# Investigating the Interaction between TRAF1 and the Cellular Inhibitor of Apoptosis Protein 2 (cIAP2)

**Fatemah Aleithan**

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS OF THE
DEGREE OF

**Master of Science**

Graduate Program of Kinesiology and Health Science

York University

Toronto, Ontario

December 2020

## Abstract:

A fundamental family of adaptor proteins termed, **t**umor necrosis factor **r**eceptor **(TNFR) -a**ssociated **f**actors **(TRAFs)** are essential for signal transduction of various receptors. However, one particular member of the TRAF family, TRAF1 stands out for its ability to have opposing effects on NF-κB activation downstream the TNFR and TLR signalling pathways. Previous studies have shown that TRAF1 is linked to an increased risk of rheumatoid arthritis (RA). Nonetheless, the mechanism concerning its role in RA is unclear. Autoimmune diseases such as RA and other inflammatory diseases are complex diseases that require the activation of multiple immune signalling pathways. In lymphocytes where TNFR pathway is predominant, TRAF1 recruits c-IAP2 to activate NF-κB leading to survival and proliferation of these cells. In inflammatory cells like macrophages, TLR signalling pathway is more predominant in activating NF-κB leading to the production of cytokines, where and TRAF1 inhibits this pathway through binding to the LUBAC complex. Since TRAF1 has opposing roles on NF-κB activation downstream of TLR and TNF receptors, it is important to study the role of TRAF1 in regulating these pathways one at a time. This study aims to investigate the role of TRAF1 downstream of TNFR signalling pathway through studying its interaction with c-IAP2. We have developed mutated versions of TRAF1 and through the use of co-immunoprecipitation determined the exact amino acids that are responsible for the interaction between TRAF1 and cIAP2. Ultimately, we identified a version of TRAF1 that does not interact with cIAP2 while maintaining its interaction with other proteins such as TRAF2 and the LUBAC complex. Moreover, we have demonstrated for the first time that TRAF's direct interaction with cIAP2 protects it from proteasomal degradation. These results provide important insights into TRAF1/cIAP2 interaction, which may ultimately lead to development of new therapeutics that target it.

## Acknowledgments

My deep gratitude and thanks goes first to my supervisor Dr.Ali Abdul-Sater for giving me the opportunity to be a part of his lab as a Master's student as well as granting me this project. Your consistent guidance, support, and encouragement helped me massively throughout this journey. I have grown tremendously not just as a researcher but as an individual and I will take these valuable lessons throughout the rest of my future career. You are truly the best mentor that I ever had in my entire academic journey.

My appreciation also extends to my lab mates, Safoura Z, Sunpreet S, Mayoorey M, Jonathan R, Ali A, Bipandeep D, Amandeep A, and Ardi S. The incredible support and care that I have gotten by every single one of you has always encouraged me to never give up during the frustrating and hard times, and for that, I thank you all.

Above all, I am indebted to my family, whose love, assistance, care and encouragement has left an everlasting impression on me. Without my family's sacrifice I would not have been able to pursue my passion in research and especially in the immunology field. Regardless of every setback, my family has stood by my side throughout this degree. A heartfelt thank you to everyone mentioned in this letter.

# Table of Contents

## List of Tables:

## List of Figures:

## List of Abbreviations:

| | |
|---|---|
| TCR | T-cell receptor |
| NF-κB | nuclear factor-κB |
| APCs | antigen presenting cells |
| MHC | major histocompatibility complex |
| ITAMs | immunoreceptor tyrosine-based activation motifs |
| Lck | lymphocyte protein tyrosine kinase |
| Zap-70 | Zeta-chain associated protein kinase |
| MAPK | mitogen- activated protein kinase |
| TNF | the tumor necrosis factor |
| NK | natural killer cells |
| TNFR | Tumor necrosis factor receptor |
| TRAF | tumor necrosis factor receptor -associated factor |
| BIR | baculoviral IAP repeat |
| TAK | Transforming growth factor-β activated kinase |
| TAB | Transforming growth factor-β activated kinase-binding protein |
| IAPs | inhibitor of apoptosis proteins |
| cIAP | cellular inhibitor of apoptosis protein |
| AnkRs | Ankyrin repeats |
| PRRs | pattern-recognition receptors |
| IKK | IκB kinase complex |
| RIP1 | receptor-interacting protein I |
| TRADD | Tumor necrosis factor receptor type 1-associated DEATH domain protein |
| RHD | Rel Homology domain |
| IL-1R | interleukin-1 receptor |
| TLR | Toll-like receptor |
| NOD | nucleotide binding-oligomerization domain |
| NLRs | NOD -like receptors (NLRs), |
| RIG-I | retinoic acid-inducible gene I |
| RLRs | RIG-I-like receptors |
| BCR | B-cell receptor |
| BIR | Baculoviral IAP-repeat |
| (HLA)-DRB1 | human leukocyte antigen |
| RA | Rheumatoid Arthritis |
| RF | Rheumatoid Arthritis Factor |
| ACPA | Citrullinated protein antibody |
| LUBAC | Linear ubiquitin chain assemble complex |
| ALRs | AIM-2 like receptors |
| CLRs | C-type receptors |

| | |
|---|---|
| LRRs | Leucine-rich repeats |
| TIR | Toll/IL-1 receptor |
| IRAK1/4 | Interleukin receptor-associated kinase 1/4 |
| IL-1β | Interleukin-1β |
| IL-6 | Interleukin-6 |

# 1.0 Literature Overview:

## *1.1 Autoimmune diseases*

Normally, autoimmune diseases result from a hyperactive immune system where lymphocytes mistakenly attack the body's own cell[1] . Autoimmunity indicates a state of imbalance between effector and regulatory immune responses as well as the body's inability to eliminate and/or control self-reactive lymphocytes [1]. Therapies, such as cytokine antagonists and TNF-α antagonists, have demonstrated great results in treating some of these diseases especially rheumatoid arthritis [1,2]. A number of genomic studies have suggested a role for many genetic polymorphisms (such as the human leukocyte antigen (HLA)-DRB1) in different autoimmune diseases [2]. HLA-DRB1 alleles encode for an amino acid sequence in third hypervariable region of DRβ chains, known as the shared epitope (SE), which is linked to an increased risk of RA [3]. Furthermore, T-cell dependent inflammatory autoimmune diseases, such as rheumatoid arthritis or multiple sclerosis, also show an imbalance between effector T-cells and T-regulatory cells and multiple mouse models of autoimmunity further support this premise [2,3]. Recent studies suggest that in rheumatoid arthritis the function of these regulatory T cells is extremely impaired [4].

Rheumatoid Arthritis (RA) is an autoimmune disease identified as a chronic inflammation of the synovial fluid as well as the destruction of the cartilage and the underlying bone [4,5]. RA could ultimately lead to the destruction of the joint causing an array of symptoms including pain, severe disability, poor quality of life, systemic complications and increased mortality [5]. Generally, the onset of this disease happens between the ages of 30 and 50[5,6]. RA is a complex disease that involves improper responses from multiple immune cell types such as macrophages, T-cells and B-cells [7,8]. Patients with RA produce (or have an upregulation of)

certain auto-antibodies such as Rheumatoid Factor (RF) and citrullinated protein antibody (ACPA)[8,9]. However, the exact mechanism of how these autoantibodies lead to an increased inflammation is still subject for further research [9].

Other genetic risk factors in RA include immune regulation (CD28), NF-κB dependent signalling pathway (TRAF1), control of T-cell activation (PTPN22), and functional differentiation (CTLA-4) [10]. Synovial inflammation arise from an infiltration of leukocytes including monocytes/macrophages, granulocytes, natural killer cells (NK), B-cells, and especially CD8+ and CD4+ T-cells all leading to the production of chemokines and pro-inflammatory cytokines[3,10]. A study by Cope and his colleagues proved that therapies that block T-cell co-stimulation are very efficient in both early as well as advanced disease stages [3,11]. Hence targeting the activation and regulation of T-cells, especially through co-stimulation pathways, could possibly lead to more effective autoimmune therapies [11,12]. One possible pathway that could be targeted is the 4-1BB:4-1BBL pathway that regulates the activation of nuclear factor-κB (NF-κB) which in turn governs a plethora of genes involved in both innate and adaptive immune responses (see figure 2.0)[3,8].

### *1.2 Nuclear Factor-κB (NF-κB)*

The transcription factor Nuclear factor-κB (NF-κB) controls multiple transcription programs of the innate and the adaptive immune systems and acts as a mediator of inflammatory responses[13,14]. In innate cells like macrophages, NF-κB promotes the expression of numerous pro-inflammatory genes, including the ones that encode cytokines and chemokines, and also contributes to inflammasome activation and regulation [13,15]. Moreover, NF-κB is critical in regulating the survival, activation and differentiation of adaptive immune cells, like T-cells and

B-cells[15]. NF-κB denotes a family of inducible transcription factors and it contains five structurally related members including NF-κB1 (or p50), NF-κB2 (or p52), RelA (or p65), RelB and c-Rel[14–16]. The Rel Homology domain (RHD) is shared between the members of this family which enables them to form hetero- or homo- dimers with each other and allows them to bind to the κB enhancer element[15,16]. In the cytoplasm, the NF-κB proteins are normally inhibited by a family of inhibitory proteins, including IκB family members that contain Ankyrin repeats (AnkRs) and which bind to NF-κB and inhibit its activity[16,17]. Activation of NF-κB occurs in response to various stimuli such as cytokine receptors, pattern-recognition receptors (PRRs), TNF receptor (TNFR) superfamily members, as well as T-cell and B-cell receptors (TCR/BCR)[16,17]. The principal mechanism for NF-κB activation is the degradation of IκBα triggered through its phosphorylation by IκB kinase (IKK) complex[18]. Upon activation, IKK phosphorylates IκBα at exactly two N-terminal serines thus generating ubiquitin-dependent (K48-Ub) IκBα degradation in the proteasome which results in the nuclear translocation of canonical NF-κB members mainly p50/RelA and p50/c-Rel dimers[17–19].

### *1.3 TNFR-associated factor (TRAFs) and TRAF1 signalling downstream of TNFR and 4-1BB receptor in T-cells*

The tumor necrosis factor (TNF) receptor-associated factors (TRAFs) were identified as the main signals for the TNF receptor superfamily (primarily TNFR2 signalling) and the interleukin-1 receptor/Toll-like receptor (IL-1R/TLR) superfamily[20]. Furthermore, multiple lines of evidence demonstrated that TRAFs are involved in a cluster of immune receptor signaling pathways , other than TLRs and TNFR, such as nucleotide binding-oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and cytokine receptors [20,21]. Adaptive and innate immunity, embryonic development, stress response

and bone metabolism are all regulated by TRAFs through stimulating cell survival, proliferation, differentiation and death[21]. Some TRAF members have also been documented to act as E3 ubiquitin ligases; hence they are able to lead to the activation of transcription factors such as NF-κB, mitogen-activated protein kinases (e.g. MAPK, ERK-1, ERK-2, JNK and p38) and interferon-regulatory factors (e.g. IRF3 and IRF7)[22]. There are six members in the mammalian TRAF family and all six TRAFs contain a highly conserved C-terminal domain (TRAF-C) that folds into a β-sandwich structure, an N-terminal coiled-coil region (TRAF-N) and a RING-finger domain, except for TRAF1which lacks the RING domain (see figure 1.0) [22,23]. Even though TRAF1 lacks a RING-finger domain, it is a critical regulatory protein in the NF-kB as well as the MAPK signalling pathways[23] Moreover, multiple studies have shown that TRAF1has both positive and negative effects on the activation of NF-kB signalling cascade depending on which receptor is being stimulated[23].

TRAF1 is a key adaptor protein in the TNFR1 and 4-1BB signalling pathways in T-cells (see figure 2.0 and 3.0)[21]. Upon activation of both of these receptors, TRAF2 gets recruited to the site of interaction along with TRAF1 in order to create a heterotrimer TRAF1:$(TRAF2)_2$ that induces NF-kB activation[21]. A previous study demonstrated that T-cells of TRAF1 deficient mice (TRAF1-/-) displayed an elevated growth and proliferation when stimulated with anti CD3 compared to wild type T-cells[23,24] . Furthermore, another study demonstrated that overexpression of TRAF1 in a T-cell antigen receptor transgenic (TCR-tg) mouse model led to a reduction in antigen-induced apoptosis of CD8+ cells[25,26]. Under those circumstances, it was evident that TRAF1's role in NF-kB activation and regulation is highly dependent on the cell type that is being stimulated as well as which TNFR pathway is being activated[26]. For example, TRAF1 has been shown to positively regulate 4-1BB signalling pathway in T-cells[26]. Certainly, TRAF1

knockout (TRAF1-/- or TRAF1-KO) CD8+ T-cells express abnormal 4-1BB induced NF-κB activation[25,26]. All in all, a great body of literature does indeed propose that TRAF1 positively regulates the canonical NF-κB signalling pathway in T-cells.

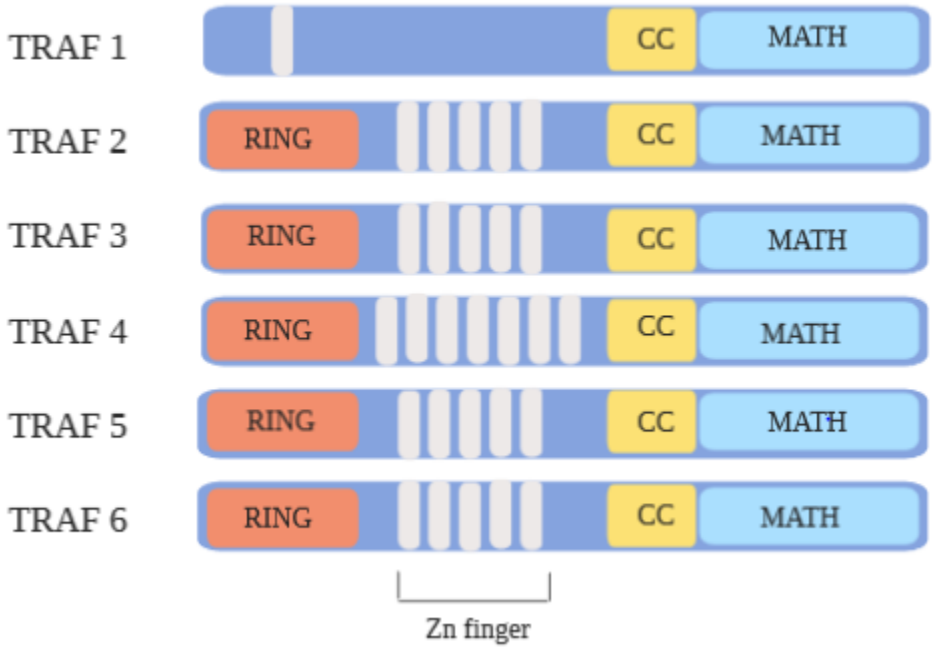


**Figure 1 A schematic of TRAF members and their overall structure. Image adapted from (22). Image created on BioRender.com**

### *1.4 T-cell activation and 4-1BB:4-1BBL signalling pathway*

The adaptive immune response, which includes T-cells, is responsible for fighting various pathogens and cancer cells[11]. These cells are produced in the bone marrow, and complete their development to become mature in the thymus[11,27]. Naïve T-cells are circulating mature T-cells that have not yet encountered their specific antigen, however once they do encounter their antigen in the lymph nodes, which is usually presented on antigen presenting cells(APCs), they get activated and become effector T-cells [11,27]. Effector T-cells include an array of different T-cells which include: T-helper cells (CD4+ T-cells), cytotoxic T-cells (CD8+ T-cells), regulatory

T-cells (Treg), and finally Memory T-cells [11,27,28]. Activation of the T-cells requires two main signals where the first signal is initiated through the interaction between the T-cell receptor (TCR) and the antigenic peptide bound to the major histocompatibility complex (MHC) class I or class II that are presented on APCs[11]. In contrast, the second signal is provided through the CD28 on the T-cell surface and its ligands CD80 and CD86 on APCs[11]. An early event in the activation of the TCR: MHC signalling is the phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytosolic side of the TCR/CD3 complex by the lymphocyte protein tyrosine kinase (Lck)[29,30]. Subsequently, Zeta-chain associated protein kinase (Zap-70) is recruited to the TCR/CD3 complex, where is activated, stimulating the recruitment and phosphorylation of downstream proteins in the mitogen- activated protein kinase (MAPK/ERK) pathway which eventually leads to NF-κB activation [29,30]. The recognition of CD28 as the main co-stimulatory signalling pathway to activate T-cells has been documented in many studies; however, numerous bodies of research have suggested that there are other inflammatory signals that are also necessary to lead to a full activation of T-cells and a formation of memory cells including: OX40/OX40L , CD27/CD70, 4-1BB/4-1BBL, and CD30/CD30L [11,27].

Human 4-1BB/4-1BBL (also known as CD137 or TNFRSF9) is part of the tumor necrosis factor (TNF) receptor superfamily and is located on the 1p3613 chromosome along with a bundle of other related genes including TNFR2, CD30, and OX40 which have been shown to be mutated in multiple malignancies [28,31]. 4-1BB is a T-cell costimulatory receptor that contains a signal peptide, an extracellular domain, a transmembrane region, and a short intracellular domain[31]. The ligand of 4-1BB —which is expressed primarily on APCs, B cells, monocytes, natural killer cells (NK), neutrophils, and macrophages— binds with 4-1BB receptor to induce

signalling through tumor necrosis factor receptor (TNFR)-associated factor1 (TRAF1) and TNFR-associated factor2 (TRAF2) and ultimately leads to the activation of NF-κB, AKT, p38 MAPK, and ERK pathways [31,32]. The activation of 4-1BB pathway leads to T-cell growth and proliferation, cytokine induction, upregulation of anti-apoptotic genes, and finally inhibiting activation-induced cell death[32]. Stimulating this receptor has also been used for cancer immunotherapy[32]. Additionally, multiple clinical studies have established an increase in serum levels of 4-1BB in multiple sclerosis and rheumatoid arthritis patients[31]. Once the 4-1BB binds to its ligand (4-1BBL), one unit of TRAF1 and 2 units of TRAF2 are recruited forming a heterotrimer (TRAF1:$(TRAF2)_2$) which directly interact with the baculoviral IAP repeat (BIR) domain on cellular inhibitor of apoptosis1/2 (cIAP1/2) proteins[33]. cIAP1/2 are E3 ubiquitin ligases that catalyze the K63-Ub of Transforming growth factor-β activated kinase (TAK) and Transforming growth factor-β activated kinase-binding protein (TAB) which in turn leads to the phosphorylation of the IKK complex (made up of three subunits IKKα, IKKβ, and NEMO) and eventually leads to NF-κB activation ( see figure 2)[33,34].

### *1.5 Cellular inhibitor of apoptosis protein (cIAP1/2)*

cIAP1, cIAP2, and the X chromosome –linked IAP (XIAP) are members of an extremely important family of inhibitor of apoptosis proteins (IAPs) that operate to weaken intrinsic and extrinsic death signalling[35]. In vitro, they work by competitively inhibiting caspase activity through their baculoviral IAP repeat (BIR) domains[35]. Nonetheless, recent studies have shown that the ubiquitin ligase activity within cIAP1/2 has a critical part in the regulation of NF-$\kappa$B signalling as well as apoptosis through ubiquitination of main proteins of the TNF receptor pathway[35,36]. The activation of the cellular inhibitor proteins are regulated through the release of

Smac, the second mitochondria-derived activator of caspases, which is an endogenous antagonist with an AVPI binding motif that binds to the BIR domains of these proteins and inhibit their activity [36,37]. cIAP1/2 are considered E3 ubiquitin ligases; in other words, these proteins lay down a specific type of ubiquitin chains termed K63 Ub chains [36]. Ubiquitin is a protein that consists of 7 lysine residues and each one can be linked to create polyubiquitin chains, notably the most common ones are Met-1linked or linear Ub chains, K48-linked Ub chains, and K6-linked Ub chains[37–39]. Inhibitor of apoptosis proteins, cIAP1/2 in this case, is involved in two main signalling pathways which are the Tumor necrosis factor receptor 1 (TNFR1) and the 4-1BB:4-1BBL pathways [40,41]. Upon TNFR1 stimulation with its own ligand (TNF), receptor-interacting protein I (RIPI) along with Tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD) are recruited to the receptor's site[42]. These proteins then recruit TRAF1 and TRAF2, forming a heterotrimer TRAF1:$(TRAF2)_2$, which in turn recruits c-IAP1/2 proteins [14,42]. As a result, TAK1 complex is recruited through the binding of the K63-Ub chains to the TAB2 subunit laid down by cIAP1/2 and ultimately triggering NF-κB signalling cascade (see figure 3.0) [14]. c-IAP1/2 also participates in the 4-1BB pathway performing the same function in the TNFR1 pathways except it ubiquitinates the TAK complex directly and not through RIPI [43,44]. In other words, the TRAF1: $(TRAF2)_2$ heterotrimer, which also forms following the stimulation of the 4-1BB receptor, recruits cIAP1/2 leading to the ubiquitinates the TAK1 complex resulting in NF-κB activation [43,44].

