the dying cell, and nucleus and chromatin condensation.[1 2] Apoptosis was first documented in 1972 by Kerr *et. al.*, where it was described as an "important basic biological phenomenon."[3] Indeed, apoptosis is just that – its many functions include maintaining cell populations in tissues, acting as a defense mechanism during immune reactions, and clearing cells that were damaged through disease or other processes. For the purposes of my thesis, the most important apoptotic pathways are the intrinsic and the extrinsic apoptotic pathways.

The intrinsic apoptosis pathway occurs in response to non-receptor-mediated stimuli: the withdrawal of factors such as cytokines, growth factors, and hormones; loss of apoptotic suppression; and cell stressors such as toxins, radiation, hypoxia, and viral infections. These factors result in the permeabilization of the mitochondrial membrane and the subsequent release of pro-apoptotic proteins from the intermembrane space. These proteins include SMAC (Secondary Mitochondrial Activator of Caspases), Cytochrome *c*, and serine protease HrtA2/OMI.[1] SMAC works by inhibiting the Inhibitor of Apoptosis (IAP) proteins, thereby allowing caspase-9 to form the apoptosome along with Apaf-1and Cytochrome *c*.[1 4 5] Once the cell is committed to following apoptosis to completion, AIF, endonuclease G, and CAD are also released from the mitochondria, where they translocate to the nucleus to condense the chromatin and fragment the DNA.[1 6 7] This pathway is primarily regulated by the Bcl-2 family of proteins.[8]

The extrinsic apoptosis pathway occurs via death receptors belonging to the tumour necrosis factor (TNF) receptor gene superfamily. The death domain (DD) on the cytoplasmic portion of the TNF receptor recruits protein complexes to initiate the signalling cascade for the given receptor.[1]

[9] For my thesis, the interaction between Tumor Necrosis Factor-Receptor 1 (TNF-R1) and TNF-alpha (TNF-α) is the pathway I will focus on. This pathway is described in greater detail in regard to the canonical NF-κB transcription factor pathway described in in the section "*Canonical and non-canonical NF-κB pathways*."

The intrinsic and extrinsic apoptotic pathways converge to the execution phase – in this phase there is a shift from activation of initiator caspases-8 -9 and -10 to activation of executioner caspases-3 -6 and -7.[10] Executioner caspases cleave various substrates such as cytoskeleton proteins, thereby leading to the morphological changes seen in apoptosis. Out of the executioner caspases, caspase-3 is considered to be the most important since it activates CAD by releasing it from its inhibitor, ICAD, allowing CAD to translocate into the nucleus to degrade chromosomal DNA.[11] Caspase-3 is also responsible for the "blebbing" seen in apoptotic cells by causing changes to and degradation of the cytoskeleton.[1]

Ubiquitination

Ubiquitination is an essential cellular process, as it regulates the cell cycle, oncogenesis, immune responses, transcriptional regulation, embryonic development, apoptosis, and intracellular signalling pathways.[12] [13] [14] Ubiquitination regulates protein half-life, alters the formation of signalosomes, and clears misfolded or otherwise defective proteins. Proteins are turned over at different rates, varying from minutes, to weeks, to months. The process involves the addition of Ubiquitin, a 76 amino acid protein, to lysine groups of cellular proteins.

Ubiquitination is a tightly regulated posttranslational modification. There are three enzymes involved in ubiquitination: the E1 ubiquitin-activating enzyme, the E2 ubiquitin conjugating enzyme, and the E3 ubiquitin ligase enzyme. E1 and E2 are responsible for initiating

the ubiquitination reaction, while the E3 ligase catalyzes the transfer of ubiquitin from E2 to its target substrate (Figure 1).[12 13] Ubiquitination is started by the ATP-dependent formation of a thiol ester bond from the C-terminus of the ubiquitin molecule to an active cysteine residue on the E1 enzyme. The ubiquitin molecule is then transferred to the E2 enzyme to for an E2-ubiquitin intermediate by a thioester linkage. Lastly, the E3 enzyme, which hold the specificity for the reaction, catalyzes the transfer of the ubiquitin molecule to the target protein for the reaction.[12 15] Ubiquitin has seven lysine residues on which ubiquitination can occur: K6, K11, K27, K29, K33, K48, and K63.[16 12 13] The type of lysine linkage affects the fate of the target protein following ubiquitination.[16 12] The two most common types of ubiquitination are K48- and K63-linked poly-ubiquitination, with K48-linked ubiquitination being the most abundant form.[17] Poly-ubiquitination is typically responsible for marking proteins for degradation or for recruitment into signalling complexes. These processes are mediated by K48-linkages and K63-linkages, respectively.[16 12 13] Mono-ubiquitination signals for DNA repair, vesicle sorting, signal transduction responses, and receptor-mediated endocytosis.[12]

Cellular Inhibitor of Apoptosis protein 1 and 2

The Inhibitor of Apoptosis (IAP) proteins are a highly conserved group of anti-apoptotic proteins found in yeast, nematodes, insects, fish, and mammals.[18 16] The IAP gene was originally discovered as a 31kDa protein with a zinc finger-like motif in SF-21 cells infected by a baculovirus.[19] The expression of the IAPs are ubiquitous and highly regulated.[20] There are eight known mammalian IAPs: BIRC1, also known as Neuronal IAP or NAIP; BIRC2, also known as cellular IAP 1 (cIAP1) or HIAP2; BIRC3, also known as cellular IAP 2 (cIAP2) or HIAP1; BIRC4, also known as X-linked IAP (XIAP) or IAP-like protein 1(ILP-1); BIRC5, also known as Survivin; BIRC6, also known as Apollon or BRUCE; BIRC7, also known as Livin, Melanoma-

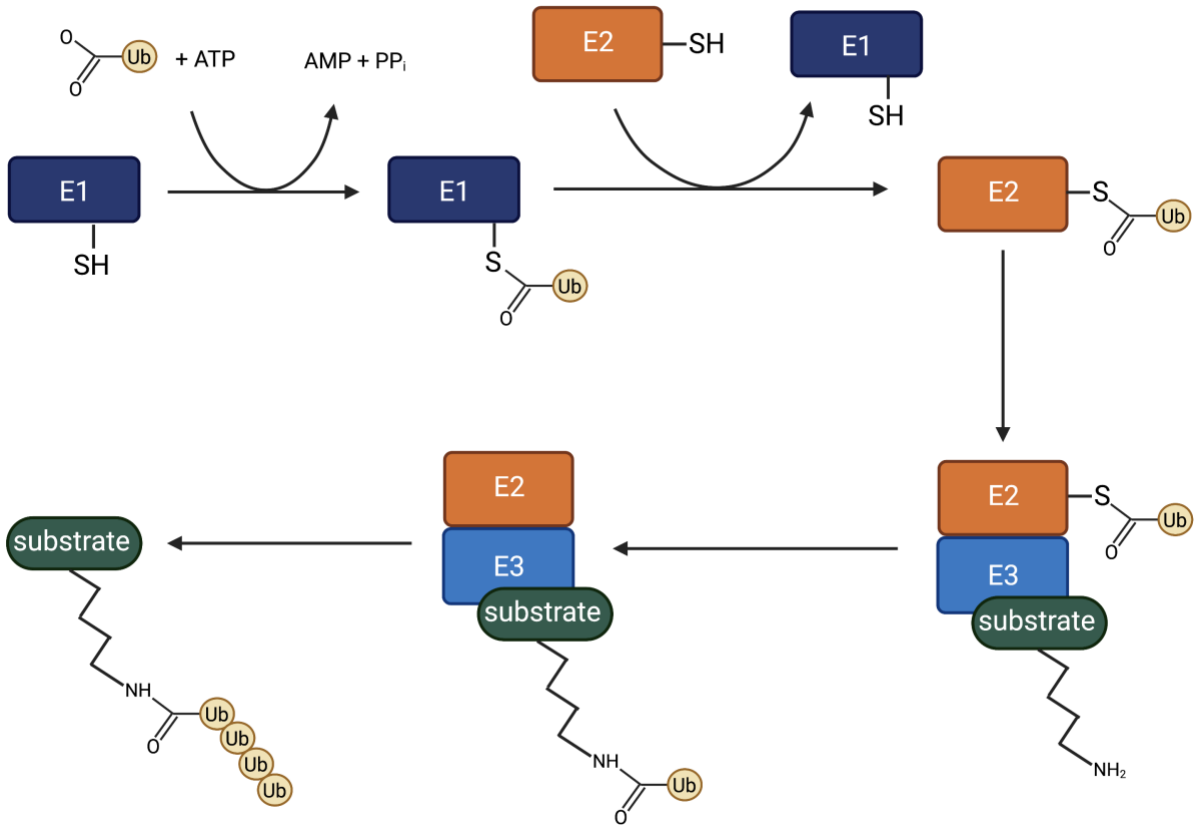


Figure 1: The Ubiquitination Cascade. The three enzymes involved are the E1 ubiquitin-activating enzyme, the E2 ubiquitin conjugating enzyme, and the E3 ubiquitin ligase enzyme. E1 and E2 are responsible for initiating the ubiquitination reaction in an ATP dependent manner through the formation of a thiol ester bind from the C-terminus of the ubiquitin molecule to an active cysteine residue on the E1 enzyme. The ubiquitin molecule is then transferred to the E2 enzyme to form an E2-ubiquitin intermediate with a thioester linkage. The E3 ligase then catalyzes the transfer of ubiquitin from E2 to its target substrate.

IAP, or ML-IAP; and BIRC8, also known as Testis-specific IAP (TsIAP) or IAP-like protein 2 (ILP-2) (Figure 2).[21 22 23]

cIAP1 and cIAP2 functional domains

The IAP proteins are defined by the presence of a Baculovirus IAP repeat (BIR) domain, which is a 70 amino acid sequence at the N terminal of the protein.[18 24 25] These domains are structured with three β-strands and between four and five alpha-helices.[16] The core of the BIR domain has a cysteine and histidine motif which interacts with a zinc ion.[18 25] There are two types of BIR domains, named Type II and Type I. Type II BIR domains have a hydrophobic groove that bind to the IAP binding motifs (IBMs) in the N-terminus of caspase subunits and IAP antagonists.[18 16] An N-terminal alanine or a serine residue is necessary for binding the IBM in a BIR domain. IBMs are present on many different proteins, including Smac and other mitochondrial proteins.[16] Caspases must be cleaved to its active form in order for the IBM to be exposed.[18] Type I BIR domains do not bind to IBMs like Type II BIR domains, instead they interact with adaptor proteins to activate pro-survival signalling pathways.[26] The IAP proteins contain one to three BIR domains.[24]

Five of the IAPs, including cIAP1 and cIAP2, contain a Really Interesting New Gene (RING) domain at the C-terminus. [24] The RING domain allows the IAPs to function in the ubiquitination pathway as an E3 Ubiquitin ligase through conjugating Ubiquitin to lysine residues on the target protein.[18 27 24 25] Several of the IAP proteins can also contain a Caspase Recruitment Domain (CARD), Ubiquitin Associated (UBA) Domain, and a Ubiquitin Conjugating (UBC) Domain.[18 24] cIAP1 and cIAP2 contain three BIR domains, a UBA domain, a CARD domain, and a C-terminal RING domain (Figure 2). When inactive, cIAP1 has a closed configuration where the

RING domain is protected in the middle of the molecule. Its BIR3 domain and its CARD domain are crucial for maintaining this closed configuration. When the BIR3 domain is engaged by a synthetic molecule, such as SMAC mimetics, it triggers a conformational change where cIAP1 will unfold itself into its active form, dimerize, and auto-ubiquitinate.[28] This will lead to the degradation of cIAP1.[16] TNFR-associated factor 2 (TRAF2) is a key regulatory partner of cIAP1 and cIAP2 that serves to block auto-ubiquitination, stabilize cIAP1/2, and regulate the cellular localization of cIAP1/2.[29 30 31]

NF-κB pathways

Canonical and non-canonical NF-κB pathways

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a family of master transcription regulation factors that control gene expression of anti-apoptotic genes, cytokines, chemokines, and other factors involved in proinflammatory immune responses.[16] There are five transcription factors in the NF-κB pathway: p50, p52, p65/RelA, RelB, and c-Rel.[27 14] NF-κB is classically grouped into two pathways, termed the canonical and non-canonical pathways. cIAP1and 2 activate the canonical pathway and conversely supress the non-canonical pathway (Figure 3).[21 27] Prior to activation of the NF-κB pathways, NF-κB dimers sit unactive in the cytoplasm due to their association with the Inhibitor of κB (IκB) proteins.[32]

In the canonical pathway, when TNF-Receptor 1 (TNF-R1) is bound by TNF-α, TRAF2 and Receptor Inducing Protein Kinase 1 (RIPK1) are recruited to the receptor.[21 14] cIAP1 and cIAP2, which are associated with TRAF2 and TRAF3, are also recruited to TNF-R1.[21 27 24 14] This forms a complex termed Complex I, which includes TNF Receptor Associated Death Domain

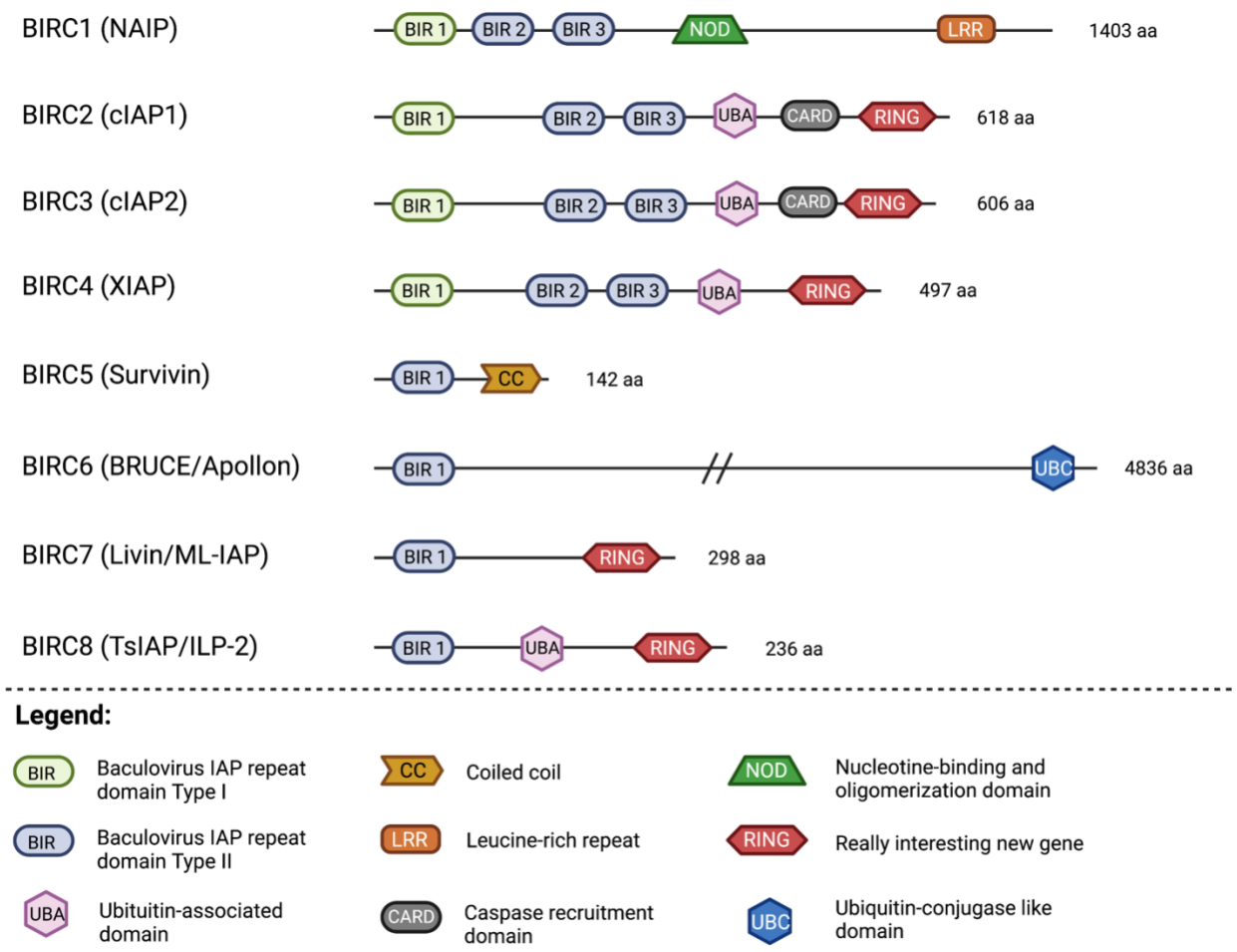


Figure 2: The domains of the IAP proteins. Members of the IAPs are classified by possessing at least one BIR domain. BIR domains mediate protein-protein interactions between the IAPs and signalling proteins such as TRAFs (tumor necrosis factor receptor-1-associated factors), caspases, and SMAC. Some IAPs contain a RING domain that functions as an E3 ubiquitin ligase, a CARD domain that suppresses E3 ligase activity, and/or a UBA domain that binds mono- and poly-ubiquitin chains. The UBC domain on BIRC6 catalyzez the addition of ubiquitin to proteins in a covalent manner. The Coiled coil domain on BIRC5 can interact with microtubules. The NOD domain on BIRC1functions as a NOD-like receptor (NLR), thereby making BIRC1 a member of the NLR super family. The LRR on BIRC1 provides a versatile domain for protein-protein interactions.

