Regulatory Dynamics of XIAP and RIPK2 Ubiquitination in the NF-κB Signaling Pathway

A thesis submitted by Yangyang Liu

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Abstract

In this study, we employed immunoprecipitation (IP) techniques to elucidate the roles of key proteins RIPK2 and XIAP within a specific pathway, focusing on their expression and regulation under various stimuli including INF-γ, L18-MDP, and the presence of small molecule inhibitors. Our findings indicate that specific stimuli are crucial for the activation of the NF-κB signaling pathway and the subsequent expression of these proteins, with the inhibitor 9332 demonstrating the capacity to modulate this activation. Furthermore, we delved into the post-translational modifications of RIPK2 and XIAP using λ-Phosphatase (λ-PPase) and USP2, providing a clearer insight into the changes these proteins undergo following ubiquitination. Our results suggest that NEMO regulates the ubiquitination of XIAP and RIPK2, and that the ubiquitination of XIAP is, to some extent, influenced by RIPK2. This comprehensive exploration sheds light on the intricate regulatory mechanisms governing key components of the NF-κB signaling pathway, highlighting the importance of post-translational modifications in cellular responses to external stimuli.

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application season and beyond. I am blessed with their friendship and hope to support them in their endeavors as they have supported me.

This project, while an academic pursuit, has been a journey of personal growth, made possible by the collective support and faith of everyone mentioned above. Thank you all for your invaluable contributions to my life and this project.

Contents

List of Abbreviations

RIPK2	Receptor-interacting serine/threonine-protein kinase 2
XIAP	X linked Inhibitor of Apoptosis Protein
NOD	Nucleotide-binding Oligomerization Domain
Ub.	Ubiquitin
L18	L18-Muramyl dipeptide
IP	immunoprecipitation
WB	Western Blot
USP2	Ubiquitin carboxyl-terminal hydrolase 2

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1 Introduction

1.1 RIPK2: Orchestrating Innate Immunity Beyond Cell Death Pathways

Receptor-interacting serine/threonine-protein kinase 2 (RIPK2) is a pivotal kinase in the Nucleotide-binding Oligomerization Domain (NOD) signaling pathway, an essential component of the innate immune response. (Nachbur et al., 2015)Unlike its well-documented relatives, RIPK1 and RIPK3, RIPK2 lacks the death domain and the RHIM necessary for interacting with death receptor complexes, steering it clear from direct involvement in cell death pathways. Instead, RIPK2 is comprised of an N-terminal kinase domain and a C-terminal Caspase Activation and Recruitment Domain (CARD), which facilitates its interaction with the NOD1 and NOD2 proteins through CARD-CARD interactions, subsequently orchestrating the activation of NF-κB and MAPK pathways. The structurally dynamic intermediate domain of RIPK2 and its posttranslational modifications hint at a nuanced regulatory role in immune signaling.(Suebsuwong et al., 2018)

1.2 The Crucial Interplay of Ubiquitination in NOD Signaling

The modulatory role of ubiquitination in NOD signaling is underscored by the involvement of the X-linked Inhibitor of Apoptosis Protein (XIAP). (Bertrand et al., 2009)As a key mediator of RIPK2 ubiquitylation, XIAP has been implicated in the direct attachment of K63-linked ubiquitin chains on RIPK2, a posttranslational modification critical for the full activation of the NF-κB and MAPK pathways.(Panda & Gekara, 2018) Studies revealing the interaction between XIAP's Baculoviral IAP Repeat (BIR) domain and the kinase domain of

RIPK2 have positioned XIAP as a crucial component in the NOD signaling cascade. Understanding the nuances of this interaction, the implications of different ubiquitin linkages, and the specific roles of ubiquitination sites on RIPK2 could provide fresh insights into the regulation of innate immunity.

Ubiquitin itself, a small regulatory protein, is central to myriad cellular processes, including protein degradation, cell cycle regulation, and immune responses. (Damgaard et al., 2012)The versatility of ubiquitin-mediated signaling is largely due to the diversity of ubiquitin chain types and linkages, which can be recognized by different downstream effector proteins. Ubiquitination's role in shaping the outcome of NOD signaling, particularly through the formation of polyubiquitin chains on RIPK2.(Yang et al., 2013)

Ubiquitin as a multifaceted modifier capable of fine-tuning inflammatory responses. This posttranslational modification acts as a scaffold for assembling signaling complexes that are integral for activating key inflammatory pathways.