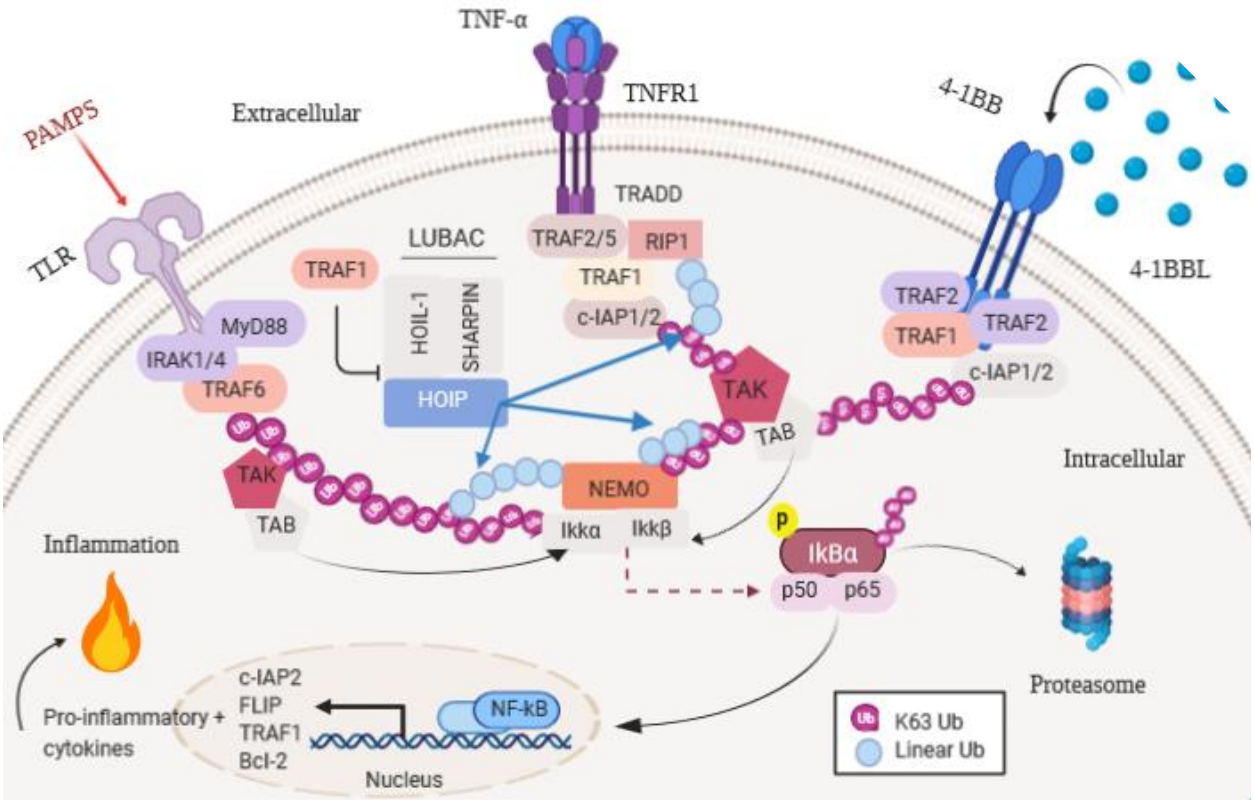


**Figure 2 An overview of NF-κB activation downstream of three key receptors: TLR, 4-1BB, and TNFR1. Image adapted from[45]. Image created on BioRender.com**

### *1.6 Toll-Like receptors (TLR) in innate immune cells*

Pattern-recognition receptors (PRRs) are mainly expressed on innate immune cells for the primary detection of foreign bodies [46,47]. PRRs detect pathogen-specific molecules known as pathogen-associated molecular patterns (PAMPs), as well as signals that are released by damaged cells known as damage-associated molecular patterns (DAMPs) [46,47]. There are different classes of PRRs including Toll-like receptors (TLRs), RIG-like receptors (RLRs), Nod-like receptors (NLRs), AIM2-like receptors (ALRs), C-type receptors (CLRs), and intracellular DNA sensors [47]. As mentioned previously, TLRs are part of the PRR receptors and they comprise 10 different members (TRL1-TLR10) generally localized on cellular membranes or

intracellular surfaces such as endosome, lysosome, ER, or the endolysosome[12,46]. Each Toll-like receptor contains a transmembrane, an ectodomain an extracellular domain with leucine-rich repeats (LRRs) which recognizes pathogenic signals such as PAMPs and DAMPs, and a Toll/IL-1 (TIR) receptor domain which recruits downstream signalling proteins[46–48].

Each TLR recognizes a different signal, TLR1, TLR2, and TLR6 respond to lipopeptide, lipoproteins, and also Gram-positive bacterial peptidoglycan [12,49]. TLR5, on the other hand, responds to bacterial flagellin and TLR4 responds to LPS of Gram-negative bacteria[46,49]. Moreover, TLR3, TLR7, TLR8, and TLR9 are stimulated by viral molecules [46,49]. Stimulation of different TLRs leads to the recruitment of TIR domain-containing adaptors including: MyD88, TRIF, TIRAP/MAL, or TRAM [46,47,50]. Upon stimulation of TLR4, MyD88 gets recruited to the TIR domain of the receptor which then leads to the recruitment of interleukin receptor-associated kinase 1/4 (IRAK1/4) [49,50]. Functionally, IRAK adaptor proteins transmit signals from the receptor to the downstream effector such as proteins TRAF6 [51]. Subsequently, TRAF6 acts as an E3 ubiquitin ligase and induce K63-linked poly-ubiquitination of itself as well as other substrates such as TAK1, TAB1, TAB2, and the IKK complex, which is made up of IKK-α, IKK-β, and NEMO [45,50]. The IKK complex in turn phosphorylates the NF-κB inhibitory protein, IκB-α, causing NF-κB to translocate into the nucleus and induce pro-inflammatory gene expression[45,52].

### *1.7 TRAF1's role downstream of Toll-Like Receptors (TLRs)*

TRAF1's action downstream of Toll-like receptors has not been thoroughly examined compared to its role downstream of other receptors such as the TNFR2. However, recently, the importance of TRAF1's role as an adaptor protein downstream of TLRs, specifically TLR4, has been reported [52]. A previous study showed that TRAF1 acts as a negative regulator of TLR-

dependent activation of NF-κB [52,53]. Functionally, TRAF1 interacts through its MATH domain, with a protein complex termed the Linear Ubiquitin chain assembly complex (LUBAC) [52]. The LUBAC complex is responsible for forming M1-Ub chains that leads to the recruitment of the IKK complex and ultimately the activation of NF-kB [45,52,53]. The same study also showed that shTRAF1 THP-1 cells have an increase in M1-Ub chains, but not K63-Ub or K48-Ub of the IKK complex [52]. This suggests that TRAF1 inhibits the LUBAC protein complex by interacting with all three components: HOIL-1, HOIP, and SHARPIN, leading to a significant decrease in NF-kB activation [53,54].

### *1.8 Ubiquitination and protein regulation*

Ubiquitin (Ub) is made up of 76-amino acids and a key feature in this protein is that it possesses seven Lys residues that can be ubiquitinated [55]. Ubiquitination is considered a post-translational modification that sends proteins for degradation by either the proteasome or the lysosome[55]. Ubiquitination (or adding Ub chains) is used in numerous cellular processes, which include: DNA repair, transcriptional regulation, signal transduction, inflammatory responses, and protein to protein interaction [55,56]. Ubiquitin (Ub) is composed of seven lysine amino acids, and each Ub can also be ubiquitinated creating poly-ubiquitin chains that are usually linked through K48 and K63 [55]. The process of ubiquitination occurs through the subsequent action of three major enzyme classes: ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin ligase (E3)[56]. Ubiquitin gets activated when an E1 enzyme goes through conformational change in its active-site cysteine residue, then ubiquitin is transported to the active site cysteine of an E2 ligase. Consequently, the E2 interacts with an E3 ligase which enables the locating and transfer of Ub onto the proteins [56]. The process of Ubiquitination can be reversed by the deubiquitination

enzymes (DUbs) [56]. These enzymes are important in the cell since they help release Ub from the tagged proteins in order to be recycled in the cell [55,56]. Proteins that are tagged for degradation by either the proteasome or the lysosome need to be deubiquitinated before their degradation for the purpose of recycling the ubiquitin by the cell. This allows for the free Ub pool to stay at steady-state in the cell [56] .

There are many different types of ubiquitination, the first type is a Met1-linked chain (or linear) which is created when Ub is attached to the N-terminus of another Ub in a linear fashion [57]. Common complexes that catalyze this type of ubiquitin chains include: the linear ubiquitin chain assembly complex (LUBAC) which indirectly regulates the downstream signaling cascade of tumor necrosis factor receptor1 (TNFR1) through NEMO and RIPK1, as well as other related inflammatory cytokines [57].The second type of Ub chains is the Lys48-linked chains (or K48) which is the main type of linkage in the cell for proteasomal degradation [57,58]. This type of UB chains is assembled when the Ub interacts with another Ub at the Lysine 48 position creating a K48 ubiquitin chain [59] . The third common type of Ub chains if the Lysine 63-linked chains (orK63) which is created through linking or attaching Ub proteins at the Lys63 sites [59]. This type of Ub chains is not only used for both lysosomal and proteasomal protein degradation, but also for creating signaling platforms for proteins downstream of immune receptors [55,58]. Recently, research has documented other types of “atypical” ubiquitin variations that are linked at the following lysine sites: Lys6, Lys11, Lys27, Lys29, Lys33 [59]. However, the specific function of each of these ubiquitin chains in the cell is still unclear [59] .

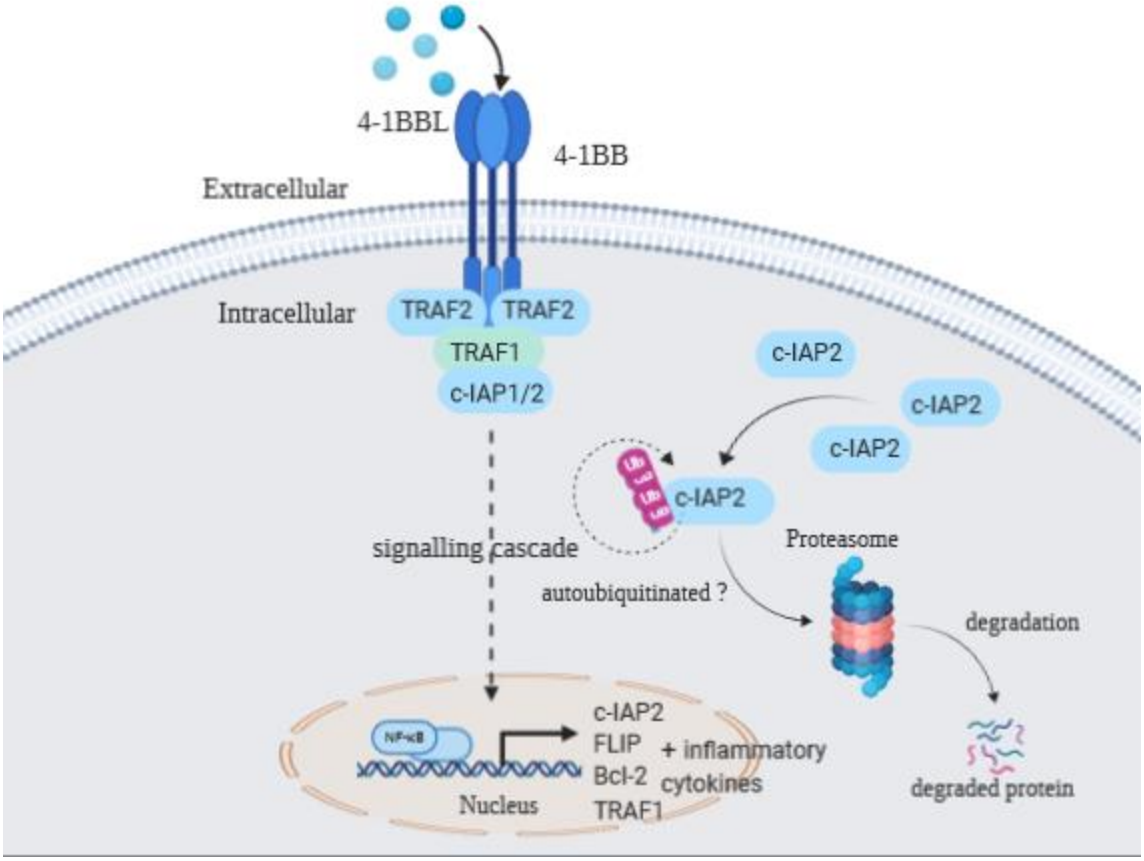


**Figure 3 An overview of cIAP2 recruitment, activation, and degradation downstream of 4-1BB receptor. Image adapted from[59]. Image created on BioRender.com.**

## 2.0 Rationale and Objectives:

## 2.1 Rationale:

RA is complex autoimmune disease that involves improper cytokine production from macrophages and auto-activation of lymphocytes. Previously published work from our lab showed that TRAF1 is involved in the pathophysiology of RA. TRAF1 promotes lymphocyte survival by positively regulating NF-κB activation downstream the TNF receptor family through its interaction with cIAP2. Contrastingly, in macrophages TRAF1 acts as a negative regulator of NF-κB to limit cytokine production. Therefore, targeting TRAF1 indiscriminately would not be an effective strategy given the dichotomous role for TRAF1 in RA pathogenesis by promoting lymphocyte survival while limiting inflammation. Hence, specifically targetingTRAF1's role downstream of TNFR signalling pathway in lymphocytes while leaving its ability to limit inflammation intact could eventually lead to more effective therapies for RA.

Intriguingly, my preliminary findings demonstrated that in addition to its role in recruiting cIAP2 in the 4-1BB signalling pathway leading to NF-κB activation, TRAF1 plays another important role in this pathway, where it stabilizes cIAP2 levels. Therefore, we investigated the mechanism of how TRAF1 protects cIAP2 from degradation.

## 2.2 Objectives:

1. Determine the specific sites of interaction (amino acid sequence) between TRAF1 and cIAP2

2. Generate a TRAF1 mutant that abolishes the interaction with cIAP2 while maintaining the interaction with other signalling partners for example: TRAF2 and LUBAC protein complex.

3. Examine how TRAF1 protects cIAP2 from degradation.

## 2.3 Hypothesis:

We hypothesized that by identifying the specific sites of interaction between TRAF1 and cIAP2 we would be able to create a TRAF1 mutant that abrogates the interaction with cIAP2 while maintaining its interaction with TRAF2 and HOIL-1. Furthermore, we hypothesized that TRAF1's interaction with cIAP2 prevents its degradation.

# 3.0 Methods

## 3.1 Cell culture:

### 3.1.1 HEK293T/17 (Human embryonic kidney 293) cell line:

HEK 293T/17 cells were used for most of the experiments listed in this document. They were kept in DMEM (SIGMA) enhanced with 10% heat inactivated fetal bovine serum (FBS), to ensure that the complement cascade is deactivated, and 1% L-Glutamine-penicillin-Streptomycin (GPS). In addition, the cells were incubated in a T-75 tissue culture flasks at 37°C supplemented with 20% O2 and 5% CO2. The media was replenished every 48hrs and cells were grown to 60% confluency before being split. Furthermore, 293T/17cells were passaged by trypsinizing them with 5ml of 1x trypsin for 5 minutes followed by centrifuging the cells at speed 1500 RPM for 5 minutes at 4°C. Lastly, the cells were re-suspended in fresh DMEM media and were placed into new T-75 flasks for maintenance.

### 3.1.2 HEK293T/17 MCSI-TRAF1and MCSI-c-IAP1 stable lines:

HEK293FT cells were transfected with 2ug of either a Flag-tagged TRAF1 or c-IAP1 (pEBB HA cIAP1) plasmid. The cells were plated and transfected exactly as mentioned in the previous section (section 3.1.2) . The cells were then treated with puromycin at 2.5μg/ml concentration for 48hrs. After a successful selection of the cells that took up the selected plasmid, the cells were kept in DMEM (SIGMA) enhanced with 10% heat inactivated fetal bovine serum (FBS), 1% L-Glutamine-penicillin-Streptomycin (GPS), and 2.5μg/ml puromycin for constant selection. The cells were then treated with 2 μg/μl of Doxycycline in order to induce

of the cloned plasmids. These cells follow the same steps as mentioned above for incubation, media change, as well as, splitting the cells**.**

### 3.1.3 shCTRL Raji / shTRAF1 Raji cell lines:

Raji is human Brukitt's B-cell lymphoma cells lines[59,60]. Compared to the HEK293T/17 cells, these cells have a high expression level of our proteins of interest endogenously, including TRAF1 and cIAP2. To mimic the experiments done on HEK293T/17 cells we used two different RAJI cell lines; ShTRAF1 RAJI cells and shCTRL cells. TRAF1 expression was knocked down in Raji cells using short hairpin RNA (shRNA). shCTRL cells, on the other hand, are control shRNA treated cells. These cells act as wild-type cells for the purpose of our experiments. Both of these cell lines were made by Dr.Ali Abdul-Sater in Tania Watts' lab at the University of Toronto (data unpublished).

The cells were cultured in T-75 flasks at 37°C incubator complemented with 20% O2 and 5% CO2. They were incubated in RPMI media (SIGMA) supplemented with 10% deactivated FBS, 1% Non-essential amino acids (NEAA), 1% L-Glutamine-penicillin-Pyruvate-Streptomycin (GPPS), 0.1% 2-Mercaptoethanol, and 2.5µg/ml. Media was replenished every 48hours and the cells were grown to confluency before split. The cells were removed from their T75flasks and placed into 50ml conical tubes then centrifuged at 1500 RPM speed for 5 minutes and at 4°C. Following centrifugation, the media is removed then the cells were re-suspended in fresh media and placed into fresh T75flasks for maintenance.

## 3.2 Experimental design

### 3.2.1 plasmids:

Human TRAF1 ORF was amplified from RAJI cells cDNA and cloned into pENTR-D-TOPO vector (Invitrogen) following manufacturer's instructions. This version of TRAF1 FL was used as the template plasmid to perform all the different mutations (TRAF1V203A, TRAF1E207A, TRAF1V203AE207A, TRAF1E209A, TRAF1E207AE209A, TRAF1I200A, TRAF1V201A, TRAF1N205A, TRAF1K206A, TRAF1L204A, TRAF1V208A) and the truncated TRAF1 (TRAF1d201-209) were used in the different experiments. All the details on how these mutants/truncations are detailed below. Human cIAP2 (Flag-cIAP2/pRK5) and human c-IAP1(pEBB HA cIAP1) were purchased from Addgene. These plasmids were cloned into the pCW57-MCS1-2A-MCS2 vector (Addgene # 71782). Human HOIL1 plasmid, that was cloned by a member in our lab, Bipandeep Dhillon, will also be used in this project to ensure that the TRAF1 mutant only affects the interaction with cIAP2 Finally, the 4-1BB DNA sequence was cloned from human T-cell cDNA, and then it was sub-cloned into the c-flag pcDNA3 vector (Addgene #20011) .