(TRADD), RIPK1, TRAF2, and cIAP1/2.[33] cIAP1 and cIAP2 ubiquitinate RIPK1, thereby initiating a signalling scaffold resulting the phosphorylation and degradation of IκB, which is part of the Inhibitor of κB Kinase (IκK) complex in the cytoplasm.[14] The IκK complex phosphorylates IκB and the NF-κB dimers are released from IκB and translocate to the nucleus and begins transcription of pro-inflammatory genes.[21 27 14] If stimulation of TNF-R1results in activation of the apoptotic pathway, TRADD and RIPK1 instead form Complex II which includes Fas-associated Death Domain (FADD) and Caspase-8. FADD activates Caspase-8 from Procaspase-8 and Caspase-8 activates downstream Caspases that execute the apoptotic pathway.[33] Apoptosis can be inhibited by Caspase-8 forming a complex with cellular FLICE-like inhibitory protein (cFLIP).[34] Alternatively, after the formation of Complex I necroptosis can be induced via the formation of a RIPK1/RIPK3 heterodimer and oligomerization of Mixed lineage kinase domain-like (MLKL). The oligomeric form of MLKL forms pores in the plasma membrane, which initiates an inflammatory response and kills the cell.[34]

In the non-canonical NF-κB pathway, NF-κB-inducing-kinase (NIK) catalyzes the phosphorylation of an IκBα homodimer. cIAP1 and 2 induce K48-linked ubiquitination of NIK in the absence of TNFR Super Family (TNFRSF) signalling, leading to the degradation of NIK and the inhibition of the non-canonical NF-κB pathway.[21 14] The non-canonical pathway can be triggered by several members of the TNFRSF, including B cell activating factor receptor (BAFFR), Receptor activator for NF-κB (RANK), TNFR2, and Fibroblast growth factor-inducible factor 14 (FN14), and CD40.[35] Binding of TNFSFs to their cognate receptor results in the cIAPs being unable to ubiquitinate TRAF3, which ubiquitinates NIK, allowing for NIK to accumulate in the cell. NIK phosphorylates and activates IKKα, which in turn phosphorylates p100. This triggers

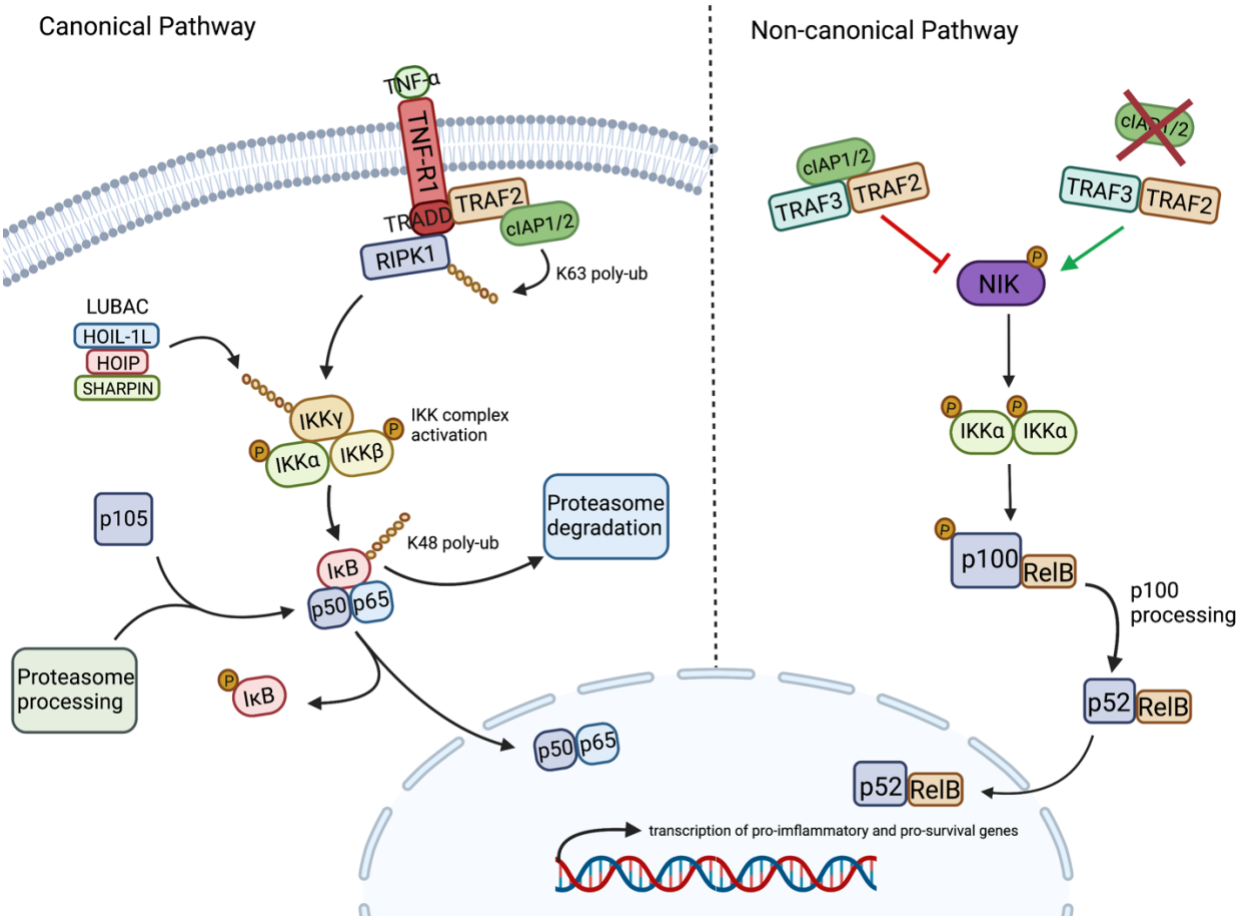


Figure 3: The canonical and the non-canonical NF-κB pathways.[36] cIAP1/2 activate the canonical pathway, and supress the non-canonical pathway. In the canonical pathway, when Tumor Necrosis Factor-Receptor 1 (TNF-R1) is bound by Tumor Necrosis Factor alpha (TNF-α), TNF-R1-associated factor 2 (TRAF2), cIAP1/2, and Receptor Inducing Protein Kinase 1 (RIPK1) are recruited to the receptor. cIAP1/2 ubiquitinate RIPK1, initiating a signalling scaffold resulting the phosphorylation and degradation of Inhibitor of κB (IκB), which is part of the Inhibitor of κB Kinase (IκK) complex. The NF-κB dimers translocate to the nucleus and begins transcription of pro-inflammatory genes. In the non-canonical NF-κB pathway, NF-κB-inducing-kinase (NIK) catalyzes the phosphorylation of an IκBα homodimer. The phosphorylation of IκBα initiates the phosphorylation, processing, and translocation of NF-κB into the nucleus. cIAP1/2 negatively regulate this pathway through the ubiquitination and subsequent degradation of NIK. Loss of cIAP1/2 or their function results in accumulation of NIK and activation of the non-canonical pathway.

the degradation of the C-terminal IκB-like structure on p100, leading to the generation of p52 and the nuclear translocation of the p52-RelB dimer to the nucleus.[35]

NF-κB in health and disease

In healthy states, the NF-κB pathways play important roles in many different cell types. The canonical NF-κB pathway is involved in initiating inflammation, as well as in the maturation process for innate and adaptive immune cells.[37] Activation of the canonical NF-κB pathway induces transcription of pro-inflammatory genes encoding for cytokines, chemokines, other mediators of inflammation found in immune cells.[38] NF-κB regulates T and B lymphocyte development and selection through induction of anti-apoptotic genes.[37] Some of these genes include TRAF1, TRAF2, cIAP1 and cIAP2. In T-cells, signalling through IKKβ and IKKα protects them from TNF-α induced apoptosis during their development.[32] [37] [39] Additionally, NF-κB is critical for the production of IL-12, which directs T-cell responses.[32] NF-κB has been known to be important in B-cells since its initial discovery. It was initially discovered through its binding compatibility with the κB site in the immunoglobulin kappa light chain enhancer, and its general presence in B-cells overall. The p50/c-Rel dimer has been found to be important for immunoglobulin class switching in mature B-cells.[32]

Due to the importance of NF-κB pathways, dysregulation of NF-κB signalling can lead to many disease states, including the development of cancer and inflammation. Both are associated with heightened NF-κB activity. The transcription of genes controlled by NF-κB in cell proliferation, cell survival, angiogenesis, metastasis, and inflammation lead to these conditions.[40] Constitutively active NF-κB or mutations in NF-κB associated oncogenes leads to cancer via promotion of cell proliferation and protecting cancer cells from conditions that would otherwise

lead to apoptosis.[40 41 42] A good example of this is in the interaction of p65 with STAT3. STAT3 helps control the interaction between p65 and the acetyltransferase p300, leading to p65 acetylation and a longer retention of p65 in the nucleus.[43] A longer retention of p65 in the nucleus leads to increased transcription of genes under its control. Dysregulation of this process can lead to higher expression of genes that will lead to increased inflammation and tumour cell survival. The tumour microenvironment also leads to increased NF-κB activity through stimulation of tumour cells with proinflammatory cytokines.[41 44]

In addition to leading to the development of cancer, chronic activation of NF-κB is associated with the development of inflammatory disorders such as Inflammatory Bowel Disease, arthritis, gastritis, and asthma, among many more.[40] In a study of patients with primary immunodeficiency, it was discovered that most of genes responsible for autoimmunity in these patients were linked to NF-κB, whether it was upstream, downstream, or a direct link to one of the NF-κB subunits. For example, patients with an autosomal dominant mutation to NF-κB2/p100/p52 had recurrent respiratory infections, adrenal insufficiency, and low serum antibody levels known as hypogammaglobulinemia. When looking into the cause of these issues, these patients were found to have a poor antibody response, unswitched naïve B cells, and defective T cell and NK cell activation. In these cases, p100 was unable to dimerize with p65.[45] Cancer and chronic inflammation seem to be comorbid as well, with approximately one in five cancers arising from chronic inflammation.[45]

The cIAPs in NF-κB signalling and cell death

cIAP1 and cIAP2 are functionally redundant members of the IAP family. The cIAPs interact with members of the TRAF family, including TRAF2, and bind to TNF receptors.[46 47]

cIAP1 is often thought of as the more dominant protein of the two; cIAP1 is more abundant and ubiquitous in cells. cIAP1 is the primary regulator of TNF-α mediated NF-κB activation.[46] cIAP2 is believed to act either as a backup for if cIAP1 is dysregulated or as a fine-tuning mechanism of the NF-κB response.[48] The latter is supported by the *ciap2* promoter being highly reactive to canonical NF-κB activation while the cIAP1 promoter does not respond in the same manner. cIAP2 is also a direct target for cIAP1-mediated ubiquitination and degradation.[46]

The cIAPs are critical regulators of the canonical and non-canonical NF-κB pathways. In the canonical pathway, cIAP1 and 2 positively regulate TNF-α mediated NF-κB activation through polyubiquitination of RIPK1.[46] [49] The ubiquitination of RIPK1 serves two functions: to inhibit apoptosis and to activate pro-survival pathways. Ubiquitination of RIPK1 blocks Caspase-8 binding, thereby inhibiting the activation of Caspase-8 from its inactive form.[49] [50] [46] RIPK1 polyubiquitination leads to engagement of the NF-κB pro-survival pathway by creating a scaffold for the assembly of the TAB2-TAB3-TAK1 and IKKα-IKKβ-IKKγ signalling complexes. These signalling complexes control NF-κB activation and promote cell survival.[49] [50] [46] The cIAPs play a critical role in NF-κB promotion of cell survival; in the simultaneous absence of cIAP1 and cIAP2 TNF-α stimulation will fail to activate the pro-survival pathway and cells will default to the extrinsic apoptotic pathway.[46] This has been shown *in* vivo, double deletion of the cIAPs in mice causes Caspase-8 dependent cell death.[47] cIAP1 and cIAP2 negatively regulate the non-canonical NF-κB pathway. TRAF3 brings TRAF2 and NIK together, and cIAP1/2 bound to TRAF2 induce K48 ubiquitination of NIK resulting in its proteasomal degradation.[46] [47] In the absence of the cIAPs NIK is no longer degraded, leading to its accumulation and the subsequent activation of the non-canonical pathway.[21] [14] Activation of the non-canonical NF-κB pathway through stimulation of its receptors mentioned in the section *Canonical and non-canonical NF-κB pathways* leads to the

degradation of TRAF3 following cIAP1 and 2 mediated K48 ubiquitination. This stabilizes NIK, and thus NIK accumulates in the cell and activates the non-canonical NF-κB pathway.[35]

Proximity labeling of protein-protein interactions

Enzyme-catalyzed proximity labeling (PL) is a technique for mapping protein-protein interactions within the cell. Enzymes catalyze the conversion of small molecules to a highly reactive intermediate that diffuses from the enzyme and covalently labels surrounding molecules in an efficient, proximity-dependent manner.[51] The two types of PL are peroxidase-based and biotin ligase-based.