Given the intricate role of ubiquitination in modulating RIPK2 activity, current researches focus on delineating the precise molecular mechanisms through which ubiquitin chains orchestrate the recruitment and activation of downstream signaling proteins(Zhang et al., 2023). This might involve elucidating the interplay between different E3 ligases, such as the IAPs, and the deubiquitinases that meticulously regulate RIPK2's ubiquitin landscape.

The functional exploration of RIPK2, alongside the detailed study of XIAP and ubiquitination, provides a fertile ground for research aimed at dissecting the complexities of immune regulation. With advancements in structural biology and

proteomics, contemporary research may pave the way to new therapeutic interventions targeting the NOD pathway, with the potential to mitigate the dysregulation observed in chronic inflammatory conditions and cancers(Jost & Vucic, 2020).

1.3 Ubiquitination of NEMO: Orchestrating NF-κB Activation

NEMO (NF-κB Essential Modulator, also known as IKKγ) is the regulatory subunit of the IκB kinase (IKK) complex and plays a crucial role in the activation of NF-κB (nuclear factor-κB). Upon various stimuli, such as genotoxic damage, NEMO is activated through a specific ubiquitination process. This ubiquitination prompts the export of NEMO from the nucleus and its association with the catalytic subunits of IKK, IKKα, and IKKβ, forming a complex. The formation of this complex leads to the activation of IKK, which subsequently triggers the phosphorylation and degradation of IκBα. The degradation of IκBα releases NF-κB, allowing it to enter the nucleus and activate the transcription of target genes. Thus, the ubiquitination of NEMO is a prerequisite for the IKK-dependent activation pathway of NF-κB, serving as a key link between intracellular signaling transduction and NF-κB activation. XIAP promotes the activation of NF-κB under genotoxic stress conditions by activating the upstream IKK kinase TAK1 and connecting it to the IKK complex(Jin et al., 2009). When NOD2 recognizes specific intracellular bacterial components (such as MDP), NOD2 activates and interacts with RIPK2, which subsequently promotes the ubiquitination of NEMO. Ubiquitination of NEMO is a necessary step for the activation of the IKK

complex, which in turn leads to the activation of NF-κB and its entry into the nucleus(Abbott, Wilkins, Asara, & Cantley, 2004).

1.4 Deciphering the NF-κB Pathway: Post-Translational Modifications in Cellular Signaling

In this study, immunoprecipitation (IP) techniques were employed to pull down related proteins, utilizing these proteins as markers to investigate the relationships among XIAP, RIPK2, and Ubiquitin. The application of λ-Phosphatase (λ-PPase) and USP2 underscores the importance of phosphorylation and ubiquitination in protein stability and signaling involvement. λ-PPase, a universal phosphatase, removes phosphorylated residues, while USP2, a deubiquitinating enzyme, trims ubiquitin chains. The deubiquitination treatment effectively eliminates the size variability and smearing caused by ubiquitination, thereby rendering the detection of RIPK2 or XIAP more precise and quantifiable. (Wei, Zhao, Zhang, & Wang, 2023).

The varied expression levels of RIPK2 and XIAP across treatment groups, coupled with the influence of post-translational modifications, underscores a complex regulatory network. The absence of RIPK2 and XIAP expression in untreated wild-type cells compared to the more pronounced expression following specific treatments. These findings dovetail with the observed post-translational modifications, accentuating the function of RIPK2 and XIAP within the cellular milieu.

Through meticulous experimentation, our work shines a spotlight on the fine-tuned interplay between cellular signaling proteins and the scaffold upon

which they operate. The NF-κB pathway, pivotal in orchestrating immune and inflammatory responses, emerges as a tapestry of intricate molecular interactions, modulated by both external stimuli and internal post-translational machinations. By delving into the regulatory roles of NEMO, RIPK2, and XIAP, and their dynamic responses to different stimuli, we contribute to the expanding vista of knowledge necessary for innovating targeted therapies against inflammatory and immune-related diseases. This study paves the way for future research to further decode the complexities of the NF-κB signaling pathway.