### 3.2.2 Cloning:

Polymerase chain reaction (PCR) was used to create the different TRAF1 mutated plasmids as well as the 4-1BB plasmid in order to clone it into and the c-flag pcDNA3 vector and TRAF1, cIAP2, cIAP1 to be further cloned into the pCW57-MCS1-2A-MCS2 vector. The

forward primer for 4-1BB consists of an XbaI restriction site followed by the first 18 base pairs of the gene. The reverse primer, however, contains a KpnI site followed by the last 18 complementary base pairs (refer to Table 7.0). Conversely, the forward primers for the other plasmids: TRAF1, cIAP2, and cIAP1, consisted of an ECORI restriction site and the first 18-26 base pairs. The reverse primers include a MluI site along with the last 21 complementary base pairs including their stop codons of their specific DNA sequences (refer to Table 7.0). Finally, the TRAF1 mutations were designed using the NEBase Changer and were ordered from IDT (Table 7.0)

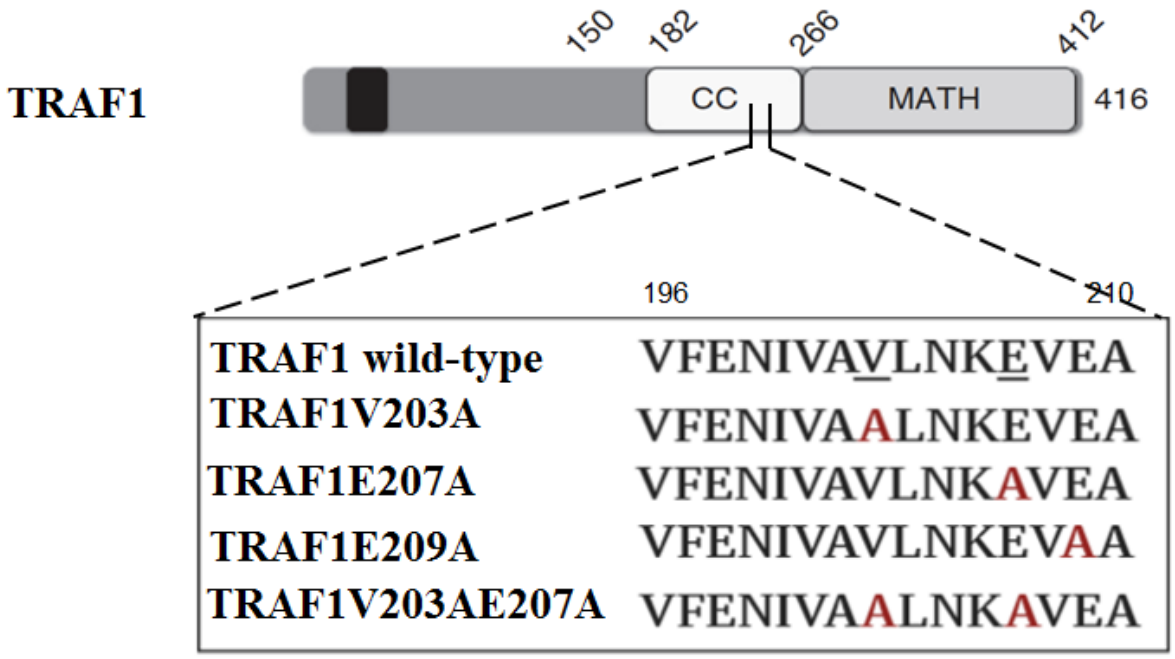


**Figure 4 The 15 amino-acid site of interaction between cIAP2 and TRAF1 along with their respective one letter codes. Image adapted from[61]. Image created on BioRender.com.**

### 3.2.3 PCR Amplification:

In order to clone TRAF1-FL, cIAP2, cIAP1 into the pCW57-MCS1-2A-MCS2 plasmid, PCR product amplification was implemented first. Table 1.0 specifies the amount of each reagent along with the quantity of template DNA used. The PCR cycle conditions are also provided in Table 2.0. Conversely, 4-1BB DNA sequence was amplified using PCR where human T-cell cDNA was used as template. Following a successful PCR amplification, the products were sub-cloned into c-flag pcDNA3 vector. The specific measurements of each reagent used as well as the PCR conditions are outlined in table 3.0 and table 4.0. Prior to cloning the PCR products into their outlined vectors, they were run on 1% Agarose gel at 70V and were extracted from the gel using the GenElute Gel Extraction Kit (Sigma) then quantified.

**Table 1.0 Platinum Superfi II PCR Amplification for c-IAP2, TRAF1, c-IAP1, and pCW57-MCSI-2A-MCS2 vector (Thermo Fisher)**

| **Components** | **50µl** | **Final concentration** |
|---|---|---|
| 5X Superfi II Buffer | 10µl | 1X |
| 10µm Forward primer | 2.5µl | 0.5µM |
| 10µm Reverse primer | 2.5µl | 0.5µM |
| 10 mM dNTPs | 1µl | 200µM each |
| Template DNA | 1µl | 10ng |
| Platinum Superfi II DNA Polymerase | 1µl | 1X |
| Nuclease free water | 32µl | _ |

**Table 2.0 Platinum Superfi II PCR cycle conditions:**

| **Step** | **Temperature** | **Time** | |
|---|---|---|---|
| Initial Denaturation | 98°C | 30 seconds | |
| 25 Cycles | 98°C | 10 seconds | |
| | 60°C<br><br>56.9°C (cIAP2) | 10 seconds | |
| | 72°C | 15-30seconds per 1kb | |
| Final Extension | 72°C | 5 minutes | |
| Hold | 4°C | Infinite Hold | |

**Table 3.0 Platinum Superfi I Green DNA Polymerase reaction components (ThermoFisher).**

| **Components** | **50µl** | **Final concentration** |
|---|---|---|
| Platinum Superfi DNA Polymerase | 0.5µl | 0.02 U/µl |
| 5x Superfi GC Enhancer | 10µl | 1x |
| Template DNA | 1µl | 10ng |
| 10 Forward Primer | 2.5µl | 0.5µM |
| 10 Reverse Primer | 2.5µl | 0.5µM |
| 5x Superfi Green Buffer | 10µl | 1x |
| 10mM dNTP mix | 1µl | 0.2mM |
| Nuclease-free water | 22.5µl | |

**Table 4.0 Platinum Superfi I Green PCR cycle conditions:**

| **Step** | **Temperature** | **Time** | |
|---|---|---|---|
| Initial Denaturation | 98°C | 30 seconds | |
| 40 Cycles | 98°C | 10 seconds | |
| | 58°C | 30 seconds | |
| | 72°C | 90 seconds | |
| Final Extension | 72°C | 5 minutes | |
| Hold | 4°C | Infinite Hold | |

### 3.2.4 Restriction Enzyme digestion:

The purified PCR product and the selected vector (c-Flag pcDNA3/ pCW57-MCS1-2A-MCS2) were restriction enzyme (RE) digested with KpnI-HF and XbaI (for 4-1BB) and with MluI-HF and ECORI-HF (TRAF1, c-IAP1) for an hour at 37°C and subsequently purified using the GenEltute PCR Clean-Up Kit (Sigma). In this reaction, 43 µL of the PCR product and 5µl of the vector was used and to it 1 µL of each KpnI-HF (NEB) and XbaI (NEB) or MluI-HF (NEB) and ECORI-HF (NEB), 5 µL of 10X Cutsmart Buffer (NEB) and 38 µL of MBG $H_2O$ were added to the vector reaction but not the PCR .

### 3.2.5 Ligation:

The purified and digested PCR product was ligated into the purified and digested vector. 65 ng of the vector (c-Flag pcDNA3) was ligated with an exact amount of insert calculated using the Insilico ligation calculator at a 3:1 vector: insert ratio. Similarly, 65ng of pCW57-MCS1-2A-MCS2 was ligated with its selected PCR products at a 3:1 vector: insert ratio. In both reactions, 1 μL of T4 DNA Ligase (NEB) and 4 μL of the 5X Ligase Buffer (NEB) were added, and the corresponding amount of MBG H2O to make the total volume of the reaction 20 μL. The reaction was left to incubate at room temperature for 4 hours and then transformed into DH5-α for TRAF1-FL, c-IAP2, 4-1BB, and c-IAP1 and NEB stable bacteria (NEB) for both MCSI-TRAF1 and MCSI-cIAP1, using the protocol described in the following sections.

## 3.3 Bacterial culture:

The bacterial cultures that were used for the various experiments were DH5-α - made in house and NEB stable bacteria (NEB). The main purpose of these bacteria is to amplify the amount of plasmid.

### 3.3.1 Transformation:

DH5-α bacteria was taken out from -80°C storage and thawed on ice for 20-30 minutes. 100ng of each DNA plasmid was added to 100ul of DH5α bacteria and left to incubate for 30 minutes. After the incubation is over, the bacteria was heat-shocked at 42°C for 45seconds then placed immediately back on ice for 3 minutes. 1ml of LB broth was added to the bacteria and the

tubes were placed in an orbital shaker for 1 hour at 37°C at 250 RPM. While the bacteria is incubating in the orbital shaker [agar plates with the appropriate antibiotic] were removed from storage at 4°C and were left to warm up to room temperature. Subsequently, 100ul of the transformed bacteria was spread onto the agar plates and left to incubate overnight at 37°C.

The NEB stable bacteria follow a similar protocol to the DH5-α bacteria except that the bacteria was left for 5 minutes after the addition of the DNA plasmid instead of 30 minutes. These bacteria were also heat-shocked at 42°C for 30 seconds instead and 950µl of room temperature NEB 10-beta/Stable Outgrowth Medium was used in place of the LB broth. Nonetheless, all the other steps are exactly the same as the DH5-α bacteria.

### 3.3.2 Plasmid DNA Miniprep:

To start the process of making mini-preps of the plasmids of interest, a single colony was picked from the agar plates that contained the transformed bacteria from the step above and placed in snap cap tubes with 3ml of LB broth and 3µl of the appropriate antibiotic (1:1000 dilution). The tubes were left to incubate in the orbital shaker overnight at 37°C and 250 RPM. The following morning, 850µl of the culture was used to make glycerol stocks (kept at -80°C) and the rest of the culture was used to make the mini-preps using the GenElute HP Plasmid Miniprep kit (Sigma-Aldrich).

### 3.3.3 Plasmid DNA Midiprep:

This step was used in order to get higher quality of the plasmids of interest (higher concentrations and higher volumes). The protocol follows the same one stated previously for the

mini-prep with the only difference being the starter culture. For this step, a starter culture of transformed DH5-α (single colony) was placed in the incubator for 6-8 hours using the same parameters as listed above, then 100µl of the starter culture was added to 100ml of LB broth and put back in the orbital shaker overnight. Subsequently, plasmids will be extracted and purified using the PureLink Fast Low-Endotoxin Midi Plasmid Purification Kit (ThermoFisher).

## 3.4 Site Directed Mutagenesis:

In order to make the different human TRAF1 mutations, TRAF1V203A, TRAF1E207A, TRAF1V203AE207A, TRAF1E209A, TRAF1E207AE209A, TRAF1I200A, TRAF1V201A, TRAF1N205A, TRAF1K206A, TRAF1L204A, TRAF1V208A) along with the truncated TRAF1 (TRAF1d201-209), Q5 Site Directed Mutagenesis Kit (NEB) will be used. The full length of the human TRAF1 gene is going to be used as a template to perform the mutations. Polymerase chain reactions (PCR) will be performed with forward and reverse primers that will have the intended mutations in them. The primers will be designed using software, NEB Base Changer (https://nebasechanger.neb.com), where the amino acids in the designated areas will be changed into alanine. After the PCR reactions are done, the new DNA fragments will then be kinased, ligated, and digested. The specific steps are mentioned below.

### 3.4.1 PCR amplification:

The first step in creating these TRAF1 mutations was to amplify the PCR product. Human TRAF1 coding DNA sequence cloned into a c-Flag pcDNA3 vector was used as the template. Table 5.0 shows the amount of each reagent used and Table 6.0 provides the PCR

conditions. Following PCR amplification, the PCR products were run on a 1% Agarose gel and then extracted from the gel using the GenElute Gel Extraction Kit (Sigma).

**Table 5.0 Q5 SDM PCR reaction components (NEB):**

| | **25µl Reaction** | **Final concentration** |
|---|---|---|
| Q5 Hot start high fidelity 2X Master mix | 12.5µl | 1X |
| 10µM Forward primer | 1.25µl | 0.5µM |
| 10µM Reverse primer | 1.25µl | 0.5µM |
| Template DNA (10ng/µl) | 1µl | 10ng |
| Nuclease-free water | 9 µl | |

**Table 6.0 Q5 SDM PCR cycle conditions:**

| **Step** | **Temperature** | **Time** | |
|---|---|---|---|
| Initial Denaturation | 98°C | 30 seconds | |
| 25 Cycles | 98°C | 10 seconds | |
| | X°C (varied, see table 7.0) | 30 seconds | |
| | 72°C | 90 seconds | |
| Final Extension | 72°C | 2 minutes | |

| | | |
|---|---|---|
| Hold | 4°C | Infinite Hold |

### *3.4.2 KLD reaction, ligation, and bacterial transformation:*

This step was done by combining 1µl of the PCR product, 5µl of the 2x KLD reaction buffer, 1ul of the 10x KLD enzyme mix and 3µl of nuclease-free water together. The mixture was then left to incubate at room temperature for 45minutes. Following the incubation, 5µl of the reaction mixture was mixed with 50µl of competent bacterial cells (that came in the kit) and left to incubate on ice for 30 minutes. The bacteria were then heat-shocked for 30 seconds at 42°C and immediately placed back on ice for 5 minutes. 950µl of super optimal broth (SOC) was added to snap cap tubes along with the KLD reaction mix and left to incubate in the orbital shaker for 1 hour at 37°C and 250 RPM. Finally, 100µl of the culture was spread on agar plates with the appropriate antibiotic and placed in the incubator overnight at 37°C.

**Table 7.0 List of primers for each TRAF1 mutation as well as the primers for 4-1BB, c-IAP2, cIAP2mut, TRAF1, cIAP1, and c-IAP1mut and their respective annealing temperatures:**

| **TRAF1 mutations/ deletions** | **Forward primers (5'- 3')** | **Reverse primers (5'- 3')** | **Annealing temperatures (Ta)** |
|---|---|---|---|
| TRAF1E207A | CTCAACAAGGcGGTGGAGGCC | GACAGCAACAATGTTCTCAAAC | 63°C |
| TRAF1E209A | AAGGAGGTGGcGGCCTCCCAC | GTTGAGGACAGCAACAATGTTCTC | 67°C |
| TRAF1E207AE209A | ggcgGCCTCCCACCTGGCCCTG | accgcCTTGTTGAGGACAGCAACAATGTTCTCAAACAC | 72°C |

| | | | |
|---|---|---|---|
| TRAF1V203A | TTCTCAAACACACGCAGC | CATTGTTGCTgccCTCAACAAGG | 63°C |
| TRAF1I200A | GTTTGAGAACgctGTTGCTGTCCTCAACAAGGAGG | ACACGCAGCTTCCCCTCC | 68°C |
| TRAF1V201A | TGAGAACATTgccGCTGTCCTCAACAAGG | AACACACGCAGCTTCCCC | 65°C |
| TRAF1L204A | TGTTGCTGTCgccAACAAGGAGG | ATGTTCTCAAACACACGC | 60°C |
| TRAF1N205A | TGCTGTCCTCgccAAGGAGGTGG | ACAATGTTCTCAAACACAC | 58°C |
| TRAF1K206A | TGTCCTCAACgcaGAGGTGGAGG | GCAACAATGTTCTCAAAC | 57°C |
| TRAF1V208A | CAACAAGGAGgcgGAGGCCTCCC | AGGACAGCAACAATGTTCTCAAACAC | 68°C |
| TRAF1V203AE207A (TRAF1V203A was used as a template and TRAF1E207A primers were used) | CTCAACAAGGcGGTGGAGGCC | GACAGCAACAATGTTCTCAAAC | 63°C |
| TRAF1 deletion 201-209 | CTCAAACACACGCAGCTTCCCC | GCCTCCCACCTGGCCCTG | 72°C |
| **Other plasmids** | | | |
| h4-1BB | h4-1BB XbaI Forward primer (5'-3'): ATATCTAGAACCATGGGAAACAGCTGTTAC | H4-1BB KpnI Reverse primer (5'-3'): ATAGGTACCCAGTTCACATCCTCCTTC | 58°C |
| hTRAF1-pCW57-MCS1-2A-MCS2 | hTRAF1 ECORI Forward primer (5'-3') ATAGAATTCACCATGGCCTCCAGCTCAGGC | hTRAF1 MluI Reverse primer (5'-3'): ATAACGCGTTAGAGTGCTGGTCTCCACAAT | 60°C |

| | | | |
|---|---|---|---|
| hcIAP2-pCW57-MCS1-2A-MCS2 | hcIAP2 ECORI Forward primer (5'-3'): ATAGAATTCACCATGAACATAGTAGAAAACAGCATATTC | hcIAP2 MluI Reverse primer (5'-3'): ATAACGCGTTGATGAAAGAAATGTACGAACTGT | 56.9°C |
| hcIAP1-pCW57-MCS1-2A-MCS2 | hcIAP1 ECORI Forward primer (5'-3'): ATAGAATTCACCATGCACAAAACTGCCTCCCAA'3 | hcIAP1 MluI Reverse primer (5'-3'): ATAACGCGTTAAAGAGAGAAATGTACGAAC'3 | 60°C |

### 3.5 Plasmid DNA Transfections into HEK293T/17 cells:

Cells were plated a day before transfection in 60mm dishes at 1.36 x$10^6$ cells/ml/dish. The following day, the cells should be at 85% - 90% confluency in order to be transfected with 2µg of cIAP2), HOIL-1, TRAF1-FL, MCSI-TRAF1, MCS1-c-IAP1, MCSI-c-IAP2, or the mutated versions of TRAF1. For the control conditions, cIAP2, HOIL1, and TRAF1-FL will be co-transfected with c-Flag pcDNA3 (empty plasmid). The media that the cells were incubating in was removed and 3.3mls of OPTI-MEM (Gibco) were added to each dish and then placed back into the incubator in order to prepare the DNA mixtures. DNA plasmids were transfected into the cells using Lipofectamine 3000 (Invitrogen). Following the protocol listed by the manufacturing company, two separate mixes were prepared - one for the DNA mixture and one for the lipofectamine reagent. 600µl of OPTI-MEM were added to two different Eppendorf tubes wherein one of the tubes 2µg of the desired plasmids were added and mixed with 4µl of the P3000 reagent (Invitrogen). The second tube contained 600µl of OPTI-MEM plus 4µl of Lipofectamine3000. After both mixtures were prepared, they were mixed together (for a total volume of 1.2mls/condition) and incubated at room temperature for 15-20 minutes then added to

the cells drop by drop. Lastly, cells were left in the incubator for 6 hours before the media was changed into full growth media and placed back into the incubator overnight.

## 3.6 Cellular treatments:

### 3.6.1 Cycloheximide treatments (CHX):

For the experiments that examined cIAP2 degradation over a specific time course, treatments of cycloheximide were added at different time points to stop the overall synthesis of proteins. Cycloheximide is usually used in experiments to determine the half-life of a protein since it inhibits protein synthesis. Prior to treatments, CHX was reconstituted in 100% ethanol (ETOH). Treatments of different amounts of CHX (3µg, 5µg, 10µg, 25µg, and 50µg) were added to the already transfected cells at 1 hr, 2hrs, 3hrs, 4hrs, 6hrs, 8hrs, 10hrs, 16hrs, and 24hrs. For the controls, however, transfected cells were treated with 100% ethanol.