Peroxidase-based PL includes enhanced Ascorbate Peroxidase (APEX/APEX2), and Horseradish peroxidase (HRP). Both peroxidase methods function off a similar mechanism in which they oxidize biotin-phenol into a highly reactive, short lived phenoxyl radical. The phenoxyl radical tags nearby proteins at side chains of electron-rich amino acids.[52] Tyrosine is the primary amino acid involved in this reaction.[53] HRP shows higher catalytic activity than APEX/APEX2 around the cell surface and the endoplasmic reticulum (ER).[54] Two drawbacks of peroxidase-based PL are the potential oxidative stress caused by the addition of H_2O_2, and that it can be challenging to apply to living tissues due to the low membrane permeability of biotin-phenol. Both HRP and APEX/APEX2 require a very short labeling time of under one minute.[55] [56]

Biotin ligase-based PL is derived from the *Escherichia coli* biotin ligase, BirA. The first biotin ligase approach to PL was dubbed "BioID" (Biotin Identification). BioID adenylates biotin to for biotin-5'-AMP, which reacts with lysine side chains on proteins within a ~10nm radius.[57] [58] BioID has an 18-hour labeling time, significantly longer than the peroxidase-based PL methods.

BioID is less toxic than peroxidase-based PL, making it a better choice when working in living systems.[57 59] Other biotin ligase-based PL includes BASU derived from *Bacillus subtilis* and AirID developed from an ancestral enzyme reconstruction algorithm.[51] The main drawback of biotin ligase-based PL is its long labeling time, which limits its applications.

TurboID is another biotin ligase-based PL that is available for use. It is a 35kDa protein generated from BioID using error-prone PCR. TurboID has 15 mutations compared to BioID, allowing it to have a significantly shorter labeling time of ~10 minutes, depending on the application. TurboID achieves a similar level of biotinylation in 10 minutes as BioID, AirID, and BASU do in 18 hours of labeling time.[60] The faster labeling time and non-toxic effects of TurboID have made it ideal for *in vivo* experiments.[51 61] The main drawback of using TurboID is that the increased promiscuity with biotin labeling also results in a larger labeling radius of 35nm.[62] Despite this, TurboID has promising applications in a variety of different work, being used in experiments involving *C. elegans*, drosophila, plants, and mammalian cells.[62 60 63]

Rationale and Objectives

Rationale

While cIAP1 and cIAP2 have been well-studied, there are many holes in our knowledge. The role and mechanism of the IAPs in ubiquitination are not yet completely understood due to the complexity of this posttranslational modification process. Additionally, The IAPs are involved in a myriad of cellular processes, and many of the process-specific mechanisms by which the IAPs are involved is unknown.

Specific objectives

We currently have an incomplete understanding of how cIAP1 and cIAP2 regulate inflammation. I aim to delve deeper into the signalling pathways that are controlled by cIAP1 and cIAP2 by discovering currently unknown protein-protein interactions. In doing so, I will determine which proteins interact with the cIAPs and what signalling pathways these proteins are involved in. The specific objectives of my research are to:

1. Generate and validate TurboID-IAP fusion constructs
2. Utilize the TurboID-IAP constructs to map IAP-protein interactions
3. Identify and explore the physiological relevance of the novel IAP-protein interactions

Chapter 2: Materials and methods

Plasmid construction

To create TurboID-IAP fusion constructs, overlapping primers were designed to insert V5-TurboID, cIAP1, cIAP2, XIAP, and Livin on a pcDNA5/FRT/TO backbone (Table 1). V5-TurboID-NES_pCDNA3 was a gift from Alice Ting (Addgene plasmid #107169; http://n2t.net/addgene: 107169; RRID:Addgene_107169). V5-tagged TurboID protein was inserted at the N-terminus of the given IAP as not to interfere with the function of the RING domain. The DNA fragments were purified via PCR amplification using PFX polymerase and assembled with the NEB HiFi DNA Assembly kit (New England BioLabs) according to kit directions. These DNA fragments were inserted on the pcDNA5/FRT/TO plasmid, overlapping with the TO (tetracycline operator) region. Bacterial transformations were performed according to NEB kit protocol with NEB DH5-alpha *E. coli* (NEB c2987).

A single digest using HindIII was used to confirm the assembled plasmids were the correct sizes according to the fragments that were assembled. Plasmids were purified with the GenElute HP Plasmid Miniprep kit (Sigma) and verified via DNA sequencing at The Centre for Applied Genomics (Toronto, ON). Confirmed plasmids were expanded in NEB DH5-alpha *E. coli* and purified via Maxiprep using the GenElute HP Plasmid Maxiprep kit (Sigma-Aldrich).

Cell culture and stable cell line generation

HEK 293T cells were obtained from the American Tissue Culture Collection (ATCC) and cultured in DMEM containing L-glutamine, non-essential animo acids, and supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), in a humidified incubator with 5% CO_2 at 37˚C. Stable TurboID clones expressing TurboID-cIAP1 or TurboID-cIAP2 were generated using the

Primer Name	Direction	Sequence
pcDNA5	Forward	5’-GGA TCC ACT AGT CCA GTG TGG-3’
	Reverse	5’-AAG CTT AAG TTT AAA CGC TAG-3’
TurboID	Forward	5’-ACG GTT TAA ACT TAA GCT TGC CAC CAT GGG CAA GCC CAT CC-3’
	Reverse	5’-TGG CGC GCC GCC GCC GGG GCC AGA TCC CCC CTG CAG CTT TTC GGC AGA CCG-3’
cIAP1	Forward	5’-GGG GGA TCT GGC CCC GGC GGC GGC GCG CCA CAC AAA ACT GCC TCC CAA AGA-3’
	Reverse	5’-CCA CAC TGG ACT AGT GGA TCC TTA AGA GAG AAA TGT ACG AA-3’
cIAP2	Forward	5’-GGG GGA TCT GGC CCC GGC GGC GCG CCA AAA AGC GCC AAC ACG TTT GAA-3’
	Reverse	5’-CCA CAC TGG ACT AGT GGATCC TTA TCA TGA AAG AAA TGT ACG-3’
XIAP	Forward	5’-GGG GGA TCT GGC CCC GGC GGC GGC GCGCCA ACT TTT AAC AGT TTT GAA GGA-3’
	Reverse	5’-CAC TGG ACT AGT GGA TCC TTA AGA CAT AAA AAT TTT TTG CTT-3’
Livin	Forward	5’-GGG GGA TCT GGC CCC GGC GGC GGC GCG CCA GGC CCT AAA GAC AGT GCC AAG
	Reverse	5’-CCA CAC TGG ACT AGT GGA TTC CTA GGA CAG GAA GGT GCG CAC-3’

Table 1: primer sequences for isolation of PCR fragments.

Flp-In Complete System Kit (ThermoFisher Scientific) as outlined below.

HEK-293T cells were transfected with pFRT using PEI and OptiMEM. Cells were cultured in complete DMEM supplemented with 250 µg/mL Zeocin. Select populations of HEK-293T cells were isolated using cloning rings two weeks after transfection with pFRT and the addition of Zeocin and expanded up to 10cm plates. Each population was assigned a unique name from 1A to 1X to differentiate them from one another. Flp-In clones were identified via B-gal assay (ThermoFisher Scientific).

TurboID-cIAP1/2 containing 293T cells were generated via the co transfection of pOG44 (Flp recombinase) and pcDNA5/FRT/TurboID-cIAP1/2 cultured in complete DMEM supplemented with 100µg/mL Hygromycin B (ThermoFisher Scientific). Populations 1H, 1J, and 1K were transfected with V5-TurboID-cIAP1 with or without pOG44 in. Population 1C was culled due to contamination prior to this transfection. Populations of cells were isolated with cloning rings after two weeks of selection with Hygromycin B and grown from 24-well plates to 10cm plates. Plates of clonal populations were frozen down to preserve the different populations. To reduce background biotinylation, TurboID-cIAP1/2 293T cells were cultured in DMEM supplemented with dialyzed FBS (Sigma Aldrich).

Western blotting

Cells were lysed with RIPA-SDS lysis buffer (10mM Tris pH 7.4, 150mM NaCl, 10mM KCl, 1mM EDTA 0.5% deoxycholic acid, 0.5% Tween-20, 0.5% NP-40, 0.1% SDS). The lysates were incubated on ice for 10 minutes, then sonicated at 20% amplitude for 10 seconds. The sonicated lysates were spun down at 14,000 RPM for 10 minutes and moved to a fresh tube to

remove residual debris. The lysates had their protein concentrations quantified with a colorimetric Bio-Rad DC protein assay, and the remaining lysate was stored at -80˚C until further use.

Samples were mixed with 5X SDS loading buffer and loaded onto 9% 1.5mm SDS-PAGE gels. The gels were run at 80V for 15 minutes, then 120V until completion. Proteins were transferred to a nitrocellulose membrane with 0.2nm or 0.45nm pores, depending on protein size using the wet transfer method. The membranes were blocked with LICOR blocking buffer (TBS based) for one hour at room temperature. The membranes were incubated with the primary antibody overnight at 4˚C. The following day, the primary antibody was removed, and the membranes were washed twice for ten minutes with TBS/T. The secondary antibody was put on and incubated in the dark for one hour at room temperature. Following the one-hour incubation, the membranes were kept in the dark and washed three times for 15 minutes with TBS/T then twice for five minutes with PBS. The membranes were scanned on the LICOR Odyssey. The following primary antibodies were used: anti-cIAP1/2, Medical and Biological Laboratories Co., LTD (MBL), CY-P1041, polyclonal; anti-TRAF2, Cell Signalling, 4172S, polyclonal; anti-RIPK1, BD Biosciences, 610549, clone 38; anti-B-Tubulin, Developmental Studies Hybridoma Bank (DSHB), E7; anti-XIAP, Dr. Robert Korneluk lab; anti-Livin, ABR, MA1-20331. Streptavidin IRDye 680 and 800 (P/N 926-68079 and P/N 926-32230) from LICOR was used for biotin detection.

TurboID proximity labeling

Stable TurboID clones in HEK 293T cells were cultured in DMEM containing L-glutamine and non-essential amino acids, and supplemented with 10% heat-inactivated dialyzed FBS, in a humidified incubator with 5% CO_2 at 37˚C. Cells were treated in 15 cm plates with 50μM Biotin, 10ng/ml TNF-α, and 100nM PRX-003 in conjunction with each other or alone to create different

sample groups and controls. Cells were treated with biotin for 35 minutes, and TNF-α and PRX-003 for 30 minutes prior to collection. Cells were washed with 5mL cold DPBS and collected in 1.5mL Eppendorf tubes. Samples were centrifuged at 13,000 x g for 1 minute, and the PBS was aspirated. All samples were stored at -80˚C prior to lysis.

Sample processing for Mass Spec analysis

Lysis was performed using the protocol above in the "Western Blotting" section. 500µL of lysis buffer was added to each sample, totalling 1500µL of lysis buffer since the samples had to be split between three tubes. The RIPA-SDS lysis buffer also included IAA, NEA, and a protease and phosphatase inhibitor cocktail. 50µL of lysate was kept for western blotting purposes, the remainder was frozen for immunoprecipitation and LC/MS analysis. Samples were normalized to 20µg of protein for western blotting, apart from the elution sample which was normalized to 5µL per sample.

The lysate for LC/MS analysis was added to 400µL suspended beads in a 25% slurry, and the samples were rotated at 4˚C overnight. The beads were pre prepared by washing twice in RIPA-SDS lysis buffer. 50µL of lysate was collected prior to incubation as the "input" fraction. The next day, the remaining lysate was removed from the beads and 200µL was kept as the "flowthrough" fraction. The beads were washed twice with 50mM Tris pH 7.5. after the first wash, 15µL of beads were kept for the elution sample for western blotting purposes. The beads for western blotting were boiled for 5 minutes in 6X lysis buffer and stored at -20˚C until use. The beads for LC/MS analysis were washed twice with 2M urea in 50mM Tris pH 7.5. 50µL of 0.1% TFA was added to the beads for 5 minutes to elute off any bound biotinylated proteins, mixing every so often. 5µL

ammonium bicarbonate pH 7.8 was added to neutralize the solution. The samples were frozen and shipped to the BioZone Mass Spectrometry facility at the University of Toronto for analysis.

Liquid Chromatography-Mass Spectrometry

All LC/MS samples were run and process by Robert Flick, M.Sc. at the BioZone Mass Spectrometry Facility, Department of Chemical Engineering and Applied Chemistry, University of Toronto. All samples were processed with an in-solution Tryptic digest. 5µL per 100µL sample volume of 200mM DTT in 100mM ammonium bicarbonate was added. The samples were then vortexed and spun in a centrifuge. The samples were incubated at room temperature for 45 minutes, then 8µL per 100µL of sample volume of 0.5M iodoacetamide in 100mM ammonium bicarbonate was added. The samples were then vortexed and spun in a centrifuge, and the samples were incubated at room temperature in the dark for 45 minutes. 20µL per 100µL of sample volume of 200mM DTT in 100mM ammonium bicarbonate was added to the samples, and the samples were then vortexed and spun in a centrifuge. The samples were incubated at room temperature for 45 minutes, then 1µg of Trypsin was added. The samples were mixed by gently pipetting up and down, then incubated at 37˚C overnight.

Following the in-solution Tryptic digest, an OMNIX tip cleanup was performed on all samples. The samples were adjusted to 1.0% TFA using 2.5% TFA solution. The tip was wet with 100µl 1:1 ACN:H2O and the solution was discarded twice, then equilibrated with 100µl 0.1% TFA and the solution was discarded twice. The samples were aspirated and dispensed 3-5 times. 100µL of 0.1% TFA was aspirated, dispensed, and discarded twice. 100µl 95% ACN/0.1% formic acid was aspirated and dispensed to elute the samples. The samples were dried in a speedvac and

resuspended in MS buffer for analysis. Prior to loading, the samples were filtered through a 0.2µ filter.

The following Chromatography settings are used: NanoUHPLC: Thermo Scientific Easy nLC 1000; Column: custom packed SilicaTip Emitter (New Objective part# FS360-75-15-N-20); C18 Resin packed in column: ReproSil-Pur C18-AQ (Dr. Maisch part# r13.aq); Sample Injection: 5µL; Solvent A: 0.1% formic acid in Water; Solvent B: 0.1% formic acid in Acetonitrile; Flow Rate: 250nL/min; Gradient: start, 0%B; 0min-5min, linear gradient to 10%B; 5-53min, linear gradient to 40%B; 53-55min, linear gradient to 95%B; 55-65min, 95%B; 65-66min, linear gradient to 0%B; 66-80min, 0%B (re-equilibriation). The following settings were used for Mass Spectrometry: Mass Spectrometer: Thermo Scientific Q-Exactive; Probe: nanoESI. Ionization Mode: Positive; Spray Voltage: 2.5kV; Capillary Temp: 250˚C; S-Lens RF Level: 50. The Full MS Settings were as follows: Resolution: 70 000; AGC Target: 1e6; Maximum Injection Time: 30ms; Scan Range: 400-2000. The Data-Dependent MS2 settings were as follows: Mode: Top 10, Resolution: 17 500; Maximum Injection Time: 50ms; Isolation window: 0.4 m/z; Scan Range: 200-2000; NCE: 27. The following data processing settings were used: Software: The GPM using X!Tandem search algorithm; Fragment Mass Error: 0.4Da; Parent Mass Error: 20ppm; Ions: a,b,x,y; Database: Human Proteome.