2 Materials and Methods

2.1 Reagents

THP1 Dual cell, HCT116 pNifty2-SEAP Clone E10 Zeocin +PLVXzsgreen-3xFLAG-hRIPK2 cells, C3040 cells (Biolabs)

Antibodies for western blot analysis: Anti- RIPK2 (Cell signaling,D10B11), Anti- XIAP (Cell siganling, 3B6), Anti-ubiquitin (Thermo Fisher)

GSK2983559 (Target MOI), NUV-601(company to be confirmed), Inhibitor 9332(abcam), L18-MDP(Invivogen, tlrl-lmdp), Recombinant human his-USP2 Catalytic domain protein(Biotech, E-506-050)

Tryspin-EDTA(Gibco), Lambda Protein Phosphatase(Biolabs)

Protein assay reagent (Pierce 660nm, Thermo scientific, 22660), 37°C, 5% CO2 tissue culture incubatorTissue culture microscope(Nikon) , 100-mm tissue-culture treated dishes (Thermo Fisher), 15-ml conical (Thermo Fisher),Plastic cell scraper

Sterile Fisher Vortex Genie 2 Chilled centrifuge, Nemo magnetic beads, 3xFlag-RIPK2 magnetic beads, HAtag magnetic beads, Histag magnetic beads, Bead separation magnetic rack (Dynal Invitrogen), End-over-end rotator (Thermo Fisher), Heat block, Mini prep box (ZYMO Research)

2.2 Cell Culture

THP1 Dual cell lines were cultured in in DMEM medium (Nalgene, 10-017-CV) containing 10% Fetal Plex (FP), 1% PSA (penicillin/ streptomycin/antimycotic solution) and 1:500 Normcin. HCT cells were cultured

in DMEM medium containing 10% FBS, 1% PSA (penicillin/ streptomycin/antimycotic solution) and 1:500 Normcin .

2.3 plasmid preparation

Bacteria are initially thawed on ice for 10 minutes before 2μl of the bacteria are isolated into a clean EP tube, to which 0.5μl of plasmid is added and gently mixed. The mixture then rests on ice for 30 minutes, followed by a 30-second heat shock at 42°C, and then placed back on ice for 5 minutes. The heater is set to 30°C at 300 rpm, and 500μl of prewarmed outgrowth media is added to the tube, which is then incubated on the heater for 1.5 hours. Subsequently, 100μl of the bacteria are added to an Agar plate with 6 glass beads and gently shaken before the beads are removed and the plate is incubated at 37°C overnight. The next day, a bacterial colony is transferred into a bottle containing 100ml LB-ampicillin medium and incubated at 37°C, 250rpm for 30 hours, after which the medium is centrifuged to collect the bacterial pellet.

To isolate plasmid DNA, start by resuspending a bacterial cell pellet in 8 ml of ZymoPURE™ P1 (Red) using vortexing or pipetting for complete dissolution. Then, add 8 ml of ZymoPURE™ P2 (Blue) to the suspension, gently mixing by inverting the tube six times to avoid vortexing and allowing the mixture to sit at room temperature for 2-3 minutes until it becomes clear, purple, and viscous, indicating complete cell lysis. Next, introduce 8 ml of ZymoPURE™ P3 (Yellow) and mix gently by inverting, continuing even after the solution turns fully yellow, signaling neutralization is complete and forming a yellowish precipitate. After attaching the plug to the Luer Lock of the ZymoPURE™ Syringe Filter-X, load

the lysate and allow 5-8 minutes for the precipitate to float. Removing the Luer Lock plug, transfer the solution through the ZymoPURE™ Syringe Filter-X into a clean 50 ml conical tube by pushing the plunger in one continuous motion until about 20 ml of cleared lysate is collected, which is crucial for the subsequent plasmid DNA binding process. Mix this cleared lysate with 8 ml of ZymoPURE™ Binding Buffer thoroughly by inverting the tube eight times. Place the Zymo-Spin™ V-PS Column Assembly onto a vacuum manifold, ensuring tight connections, and with the vacuum off, add the lysate-buffer mixture into the column, turning on the vacuum to filter through all liquid. After discarding the 50 ml Reservoir, wash the column first with 5 ml of ZymoPURE™ Wash 1, then twice with 5 ml of ZymoPURE™ Wash 2, ensuring the vacuum is off between additions and turned on to pass the liquid completely through the column. Post-wash, centrifuge the Zymo-Spin™ V-PS Column in a Collection Tube at ≥ 16,000 x g for 1 minute to remove residual wash buffer, then transfer the column to a clean 1.5 ml microcentrifuge tube. Elute the plasmid DNA by adding 200 µl pure water to the column, waiting for 2 minutes before centrifuging at ≥ 16,000 x g for 1 minute to recover the purified plasmid DNA.