### 3.6.2 MG-132, Chloroquine (CQ), and Bafilomycin treatments:

MG-132 (Sigma) was reconstituted in DMSO at a concentration of 10mM and 1µl was used to treat the selected cells (10µM). Similarly, Chloroquine was reconstituted in water at a concentration of 25mM and used at 25µM for cellular treatments. Bafilomycin, on the other hand, was reconstituted in DMSO at a concentration of 100µM and used at 100nM for cellular treatments. All the reagents were aliquoted into light safe Eppendorf tubes and stored at -20. The cells were treated with the different inhibitors at different time points then lysed and the lysates were collected. Protein concentrations were then measured and a western was performed.

### 3.7 Collection of cell lysates:

Transfected HEK293T/17 cells will be removed from the incubator and placed on ice then lysed with 250ul of freshly prepared lysis buffer containing 1% NP- 40 (Igepal CA-630), 100mM Tris pH 8.0, 20% glycerol, 0.2mM EDTA, 150mM NaCl, Complete 1x Protease Inhibitor Cocktail, 1x PhosSTOP Phosphatase Inhibitor Cocktail, 1 mM DTT, and Molecular Biology Grade water (MBG H2O). Before the lysis buffer is added to the cells, they were washed with 3mls of ice cold 1x PBS to ensure that the media is fully removed- and a pH of approximately 7.4 is maintained. Lysis buffer was then added to the cells and were left to incubate flat on ice for 10 minutes. After the incubation duration was done, cells were scraped, placed into Eppendorf tubes, and left to incubate for 20 more minutes with vortexing every 5 minutes. Lastly, samples were centrifuged at 14000RPM for 15 minutes at 4°C and lysates were stored at -80°C. Lysates were then quantified using a Bradford protein quantification assay and samples were prepared for immunoblotting.

### 3.8 Co-immunoprecipitation:

Transfected HEK293T/17 cells were lysed with 500ul of lysis buffer containing 20 mM Tris HCl pH 8, 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, Complete Protease Inhibitor Cocktail, PhosSTOP Phosphatase Inhibitor Cocktail and 1 M DTT, and Molecular Biology Grade water (MBG H2O). Lysates were obtained following the same lysis procedure listed above. Then, co-immunoprecipitation was done on these lysates with Protein G DynaBeads (Invitrogen), while 15ul of the lysates were removed and saved to be used later as input. DynaBeads were washed with PBS plus 0.02% Tween and 25ul portions were used for each sample. Subsequently, 0.8µg of TRAF1 antibody (1F3) was added to the DynaBeads and

incubated on a rotator for 15 minutes at room temperature. The tubes were then placed on a magnet after the incubation time is complete to separate the beads from the solution that contains the antibody. The solution was removed, and the remaining beads were mixed with the lysates obtained earlier and placed back on the rotator for 15 minutes at room temperature. Lastly, beads were washed 3 times with diluted 1x Co-IP lysis buffer, containing no inhibitors, and with PBS twice before being eluted with 25 µL of 2X Laemmeli Buffer (BioRad).

### 3.9 Immunoblotting:

Samples prepared using the procedures mentioned earlier were heated at 100°C for 5 minutes and loaded onto 10% polyacrylamide gels. They ran at 60 V for 30 minutes and at 120 V for 90 minutes before finally being transferred to PVDF membranes. Membranes were blocked with 5% skim milk in TBST for 1 hour, and then incubated with the relevant primary antibody (1:1000 dilution) at 4°C overnight (see table 8.0) . The following day, membranes were washed 3 times with 1x TBST for 10 minutes, then incubated with the appropriate secondary antibody diluted in 10 mL of 5% skim milk in TBST (1:10000 dilution). After that, the membranes were washed three times with 1x TBST for 10 minutes. Finally, blots were imaged (Chemiluminescence) using Clarity ECL (BioRad)..

**Table 8.0List of Primary antibodies used for Western Blot Analysis:**

| Primary antibody | Origin | Dilution | Company | Catalogue No |
|---|---|---|---|---|
| cIAP2 | Rabbit | 1:1000 | Cell Signalling | 3130 |
| TRAF1 | Rabbit | 1:1000 | Cell Signalling | 4715 |
| c-IAP1 | Rabbit | 1:1000 | Cell Signalling | 7065 |
| TRAF2 | Rabbit | 1:1000 | Cell Signalling | 4724 |

| | | | | |
|---|---|---|---|---|
| RBCK1(H-1) (HOIL-1) | Mouse | 1:1000 | Santa Cruz Biotechnology | sc-393754 |
| B-actin | Mouse | 1:5000 | R&D Systems Inc. | MAB8929 |

### 3.9.1 Bradford protein Assay:

The Bradford Reagent (BioRad) was taken out of the 4°C fridge. The BSA aliquot was also taken out of the -20°C freezer and was placed on ice until thawed. In a 50ml conical tube, the 5X Bradford Reagent was diluted to 1X with dd$H_2O$. Then, six different concentrations were made using the BSA (1mg/ml) and the diluted Bradford Reagent. In one Eppendorf tube 872µl of the diluted Bradford reagent was mixed with 128µl of the BSA, then 500µl of the mixture was transferred to another Eppendorf tube and mixed with 500µl of the diluted Bradford reagent. The serial dilution was continued until concentrations of 64µg/ml, 32µg/ml, 16µg/ml, 8µg/ml, and 4µg/ml were achieved.

The standard concentrations were transferred into a flat-bottomed 96-well plate in duplicates, each well containing 200µl of the standard mixture. The samples were prepared by mixing 500µl of the diluted Bradford reagent with 2µl of lysate. For the blanks, however, 500µl of the reagent was combined with 2µl of lysis buffer. 200µl of the samples as well as the blanks were transferred to a 96-well plate, the same one as the standards, in duplicates. Absorption was then measured at 595nm wavelength. The concentrations of these proteins were then calculated using these measurements.

## 4.0 Results

### *4.1 Effects of single amino acid substitutions of TRAF1 mutants on the interaction with cIAP2.*

The first step in this project is to examine whether substituting single amino acids in the coiled-coil region of TRAF1 (highlighted in figure 4) would have an effect on the interaction with cIAP2 protein. In order to test this, we generated different TRAF1 mutants substituting each amino acid with an Alanine. We also created a TRAF1 truncation where we deleted the 9 amino acid sequence in the coiled coil domain (TRAF1Δ201-209) highlighted in figure 4. Each TRAF1 mutant/truncation was co-transfected with our protein of interest, cIAP2, which was also transfected on its own to act as a negative control, while c-FLAG (empty plasmid), was used as another control. As a positive control for the immunoprecipitation assay, full-length TRAF1 (TRAF1 FL) was co-transfected with cIAP2. Lysates were then immunoprecipitated with a TRAF1 specific antibody (I F-3) and immunoblotted for our respective proteins. The results show a significant reduction in cIAP2 co-immunoprecipitation when co-transfected with the deleted version of TRAF1, TRAF1Δ201-209 compared to TRAF1 FL (Figures 5 and 6). This consolidates the fact that TRAF1 does, in fact, interact with c-IAP2 in this region. However, when we mutated amino acids Isoleucine 200, Lysine 206, Valine 208, and Glutamic acid 209 in TRAF1 and transfected them with cIAP2, the amount of co-immunoprecipitated protein was not significantly reduced indicating that the specific interaction between cIAP2 and TRAF1 does not occur at these sites.

Interestingly, the amount of cIAP2 that was pulled down was apparently reduced when co-transfected with TRAF1 mutants that contained the V203A and the E207A substitutes signifying

that these amino acids are critical for the interaction with cIAP2 (Figures 5, 6, and 7). Figure 6 show an optimized IP condition where we were able to reduce non-specific binding of proteins to the protein-G immunoprecipitation beads. Strikingly, we show that TRAF1V203A and TRAF1E207A mutations diminish the interaction of TRAF1 with cIAP2 compared to the positive control (figure 6). Therefore, since the interaction is significantly reduced with the mutations mentioned above, a double mutated version of TRAF1, where both V203 and E207 were simultaneously mutated, was generated to examine whether or not it would further interrupt the interaction. Indeed, the interaction with c-IAP2 with co-transfected with the double mutated TRAF1 (TRAF1V203AE207A) was abrogated (Figures 5 and 6). This further suggests that amino acids V203 and E207 of TRAF1 are essential for the interaction with cIAP2.

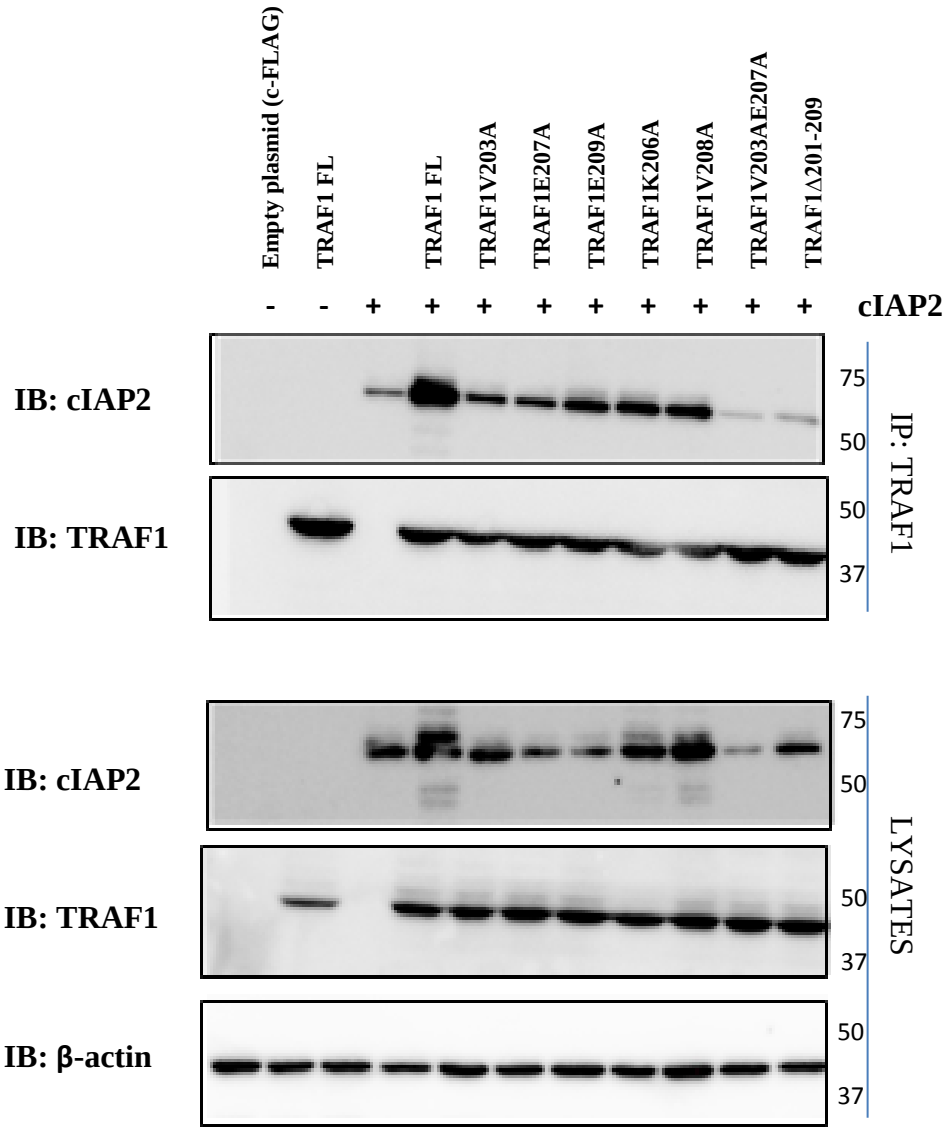


**Figure 5 effects of TRAF1 mutations/deletions on cIAP2**. Immunoprecipitation (IP) of TRAF1 from lysates of HEK293T/17 cells, followed by immunoblotting (IB) with cIAP2 and TRAF1 specific antibodies and a light-chain-specific anti-Rabbit -horseradish peroxidase. This experiment was conducted three independent times

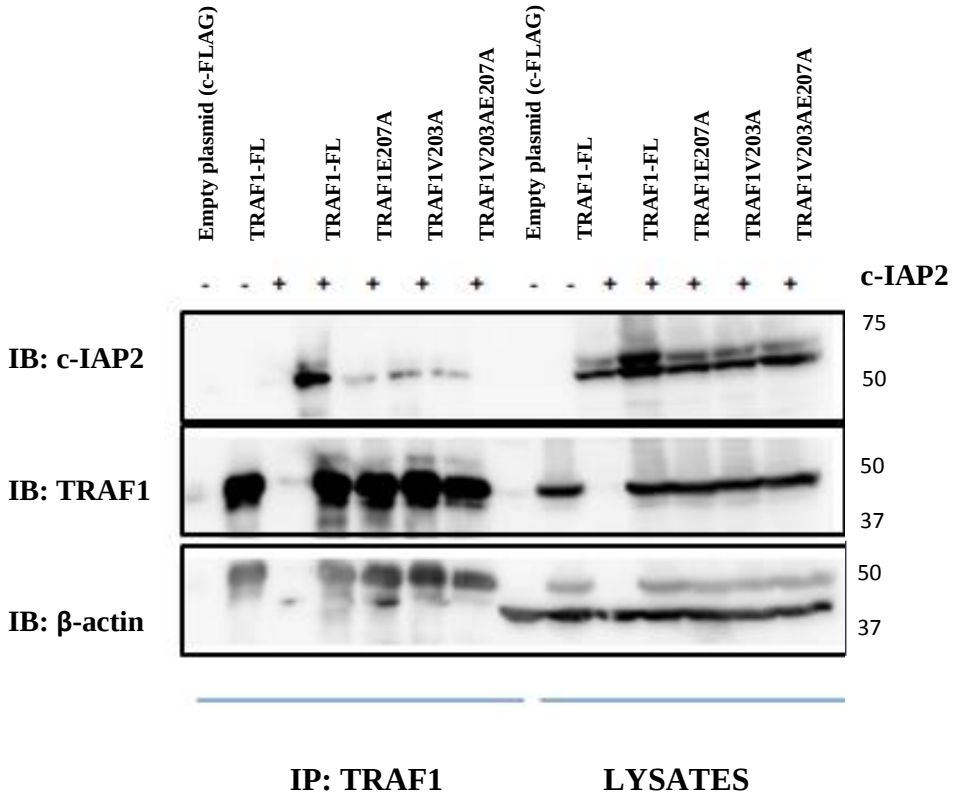


**Figure 6 TRAF1V203A , TRAF1E207A and double mutation are critical for the interaction with cIAP2**. Immunoprecipitation (IP) of TRAF1 from lysates of HEK293T/17 cells, followed by immunoblotting (IB) with cIAP2 and TRAF1 specific antibodies and a light-chain-specific anti-Rabbit -horseradish peroxidase. The blot was re-probed for β-actin and the upper band is left-over from previous probe (TRAF1). This experiment was conducted three independent times.

### *4.2 Effects of TRAF1 mutants on the interaction with HOIL-1 and TRAF2*

Even though we were successful at identifying the specific sites of interaction between TRAF1 and cIAP2, it is essential to test whether these mutants maintain the interaction of TRAF1 with other key proteins such as HOIL-1 (LUBAC component) and TRAF2. This is because we want to create a TRAF1 mutant that does in fact decrease NF-κB activation downstream of TNFR pathway while maintaining its role as a negative regulator through its interaction with the LUBAC complex, specifically HOIL-1. Hence , we co-transfected TRAF1-FL, TRAF1V203A, and TRAF1E207A with either HOIL-1 or cIAP2 in HEK293 cells and immunoprecipitated with a TRAF1- specific antibody (I F-3) then immunoblotted for the corresponding proteins which were: TRAF1, cIAP2, and HOIL-1. Figure 7A/7B as well as figure 8A/8B both show that TRAF1V203A and TRAF1E207A mutants maintained similar level of interaction with HOIL-1 whereas the interaction with cIAP2 was diminished when compared to wild-type TRAF1. In addition, these mutants did not affect the interaction with TRAF2, another important signalling partner for TRAF1. Altogether, these results show that V203 and E207 are the residues where TRAF1 interacts specifically with cIAP2 but not with other signalling partners (figure 9).

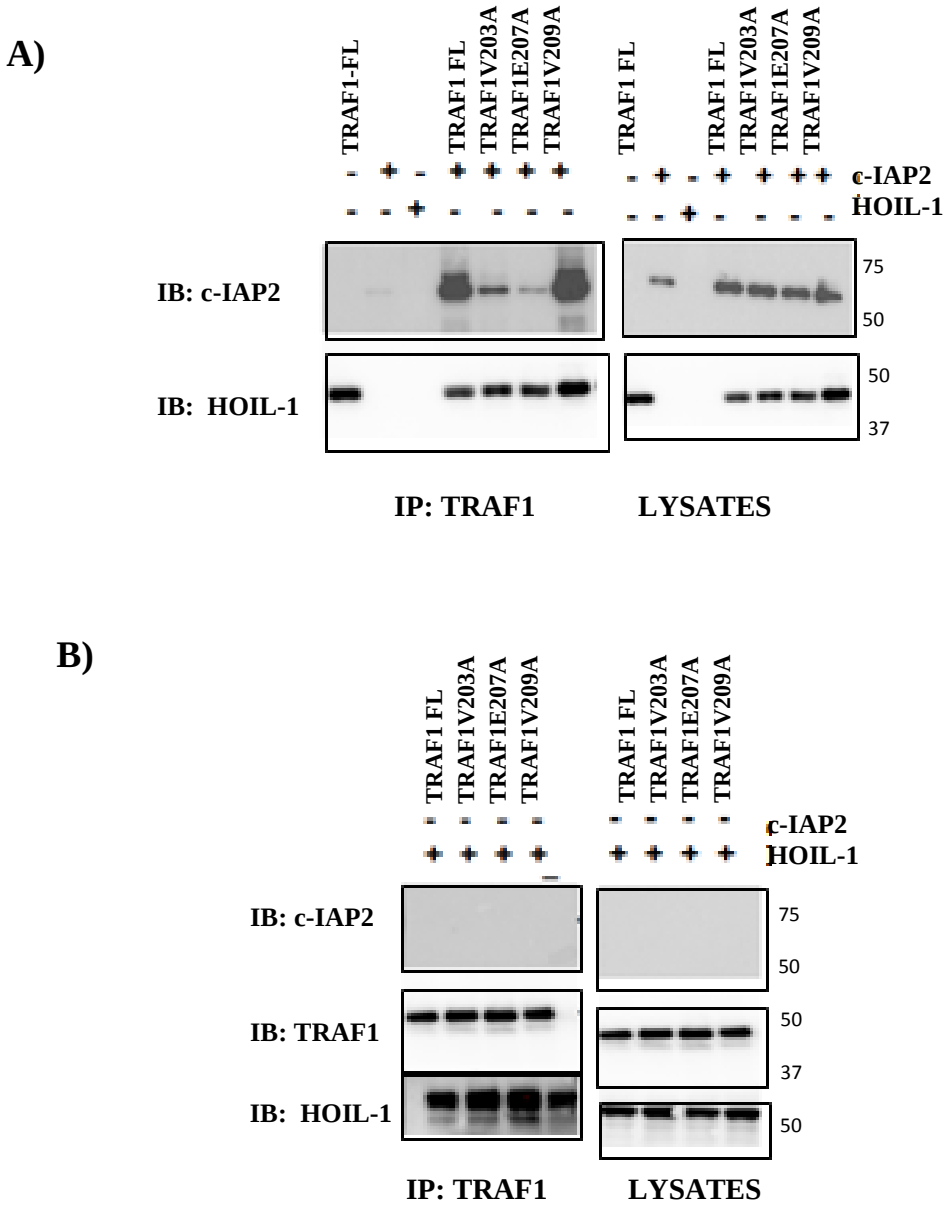


**Figure 7 Effects of TRAF1 mutants on the interaction with HOIL-1 and c-IAP2**. Immunoprecipitation (IP) of TRAF1 from lysates of HEK293T/17 cells, followed by immunoblotting (IB) with A) c-IAP2 and TRAF1 specific antibodies and a light-chain-specific anti-Rabbit -horseradish peroxidase. B) Immunoblotted with HOIL-1 specific antibody and a light-chain-specific anti-Mouse -horseradish peroxidase. This experiment was conducted three independent times.