Data analysis

LC/MS results were processed via manual exclusion of proteins that appeared in control samples in data samples. Any proteins that were found a control sample were marked in all samples and excluded from consideration as a potential cIAP1 interactor. Unmarked proteins were considered to be potential true cIAP1 interactors, and further analysis was done. These proteins

were confirmed as potential hits in a preliminary manner through ProHits, where the raw data was run through the Significance Analysis of INTeractome (SAINT) algorithm for specificity. Proteins were further checked for connections to cIAP1 and the NF-κB pathways through literature deep dives and the creation of a protein interaction network with cIAP1 using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING). The potential hits were also screened using The Biological General Repository for Interaction Datasets (BioGRID) to check for known connections.

Chapter 3: Results

Objective 1: Generate and validate TurboID-IAP fusion constructs

Generation of TurboID-IAP fusion constructs

Prior to assembly of the plasmid with the NEB HiFi DNA Assembly kit, I isolated DNA fragments of the necessary IAPs, TurboID, and pcDNA5/FRT/TO via PCR purification with primers designed for the start and the end of the gene. There was an additional linker of base pairs added to the end of the TurboID gene and the start of each IAP gene. All of the PCR amplicons were the expected size and contain no additional bands at other sizes (Figure 4A). The sizes of the DNA fragments in Figure 4A match the expected sizes of the plasmids when added together (Figure 4B, Table 2). I have assemblies of TurboID-cIAP1, -cIAP2, -XIAP, and -Livin gene fusions that are cloned into the plasmid pcDNA5/FRT/TO.

Confirmation of TurboID-IAP fusion proteins

I transfected HEK 293T cells with the pcDNA5/FRT/TurboID-IAP plasmids to determine if the TurboID-IAP fusion genes are detectable as proteins post-transfection. The predicted size of the TurboID-IAP fusion proteins is 35kDa larger than the endogenous IAP; this was reflected in the band sizes of the TurboID-IAP fusion proteins detected via Western blotting (Figure 5). Transfection of the constructs into HEK 293T cells revealed that the pcDNA5/FRT/TurboID-IAP plasmids were able to be detected by antibodies specific to the corresponding IAP (Figure 5A), as well as by an antibody targeting the V5 tag conjugated to TurboID (Figure 5B).

Generation of a stable cell line expressing TurboID-cIAP1 or TurboID-cIAP2

A low expression level is critical when using a BioID approach because high expression levels of the BioID or TurboID fusion protein can lead to artifactual subcellular localization. This can result

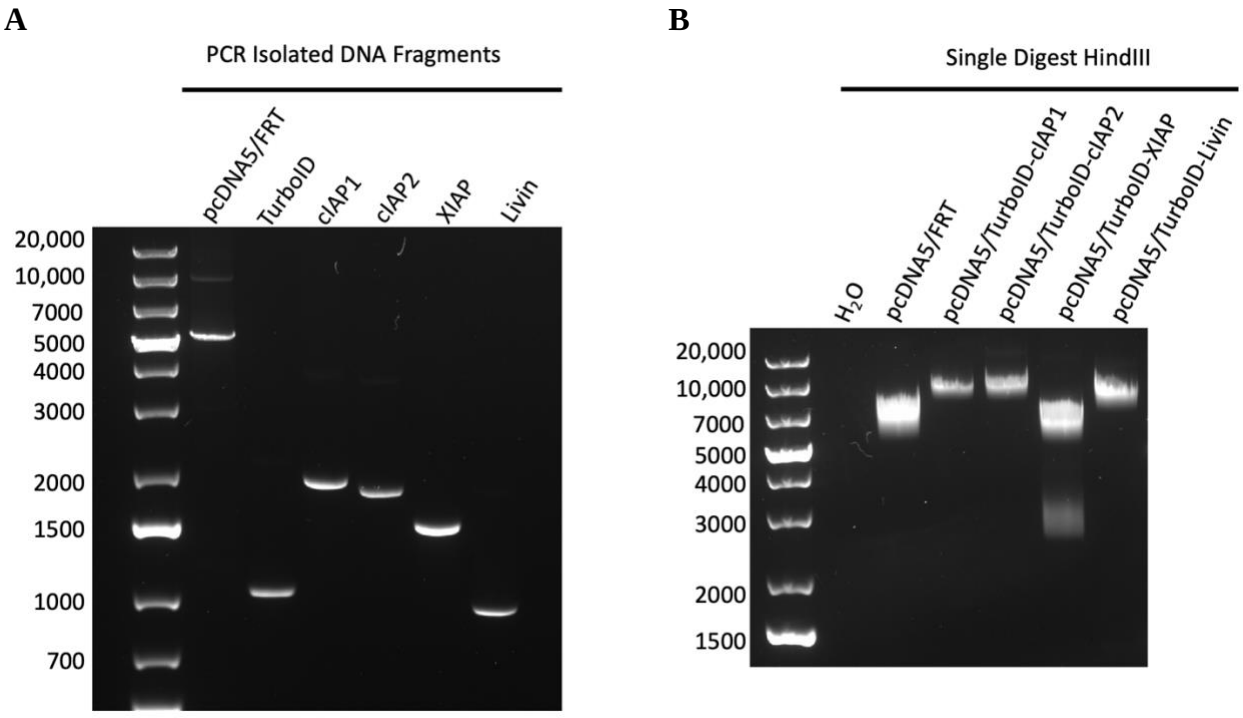


Figure 4: Assembly of pcDNA5/FRT/TurboID/IAP plasmids from purified DNA fragments. A. PCR purified DNA fragments on a 0.8% agarose gel. B. Single digest with HindIII on plasmids assembled with the NEBuilder HiFi DNA Assembly kit from purified DNA fragments in A. Expected sizes of plasmids matched the sizes of the PCR purified DNA fragments when assembled, indicating a successful construction of the plasmids.

PCR purified fragments	Fragment sizes (bp)	Plasmid	Fragment makeup	Fragment sizes before assembly (bp)	Assembled plasmid size (bp)
pcDNA5/FRT	5150	pcDNA5/FRT	pcDNA5/FRT	5150	5150
cIAP1	1854	pcDNA5/FRT/ TurboID/cIAP1	pcDNA5/FRT	5150	8024
			TurboID	1020	
			cIAP1	1854	
cIAP2	1815	pcDNA5/FRT/ TurboID/cIAP2	pcDNA5/FRT	5150	7985
			TurboID	1020	
			cIAP2	1815	
XIAP	1491	pcDNA5/FRT/ TurboID/XIAP	pcDNA5/FRT	5150	7661
			TurboID	1020	
			XIAP	1491	
Livin	894	pcDNA5/FRT/ TurboID/Livin	pcDNA5/FRT	5150	7064
			TurboID	1020	
			Livin	894	
TurboID	1020				

Table 2: TurboID-IAP fragment and plasmid sizes.

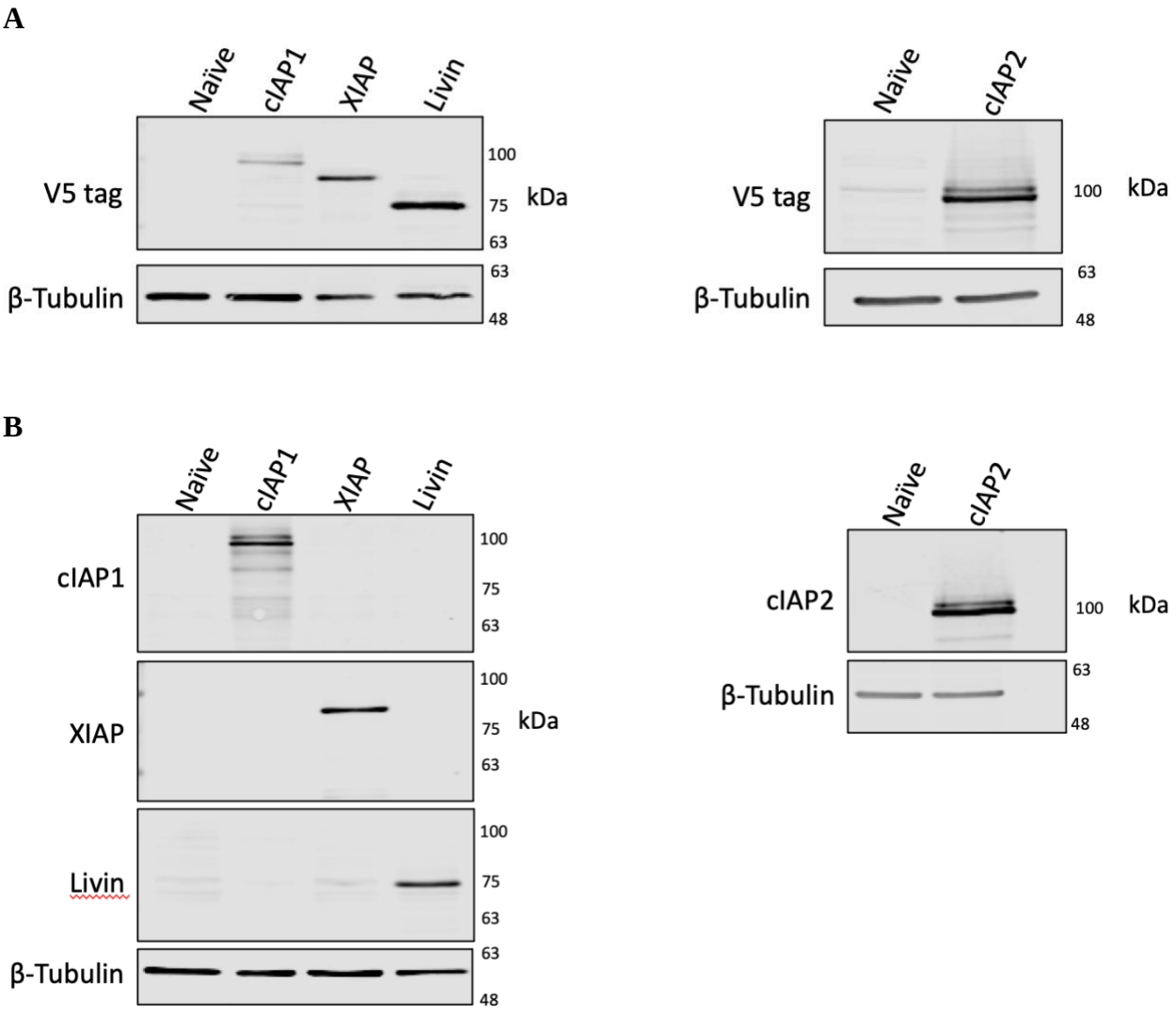


Figure 5: Assembly of TurboID-IAP plasmids was successful. HEK-293T cells were reverse transfected for 48h with pcDNA5/FRT/TurboID-IAP and processed for Western blotting. Blots were assessed for IAP expression with antibodies targeting V5 (**A**) and the corresponding IAP (**B**).

in the biotinylation of proteins that the bait protein would never usually encounter. Additionally, TurboID is a highly promiscuous biotin ligase with a very short labeling time which increases the chances of non-specific labeling. Overexpression of the TurboID-tagged bait protein will result in false positive interactions caused by the TurboID-tagged bait protein interacting with proteins that it would usually never interact with in the cell. The benefit of using the pcDNA5/FRT system is to create a stable cell line expressing your protein of interest at physiologically relevant levels without the added step of transfecting cells for every experiment. pcDNA5/FRT/TO is sold as part of a kit called the "Flp-In Complete System" and is used to generate stable cell lines that express the protein of interest at levels closer to normal physiological levels. The steps when using the kit are as follows (Figure 6):

1. Use pFRT/LacZeo to generate a cell line with an integrated FRT domain, expression of β-galactosidase (LacZ operon), and resistance to Zeocin. Zeocin is added to the media to select for cells with the domain is integrated.
2. Cotransfect pcDNA5/FRT/TurboID-IAP and pOG44 (Flp-recombinase) to generate a stable cell line expressing the TurboID-IAP fusion protein at the integrated FRT domain, are resistant to Hygromycin B, display no β-galactosidase expression and are sensitive to Zeocin. Hygromycin B is added to the media to select for cells that have integrated the TurboID-IAP gene at the FRT domain on the genome.

pFRT/LacZeo was successfully integrated into the genome of select HEK 293T cells

To generate cells that have an integrated FRT domain, are Zeocin resistant, and express β-Galactosidase, I transfected HEK-293T cells with a linearized pFRT/LacZeo plasmid using HindIII as the restriction enzyme. Linearization was performed to increase the chances of the

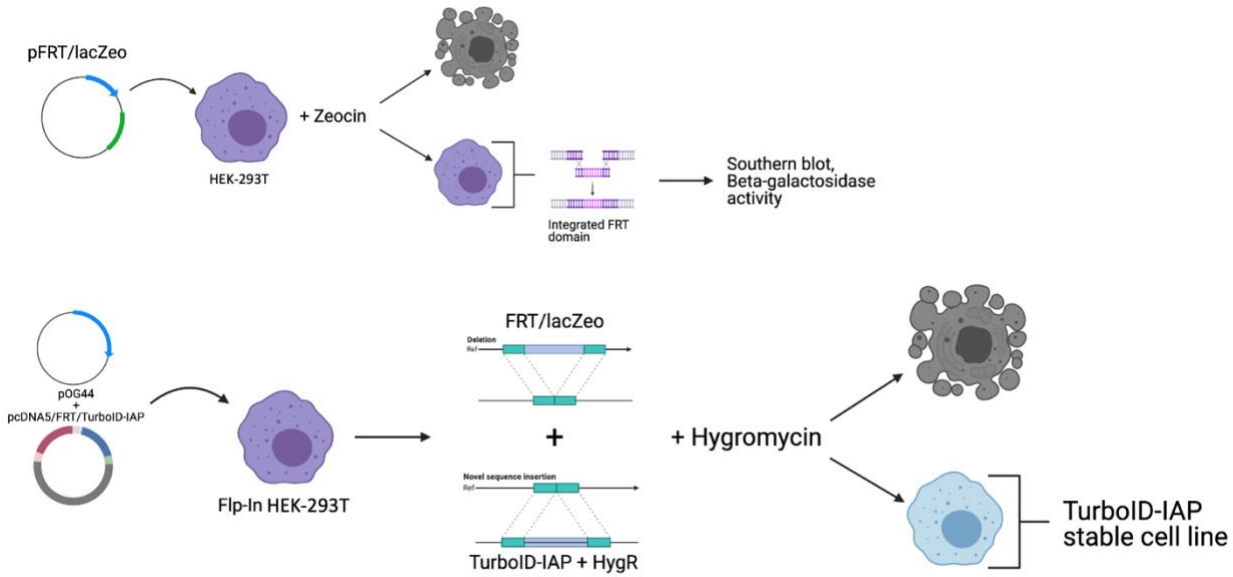


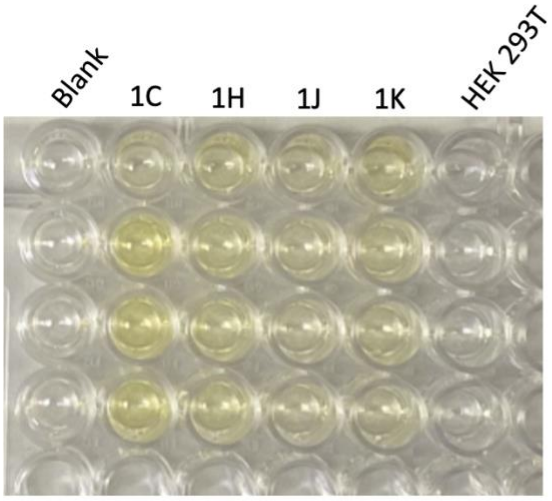
Figure 6: Creation of a stable cell line using the Flp-In Complete System kit. First, HEK-293T cells are transfected with linearized pFRT/LacZeo. Zeocin is added to the to select for cells that have integrated the FRT domain and the LacZeo domain. Stable integration into the genome is identified via β-galactosidase activity. These cells will express β-galactosidase activity and are Zeocin resistant. Next, stable Flp-In clones are selected to undergo a cotransfection of pOG44 (Flp recombinase) and pcDNA5/FRT/TurboID-IAP. Hygromycin B is added to the media to select for cells that have successfully integrated pcDNA5/FRT/TurboID-IAP and by extension the Hygromycin B resistance gene into the present FRT domain in the Flp-In cell's genome. The end goal is a cell that expresses the protein of interest, is Hygromycin B resistant, and is Zeocin sensitive.

plasmid inserting itself into the genome correctly without disruption of the FRT domain or the LacZeo fusion gene. I isolated single cell clones to ensure all clones had the integrated FRT domain and the LacZeo fusion gene in the same site on the genome to ensure the LacZeo gene is expressed at a consistent level across all cells and the Zeocin resistant cells are not preventing zeocin sensitive cells from dying by their proximity to Zeocin resistant cells. To assess for correct integration of the FRT domain I selected 4 populations grew well during the cloning process: 1C, 1H, 1J, and 1K. I observed a good signal of β-galactosidase in all populations upon analysis with the Mammalian B-Galactosidase Assay Kit from ThermoFisher Scientific (Figure 7). Clone 1C had significantly higher β-Galactoside expression when compared to the other three clones. This indicates integration of the FRT domain, since the FRT domain is upstream from a LacZ-Zeocin fusion gene.