2.4 Transfection

Begin by waiting until the cell density in each plate reaches approximately 80-90%. Once this density is achieved, replace the medium, and wait for two hours before starting the transfection process. To prepare for transfection, mix 1 ml of Opti-MEM (Gibco) with 37.5 µl of Polyethylenimine(PEI) and 12.5 ng of DNA. Allow this mixture to sit quietly for 20 minutes to ensure proper mixing.

After the waiting period, add the reagent mixture directly to the cells' plate. The transfection will be complete 16 hours after the addition of the reagent to the cells.

2.5 Pull-down preparation

For NEMO/3xFLAG RIPK2/HA tagged ubiquitin pull down preparation: Cells are washed with 1ml of PBS per plate, and this PBS, now containing the cells, is transferred sequentially through all plates to ensure complete collection.

The cell-containing PBS is then transferred to an Eppendorf tube and centrifuged at 5000rpm for 2 minutes. The supernatant is discarded, and cells are resuspended in fresh PBS. This suspension undergoes a second centrifugation at 5000rpm for 2 minutes, after which the PBS is removed. Cells are then lysed by adding lysis buffer(10% NP-40, 0.5% Triton X-100, 500mM Tris-Cl, pH7.2-7.4, 150mM Nacl, 1mM EDTA,1mM EGTA, 3mM NaF, 1mM Na3VO4, 10mM beta-glycerophosphate, 1uM aprotinin, leupeptin, pepstatin, 1uM PMSF) containing PMSF, and the mixture is incubated in a cold room for 30 minutes. Following this, a centrifugation at 1000xg for 15 minutes at 4°C is performed, and the clear supernatant is collected for protein assay and normalization.

For bead preparation, 100µl beads are placed into a 1.5ml Eppendorf tube, washed three times with 500µl of lysis buffer without PMSF, and then supernatant is removed. The beads are incubated with 1ml of 0.5% gelatin in water for one hour, followed by three washes with 500µl of lysis buffer without PMSF.

Sample preparation involves taking 75µl of the normalized lysate, adding 4xSDS, and boiling the mixture at 95°C for 5 minutes. The beads are aliquoted,

combined with the lysate, and incubated in cold room for 2 hours. After incubation, the beads are washed three times with 500µl of wash buffer(10% NP-40, 0.5% Triton X-100, 500mM Tris-Cl, pH7.2-7.4, 300mM Nacl). USP2 and DTT are prepared in a mixture containing 0.143µl USP2 and 1.588µl 0.1M DTT in 30µl of DUB buffer. Samples are then aliquoted for further processing. For IP samples, the supernatant is removed, 30µl of lysis buffer and 10µl of 4xSDS are added, and samples are boiled at 95°C for 5 minutes to collect the supernatant. USP2 samples are similarly processed, with the addition of prepared DUB buffer before incubation at 37°C for 30 minutes, followed by the addition of 10µl of 4xSDS and boiling at 95°C for 5 minutes.

For His-tagged ubiquitin pull down preparation:

To purify proteins under denaturing conditions, start by resuspending the cell pellet in 1 ml of Guanidinium Lysis Buffer(6 M Guanidine Hydrochloride, 20 mM Sodium Phosphate, pH 7.8, 500 mM NaCl). Centrifuge at 3,000 × g for 15 minutes to pellet cellular debris, and then transfer the supernatant to a fresh tube.

Proceed with protein purification as follows: Add 1 ml of the lysate to the beads that have been prepared for denaturing conditions. Allow the mixture to bind for 30 minutes at room temperature with gentle agitation, such as on a rotating wheel, to maintain the resin in suspension. After binding, settle the resin by gravity or low-speed centrifugation at 800 × g and carefully remove the supernatant.

Wash the beads by resuspending in 0.5 ml of Denaturing Washing Buffer(8 M Urea, 20 mM Sodium Phosphate, pH 6.0, 500 mM NaCl) for three times.

Finally, elute the protein by adding 30 µl of Denaturing Elution Buffer. 30µl of lysis buffer and 10µl of 4xSDS are added, and samples are boiled at 95°C for 5 minutes to collect the supernatant.