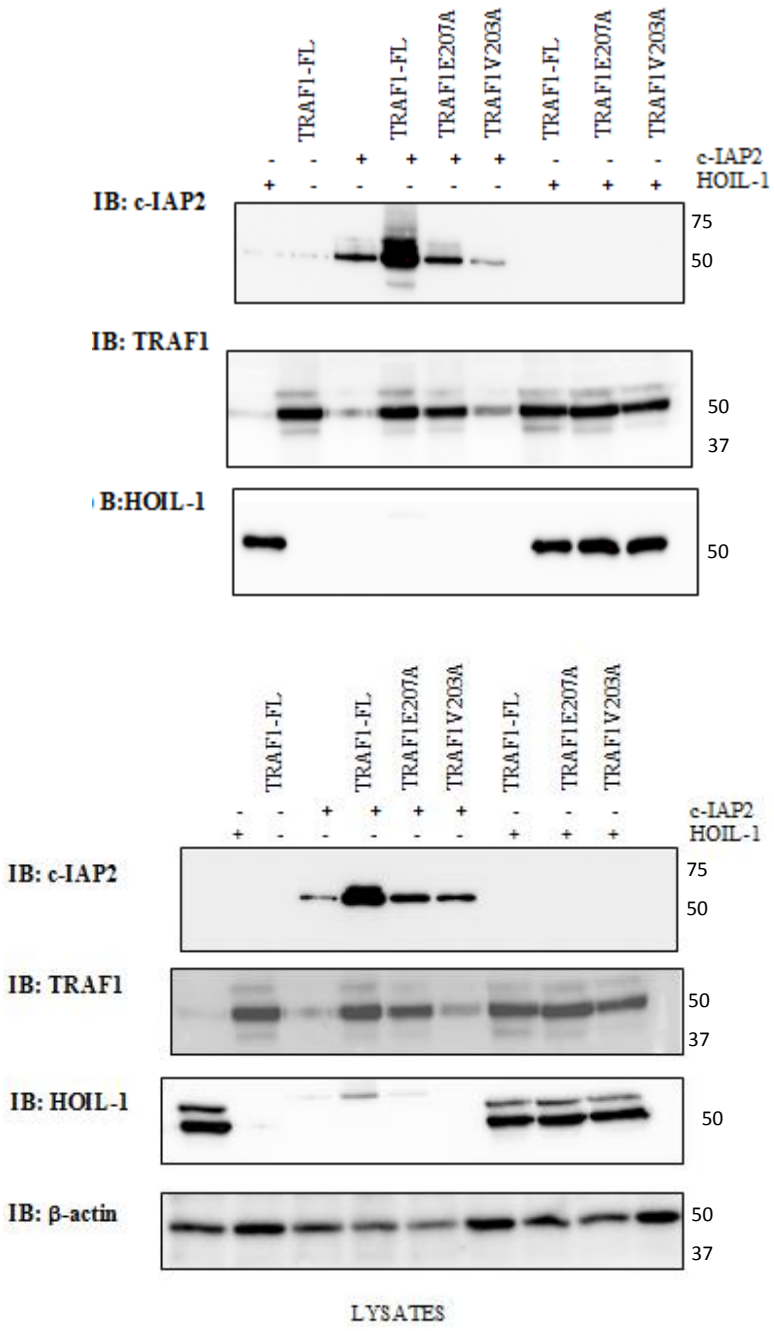


**Figure 8 Effects of TRAF1V203A and TRAF1E207A mutants on the interaction with HOIL-1.** A) Immunoprecipitation (IP) of TRAF1 from lysates of HEK293T/17 cells, followed by immunoblotting (IB) with c-IAP2, TRAF1, HOIL-1 specific antibodies and a light-chain-specific anti-Rabbit (cIAP2 and TRAF1) and anti-Mouse (HOIL1) -horseradish peroxidase. B) Lysates of HEK293T/17 cells immunoblotted with Immunoblotted with c-IAP, TRAF1, HOIL-1 specific antibodies and a light-chain-specific anti-Rabbit (cIAP2 and TRAF1) and anti-Mouse (HOIL1) -horseradish peroxidase. Experiment replicated three independent times.

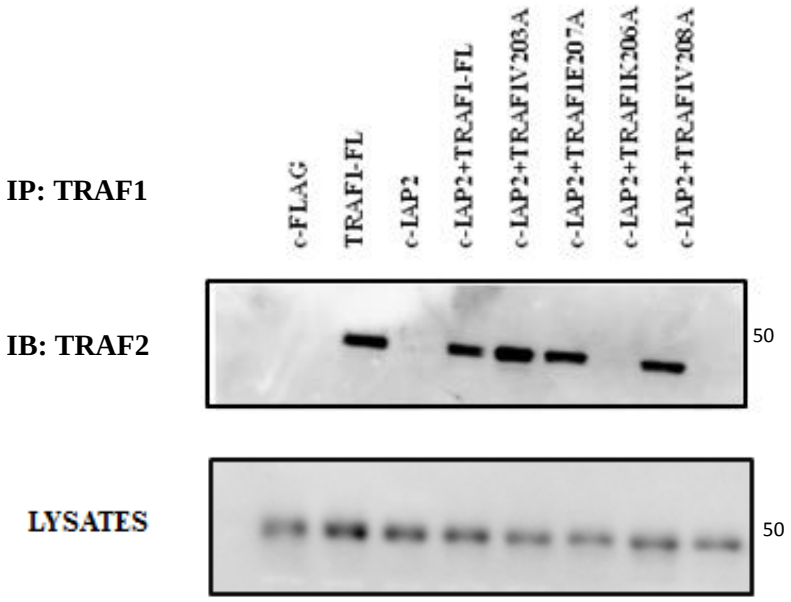


**Figure 9 Effects of single amino acid substitutions on the interaction with TRAF2.**

Immunoprecipitation (IP) of TRAF1 from lysates of HEK293T/17 cells, followed by immunoblotting (IB) with c-IAP2 and TRAF1 specific antibody and a light-chain-specific anti-Rabbit -horseradish peroxidase. This experiment was conducted three independent times.

### *4.3 TRAF1 stabilizes cIAP2*

As we were elucidating the sites of interaction between TRAF1 and cIAP2 we found that mutating residues 203 and 207 as well as the double mutation of both residues as sites of interaction with c-IAP2, we made an intriguing observation. Steady state expression levels of cIAP2 were significantly higher when co-expressed with TRAF1 FL compared to cIAP2 alone (see Figures 5, 6, 7, and 8B). This was despite loading equal amounts of total protein and transfecting equal amount of total plasmid DNA. This led us to hypothesize that TRAF1, in addition to its role in recruiting c-IAP2 to the TNFR complex, stabilizes cIAP2 by protecting it from degradation. In order to test this hypothesis, we assessed cIAP2 protein stability in the presence or absence of TRAF1 using a cycloheximide chase assay. We co-transfected cIAP2 with the empty plasmid (c-Flag) or with an equal amount of TRAF1 followed by a protein quantification to ensure equal loading. Figure 10 shows that when wild type TRAF1 is co-transfected with cIAP2, the expression levels of cIAP2were significantly higher than the cIAP2 alone at basal level without CHX treatments. Moreover, the same figure shows that in the conditions where cIAP2 was transfected by itself, the protein levels were significantly decreased after 4 and 6 hrs of cycloheximide treatments whereas this decrease is less prominent in the presence of wild type TRAF1. This further proposes that TRAF1 is protecting cIAP2 from degradation.

Next, we wanted to determine the optimal time course of TRAF1-mediated stabilization of cIAP2 by modifying the timing of CHX treatments. Thus, 293T/17 cells were transfected with c-IAP2 as well as c-FLAG in order to keep the total DNA transfected equal among all the conditions, or with equal amounts with TRAF1 FL. The cells are then treated with 5μg/μl of CHX at 1hr, 3hrs, and at 6hrs. Figure 11 shows that, just as observed in prior experiments, the

basal level of cIAP2 expression in the presence of wild type TRAF1 is significantly higher compared to the expression levels in the absence of wild type TRAF1. Also, cIAP2 expression was reduced as early as 1hr following CHX treatment in the cells that were transfected with c-IAP2 alone compared to those transfected with c-IAP2+TRAF1 FL (figure 11).

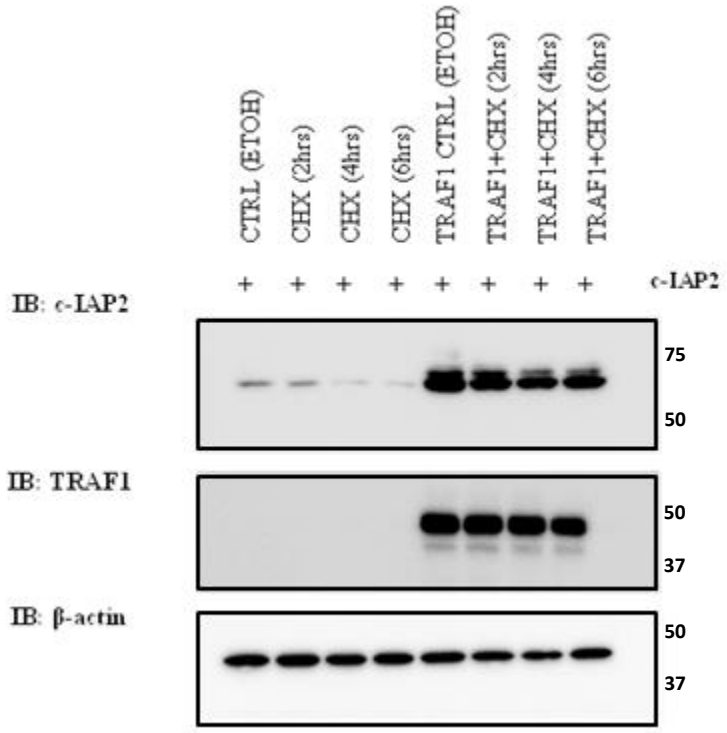


**Figure 10 Effects of TRAF1 on cIAP2 expression levels**. An over-expression in HEK293T/17 cells t treated with 5µg/µl cycloheximide (CHX) at 2hrs, 4hrs, and 6hrs, then lysed and immunoblotted with cIAP2 and TRAF1 specific antibodies and a light-chain-specific anti-Rabbit -horseradish peroxidase. This experiment was conducted three independent times

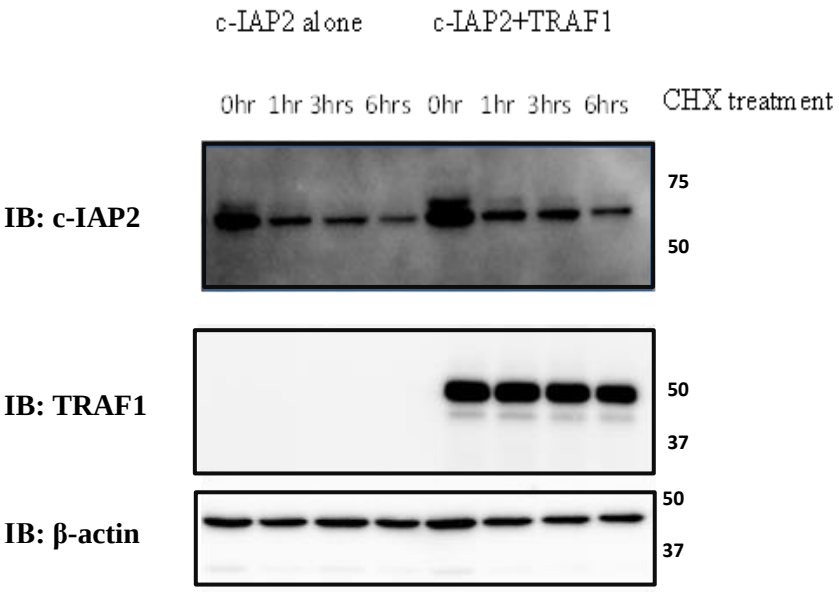


**Figure 11 TRAF1 stabilizes cIAP2**. An over-expression in HEK293T/17 cells t treated with 5μg/μl cycloheximide (CHX) at 1hr, 3hrs, and 6hrs, then lysed and immunoblotted with cIAP2 and TRAF1 specific antibodies and a light-chain-specific anti-Rabbit -horseradish peroxidase. This experiment was conducted three independent times.

### *4.4 Direct interaction between cIAP2 and TRAF is required for its stability.*

Although we have demonstrated that TRAF1 helps in limiting cIAP2 degradation, we still need to confirm that this process is mediated by the specific interaction between cIAP2 and TRAF1. To this end, we co-transfected cIAP2 with the following TRAF1 mutants: TRAF1V203A, TRAF1E207A, and TRAF1V203AE207A. These mutants were previously shown to diminish the interaction with c-IAP2. We then performed the cycloheximide chase assay. Results clearly showed that unlike WT TRAF1 (TRAF1 FL), TRAF1V203A fails to protect cIAP2 from degradation, as evident from the decrease in cIAP2 expression following 4 and 6 hrs of CHX treatments that is analogous to the rate of degradation when cIAP2 is co-transfected with the empty plasmid (Figure 12). Similarly, Figure 13 shows TRAF1E207A does not prevent cIAP2 degradation to the same extent as wild-type TRAF1. Both of these experiments have also been conducted using the 1hr, 3hrs, and 6hrs treatment time-points however the findings were similar (Figures 15 and 16). Lastly, the double mutated TRAF1 (TRAF1V203AE207A), also demonstrated lower capacity to stabilize cIAP2 expression in the presence of CHX compared to wild type TRAF1 (Figure 14). These results were consistent following prolonged treatments with CHX. Both single TRAF1 mutants as well as the double mutant showed reduced cIAP2 expression levels compared to TRAF1 FL following 16 hrs of CHX treatments (Figure 17). Since we have found from previous results that these mutants are indeed the sites of interaction with cIAP2, it further proves that it is, in fact, the specific interaction between the two proteins that prevent cIAP2 from being degraded. However, the mechanism of how the protein is being degraded in the cell is still unclear.

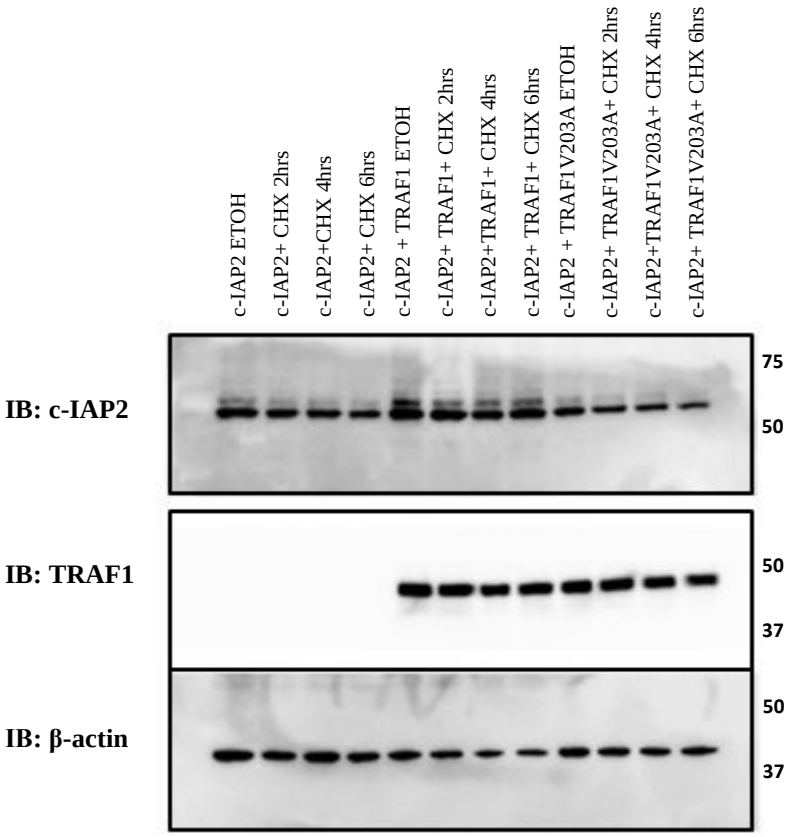


**Figure 12 Effects of direct interaction with TRAF1V203A on the expression levels of cIAP2**. An over-expression in HEK293T/17 cells t treated with 5µg/µl cycloheximide (CHX) at 2hrs, 4hrs, and 6hrs, then lysed and immunoblotted with cIAP2 and TRAF1 specific antibodies and a light-chain-specific anti-Rabbit -horseradish peroxidase. This experiment was conducted three independent times.

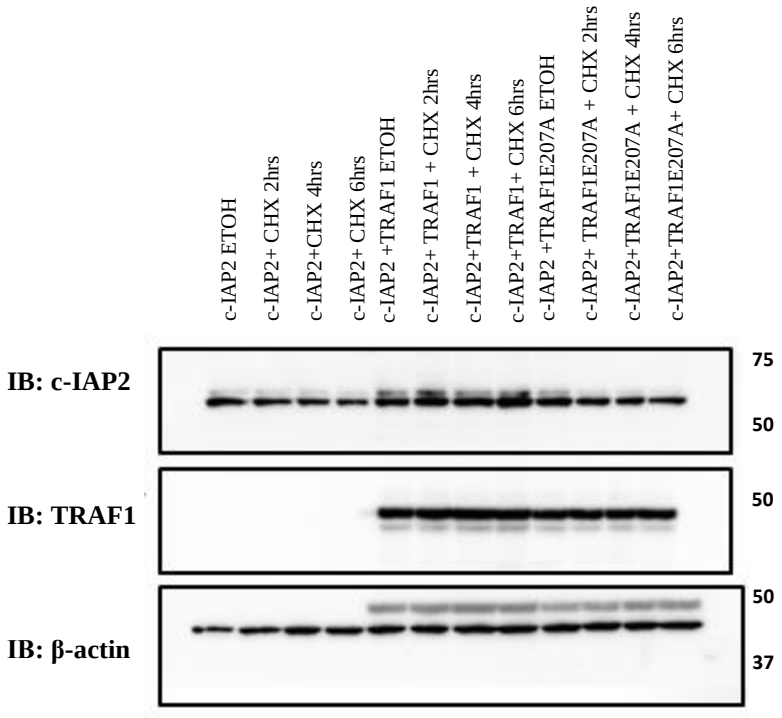


**Figure 13 Effects of direct interaction with TRAF1E207A on the expression levels of cIAP2**. An over-expression in HEK293T/17 cells t treated with 5μg/μl cycloheximide (CHX) at 2hrs, 4hrs, and 6hrs, then lysed and immunoblotted with cIAP2 and TRAF1 specific antibodies and a light-chain-specific anti-Rabbit -horseradish peroxidase. The blot was re-probed for β-actin and the upper band seen is left-over from the previous probe (TRAF1). This experiment was conducted three independent times.