Confirmation of stable cell lines expressing TurboID-cIAP1 and -cIAP2

I cotransfected the FlpIn HEK 293T clones 1H, 1J, and 1K generated in the previous section with pOG44, a plasmid that contains the FLP Recombinase gene, and pcDNA5/TurboID-cIAP1 and -cIAP2. Clone 1C was culled prior to the cotransfection due to contamination. This process is used to generate a stable cell line that expresses your protein of interest from previously generated Flp In cells. Clones 1H and 1K died upon transfection prior to the addition of Hygromycin B. One clone from clone 1J was positive for TurboID-cIAP1, clone 1J-Tc1-44 (Figure 8). Both cIAP1 and V5-TurboID were detected at the expected 100kDa. This clone was in the experimental group where FLP recombinase was included, as denoted by the suffix "-44" in the name of the clone. The identity of the clones are as follows: 1J is the Flp-In HEK-293T clone, Tc1

A



B

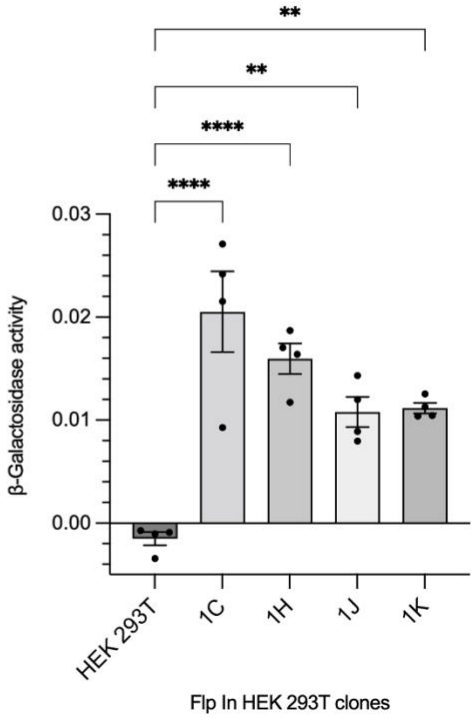


Figure 7: Identification of stable HEK293-FRT/Lac/Zeo clones through expression of β galactosidase. Flp-In HEK-293T clones were lysed with M-PER lysis buffer. Absorbance was read on a microplate reader at 405nm after 30 minutes of incubation with the β-gal assay reagent according to kit directions. **A**. Colorimetric analysis of β-galactosidase activity. There were four technical replicates for each sample arranged vertically. **B**. B-galactosidase absorbance reading. The HEK 293T cells are untransfected. Statistics were performed with an Ordinary one-way ANOVA with multiple comparisons, comparing each clone to HEK 293T cells as a negative control for β-galactoside activity. **$p<0.05$, ****$p<0.0001$

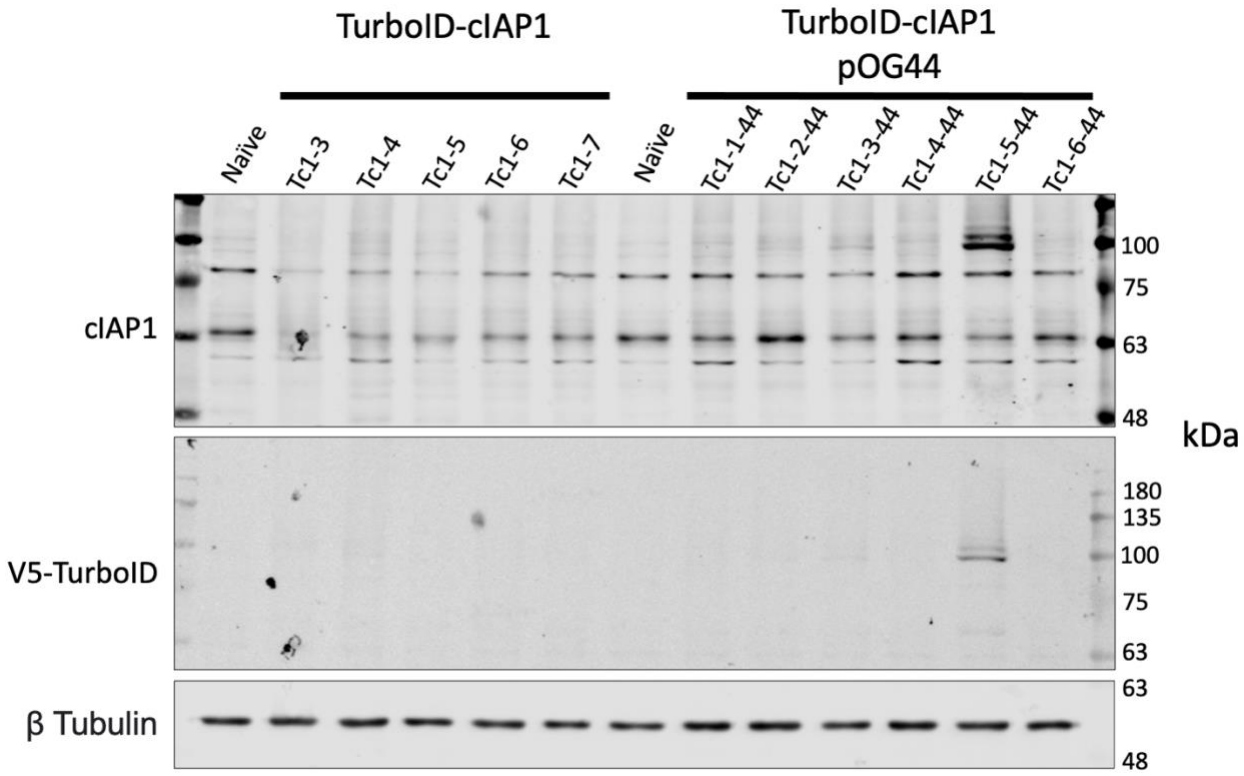


Figure 8: Generation of a TurboID-cIAP1 expressing HEK293T clone was successful. Flp In clone 1J was cotransfected with pOG44 and pcDNA5/TurboID-cIAP1 and processed for Western blotting following selection with Hygromycin B. cIAP1 was detected using a corresponding antibody (top) and an antibody targeting the V5 tag on the fusion protein (middle). The anti-V5 antibody detected the presence of a V5 tag only in clone Tc1-5-44, which confirms the expression of V5-TurboID-cIAP1 at the expected size of 100kDa.

is a shortened version of TurboID-cIAP1, Tc2 is a shortened version of TurboID-cIAP2, and 44 denotes that the clone came from a group that was co-transfected with FLP recombinase.

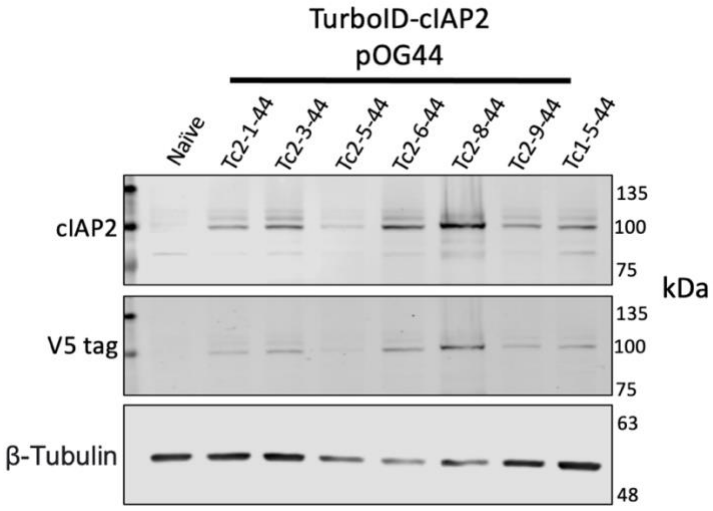
My initial attempt of generating clones that express TurboID-cIAP1 and TurboID-cIAP2 resulted in only one clone, clone 1J-Tc1-44, that stably expresses TurboID-cIAP1. Clone Tc1-5-44 expresses V5-TurboID-cIAP1 at 100kDa, as well as endogenous cIAP1 at 63kDa. A subsequent attempt resulted in the generation of several TurboID-cIAP2 clones (Figure 9). Out of these TurboID-cIAP2 clones, clones 1J-Tc2-3-44, 1J-Tc2-6-44, 1J-Tc2-10-44, and 1J-Tc2-12-44 match clone 1J-Tc1-5-44 the closest for the band strength of cIAP1/2 and of the V5 tag of TurboID. No further work has been done on TurboID-cIAP2 due to time constraints.

Objective 2: Utilize the TurboID-IAP constructs to map IAP-protein interactions

Determining biotinylation time for TurboID

BioID uses free biotin within the cell to tag nearby proteins in a process known as biotinylation. TurboID has a required labeling time of 10 minutes according to the TurboID Nature protocols paper, however, some users have found that longer labeling times are necessary for analysis applications such as Mass Spectrometry.[51 64] I performed a biotinylation time course in HEK-293T cells transfected with TurboID-cIAP1 or TurboID-cIAP2 to determine the optimum time length for biotin incubation (Figure 10). Biotinylation was sufficiently above the background level at the 30 minute incubation mark. To make sure enough biotinylation is occurring by the time TNF-α is added to stimulate the canonical NF-κB pathway for 30 minutes, an extra 5 minutes of biotin incubation time was added to make a total of 35 minutes of biotin incubation.

A



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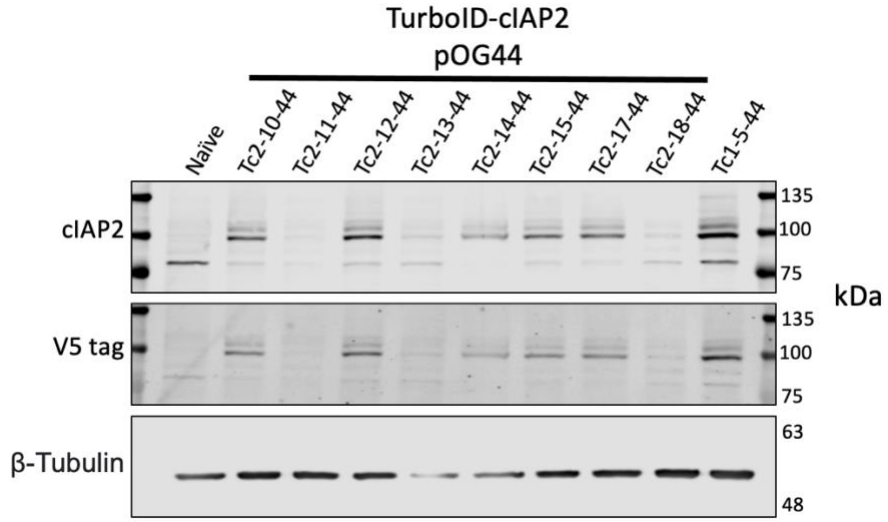


Figure 9: A TurboID-cIAP2 expressing HEK293T clone was successfully obtained using clone 1J. Flp In clone 1J was cotransfected with pOG44 and pcDNA5/TurboID-cIAP2 and processed for Western blotting following selection with Hygromycin B. Some clones were contaminated and discarded prior to processing for Western blotting, but the original numbers assigned to each clone has remained constant for consistency with labels. **A**. cIAP2 was detected in clones one to nine using a corresponding antibody (top) and an antibody targeting the V5 tag on the fusion protein (middle). The anti-V5 antibody detected the presence of a V5 tag only in clone Tc1-5-44, which confirms the expression of V5-TurboID-cIAP1 at the expected size of 100kDa. **B**. cIAP2 was detected in clones 10 to 18 using a corresponding antibody (top) and an antibody targeting the V5 tag on the fusion protein (middle). The anti-V5 antibody detected the presence of a V5 tag only in clone Tc1-5-44, which confirms the expression of V5-TurboID-cIAP1 at the expected size of 100kDa.