2.6 Western blotting

Following specified treatments, cells underwent lysis in RIPA buffer (Cell Signaling) with the addition of PMSF at a concentration of 5 mg/ml, followed by two rounds of sonication. The resulting lysate was then used for protein quantification utilizing Bio-Rad Protein Assay reagents. After equalizing protein concentrations, 4x SDS sample loading buffer was incorporated into each sample, which was then heated to 98 °C for a duration of 5 minutes before being preserved at -20 °C for future use.

Samples, normalized by protein content, were loaded onto an SDS-PAGE gel and electrophoresed at a constant 30 mA. The separated proteins were then transferred to nitrocellulose membranes supplied by Bio-Rad. These membranes were initially blocked with 5% milk (composition to be confirmed) at room temperature for half an hour. Following the blocking step, the membranes were briefly washed with blocking buffer and subsequently incubated with respective primary antibodies at a dilution of 1:1000, maintained in a 4 °C environment throughout the night.

The following day, membranes were subjected to a series of washes with 1x TBST, thrice for 10 minutes each, then incubated with secondary antibodies at a highly dilute concentration of 1:30,000 at room temperature for an hour. After antibody binding, membranes were rigorously washed thrice more, this time for

45 minutes per wash with continuous agitation. Finally, membranes were analyzed using the Li-Cor Odyssey CLx imaging system, leading to the production of developed film images.2.5. Medium collection and concentration.

3 Result

	Lysate		IP				λ-ppase + USP2			
	NEMO		WT	NEMO	NEMO	NEMO	WT	NEMO	NEMO	NEMO
INF-γ	+		+	-	+	+	+	-	+	+
L18	+		+	-	+	+	+	-	+	+
In9332	-		-	-	-	+	-	-	-	+

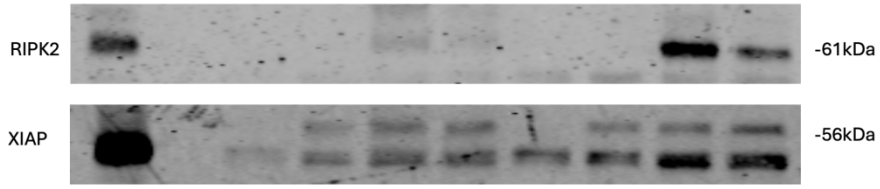

Fig3-1 Regulatory Effects of NEMO on RIPK2 and XIAP Expression

THP1 cells were stimulated with 50ng/ml INF-γ for 2 hours. Then treat the cells with 1uM inhibitor 9332, wait for 10min, treat the cells with 1uM L18 for 1hour.

In this figure, we explored the interaction between NEMO and its effect on the expression of RIPK2 and XIAP proteins under various conditions, including the presence of INF-γ, L18-MDP, and inhibitor 9332.

L18-MDP (Muramyl Dipeptide) is an immune adjuvant, which is the smallest identifying unit of peptidoglycan in the bacterial cell wall. L18-MDP has the ability to enhance immune responses and is widely used in research and development of vaccines. It triggers an immune response by activating the host's immune cells, such as macrophages. L18-MDP specifically participates in the activation of the NF-κB (nuclear factor kappa B) signaling pathway by recognizing and activating NOD2 (nucleotide-binding oligomerization domain protein 2), an intracellular pattern recognition receptor, thereby regulating Inflammatory and immune responses(Eizuru, Nakagawa, Hamasuna, & Minamishima, 1992). Inhibitor9332 is a RIPK2 molecular inhibitor.

The NF-κB signaling pathway plays a pivotal role in regulating immune responses, inflammation, and cell survival. The modulator NEMO is crucial for the activation of this pathway. Here, we investigated the effects of specific treatments on the expression of RIPK2 and XIAP proteins via the role of NEMO.

The study utilized wild-type cells and cells subjected to pull down of NEMO, treated with combinations of INF-γ, L18-MDP, and inhibitor 9332. Immunoprecipitation (IP) and Western Blot (WB) analyses were conducted to assess the expression of RIPK2 and XIAP proteins.