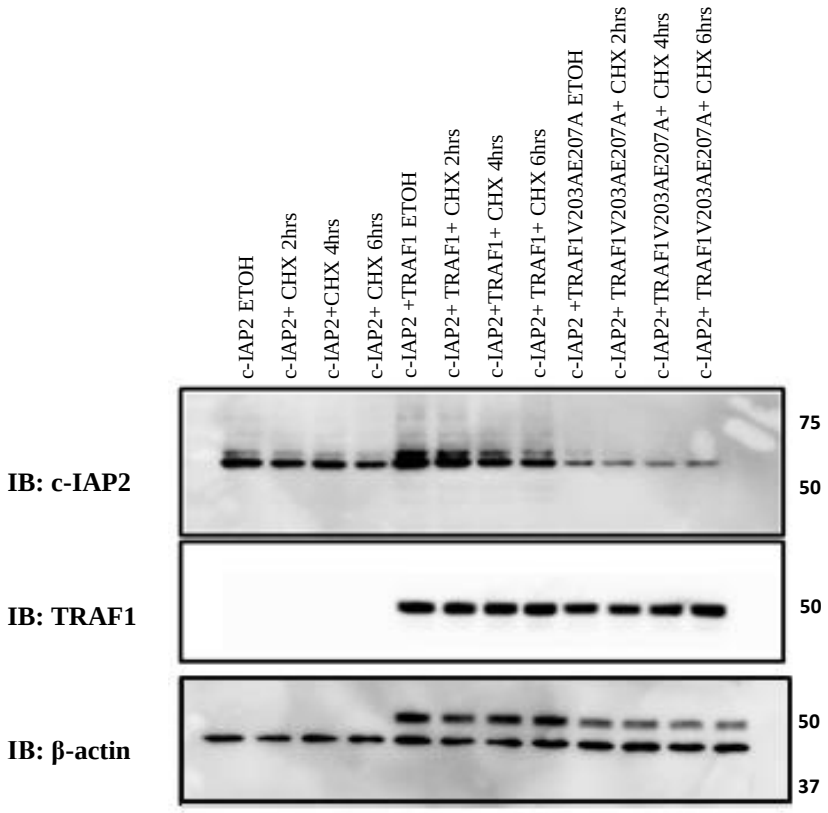


**Figure 14 Effects of direct interaction with TRAF1V203AE207A on the expression levels of cIAP2**. An over-expression in HEK293T/17 cells t treated with 5µg/µl cycloheximide (CHX) at 2hrs, 4hrs, and 6hrs, then lysed and immunoblotted with cIAP2 and TRAF1 specific antibodies and a light-chain-specific anti-Rabbit -horseradish peroxidase. The blot was re-probed for β-actin and the upper band seen is left-over from the previous probe (TRAF1). This experiment was conducted three independent times.

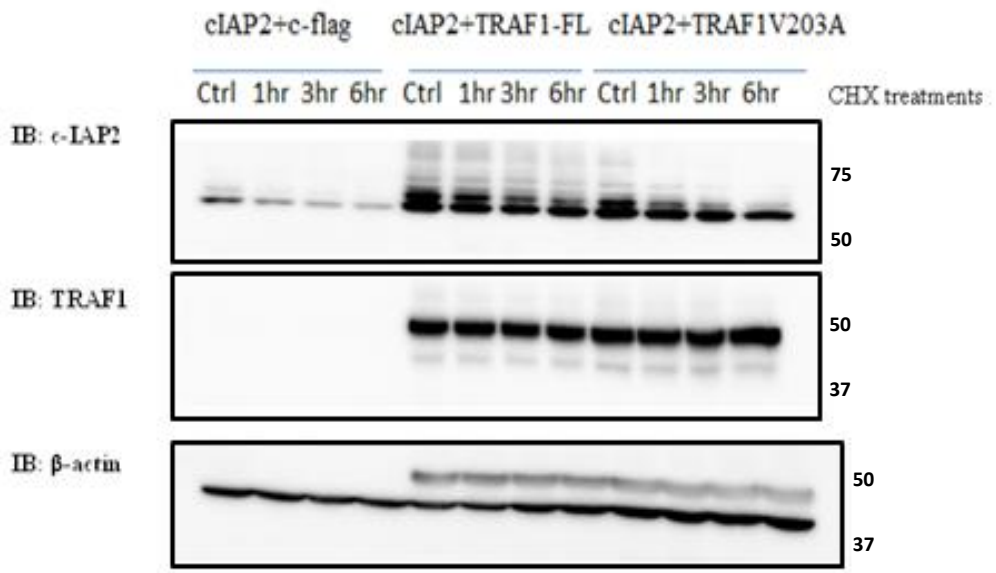


**Figure 15 Effects of TRAF1V203A on cIAP2 expression levels compared to wild-type TRAF1**. An over-expression in HEK293T/17 cells t treated with 5µg/µl cycloheximide (CHX) at 1hr, 3hrs, and 6hrs, then lysed and immunoblotted with cIAP2 and TRAF1 specific antibodies and a light-chain-specific anti-Rabbit -horseradish peroxidase. The blot was re-probed for β-actin and the upper band seen is left-over from the previous probe (TRAF1). This experiment was conducted three independent times.

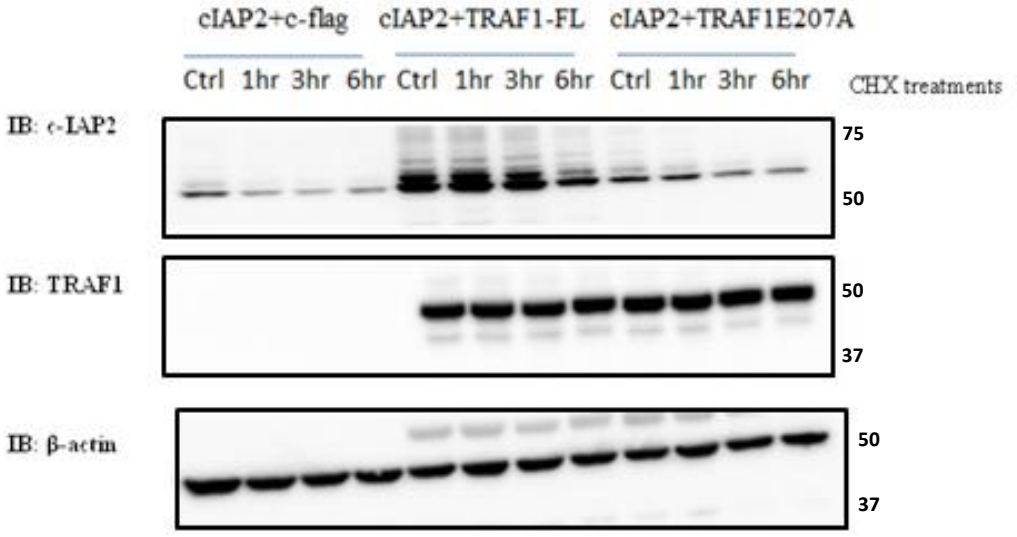


**Figure 16 Effects of TRAF1E207A on c-IAP2 expression levels compared to wild-type TRAF1.** An over-expression in HEK293T/17 cells t treated with 5μg/μl cycloheximide (CHX) at 1hr, 3hrs, and 6hrs, then lysed and immunoblotted with cIAP2 and TRAF1 specific antibodies and a light-chain-specific anti-Rabbit -horseradish peroxidase. The blot was re-probed for β-actin and the upper band seen is left-over from the previous probe (TRAF1). This experiment was conducted three independent times.

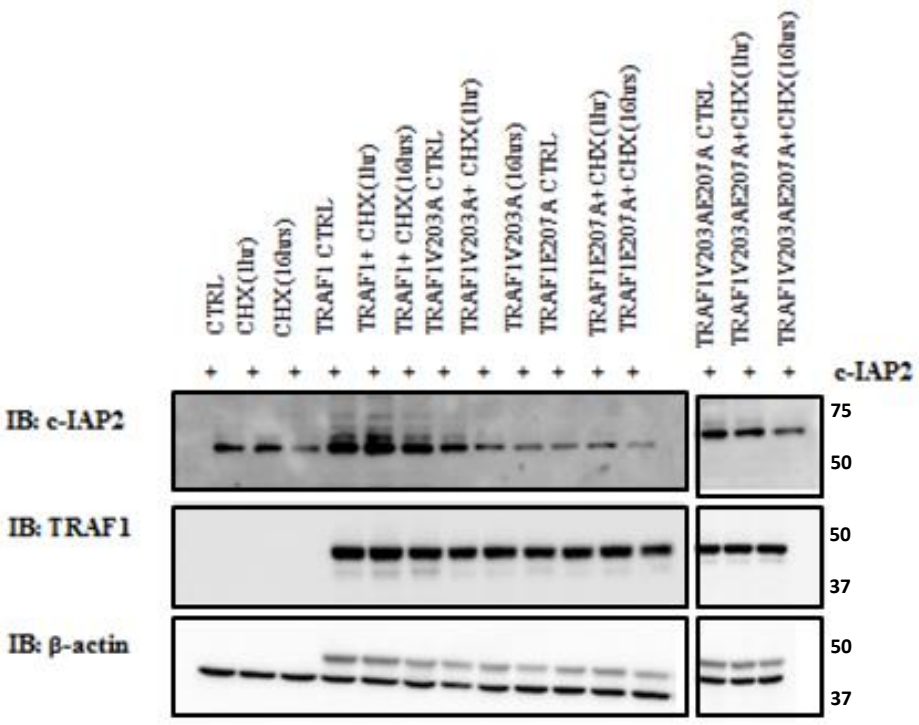


**Figure 17 Effects TRAF1 mutants on the expression levels of cIAP2 with prolonged CHX treatments.** An over-expression in HEK293T/17 cells t treated with 5µg/µl cycloheximide (CHX) at 1hr and 16hrs, then lysed and immunoblotted with cIAP2 and TRAF1 specific antibodies and a light-chain-specific anti-Rabbit -horseradish peroxidase. The blot was re-probed for β-actin and the upper band seen is left-over from the previous probe (TRAF1). This experiment was conducted three independent times.

### *4.5 Inducing TRAF1 protein levels enhances cIAP2 stability.*

Next, we generated a HEK293T/17 MCSI-TRAF1 stable cell line where TRAF1 expression was induced with doxycycline treatments over various time points and examined c-IAP2 expression. In order to create the stable cell line, we first cloned TRAF1 into the inducible plasmid, then transfected into HEK293T/17 cells and selected with puromycin. In order to determine which concentration of doxycycline is best for inducing TRAF1 we tested two different concentrations (1ug/ul and 2ug/ul). Figure 27 (in Appendix) shows that induction with 2ug/ul showed better protein expression and thus this concentration will be used for future treatments.

To examine how TRAF1 increase the steady-state levels of cIAP2 overtime, MCSI-CTRL cells (plasmid control) and MCSI-TRAF1 cells were transfected with 2ug of cIAP2 followed by 2μg/μl Doxycycline to induce TRAF1 expression for 16, 24, and 48 hrs. Consistent with our previous observations, levels of cIAP2 significantly increased with increasing TRAF1 levels at 24 and 48 hrs when compared to the plasmid control (Figure 18). This further indicates that the TRAF1 plays an important role in stabilizing cIAP2 and maintaining its steady state expression.

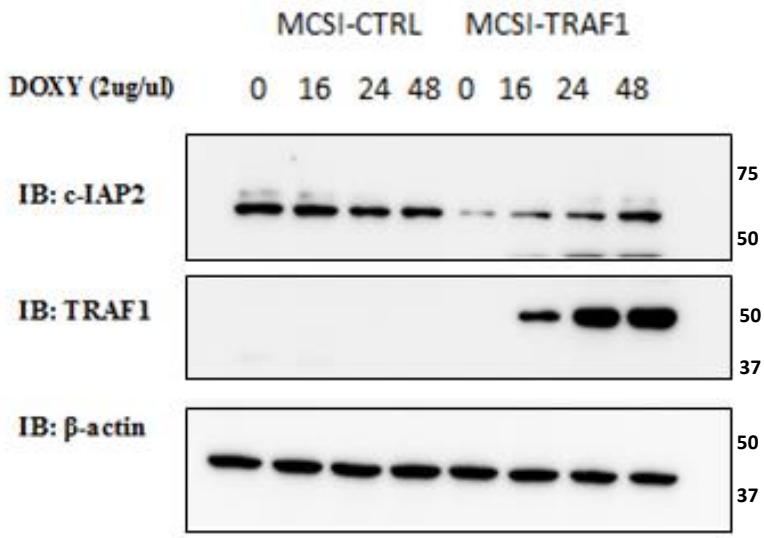


**Figure 18 Effects of inducing TRAF1 expression on c-IAP2 in MCSI-TRAF1 HEK293 cells.** An overexpression in both cell lines MCSI-CTRL and MCSI-TRAF1. Both cells were treated with 2µg/µl Doxycycline to induce TRAF1 expression at 16hrs, 24hrs, and 48hrs, then lysed and immunoblotted for c-IAP2 and TRAF1 specific antibodies and a light-chain-specific anti-Rabbit -horseradish peroxidase. This experiment was conducted three independent times.

### *4.6 Effects of endogenous production of c-IAP2 and TRAF1 in B-cell lymphoma line.*

To determine whether TRAF1-mediated stabilization of cIAP2 is physiologically relevant, we investigated if this phenomenon also occurs in cells that endogenously express both cIAP2 and TRAF1. Thus, we utilized RAJI cells, which express high levels of endogenous TRAF1 and cIAP2. To this end, we employed RAJI cells where TRAF1 expression has been knocked down by RNA interference (shTRAF1 RAJI) as well as a control knockdown (shCTRL RAJI). Remarkably, basal cIAP2 expression levels in shCTRL cells were significantly higher than those in shTRAF1 cells (see Figures 19). Furthermore, it is clear that there is a significant decrease in the levels of cIAP2 with the treatment of CHX at 4 and 6 hrs in the shTRAF1 cells (Figures 19). This decrease is not as significant when compared to the expression levels in shCTRL cells. Therefore, TRAF1 can prevent cIAP2 degradation in immune cells as well such as B-lymphoma cells.

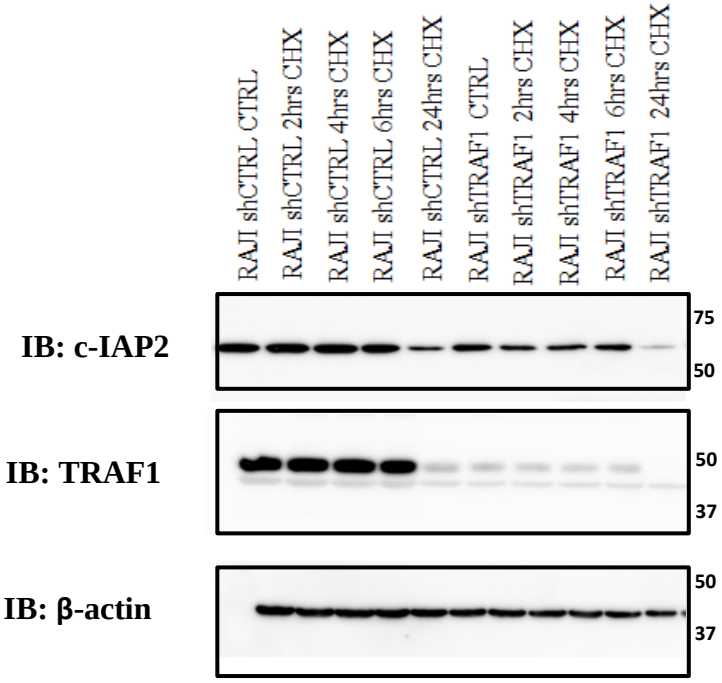


**Figure 19 Endogenous c-IAP2 expression levels in B-lymphoma cells.** An endogenous measurement of the proteins of interest in ShCTRL RAJI cells (1-5) and ShTRAF1 RAJI cells (6-10). Cells were treated with 5ug/ul Cycloheximide (CHX) at 2hrs, 4hrs, 6hrs, and 24hrs and immunoblotted for c-IAP2 and TRAF1 specific antibodies and a light-chain-specific anti-Rabbit -horseradish peroxidase. This experiment was conducted three independent times.

### *4.7 TRAF1 protects cIAP2 from proteasomal degradation.*

In order to determine where the protein degrades in the cell, different inhibitors were used that block distinct mechanisms of protein degradation. HEK 293T/17 cells were treated with either MG-132, which inhibits proteasomal degradation, Cholorquine (CQ) (which inhibits the fusion of the lysosome with autophagosome), or Bafilomycin (which is an autophagosomal inhibitor) prior to various time points of CHX treatments. Figure 21 shows that cIAP2 expression levels were increased with MG-132 treatment while there was no significant difference in the expression levels with CQ and Bafilomycin (not shown) treatments. Similarly, the expression levels of cIAP2 in the cells that are transfected with TRAF1 also exhibit a similar trend (figure 21). These findings indicate that cIAP2 is likely being degraded in the proteasome, where interaction with TRAF1 prevents this process. Given that proteasomal degradation entails tagging the protein with K48-linked ubiquitin chains, the ubiquitin ligase that causes cIAP2 degradations remains unknown.

.

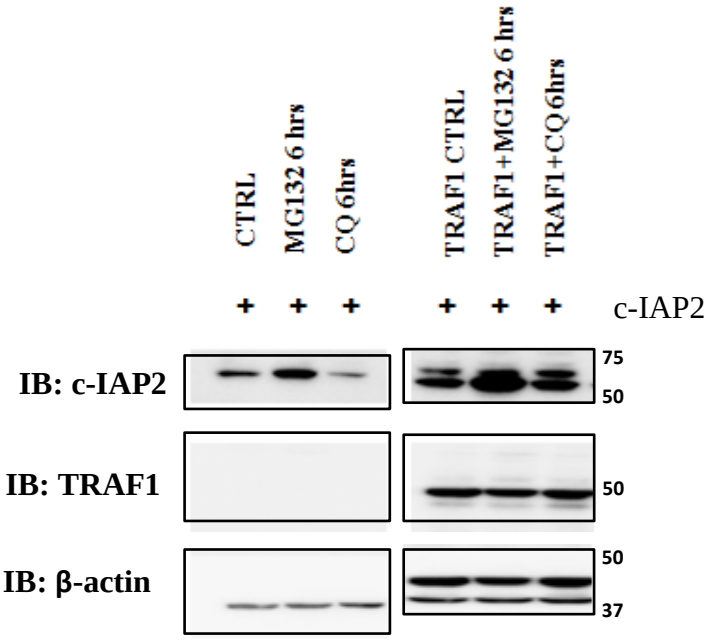


**Figure 20 c-IAP2 expression levels with different pharmacological inhibitors.** An over-expression in HEK293T/17 cells treated with MG-132 at 6hrs or with Chloroquine at 6hrs and immunoblotted for c-IAP2 and TRAF1 specific antibodies and a light-chain-specific anti-Rabbit -horseradish peroxidase. The blot was re-probed for β-actin and the upper band seen is left-over from the previous probe (TRAF1). This experiment was conducted two independent times.

## 5.0 Discussion

Previous research has shown that TRAF1 participates in multiple immune pathways and depending on the pathway activated it can either lead to an increase or a decrease in NF-**κ**B activation [34,35,37]. TRAF1 down regulates NF-**κ**B activity downstream of receptors expressed on innate immune cells (e.g. monocytes) such as TLRs by inhibiting the function of LUBAC and blocking the catalysis of linear ubiquitin chains [38–40]. However, TRAF1 enhances NF-**κ**B activation downstream of receptors expressed on adaptive immune cells (i.e. lymphocytes) such as TNF receptors by recruiting cIAP2[35,36]. These opposing roles of TRAF1 highlight the importance of this adaptor protein to the development of autoimmune diseases. Therefore, targeting TRAF1 indiscriminately by blocking its function would not be an effective strategy. This is because by doing so, we would be reducing lymphocyte survival but also at the same time, increasing inflammation from monocytes. Instead, we propose that we should design strategies to inhibit TRAF1's ability to induce recruit cIAP2 to NF-κB activation and survival of lymphocytes, while maintaining its ability in limiting inflammation by blocking LUBAC assembly in monocytes. To achieve this, we must first elucidate the sites of interaction between TRAF1 and cIAP2 then clone TRAF1 mutants that fail to interact with cIAP2 while maintaining interaction with LUBAC.

We generated multiple single mutations within the coiled-coil domain of TRAF1 (see figure 4). Mutating the residues valine 203 and glutamic acid 207 to alanine led to a substantial reduction in the interaction between TRAF1 and cIAP2. Furthermore, the double mutant of these two residues (TRAF1V203AE207A) showed a near complete abrogation of this interaction. This strongly indicates that the interaction between cIAP2 and TRAF1 occurs at these two amino

acids residues. However, it is important to confirm that these mutants only affect the interaction with cIAP2 but not with other TRAF1 signaling partners, such as TRAF2 and LUBAC. Indeed, we demonstrated that while these mutations abolished the interaction of TRAF1 with cIAP2, they retained its ability to interact with both TRAF2 and HOIL-1.