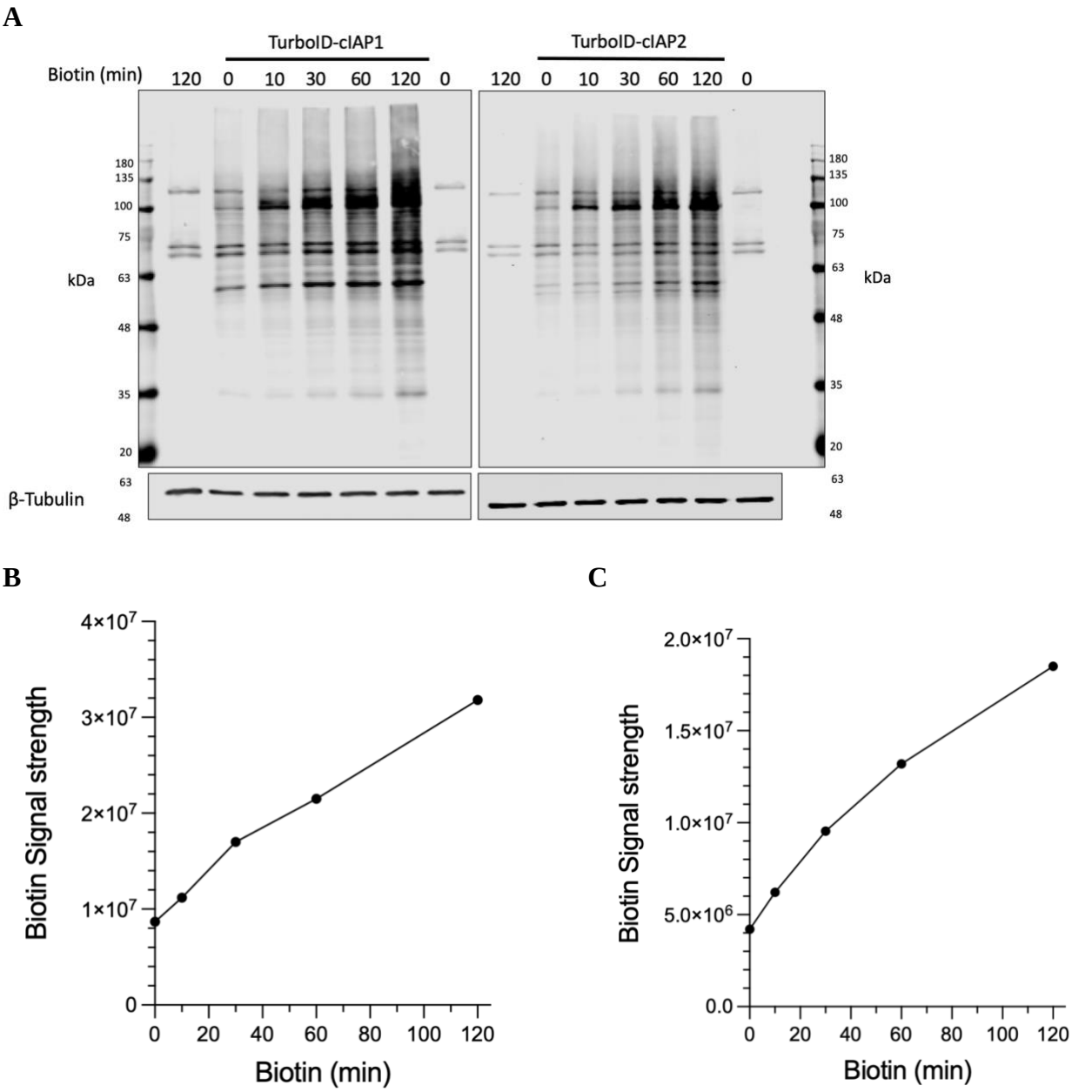


Figure 10: Biotinylation time course of TurboID-cIAP1 and TurboID-cIAP2. HEK-293T cells were reverse transfected for 24h with TurboID-cIAP1 or TurboID-cIAP2. Cells were incubated with 50µM of biotin for 0, 10, 30, 60, or 120 minutes. Naïve HEK-293Ts were used as a control. **A**. Western blot of samples. Membranes were incubated with Streptavidin-IRDye (1:3000) in the dark for 30 minutes to detect biotinylation. **B**. Biotin signal strength of TurboID-cIAP1 transfected HEK 293T cells. **C**. Biotin signal strength of TurboID-cIAP2 transfected HEK 293T cells.

Preparation of samples for mass spectrometry

I treated clone 1J-Tc1-5, HEK 293T cells transfected with pcDNA3/TurboID-NES, and untransfected HEK 293T cells with different combinations of the following conditions: 50µM biotin for 35 minutes, 10ng/mL TNF-α for 30 minutes, and 100nM PRX-003 for 30 minutes. Biotin treatment is to provide excess biotin as a substrate for TurboID to conjugate to nearby proteins. The TNF-α treatment is to stimulate the canonical NF-κB pathway and the PRX-003 treatment is to stimulate the non-canonical NF-κB pathway. PRX-003 is a SMAC mimetic. SMAC mimetics are a novel class of cancer therapy drug that mimics the N-terminal residues (AVPI) in the endogenous protein SMAC. SMAC mimetics act as IAP inhibitors.[65`] A subset of 15µL of streptavidin-coated magnetic beads from magnetic beads resuspended in 250µL wash buffer was taken for the elution fraction; the remaining beads were kept for mass spectrometry analysis.

Prior to sending samples for mass spectrometry, I analyzed my samples via Western blotting for immunoprecipitation of biotinylated proteins. Biotinylation of proteins by V5-TurboID-NES (Nuclear Export Signal) (no bait protein) and V5-TurboID-cIAP1 was successful (Figure 11 and 12). V5-TurboID-cIAP1 appears to have a greater specificity for biotinylation of interacting proteins than V5-TurboID-NES with no bait protein. There is a more defined band pattern in the V5-TurboID-cIAP1 samples (Figure 11), while in the V5-TurboID-NES samples the banding takes up the whole lane and demonstrates little to no specificity (Figure 12). V5-TurboID-cIAP1 samples with added biotin have a faint but present smear in the lanes, and stronger bands in the 48kDa to 100kDa range that are hard to see or not visible in the samples without added biotin (Figure 11C).

I assessed if TurboID-cIAP1 proteins led to biotinylation of a known interactor of cIAP1/2, RIPK1, to see if my pulldown of cIAP1 interacting proteins after proximity labelling with TurboID was

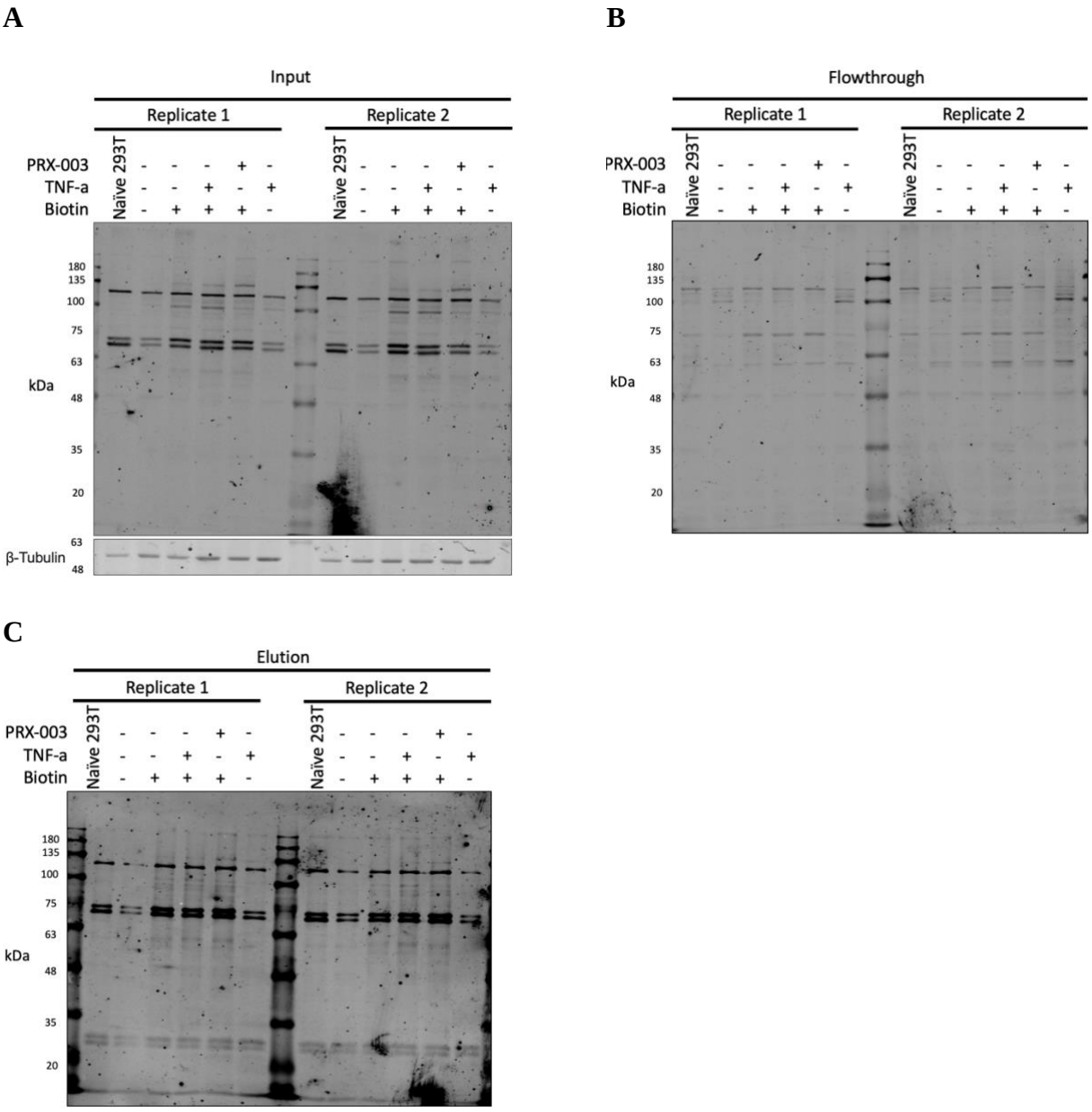


Figure 11: Biotinylation of endogenous proteins by V5-TurboID-cIAP1. Cells were treated using 50μM biotin for 35 minutes, 10ng/mL TNF-α for 30 minutes, and 100nM PRX-003 for 30 minutes. The cells were collected and processed for immunoprecipitation, then analyzed via Western blotting for biotinylated proteins. **A**. Input fraction before bionylated proteins were immunoprecipitated. **B**. Flow-through of biotinylated proteins unbound by streptavidin beads. **C**. Biotinylated proteins eluted off streptavidin coated beads used for separation of biotinylated proteins.

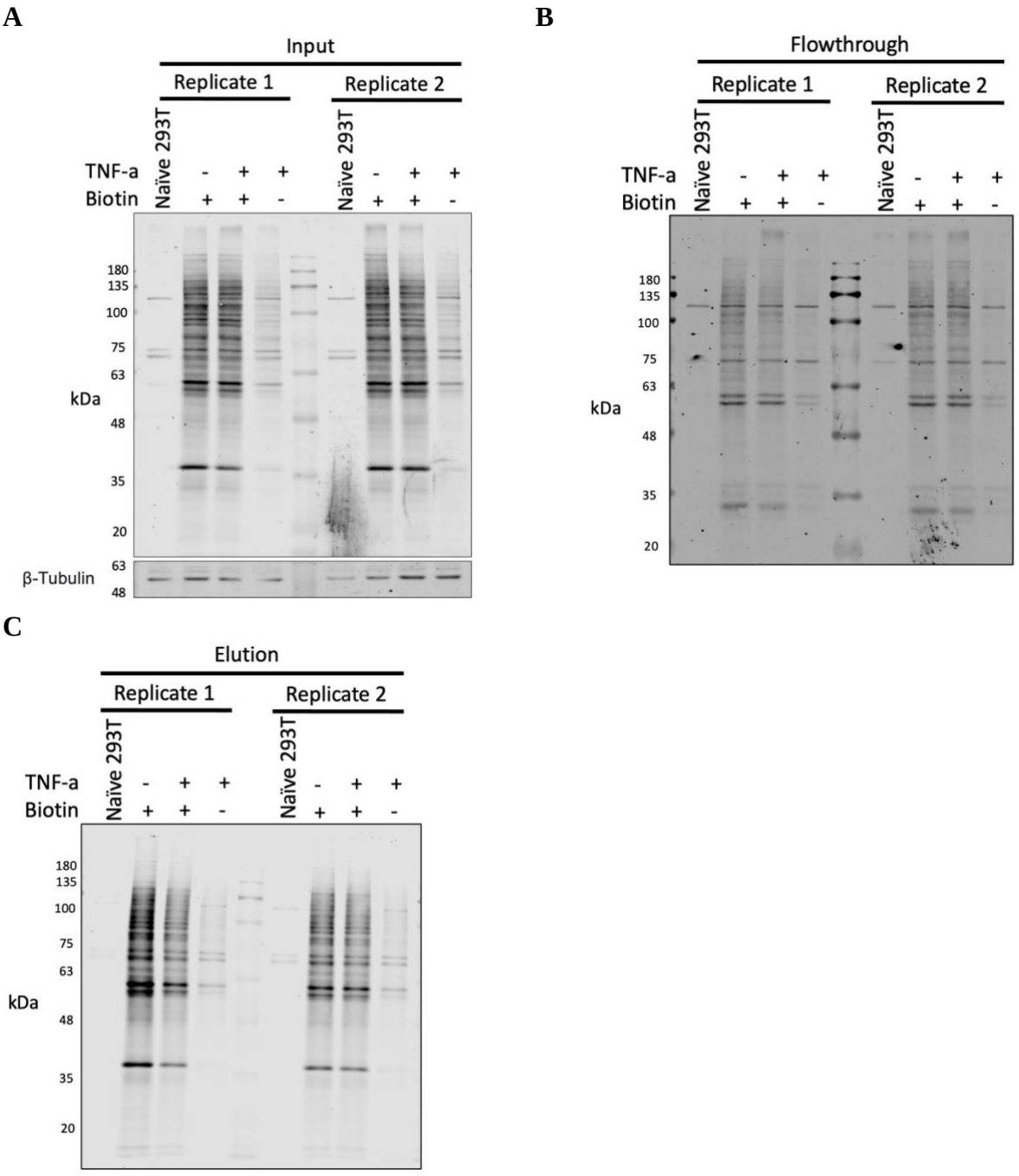


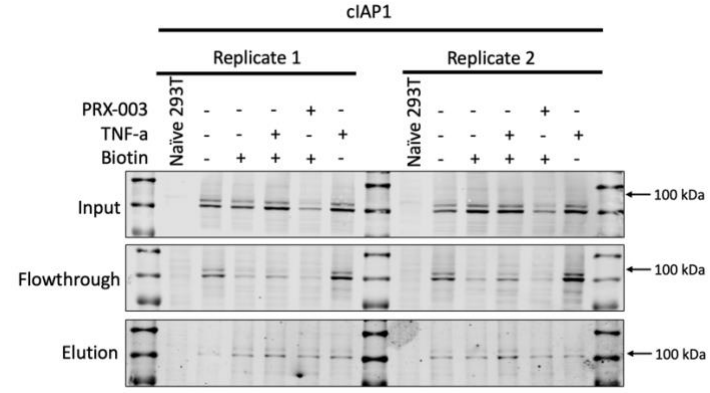
Figure 12: Biotinylation of endogenous proteins by V5-TurboID-NES. The experiment was carried out as in Figure 11. **A**. Input fraction before bionylated proteins were immunoprecipitated. **B**. Flow-through of biotinylated proteins unbound by streptavidin beads. **C**. Biotinylated proteins eluted off streptavidin coated beads used for separation of biotinylated proteins.

successful. The addition of TurboID to the N terminus of cIAP1 seems to have no impact on the function of cIAP1 in the canonical NF-κB pathway. RIPK1 was found to be present in the eluate (Figure 13B). There is variation in how much RIPK1 is eluted between replicate 1 and replicate 2, so there is potentially sample to sample variation. PRX-003 is a dimeric SMAC mimetic that mediates the interactions between the IAPs and their ubiquitination substrates. Endogenous cIAP1 ubiquitinates RIPK1 in response to TNF-α and SMAC mimetics. V5-TurboID-cIAP1 is depleted from the flowthrough samples and present in the eluted samples, so there is good indication that the pulldown was successful in eluting biotinylated proteins (Figure 12A). V5-TurboID-cIAP1 was depleted from the input samples treated with PRX-003, likely due to autoubiquitination and degradation of V5-TurboID-cIAP1.