Our IP experiments revealed distinctive patterns in the expression of RIPK2 across different treatment groups: wild-type cells with no treatment and NEMO pull down cells with no treatment showed no detectable bands for RIPK2, indicating that NEMO does not bind to RIPK2 under these conditions. NEMO pull down cells treated with INF-γ and L18-MDP displayed clear bands, suggesting that the activation of NF-κB signaling, possibly mediated by NEMO, leads to the increased expression of RIPK2. NEMO pull-down cells treated with INF-γ, L18-MDP, and inhibitor 9332 also showed bands for RIPK2, but they were fainter compared to the NEMO pull-down cells only treated with INF-γ, L18-MDP, indicating that inhibitor 9332 partially suppresses the binding between RIPK2 and NEMO.

These results underline the complex regulatory mechanisms governing the NF-κB signaling pathway and its components.

To further confirm the post-translational modifications regulating RIPK2's function and stability, we treated immunoprecipitated complexes with Lambda

Protein Phosphatase (λ-PPase) and ubiquitin-specific protease 2 (USP2). λ-PPase, a broad-spectrum phosphatase, removes phosphate groups from phosphorylated serine, threonine, and tyrosine residues, potentially altering protein activity, interaction capabilities, and cellular localization. On the other hand, USP2 specifically disassembles ubiquitin chains from proteins, impacting their degradation rate, subcellular localization, and participation in signaling pathways.

Following the treatment with λ-PPase and USP2, a more compact and intense protein band in Western Blot analysis was observed. This proves that RIPK2 is depleted and ubiquitinated after stimulation by L18, and the subsequent fading of group protein traces once again proves the role of the inhibitor.

The absence of detectable XIAP in untreated WT cells contrasts with the slight increase observed in NEMO pull-down cells, suggesting that NEMO cells bind to XIAP.

The enhanced expression of XIAP in response to INF-γ and L18-MDP treatment underscores the role of external stimuli in modulating XIAP levels. The observed decrease in XIAP ubiquitination following λ-PPase and USP2 treatment across all groups suggests that dephosphorylation and deubiquitination contribute to XIAP's increased stability and possibly its activity within the cell.

This study illuminates the intricate mechanisms by which NEMO influences the expression of RIPK2 and XIAP through the NF-κB signaling pathway, under the modulation of INF-γ, L18-MDP, and inhibitor 9332. Our experiments reveal that NEMO binds to activated and activated RIPK2 and is always bound to XIAP, and under experimental conditions, both RIPK2 and XIAP are ubiquitinated. The

application of λ-PPase and USP2 further demonstrates the crucial role of post-translational modifications, including dephosphorylation and deubiquitination, in regulating the stability and function of these proteins. These findings underscore the complex interplay between NEMO and the NF-κB pathway in immune responses, highlighting potential targets for therapeutic intervention in inflammatory and immune-related disorders.

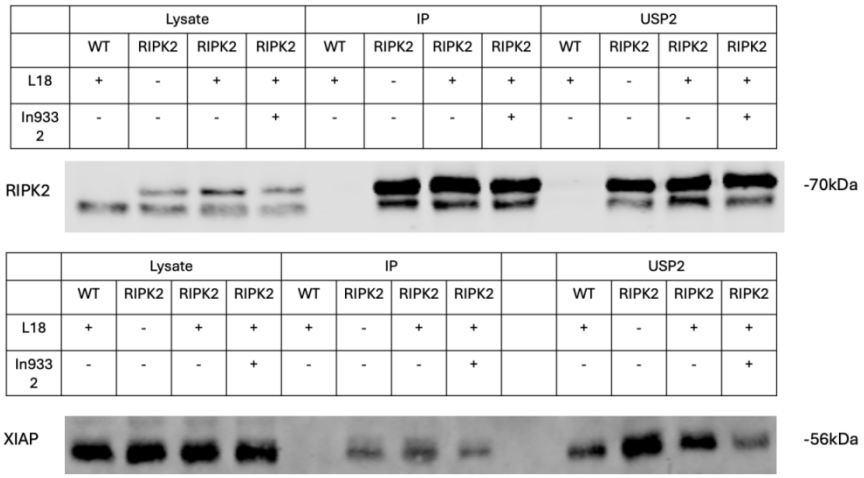

Fig3-2 Impact of RIPK2 Activation on XIAP Ubiquitination

HCT cells were stimulated with 10ng/ml Doxycycline for 12 hours. Then treat the cells with 1uM inhibitor 9332, wait for 10min, treat the cells with 1uM L18 for 1hour.