The second component to this project was to look at the effect of TRAF1 in stabilizing cIAP2. A consistent phenomenon that we kept observing while performing the immunoprecipitation experiments is that the expression of cIAP2 when co-transfected with wild type TRAF1 is significantly greater compared to the expression of cIAP2 when transfected alone. Thus, we investigated this further by determining whether TRAF1 enhances the stability of cIAP2 protein by treating the cells with cycloheximide (CHX). cIAP2 expression was lower following 1 hr of CHX treatments when expressed alone, but its expression was remarkably higher when co-expressed with TRAF1.

Next, we wanted to examine whether stabilization of cIAP2 requires direct interaction with TRAF1 or whether TRAF1 overexpression is indirectly causing this phenomenon. To this end, cIAP2 was overexpressed in 293T/17 cells either alone or along with wild type TRAF1 (TRAF1 FL) or mutant TRAF1 that do not interact with cIAP2 (i.e. TRAF1V203A and TRAF1E207A). Remarkably, cIAP2 expression levels were only stabilized when co-transfected with WT TRAF1 but not when co-transfected with the interaction-deficient mutants. As such, it is clear that it is the protein-to-protein interaction between TRAF1 and cIAP2 that aids in prolonging the half-life of cIAP2 protein and preventing its degradation. Although these findings are quite convincing, it is worth noting that they were all measured in an overexpression system in HEK293T/17 cells which are not immune cells. Consequently, it is important to look at the effects of TRAF1 on cIAP2 levels endogenously in a more relevant cell line.

To this end, we utilized RAJI cells, where TRAF1 is endogenously expressed and plays an important role in TNFR signaling. We stably knocked down TRAF1 expression in these cells by RNA interference (shTRAF1 RAJI) and used a non-targeting RNAi as a control knockdown (shCTRL RAJI). Examining the effect of TRAF1 on cIAP2 in these cells will provide a more realistic and a more relevant interpretation. The results show a similar trend as observed in previous experiments. Loss of TRAF1 expression (shTRAF1) led to a significant decrease in cIAP2 expression compared to control cells (shCTRL cells) at basal levels (no CHX treatments). Moreover, cIAP2 decreased dramatically over 4hrs and 6hrs of CHX treatments in shTRAF1 cells compared to shCTRL. This suggests that TRAF1 can protect cIAP2 from rapid degradation in immune cells as well.

After demonstrating the effects of TRAF1 on cIAP2 stabilization, we examined where the protein is being degraded in the cell. Transfected HEK293 cells were treated with different inhibitors: MG-132 (proteasomal inhibitor), chloroquine diphosphate salt (inhibit fusion of lysosome with autophagosome) and Bafilomycin (autophagosomal inhibitor) . The results show that the expression levels of cIAP2 were restored with MG-132 in comparison to chloroquine and bafilomycin treatments. Increasing cIAP2 levels upon MG-132 treatments indicates that the protein is, in all likelihood, being degraded in the proteasome. Intriguingly, the levels of cIAP2 also increased with MG-132 even when co-transfected with wild type TRAF1. Given that, we hypothesized that there are two pools of cIAP2 in the cells: one that is bound to TRAF1 and is therefore protected from degradation, while the non-TRAF1 bound pool continues to be degraded.

Previous literature note that cIAPs inhibit cell death both by binding to and inhibiting caspase activity or by ubiquitinating the pro-apoptotic mitochondrial protein Smac/DIABLO

[34,36]. Past studies have also shown that cIAP1 participates in ubiquitinating cIAP2 and cause its degradation [62–64]. A formerly published study demonstrated that upon stimulation of the TNF-receptor cIAP1 ubiquitinates TRAF2 and causes its proteasomal degradation. This study also established that overexpression of a mutated version of cIAP1 (E-3 inactive) prevented the degradation of TRAF2 and ultimately apoptosis [62,63]. Moreover, lower levels of cIAP2 witnessed in these studies was linked to the constant ubiquitination of cIAP2 by cIAP1 [64–66]. Given this information, we hypothesized that over-expressing c-IAP2 with wild-type TRAF1 will also prevent further ubiquitination of c-IAP2 by the ubiquitin protein ligase cIAP1. Nonetheless, this will be further investigated [in the future directions] using catalytically inactive mutant versions of cIAP1/2.

Lastly, we were successful at creating a TRAF1 inducible HEK293 stable cell line. The purpose of this is to establish a stable cell model where TRAF1 is induced over time rather than depleted. The benefit of this model is to examine the expression levels of our proteins of interest at longer time periods. Longer treatments with CHX increased cell death since it inhibits protein synthesis indiscriminately and thus making it challenging to examine cIAP2 levels at longer time points. Another advantage of this model is that all the cells are homogenous in culture which will aid in decreasing the chances of variability related to transfection efficiency between conditions. We predicted that cIAP2 expression levels will increase as levels of TRAF1 increase. HEK293 MCSI-TRAF1 cells were transfected with cIAP2 and treated with Doxycycline for 16hrs, 24hrs, and 48hrs. The results show a significant increase in cIAP2 expression at 24 and 48 hrs compared to 16 hrs concomitant with the increase in TRAF1 expression. These observations support our hypothesis and prove that TRAF1 does not only have a role in recruiting cIAP2to the TNFR complex but also in stabilizing it from rapid degradation. It is worth mentioning that we

also generated a cIAP1 stable cell line to test how inducing cIAP1 would affect cIAP2 levels; however the results did not show a clear trend and were arbitrary. Possibly, this is because HEK293 cells produce cIAP1 endogenously which led to a constant production of the protein.

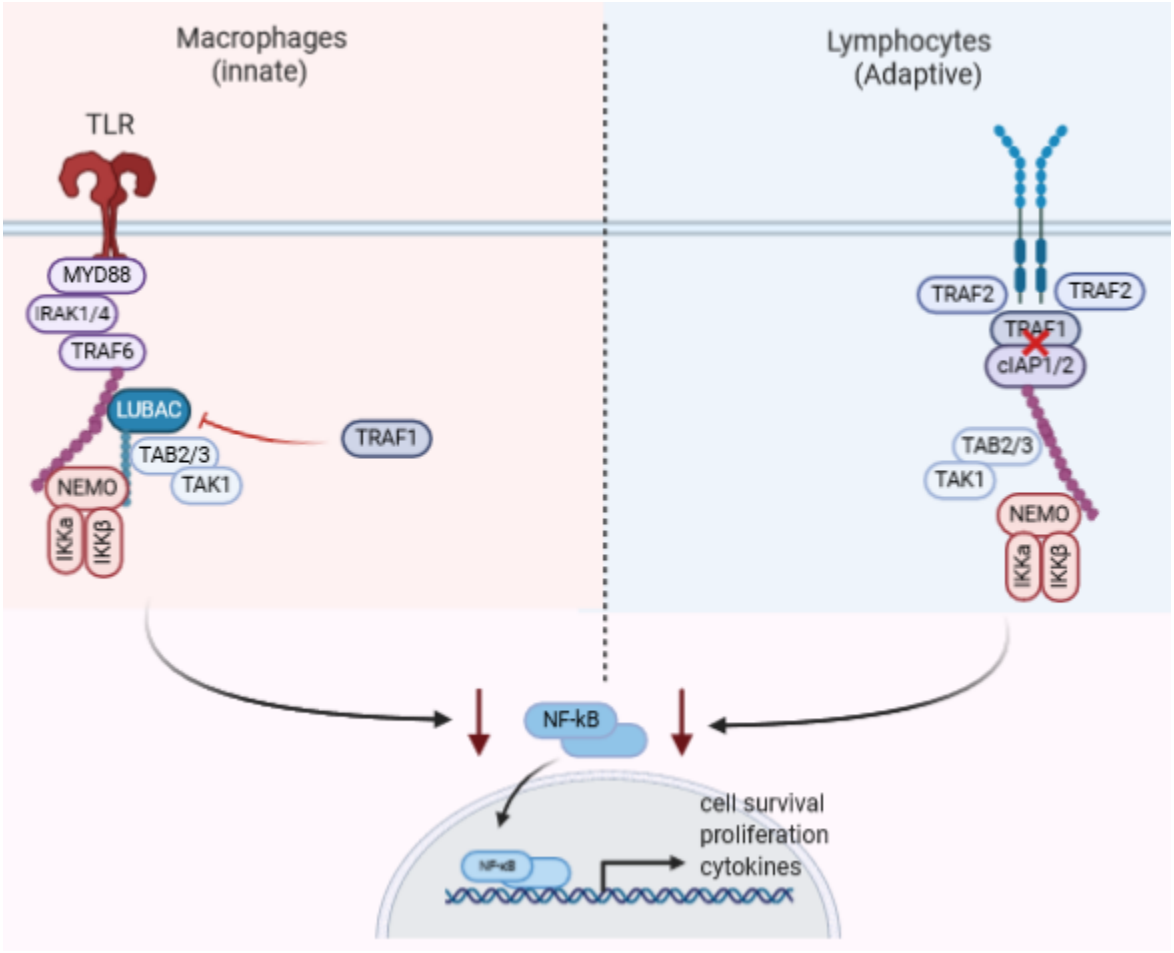


**Figure 21 A summary diagram of the different roles of TRAF1 downstream of different receptors in innate and adaptive immune cells. Image created on BioRender.com.**

## 6.0 Limitations

One major limitation of this study is the lack of proper functional assay to test NF-κB activation. Overall, the purpose of our project is to find a TRAF1 mutant that does not interact with cIAP2 downstream of TNFR signalling pathway in lymphocytes, while maintaining its negative regulation on NF-κB activation downstream of TLR pathway in innate immune cells. The NF-κB reporter assay that was performed did not give any clear results and was inconclusive. Thus, our next step is to construct a working functional assay to confirm that our TRAF1 mutants inhibit NF-κB activity downstream of TNFR signalling pathway. It is also important to mention that the study is an over-expression study in HEK293 cells which are not immune cells. Immune cells do not take up foreign DNA as readily as HEK293 cells and hence they were used to establish a cellular model to test our hypothesis. Therefore, it is important to see if the results are replicable in a more relevant immune cell line such as THP-1 or JURKAT cells.

## 7.0 Future Directions

With the insights of this study, the next step is to create a working functional assay to test the effect of these mutants on NF-κB activation. One way to achieve this is to study these mutants downstream of both the TLR and the TNFR in TRAF1 knockout THP-1 cells. Also, create a TRAF1V203A and E207A knock-in in THP-1 cells and measure NF-kB activity through measuring pro-inflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6).

Another cell line that we will be testing these mutants in is JURKAT cells, a T lymphocyte cell line. These cells have an abundance of 4-1BB receptors that when activated will activate NF-κB in a manner that is dependent on c-IAP2 and TRAF1. These cells will be electroporated in order to introduce the mutated versions of TRAF1, and NF-κB activation will be measured by Western blotting. Once we examine the effects of these changes on both cell lines the next step is to create a knock-in mouse that contains the mutated TRAF1 and observe the state of inflammation through the measurements of pro-inflammatory cytokines then ultimately use these findings to expand our understanding of autoimmune diseases including RA. Lastly, we will examine the mechanism of how cIAP2 is being degraded in the cell by using catalytically inactive forms of both cIAP1/2.

## 8.0 Conclusion

Our study is the first to find the exact sites of interaction betweenTRAF1 and c-IAP2. We present the novel finding that mutating the amino acid residues valine 203 and glutamic acid 207 into alanine significantly reduces the interaction with c-IAP2. Our findings demonstrate that these residues are specific for the interaction of TRAF1 with cIAP2 since the interaction with both TRAF2 and HOIL-1 were not affected by these mutants. Moreover, TRAF1 does not only recruit cIAP2 but also protects it from degradation. Using pharmacological inhibitors, we concluded that cIAP2 is being degraded in the proteasome. In addition, we performed similar experiments on RAJI cells and found that TRAF1-mediated stabilization of cIAP2 occurs in endogenously expressed proteins.

## 9.0 References:


1. Rosenblum, M. D., Remedios, K. A. & Abbas, A. K. Mechanisms of human autoimmunity. *J. Clin. Invest.* **125**, 2228–2233 (2015).
2. Kim, H.-J. & Cantor, H. Regulatory T cells subdue an autoimmune disease. *Nature* **572**, 443–445 (2019).
3. Cope, A. P., Schulze-Koops, H. & Aringer, M. The central role of T cells in rheumatoid arthritis. *Clin. Exp. Rheumatol.* **25**, S4-11 (2007).
4. Firestein, G. S. Evolving concepts of rheumatoid arthritis. *Nature* **423**, 356–361 (2003).
5. Köhler, B. M., Günther, J., Kaudewitz, D. & Lorenz, H.-M. Current Therapeutic Options in the Treatment of Rheumatoid Arthritis. *J. Clin. Med.* **8**, (2019).
6. Karlson, E. W., & Deane, K. (2012, May). Environmental and gene-environment interactions and risk of rheumatoid arthritis. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3402910/.
7. Karlson, E. W. & Deane, K. Environmental and gene-environment interactions and risk of rheumatoid arthritis. *Rheum. Dis. Clin. North Am.* **38**, 405–426 (2012).
8. van Halm, V. P. *et al.* Lipids and inflammation: serial measurements of the lipid profile of blood donors who later developed rheumatoid arthritis. *Ann. Rheum. Dis.* **66**, 184–188 (2007).
9. Nell, V. *et al.* Autoantibody profiling as early diagnostic and prognostic tool for rheumatoid arthritis. *Ann. Rheum. Dis.* **64**, 1731–1736 (2005).
10. Mellado, M. *et al.* T Cell Migration in Rheumatoid Arthritis. *Front. Immunol.* **6**, (2015).
11. Pennock, N. D. *et al.* T cell responses: naïve to memory and everything in between. *Adv. Physiol. Educ.* **37**, 273–283 (2013).

12. Medzhitov, R., Preston-Hurlburt, P. & Janeway, C. A. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **388**, 394–397 (1997).
13. Carlota, C., Marruecos, L., Vert, A., Bigas, A. & Liuis, E., NF-κB Members Left Home: NF-κB-Independent Roles in Cancer. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5489812/.
14. Lawrence, T. The Nuclear Factor NF-κB Pathway in Inflammation. *Cold Spring Harb. Perspect. Biol.* **1**, (2009).
15. Hayden, M. S. & Ghosh, S. Shared principles in NF-kappaB signaling. *Cell* **132**, 344–362 (2008).
16. Beinke, S. & Ley, S. C. Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. *Biochem. J.* **382**, 393–409 (2004).
17. Vallabhapurapu, S. & Karin, M. Regulation and function of NF-kappaB transcription factors in the immune system. *Annu. Rev. Immunol.* **27**, 693–733 (2009).
18. Karin, M. & Delhase, M. The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. *Semin. Immunol.* **12**, 85–98 (2000).
19. Simmonds, R. E. & Foxwell, B. M. Signalling, inflammation and arthritis: NF-kappaB and its relevance to arthritis and inflammation. *Rheumatol. Oxf. Engl.* **47**, 584–590 (2008).
20. Wajant, H., Henkler, F. & Scheurich, P. The TNF-receptor-associated factor family: scaffold molecules for cytokine receptors, kinases and their regulators. *Cell. Signal.* **13**, 389–400 (2001).
21. McPherson, A. J., Snell, L. M., Mak, T. & Watts, T. Opposing Roles for TRAF1 in the Alternative versus Classical NF-κB Pathway in T Cells. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3391120/.

22. Carpentier, I. & Beyaert, R. TRAF1 is a TNF inducible regulator of NF-kappaB activation. *FEBS Lett.* **460**, 246–250 (1999).
23. Wicovsky, A. *et al.* Tumor necrosis factor receptor-associated factor-1 enhances proinflammatory TNF receptor-2 signaling and modifies TNFR1-TNFR2 cooperation. *Oncogene* **28**, 1769–1781 (2009).
24. Tsitsikov, E. N. *et al.* TRAF1 is a negative regulator of TNF signaling. enhanced TNF signaling in TRAF1-deficient mice. *Immunity* **15**, 647–657 (2001).
25. Cannons, J. L. *et al.* 4-1BB ligand induces cell division, sustains survival, and enhances effector function of CD4 and CD8 T cells with similar efficacy. *J. Immunol. Baltim. Md 1950* **167**, 1313–1324 (2001).
26. Sabbagh, L., Pulle, G., Liu, Y., Tsitsikov, E. N. & Watts, T. H. ERK-dependent Bim modulation downstream of the 4-1BB-TRAF1 signaling axis is a critical mediator of CD8 T cell survival in vivo. *J. Immunol. Baltim. Md 1950* **180**, 8093–8101 (2008).
27. Charles A Janeway, J., Travers, P., Walport, M. & Shlomchik, M. J. T Cell-Mediated Immunity. *Immunobiol. Immune Syst. Health Dis. 5th Ed.* (2001).
28. Cheuk, A. T. C., Mufti, G. J. & Guinn, B. Role of 4-1BB:4-1BB ligand in cancer immunotherapy. *Cancer Gene Ther.* **11**, 215–226 (2004).
29. Chen, L. & Flies, D. B. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat. Rev. Immunol.* **13**, 227–242 (2013).
30. Jankowska, K. I. *et al.* Integrins Modulate T Cell Receptor Signaling by Constraining Actin Flow at the Immunological Synapse. *Front. Immunol.* **9**, 25 (2018).
31. Vinay, D. S. & Kwon, B. S. 4-1BB signaling beyond T cells. *Cell. Mol. Immunol.* **8**, 281–284 (2011).

32. Vinay, D. S. & Kwon, B. S. Immunotherapy of Cancer with 4-1BB. *Mol. Cancer Ther.* **11**, 1062–1070 (2012).
33. Sanchez-Paulete, A. R. *et al.* Deciphering CD137 (4-1BB) signaling in T-cell costimulation for translation into successful cancer immunotherapy. *Eur. J. Immunol.* **46**, 513–522 (2016).
34. Cossu, F., Milani, M., Mastrangelo, E. & Lecis, D. Targeting the BIR Domains of Inhibitor of Apoptosis (IAP) Proteins in Cancer Treatment. *Comput. Struct. Biotechnol. J.* **17**, 142–150 (2019).
35. Graber, T. E. & Holcik, M. Distinct roles for the cellular inhibitors of apoptosis proteins 1 and 2 | Cell Death & Disease. https://www.nature.com/articles/cddis201120.
36. Sun, H., Lu, J., Liu, L., Yang, C.-Y. & Wang, S. Potent and selective small-molecule inhibitors of cIAP1/2 proteins reveal that the binding of Smac mimetics to XIAP BIR3 is not required for their effective induction of cell death in tumor cells. *ACS Chem. Biol.* **9**, 994–1002 (2014).
37. Dhillon, B., Aleithan, F., Abdul-Sater, Z. & Abdul-Sater, A. A. The Evolving Role of TRAFs in Mediating Inflammatory Responses. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6369152/.
38. Li, X., Jiang, S. & Tapping, R. I. Toll-like receptor signaling in cell proliferation and survival. *Cytokine* **49**, 1–9 (2010).
39. Arch, R. H., Gedrich, R. W. & Thompson, C. B. Tumor necrosis factor receptor-associated factors (TRAFs)--a family of adapter proteins that regulates life and death. *Genes Dev.* **12**, 2821–2830 (1998).
40. Zapata, J. M. *et al.* CD137 (4-1BB) Signalosome: Complexity Is a Matter of TRAFs. *Front. Immunol.* **9**, (2018).