Objective 3: Identification of V5-TurboID-cIAP1 interactors

The samples sent for identification of putative cIAP1 interacting proteins resulted in a small list of potential interactors. Through manual exclusion of proteins found in the control samples, I narrowed the number of candidates to a select few hits (Table 3). Four of the hits have clear links to NF-κB signalling: POTEE, KAP1 (also known as TRIM28), PTBP1, and URGCP. All proteins were found in TNF-α stimulated samples, except for URGCP, which was found in the PRX-003 treatment group. GARIN1A was present in both TNF-α treatment and PRX-003 treatment. This is the only protein that was found in multiple treatment groups, excluding control samples. The candidate proteins in Table 1were analyzed for literature and database connections to cIAP1 and to each other using STRING. The STRING analysis resulted in a protein network that connected the several of the candidate proteins to proteins with connections to TNFRSF1A (Figure 14). JPT1 (HN1) is connected to TRIM28, which is connected to cIAP1 through NF-κB and a network of

A



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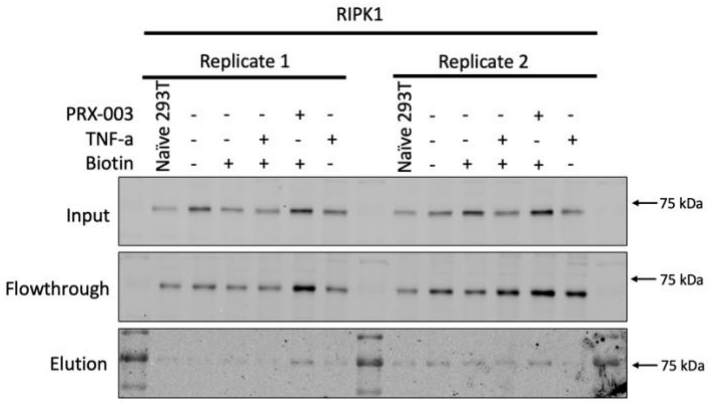

Figure 13: V5-TurboID-cIAP1 interacts with RIPK1. A. The experiment was carried out as in Figure 11. V5-TurboID-cIAP1 was detected with a corresponding anti-cIAP1 antibody. **B**. RIPK1 is present in the input, flowthrough, and elution samples.

Sample	Protein Identity	Protein Name	Protein Localization	Function in Signalling Pathways
TNF-α	POTEE	POTE ankyrin domain family, member E	Intracellular (general)	Enriched in NF-κB signalling; upregulated in cancer. Associated with poor prognosis.
	TRIM28/ KAP1	Krüppel-associated box (KRAB)-associated protein 1	Nucleoplasm	Promotes p53 degradation through MDM2
	JPT1	Jupiter microtubule associated homolog 1	Nucleoplasm, Nuclear membrane, Nucleoli	No clear link. Plays a role in regulating the cell cycle and adhesion
	DBI	Diazepam binding inhibitor	Endoplasmic reticulum, Golgi apparatus (ligand binding dependent)	No clear link
	PTBP1	Polypyrimidine tract binding protein 1	Nucleus	Associated with poor prognosis in cancer. Regulates pro-inflammatory SASP (Senescence-associated secretory phenotype) proteins
	SLC38A11	Solute carrier family 38 member 11	Cell membrane (transmembrane)	No clear link
TNF-α No added Biotin (Biotinylation still occurs but at a lesser rate – FBS has residual biotin)	MYMK	Myoblast specific protein	Cell membrane, Golgi apparatus	Myoblast fusion
	GARIN1A	Golgi-associated RAB2 interactor 1A	Cell membrane, Golgi apparatus	No clear link – poorly studied
	URGCP	Upregulator of cell proliferation	Cytoplasm, Nucleus	Upstream regulator of NF-κB signalling through promotion of IKKα/β and IκBα phosphorylation
PRX-003	GARIN1A	Golgi-associated RAB2 interactor 1A	Cell membrane, Golgi apparatus	No clear link – poorly studied
	IPO13	Importin 13	Cytoplasm, Nucleus	No clear link

Table 3: Putative interactors of V5-TurboID-cIAP1 have connections to regulatory cellular processes.

Heterogeneous nuclear ribonuclearproteins (HNRNP). PTBP1 is included in the HNRNP cluster, and it has a connection to Caspase-8. POTEE is also included through a connection with TAB2. The other proteins do not have connections to any of the network shown.

I investigated the MS hits for protein interactions via BioGRID (Table 4). TRIM28 had many relevant interactions, including several different caspases, MYC family proteins, and two members of the IAP family. PTBP1 showed interactions with cIAP2 and MYC, as well as with RAB2A. RAB2A is an interactor with GARIN1A, the protein that showed up as a hit in two treatment groups. TRIM28 also showed interactions with RAB2A and RAB2B, the two proteins that GARIN1A is associated with. IPO13 showed interactions with two members of the TNFR Super Family, MYC, Major Histocompatibility complex II (MHCII, CD74), and SMAC.

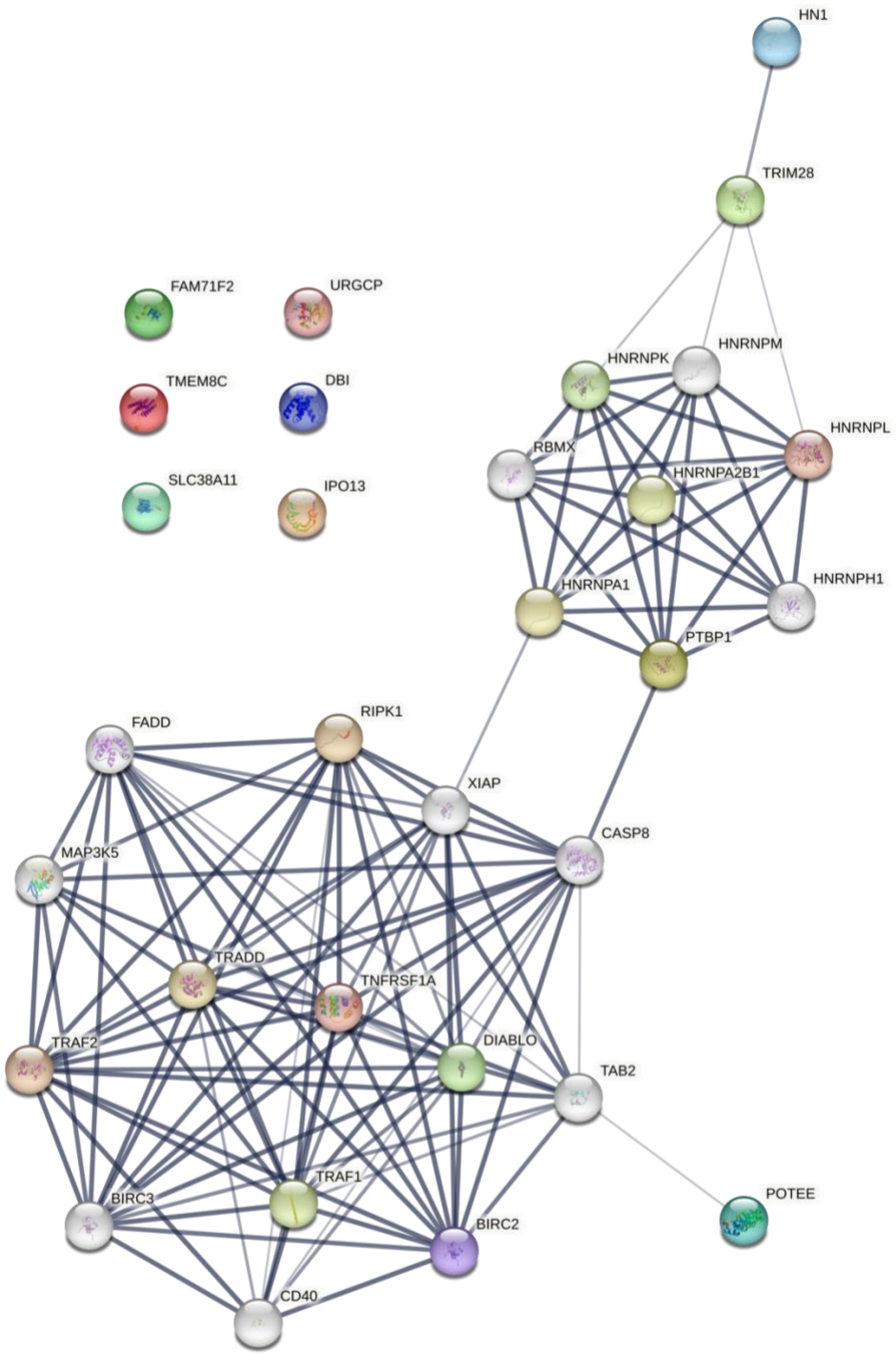

Figure 14: Several hits from LC/MS analysis show distant connections to cIAP1 and the NF-κB signalling pathways. The proteins identified in LC/MS and cIAP1 were put into STRING to create a protein interaction network. Thicker lines indicate a stronger connection. HN1 is JPT1, TMEM8C is MYMK, and FAM71F2 is GARIN1A as recognized by the STRING database.

Sample	Protein Identity	Protein Name	Relevant Known Interactions
TNF-α	POTEE	POTE ankyrin domain family, member E	p65/RelA[66]
	TRIM28/ KAP1	Tripartite Motif Containing 28/Krüppel-associated box (KRAB)-associated protein 1	Apollon/BRUCE[67] CASP1[68] CASP3[67] CASP8[69] cIAP2[70] IKBKAP[67] JPT1[71] MYC[72] MYCN[72] NF-κB1 (p50)[73] NLRP3[68] RIPK1[74] RIPK3[74] STAT3[75]
	JPT1	Jupiter microtubule associated homolog 1	TRIM28/KAP1[71]
	DBI	Diazepam binding inhibitor	TRAF3[76]
	PTBP1	Polypyrimidine tract binding protein 1	cIAP2[70] MYC[77] MYCN[72] RAB2A[78]
	SLC38A11	Solute carrier family 38 member 11	N/A
TNF-α No biotin added (Biotinylation still occurs but at a lesser rate – FBS has residual biotin)	MYMK	Myoblast specific protein	N/A
	GARIN1A	Golgi-associated RAB2 interactor 1A	RAB2A[79 80] RAB2B[79 80]
	URGCP	Upregulator of cell proliferation	N/A
PRX-003	GARIN1A	Golgi-associated RAB2 interactor 1A	RAB2A[79 80] RAB2B[79 80]
	IPO13	Importin 13	CD40[79] CD74[81] MYC[82] SMAC[83] TNFRSF9*

Table 4: The TurboID-cIAP1 interactors have relevant known interactions within the NF-κB signalling pathways and with each other. Proteins were searched on BioGRID, results were considered relevant if there were connections to NF-κB signalling pathways, apoptosis, or between other proteins identified via LC/MS. *pre-publication – no citation available.

Chapter 4: Discussion

There has been considerable research on understanding the IAPs since their discovery in 1993.[84] A BioID approach with the IAPs as bait has not been previously done. I have identified six potential cIAP1 interacting proteins that have ties to the NF-κB pathway in a pro-survival manner through a TurboID screen.

Involvement of V5-TurboID-cIAP1 interactors in NF-κB

In this study, I aimed to find novel interactors for cIAP1 within the NF-κB signalling pathways. I identified four proteins in my LC/MS experiment that can be linked directly to NF-κB signalling using literature: POTEE, TRIM28, PTBP1, and URGCP. Two other proteins, MYMK and GARIN1A, do not have literature links to NF-κB signalling but they are interesting for other reasons. These proteins will be discussed in deeper context in the next few sections. These six proteins have piqued my personal interest, but that does not mean that the other hits should be discounted. While the other hits may not have as strong of literature links, the BioGRID database search showed some interesting protein interactions. JPT1 has links to TRIM28 so future studies would be necessary to determine its relevance to cIAP1, particularly if TRIM28 shows a strong connection to cIAP1 or NF-κB signalling (Figure 14, Table 3). DBI has connections to TRAF3. This may implicate DBI in NF-κB signalling or IFN production, as TRAF3 is a negative regulator of the NF-κB pathway and a positive regulator of type II IFN production.[85] IPO13 has connections with two members of the TNFR super family, CD40 and TNFRSF9. cIAP1 is involved in both signalling pathways. In CD40 signalling cIAP1 maintains a low level of TRAF2 by triggering its proteasomal degradation, this promotes the secretion of proinflammatory cytokines following exposure to CD40 Ligand (CD40L).[86] cIAP1 and 2 are recruited to the TNFRSF9 signalling complex through their association with TRAF2. They may induce the TNFRSF9-induced K63

ubiquitination of TRAF2.[87] It is also connected to CD74 which is the Human Leukocyte Antigen (HLA) class II histocompatibility antigen gamma chain, a transmembrane protein that is part of the Major Histocompatibility Complex II (MHC II)-presentation complex.[88] Lastly, IPO13 had a connection with SMAC when BioGRID was used to probe for known interactions. Delving deeper into the listed interactions returned nothing of note as they were part of much larger studies and therefore not discussed in depth in the papers, but this opens avenues for future study.

POTEE

POTEE (POTE ankyrin domain family, member E) is a member of the POTE (Prostate, Ovary, Testes, and Embryo) gene family. The gene family was discovered more recently and has been found to be expressed in a variety of different cancers. Normal tissues have very low levels of POTEE, but is overexpressed in tumours.[89] POTEE has N-terminal cysteine rich domains, seven ankyrin repeat domains, and C-terminal spectrin-like helices. In a paper by Shen *et al.*, NF-κB signalling was found to be one of the most enriched pathways when analyzing the transcriptome via RNA-Seq in cells overexpressing POTEE.[89]

POTEE is linked to Sphingosine 1 Phosphate (S1P) signalling. In healthy cells, S1P signalling is linked to apoptosis, cell growth, and cell migration. When POTEE signalling is dysregulated, it leads to the development of cancer and inflammation disorders. Intracellular activation of S1P signalling is catalysed by Sphingosine Kinase 1 (SPHK1), as well as several other enzymes. SPHK1 is a crucial part of POTEE mediated cell growth through the POTEE/SPHK1/p65 signalling axis.[89] SPHK1 and POTEE seem to be closely linked, as their mRNA expression levels are positively correlated and when POTEE is knocked down, SPHK1 levels also decrease. Together, they regulate levels of phosphorylated p65. Phosphorylated p65

levels were reduced in cells with a POTEE knockdown and increased in cells overexpressing POTEE. Knockdown of SPHK1 in POTEE overexpressing cell lines also reduced levels of phosphorylated p65, demonstrating a correlation between p65 phosphorylation and POTEE/SPHK1 expression levels. These results were echoed in vivo, as when POTEE was knocked down in tumours there was also decreased SPHK1 expression and reduced levels of phosphorylated p65.[89]

SPHK1 and S1P are also linked to the NF-κB pathway through interactions with TRAF2. In a paper by Alvarez *et al.* in 2010, they found that SPHK1 and the production of S1P required K63-linked ubiquitination of RIPK1 through interactions with TRAF2, and that S1P influences the E3-ligase activity of TRAF2.[90] When S1P levels are depleted by inhibiting SPHK1, the TNF-α induced degradation of IKKα/β by IκBα was reduced according to the dose of SPHK1 inhibitor. The absence of SPHK1 also reduced the translocation of p50 and p65 to the nucleus and halted the phosphorylation of IKKα/β and IκBα following TNF-α stimulation. Typically TRAF2 is polyubiquitinated after TNF-α stimulation, however in the absence of SPHK1 this occurred at a much lesser rate.[90] Through these results it can be inferred that POTEE is also important for this process, since POTEE was demonstrated to be an important upstream regulator of SPHK1, and through that, influences S1P activity.