In this figure, I explored the regulatory role of RIPK2 in the NF-κB signaling pathway and its effect on XIAP expression by utilizing doxycycline to activate 3xFlag-tagged RIPK2, alongside immunoprecipitation (IP) analysis to assess XIAP levels. Remarkably, upon the addition of doxycycline, except in wild-type (WT) cells, two distinct bands were observed in the lysis group, indicating successful activation and expression of RIPK2. The presence of two bands suggests the existence of RIPK2 in multiple post-translational modification states,

affecting its mobility in gel electrophoresis. This finding underscores the efficacy of doxycycline in inducing specific gene expression and hints at the multifaceted involvement of RIPK2 in cellular signaling.

Concerning XIAP, the IP results revealed a nuanced pattern of expression across different treatment groups. In the WT cells, no XIAP expression was detected, likely due to the absence of activated RIPK2, which may influence XIAP levels. Slight XIAP expression was observed in the untreated group, suggesting baseline XIAP expression potentially modulated by signals other than RIPK2 activation. Enhanced XIAP expression was noted in groups with activated RIPK2, with a notable reduction in the group subjected to additional treatments, reflecting the importance of inhibitors.

The introduction of USP2, a deubiquitinating enzyme, led to more pronounced and compact bands for XIAP across all groups, including the WT group where XIAP was previously undetectable. This indicates that XIAP is previously ubiquitinated. These observations provide new insights into the interaction between RIPK2 and XIAP within cellular signaling pathways. The activation state of RIPK2 not only affects its own expression and potential functionality but also indirectly influences the ubiquitination level of XIAP.

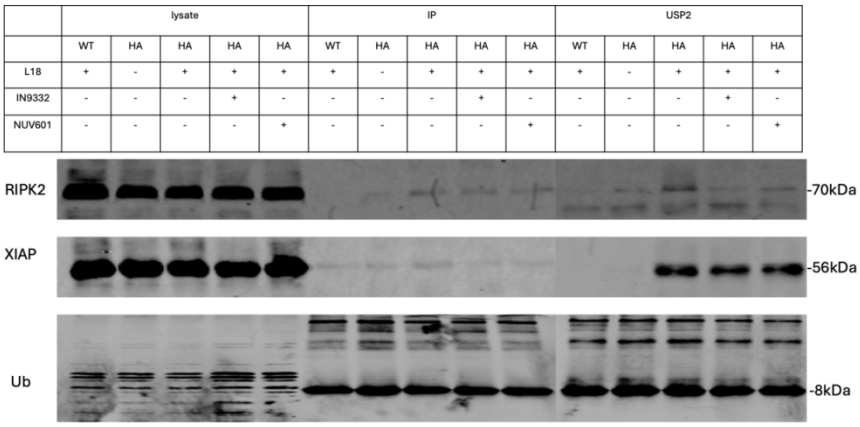

Fig3-3 HA tagged Ubiquitination Status and Its Impact on RIPK2 and XIAP Detection

Treat the cells with 1uM inhibitor 9332/1uM NUV601, wait for 10min; then treat the cells with 1uM L18 for 1hour. Two gels were used to run the samples separately, due to the limitation of a single gel not being able to accommodate so many samples simultaneously.

In this figure, HA-tagged ubiquitin proteins were pulled down, followed by different cell treatments, and the expression of XIAP and RIPK2 was analyzed WB.

In the IP experiments, both XIAP and RIPK2 showed no significant traces across all treatment groups. This suggests that these proteins are less stable in their ubiquitinated state, leading to degradation or loss during the IP process.

The treatment with USP2 significantly impacted the detection of RIPK2 and XIAP. For RIPK2, almost no traces were observed in the first two groups (WT and untreated HA-tagged ubiquitin cells), while a clear trace was evident in the third group (HA-tagged ubiquitin pull-down cells treated with L18-MDP), indicating that the addition of L18-MDP promotes the expression or stability of RIPK2, and the deubiquitinating effect of USP2 may further increase the stability and detectability of RIPK2. In the HA-tagged ubiquitin pull-down cells treated

with L18 and either Inhibitor 9332 or NUV601, the traces of RIPK2 weakened, possibly reflecting the negative impact of these inhibitors on RIPK2 or their alteration of RIPK2's ubiquitination status, affecting its response to USP2 treatment.