41. MacEwan, D. J. TNF ligands and receptors – a matter of life and death. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1573213/.
42. Lalani, A. I., Zhu, S., Gokhale, S., Jin, J. & Xie, P. TRAF Molecules in Inflammation and Inflammatory Diseases. *Curr. Pharmacol. Rep.* **4**, 64–90 (2018).
43. Deshaies, R. J. & Joazeiro, C. A. P. RING domain E3 ubiquitin ligases. *Annu. Rev. Biochem.* **78**, 399–434 (2009).
44. Liu, T., Zhang, L., Joo, D. & Sun, S.-C. NF-κB signaling in inflammation. *Signal Transduct. Target. Ther.* **2**, 1–9 (2017).
45. Abdul-Sater, A. A. *et al.* The signaling adaptor TRAF1 negatively regulates Toll-like receptor signaling and this underlies its role in rheumatic disease. *Nat. Immunol.* **18**, 26–35 (2017).
46. Ostuni, R., Zanoni, I. & Granucci, F. Deciphering the complexity of Toll-like receptor signaling. *Cell. Mol. Life Sci. CMLS* **67**, 4109–4134 (2010).
47. Vollmer, S. *et al.* The mechanism of activation of IRAK1 and IRAK4 by interleukin-1 and Toll-like receptor agonists. *Biochem. J.* **474**, 2027–2038 (2017).
48. Berglund, N. A., Kargas, V., Ortiz-Suarez, M. L. & Bond, P. J. The role of protein-protein interactions in Toll-like receptor function. *Prog. Biophys. Mol. Biol.* **119**, 72–83 (2015).
49. Kollewe, C. *et al.* Sequential autophosphorylation steps in the interleukin-1 receptor-associated kinase-1 regulate its availability as an adapter in interleukin-1 signaling. *J. Biol. Chem.* **279**, 5227–5236 (2004).
50. Xia, Z.-P. *et al.* Direct activation of protein kinases by unanchored polyubiquitin chains. *Nature* **461**, 114–119 (2009).

51. Vollmer, S. *et al.* The mechanism of activation of IRAK1 and IRAK4 by interleukin-1 and Toll-like receptor agonists. *Biochem. J.* **474**, 2027–2038 (2017).
52. Guo, H., Callaway, J. B. & Ting, J. P.-Y. Inflammasomes: mechanism of action, role in disease, and therapeutics. *Nat. Med.* **21**, 677–687 (2015).
53. Wajant, H. & Siegmund, D. TNFR1 and TNFR2 in the Control of the Life and Death Balance of Macrophages. *Front. Cell Dev. Biol.* **7**, (2019).
54. Stringer, D. K. & Piper, R. C. Terminating protein ubiquitination: Hasta la vista, ubiquitin. *Cell Cycle Georget. Tex* **10**, 3067–3071 (2011).
55. Ellgaard, L., Laugesen, S. & McCellan, A. J. Cellular functions and molecular mechanisms of non-lysine ubiquitination - PubMed. https://pubmed.ncbi.nlm.nih.gov/31530095/.
56. Swatek, K. N. & Komander, D. Ubiquitin modifications. *Cell Res.* **26**, 399–422 (2016).
57. Mallette, F. A. & Richard, S. K48-linked ubiquitination and protein degradation regulate 53BP1 recruitment at DNA damage sites. *Cell Res.* **22**, 1221–1223 (2012).
58. Edilova, M. I., Abdul-Sater, A. A. & Watts, T. H. TRAF1 Signaling in Human Health and Disease. *Front. Immunol.* **9**, (2018).
59. Karpova, M. B. *et al.* Raji revisited: cytogenetics of the original Burkitt's lymphoma cell line | Leukemia. https://www.nature.com/articles/2403534.
60. Hsum, Y. W. *et al.* Cancer chemopreventive activity of maslinic acid: suppression of COX-2 expression and inhibition of NF-κB and AP-1 activation in Raji cells. *Planta Med.* **77**, 152–157 (2011).
61. Kim, C. M. *et al.* Crystal structure of TRAF1 TRAF domain and its implications in the TRAF1-mediated intracellular signaling pathway. *Sci. Rep.* **6**, 25526 (2016).

62. Darding, M. *et al.* Molecular determinants of Smac mimetic induced degradation of cIAP1 and cIAP2. *Cell Death Differ.* **18**, 1376–1386 (2011).
63. Varfolomeev, E. *et al.* c-IAP1 and c-IAP2 Are Critical Mediators of Tumor Necrosis Factor α (TNFα)-induced NF-κB Activation. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3259840/.
64. Conze, D. B. *et al.* Posttranscriptional downregulation of c-IAP2 by the ubiquitin protein ligase c-IAP1 in vivo. *Mol. Cell. Biol.* **25**, 3348–3356 (2005).
65. Bertrand, M. J. M. *et al.* cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. *Mol. Cell* **30**, 689–700 (2008).
66. Wang, L., Du, F. & Wang, X. TNF-alpha induces two distinct caspase-8 activation pathways. *Cell* **133**, 693–703 (2008).

## 10.0 Appendix

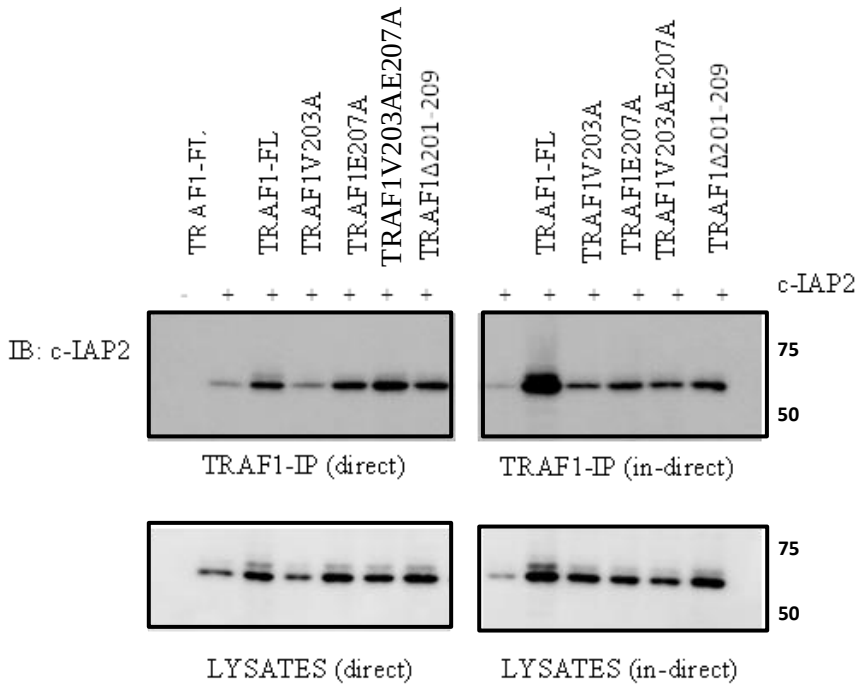


Figure 22 mutant TRAF1 do not pull downcIAP2. Immunoprecipitation with TRAF1 specific antibody using HEK293 lysates. A) IP was done through incubating the antibody with the magnetic beads. B) IP was done through incubating the antibody with the lysates. Both were immunoblotted for a cIAP2 specific antibody and a light-chain-specific anti-Rabbit -horseradish peroxidase. Experiment was performed three independent times.

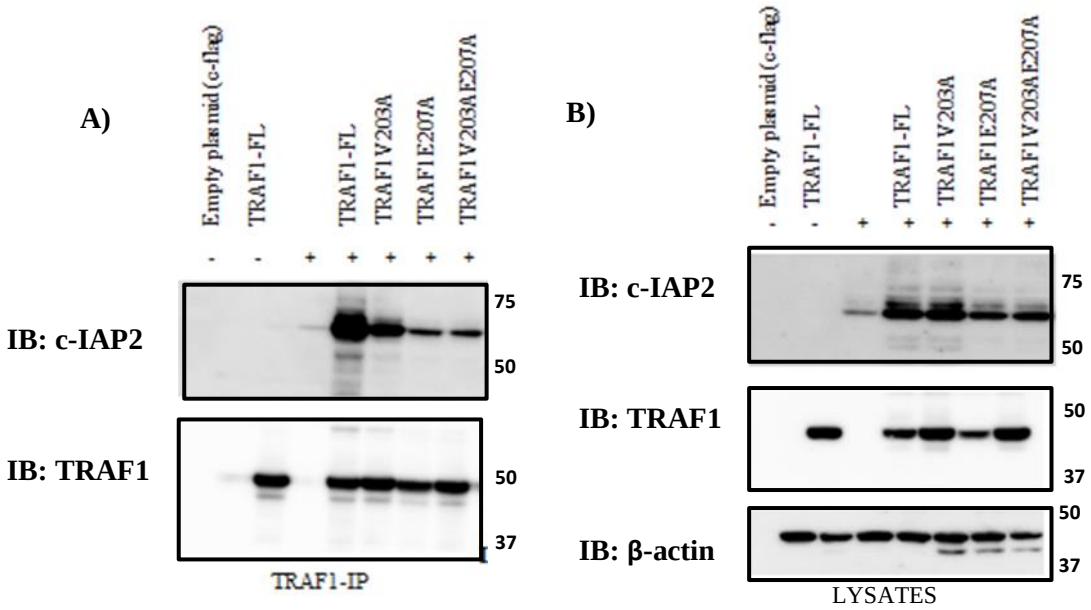


Figure 23 A) optimized IP conditions to reduce non-specific binding of cIAP2 to the magnetic beads. Immunoprecipitation (IP) with a TRAF1 specific antibody (IF3) of HEK 293 lysates transfected with the plasmids indicated below and B) Input of the same lysates then immunoblotted for cIAP2 and TRAF1 specific antibody and a light-chain-specific anti-Rabbit - horseradish peroxidase. Experiment was performed three independent times.

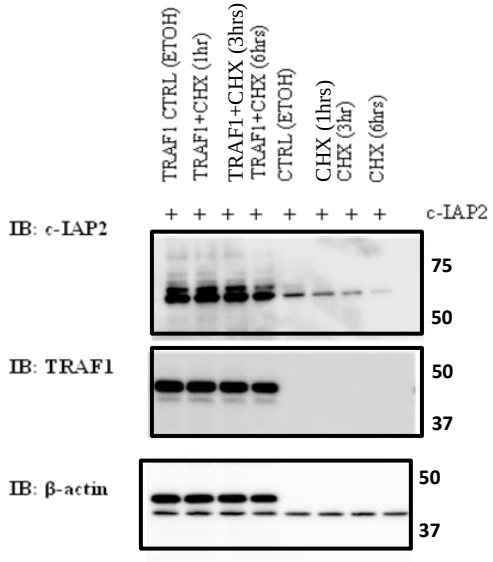


Figure 24 TRAF1 stabilizes cIAP2. An overexpression in HEK 293 cells transfected with c-IAP2 alone or c-IAP2 with TRAF1 FL. Cells were treated with 5ug/ul CHX at: 1hr, 3hrs, and 6hrs then immunoblotted with c-IAP2 and TRAF1 specific antibody and a light-chain-specific anti-Rabbit -horseradish peroxidase. Experiment was performed three independent times.

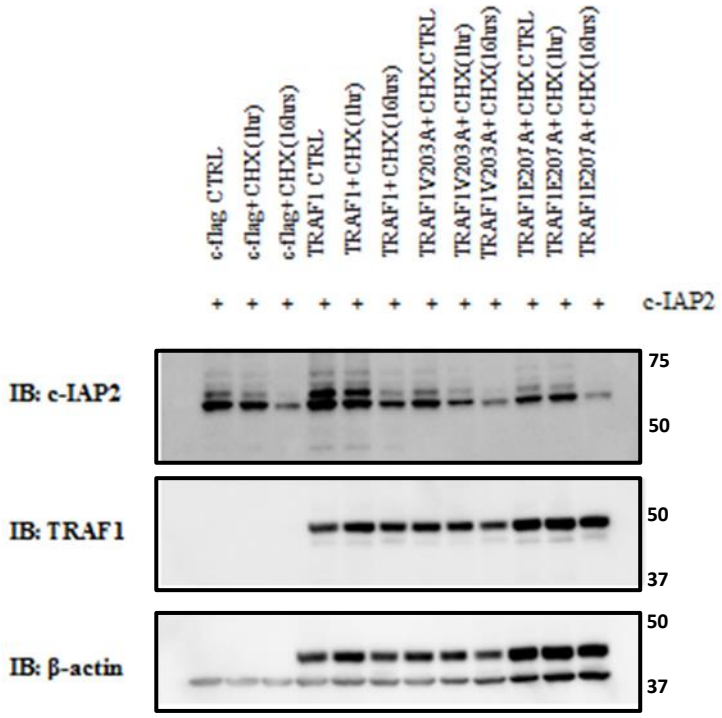


Figure 25 An over expression in HEK293 cells transfected with either c-IAP2 alone, or c-IAP2 with TRAF1 FL or c-IAP2 with either TRAF1V203A or TRAF1E207A. Cells were then treated with CHX at 1hrs and 16 hrs then immunoblotted with c-IAP2 and TRAF1 specific antibody and a light-chain-specific anti-Rabbit -horseradish peroxidase. Experiment was performed three independent times.

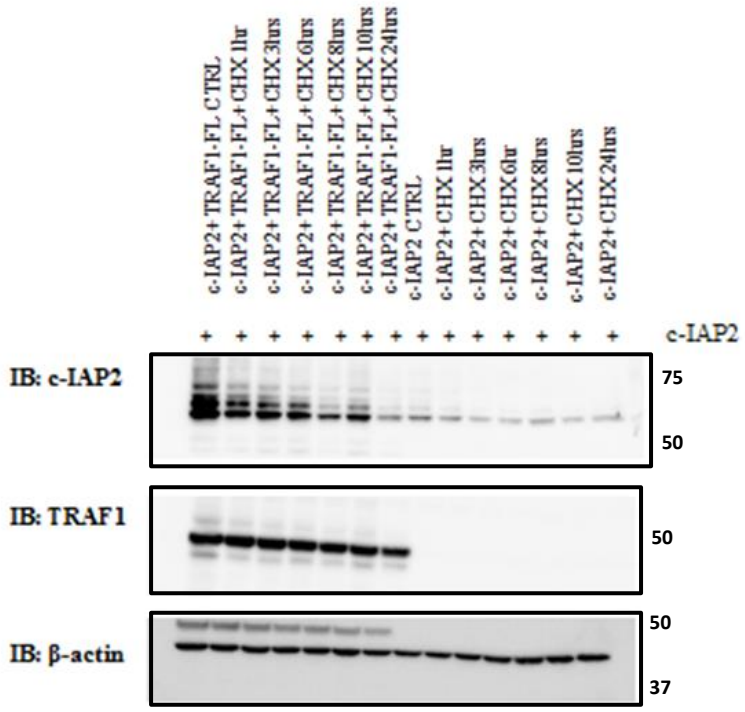


Figure 26 optimization of the CHX chase assay. An over expression in HEK293 cells transfected with either c-IAP2 alone or c-IAP2 with TRAF1 FL. Cells were then treated with CHX at an extended time points: 1hr, 3hrs, 6hrs, 8hrs, 10hrs, and 24hrs then immunoblotted with c-IAP2 and TRAF1 specific antibody and a light-chain-specific anti-Rabbit -horseradish peroxidase. Experiment was done three independent times.


Figure 27 TRAF1 expressions with various amount of DOXY. An over expression in MCSI-TRAF1 inducible cell line transfected with c-IAP2 and treated with either 1ug/ul or 2ug/ul of Doxycycline then immunoblotted with TRAF1-specific antibody and a light-chain-specific anti-Rabbit -horseradish peroxidase. Experiment was performed three independent times.

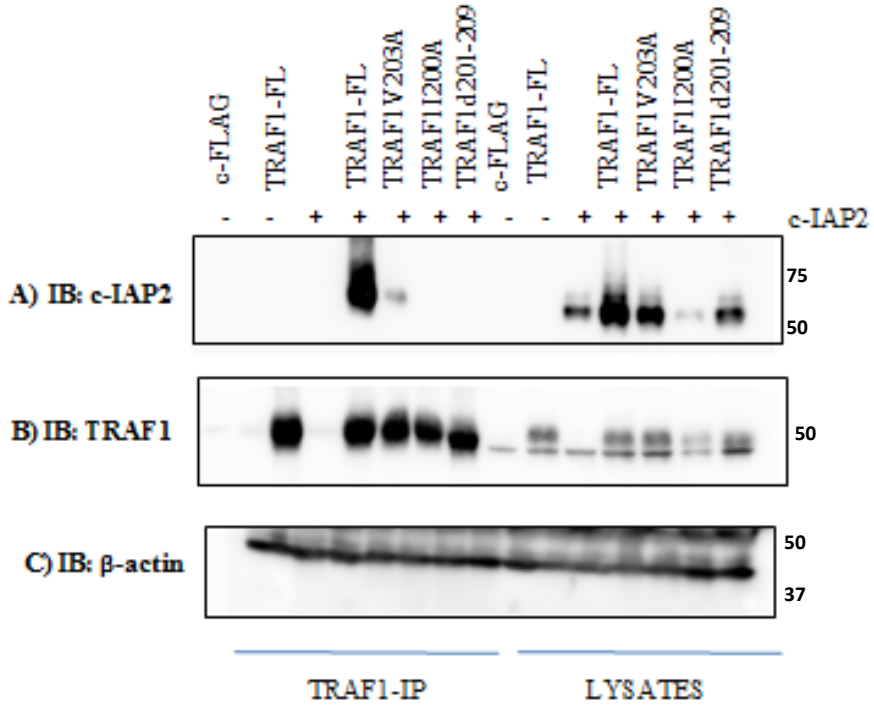


Figure 28 Effects of TRAF1I200A on the interaction with c-IAP2. Immunoprecipitation (IP) with TRAF1-specific antibody of lysates of HEK 293T/17 cells transfected with 1) c-Flag pcDNA3 vector, 2) TRAF1 FL and c-flag, 3) c-IAP2 and c-Flag pcDNA3 vector, 4) c-IAP2 and TRAF1 FL, 5) c-IAP2 and TRAF1V203A, 6) c-IAP2 and TRAF1I200A,7) c-IAP2 and TRAF1Δ201-209 followed by immunoblotting (IB) with c-IAP and TRAF1 specific antibody and a light-chain-specific anti-Rabbit -horseradish peroxidase. This experiment was conducted three independent times.

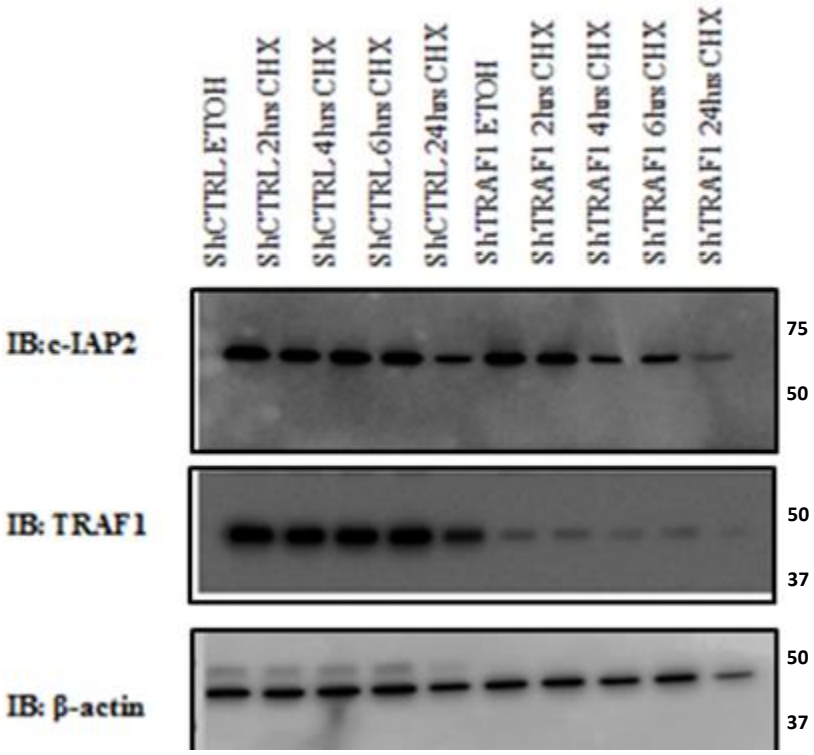


Figure 29 shTRAF1 show reduced expression levels of c-IAP2.
An endogenous measurement of the proteins of interest in ShCTRL RAJI cells (1-5) and ShTRAF1 RAJI cells (6-10). Cells were treated with 5ug/ul cycloheximide (CHX) at 2hrs, 4hrs, 6hrs, and 24hrs and immunoblotted for c-IAP and TRAF1 specific antibodies and a light-chain-specific anti-Rabbit -horseradish peroxidase. This experiment was conducted three independent times.