TRIM28/KAP1

TRIM28 (Tripartite Motif Containing 28), also known as KAP1 (Krüppel-associated box (KRAB)-associated protein 1), is a transcriptional regulator that contains several well-preserved motifs. TRIM28 has an N-terminal RING domain, two B boxes for higher order oligomerization, and an antiparallel coiled-coil domain.[91] It has links to NF-κB signalling through its interaction

with the STAT3/p300 complex and the p65 subunit of NF-κB.[92] Signal Transducer and Activator of Transcription 3 (STAT3) is known to play a role in multiple cancers, and has been named as a possible inhibitor of IKK in immune cells. Considering STAT3 and NF-κB are both involved in controlling an overlapping set of pro-survival and pro-cell proliferation genes it would make sense for the two to have crosstalk to coordinate responses.[93]

Kamitani *et al.* investigated the interaction between STAT3, p300, and p65/RelA of the NF-κB family.[92] STAT3 regulates p300, which is an acetyltransferase that regulates the acetylation of p65. Acetylation of p65 and other NF-κB subunits increases their retention in the nucleus by affecting their nuclear export, which in turn will increase translation of genes connected to NF-κB signalling.[92 93] Hyperacetylation of p65 by STAT3 leads to NF-κB activation in tumour cells and the tumour microenvironment.[93] Following TNF-α stimulation, TRIM28 negatively regulates the phosphorylation of STAT3 at Ser^{727} and the acetylation of p65. TRIM28 was found to directly interact with p65 over all other NF-κB subunits from a western blot performed by Kamitani *et al.* In fact, after TNF-α stimulation TRIM28 had an increased affinity for binding to p65, which indicated that nuclear translocation of p65 was dependent on its interaction with TRIM28. When investigating the role of TRIM28 in the NF-κB-STAT3 complex, it was shown that TRIM28 negatively regulates the interaction between the STAT3/p300 complex and p65 by inhibiting p300 binding. In the presence of TRIM28, p300-bound p65 were less abundant compared to when TRIM28 was absent.[92] This suggests that TRIM28 may play a sort of regulatory function in NF-κB signalling driven by its interaction with STAT3.

PTBP1

PTBP1 (Polypyrimidine tract binding protein 1) is a protein that regulates the splicing of different genes, including *Ripk1*. Heightened PTBP1 expression occurs in various cancers, it is a marker for poor prognosis.[94] PTBP1 initiates Nonsense-mediated mRNA decay (NMD) targeted splicing in *Ripk1*, which in turn leads to suppressed nuclear translocation of p65 in endothelial cells. In a paper by Hensel *et al.* in 2022, PTBP1 was identified in an RNAi screen for a SASP (Senesence-associated secretory phenotype) response that requires NF-κB signalling to induce the SASP response.[95] It is not yet known how PTBP1 functions in this pathway, but it is hypothesized that RNA splicing is likely to play a large part in inducing the SASP response that requires NF-κB signalling for its induction.[95] PTBP1 was also found in conjunction to another SASP response, where its knockdown interfered with tumour growth.[94] PTBP1 is additionally involved in T cell expression changes through RNA decay.[96] This process is critical for T cell proliferation. Embryonic stem cells that lack PTBP1 were blocked in their G2/M progression in the cell cycle, and PTBP1 knockdown in PTBP1-overexpressing tumour cells showed impairment in their growth and their invasiveness. T cells with reduced levels of PTBP1 showed impaired NF-κB function as shown by their inability to degrade IκBα and due to p65 activation not being observed after stimulation with anti-CD3 and anti-CD28 beads.[96] This connection has not been explored in depth, so it is unknown how the effect PTBP1 has on NF-κB function connects to the role PTBP1 in cell proliferation; However, both play a role in the proliferation of T cells, so it would be worthwhile to parse out how the two may be connected since PTBP1 influences the splicing of *Ripk1*.

URGCP

URGCP (Upregulator of cell proliferation), also known as URG4, helps to regulate cell proliferation in healthy tissues. It is also recognized as an oncogene in many cancers, promoting the proliferation and tumorigenicity of tumours.[97] The upregulation of URGCP in non-small cell lung cancer (NSCLC) is correlated with poor patient prognosis due to URGCP increasing the invasiveness of NSCLC cells.[98] URGCP regulates NF-κB signalling upstream by promoting phosphorylation of IKKα/β and IκBα, and consequently increases metalloproteinase-9 (MMP-9) production. The exact mechanism behind this is currently unknown, however it appears that URGCP increases the recruitment of NF-κB subunits to the MMP-9 promoter. This is supported by URGCP silencing in NSCLC cells inhibiting IKK activation, leading to the suppression of NF-κB signalling.[98] In hepatocellular carcinoma (HCC) cells, overexpression of URGCP increases the angiogenic capacity. URGCP expression was found to correlate directly to NF-κB transcriptional activity. Several NF-κB target genes including TNF-α, IL-6, IL-8, and MYC were upregulated when URGCP was overexpressed, and downregulated when URGCP was silenced in HCC cells. Additionally, when NF-κB was inhibited by blocking IκBα degradation in URGCP overexpressing HCC cells there was a reduction in the angiogenic capacity compared to when IκBα was able to be degraded.[97] Despite knowing that URGCP is an upstream regulator of NF-κB, it is unknown where URGCP acts with the NF-κB pathways. Future studies are needed to determine the mechanism for this regulation.

MYMK

MYMK, also known as myomaker or Transmembrane Protein 8C (TMEM8C), is a highly conserved cell membrane protein specific to muscle cells. The conservation of myomaker is

prevalent across all vertebrates which suggests it is an extremely important protein; within vertebrates there is approximately 60% of the protein conserved as a direct sequence.[99] [100] It is involved in myoblast fusion, which is required for the formation of multinucleated myofibers and the formation of skeletal muscle. The C-terminus of myomaker is a cytoplasmic domain that drives myoblast fusion.[99] Myoblast fusion has multiple steps starting off with cell-cell interaction and followed by the merging of the cells. The membrane fusion event is driven by actin-cytoskeletal dynamics.[100] Myomaker an absolute requirement for myoblast fusion – in myomaker knockout mice there was an absence of multinucleated myofibers.[100] This is also seen *in vitro,* where C2C12 cells without myomaker in showed an absence of myoblast fusion.[99] Myoblast fusion is linked to cIAP1 and the NF-κB pathways as discussed in the paper by Enwere *et al.* in 2012.[101] Differentiation of myoblasts is blocked by the canonical NF-κB pathway, and activated by the non-canonical NF-κB pathway.[101] [102] Further work can be done to determine the if and how myomaker-mediated myoblast fusion and the NF-κB pathways intersect.

GARIN1A

Golgi-associated RAB2 interactor 1A (GARIN1A) is the only protein that showed up under two treatment groups in my LC/MS experiment. This gives good confidence that this protein is a true interactor, and that it has the potential to be important in NF-κB signalling. Despite this, GARIN1A is not very well studied. As its name suggests, it is an interactor of RAB2A (Ras-related protein 2A) and RAB2B (Ras-related protein 2B). PTBP1 may have functions relating to GARIN1A, as PTBP1 was found to interact with RAB2A. RAB2A and RAB2B do not have literature links to NF-κB signalling, however RAB2A is implicated in breast cancer development through inactivation of ERK1/2 (Extracellular-signal Regulated Kinase 1/2) through MKP3

(Mitogen Activated Protein Kinase Phosphatase 3). Further studies are necessary to determine the exact function of GARIN1A, and what signalling pathways it is involved in.

Validation of V5-TurboID-cIAP1 interactors

While the results from the LC/MS experiment are very promising, it is crucial to confirm the hits in multiple ways to increase the confidence that the findings are real. Two techniques that are good to start with when confirming a protein-protein interactions are Proximity Ligation Assays (PLA) and co-immunoprecipitation (co-IP). Following good results from these, the specifics for the interaction between the identified hit and the bait protein should be confirmed using domain changes within the bait protein. One potential method for this is Far Western blotting.

PLA allows for proteins to be detected within a radius of 40nm at endogenous levels.[103 104] This technique uses two primary antibodies, one for each protein of interest, of different animal species to detect the proteins. Secondary antibodies conjugated with a plus or a minus PLA probe are then used to detect the primary antibodies. If the proteins of interest interact with one another, the PLA probes hybridize into a circular DNA strand. The DNA is then amplified and detected with fluorescent or HRP labeled complimentary oligonucleotide probes, and visualized with fluorescent microscopy.[103 104] This technique has the added benefit of allowing for the localization of the protein interaction to be visualized, since it is a microscopy based technique.[104] This technique can be applied to further my work by checking for interactions between cIAP1 and each hit. The drawback of this method is that it relies on antibodies being readily available for each hit. Considering some of the hits are currently not well studied, they may not have good antibodies available.

Immunoprecipitation is a technique where a molecule such as a protein is precipitated out of a mixture, for example out of a cell lysate, using an affinity capture method. This can be done using an antibody specific to the target or to amino acid sequences for short peptide tags such as hemagglutinin or myc tags.[105] [106] The antibodies can be coupled to agarose or magnetic beads if they have not been purchased already coupled, otherwise an antibody can be added to the protein lysate and Protein A or G coupled beads can be used to pull it down.[105] Co-IP uses the same principle as immunoprecipitation, but as the "co-" in its name suggests the goal of co-IP is to precipitate a complex of molecules out of a mixture. After the lysate is isolated and the antibodies are coupled to the beads, The precipitated mixture is run on an SDS-PAGE gel much like a regular Western blot, but only proteins that bind to the antibody, or proteins that associate with that protein will be present on the blot. Thus, it can be inferred that proteins detect on the Western blot have formed a protein complex with the immunoprecipitated protein.[106] Much like PLA, each hit can have their interaction with cIAP1 verified with co-IP. Since peptide tags can be conjugated to proteins as a way of detection, this method may help bypass the need for antibodies specific to the targets. This is useful for less well studied proteins that may not have good, specific antibodies available.

Far Western blots are a variation on the traditional Western blot. Western blotting is very useful for determining whether a protein is present in cell lysates, but it lacks the ability to detect interactions between proteins. Far Western blotting is a technique used to search for interactions between a protein of interest and bait proteins immobilized in a solid support membrane such as nitrocellulose.[107] [108] This technique can also aid in determining the protein sites necessary for protein-protein interaction by changing protein domains and amino acid residues. The relative binding capacity for the protein of interest can also be determined by varying the concentrations

of protein used.[107] [108] This technique is also useful for detecting proteins that do not have antibodies readily available for detection, as a non-antibody protein probe can be used for detection.[107] This technique can be applied to the potential cIAP1 interactors identified in the LC/MS experiment initially to look for an interaction, then the proteins can be titrated to determine the cut off concentration and binding capacity for interaction with cIAP1 if they come back positive for interactions with cIAP1. From there, the domains and binding sites in cIAP1 can be altered to determine the domain requirements for protein-protein interaction.

Limitations

Despite taking careful measures to ensure my results are as well obtained as possible, every study will have its limitations. Some of the key limitations within my project include the promiscuity of TurboID as a biotin ligase, the cell line choice, and cellular localization of the potential cIAP1 interactors I found with LC/MS.

As discussed previously, TurboID is a highly promiscuous biotin ligase that was deliberately mutated from BioID.[60] TurboID was selected for its efficiency – The canonical NF-κB pathway activates and runs to completion within 30 minutes, which is substantially longer than the18 hour labeling time of BioID. The short labeling time can also be a drawback. The higher efficiency comes with a larger labeling radius of 35nm, which means nearby proteins that are not involved in the signalling pathway may be misidentified as a potential interactor. Due to this it is very important to thoroughly investigate the hits from the LC/MS experiment with a variety of methods to increase the confidence that it is a true interaction.

This point ties into the cellular localization of the proteins identified via LC/MS analysis. Several of the proteins, including POTEE, IPO13, and URGCP have general intracellular or

cytoplasmic localization according to the Human Protein Atlas. These proteins, as well as protein that get packaged into extracellular vesicles, tend to be highly abundant. Highly abundant proteins are more likely to be misidentified in proximity interaction studies, so it is especially important to use multiple ways of showing that the interaction is real with these proteins. Despite URGCP and POTEE having ties to the NF-κB pathways, if more work is done on these proteins with respect to their interaction with cIAP1, it will be crucial to be very thorough when studying them.

The cell line choice may have had an impact on results. This work was done in HEK293T cells, which are a Human Embryonic Kidney cell line expressing an SV40 large T antigen established in the 1970s.[109] Its fast growth rate and adherent properties made it an ideal choice for cloning and large-scale experiments. The fast growth rate allowed for experiments to be scaled up without losing too much time or using excessive amounts of media during repeated subcultures. For the LC/MS experiments, dialyzed FBS was used in replacement of regular FBS since it has a lower biotin content. This would allow for less accidental biotinylation by TurboID-cIAP1. Dialyzed FBS proved to be difficult to acquire reliably, so it was necessary to avoid using too much media when culturing cells. Additionally, HEK293T cells being an adherent cell line allowed for easier differentiation between live and dead cells during selection processes when cloning the TurboID-cIAP1 expressing HEK293T cells. These factors influenced the shift away from using a more biologically relevant immune cell line. Immune cell lines are very difficult to transfect and much slower to grow. When I attempted to use an immune cell line, it would not take up plasmid during transfection, and it performed poorly on a preliminary kill curve with Hygromycin B when attempting to begin the cloning process described in the sections *Cell culture and stable cell line generation* (Materials and Methods) and *Stable cell line generation* (Results). This prompted a switch to HEK293T cells due to the reasons stated above. Due to the shift towards a more generic

cell line, cIAP1 activity could not be observed within immune cells. Therefore, it cannot be inferred from the data if the connections made between cIAP1 and immunologically relevant or tissue specific proteins in the LC/MS experiment would occur within immune cells or within that tissue type without further experimentation.

Significance

The NF-κB family of transcription factors are involved in a diverse range of cellular functions. The canonical and non-canonical NF-κB pathways are implicated in many processes, from immune responses to skeletal muscle formation to cell death. The work done in my thesis sheds light on how seemingly different cell functions and proteins are interconnected. For example, myomaker and TRIM28 have a common connection of Cyclin D1 signalling regulated by NF-κB.[110 102] A few of the hits from my LC/MS experiment also have literature connections to the p65 subunit of NF-κB, including POTEE and Trim28.[89 92] Additionally, many of the proteins identified in the LC/MS experiment have links to the development of different types of cancer and are diagnostic markers for poor prognosis of patients.[89 98 94] This suggests that while there is a lot of complexity to how cancer develops, the NF-κB pathways may be a common point where dysregulation in cellular functions converge. To better treat cancer it may be effective to use targeted approaches that focus on not only the NF-κB pathways as SMAC mimetics do, but also treat the surrounding dysregulation in the cell as a form of combination immunotherapy. Combination immunotherapies are currently emerging with immune checkpoint inhibitors. For example, an anti-PD-L1 antibody called atezolizumab is being combined with bevacizumab, an anti-vascular endothelial growth factor antibody, as a clinically approved method to treat HCC.[111] Perhaps using SMAC mimetics in combination with other immunotherapy in a specific and

targeted manner to the individual case would yield success in treating cancers that currently have a poor prognosis.

Conclusions

In this study I have explored the interactions between cIAP1 and the NF-κB signalling pathways. I have found potential interactions with several proteins that have literature links to NF-κB signalling, and to processes like cell growth and differentiation that cIAP1 is known to have a role in. Further work needs to be done to confirm these interactions and to discover the mechanisms by which these interactions occur. NF-κB signalling is known to have widespread function within the cell and within diseases such as cancer; it will be beneficial to study these interactions to better understand how cancer develops and how to treat it best, especially in patients with a poor prognosis.

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