For XIAP, after USP2 treatment, almost no traces were observed in the first two groups, whereas in the latter three groups, distinct and compact traces appeared, indicating a positive effect of L18-MDP addition and inhibitor usage on the stability and expression of XIAP post-deubiquitination. The lack of significant differences in XIAP expression among these groups may suggest that these treatments affect XIAP through similar mechanisms or that the effect of USP2 on XIAP has reached a saturation point under these conditions.

Ubiquitin showed significant traces in all groups, with no significant difference between IP and post-USP2 treatment, indicating that ubiquitin protein is relatively stable under these conditions, and the deubiquitinating action of USP2 on ubiquitin itself may not be sufficient to affect its detection level in WB.

The results of this experiment reveal the impact of L18-MDP and different inhibitors on the expression of RIPK2 and XIAP in cells subjected to HA-tagged ubiquitin pull-down, and how USP2 deubiquitination treatment can significantly alter the detectability of these proteins. Specifically, USP2 treatment highlights the importance of considering protein ubiquitination states in cellular signaling research.

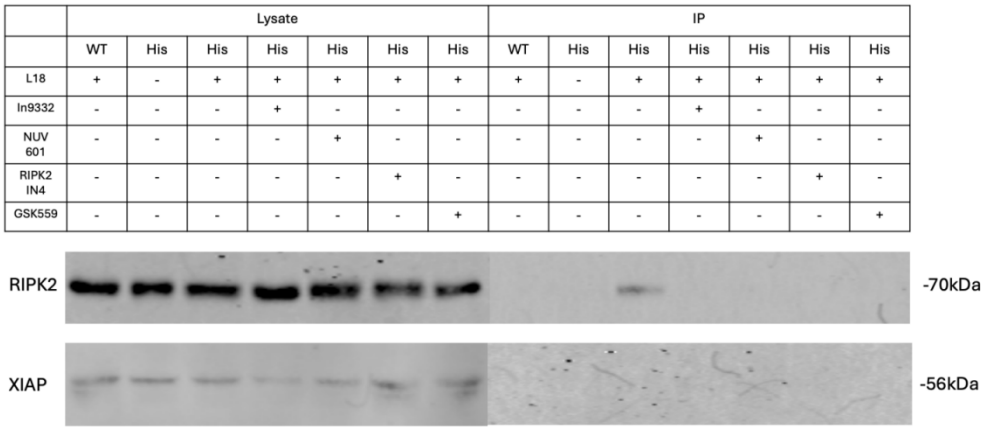

Fig3-4 His tagged Ubiquitination Status and Its Impact on RIPK2 and XIAP Detection

Treat the cells with 1uM inhibitor 9332/NUV601/RIPK2 inhibitor4/GSK559, wait for 10min; then treat the cells with 1uM L18 for 1hour.

This figure explored the impact of ubiquitination on specific cellular proteins (RIPK2 and XIAP) under various treatment conditions by utilizing His-tagged ubiquitin proteins and conducting a pull-down assay with beads. The objective was to investigate how different treatments affect the ubiquitination and subsequent expression of RIPK2 and XIAP.

WT cells and His-tagged ubiquitin pull-down cells with no treatment showed almost no traces of RIPK2, suggesting that under these conditions, RIPK2 either has low expression levels or is not ubiquitinated sufficiently to be captured by the His-tagged ubiquitin pull-down assay.

His-tagged ubiquitin pull-down cells treated with L18-MDP showed traces of RIPK2, indicating that adding L18-MDP may promote the ubiquitination or enhance the expression level of RIPK2, enabling its capture in the pull-down assay.

The subsequent four groups treated with L18 and various inhibitors showed no traces of RIPK2. This suggests that these specific inhibitors (Inhibitor 9332, Nuvation601, RIPK2 inhibitor4, GSK559) may prevent the ubiquitination of RIPK2 or directly affect its expression or stability, rendering it undetectable in the pull-down assay.

All groups showed no traces of XIAP, which could mean that XIAP did not undergo significant ubiquitination under these experimental conditions or the level of ubiquitination was insufficient for detection via the His-tagged ubiquitin pull-down method. Another possibility is that XIAP's expression levels are inherently low under these conditions.

These findings suggest that L18-MDP may promote the ubiquitination or expression of RIPK2, while the four different inhibitors might inhibit the ubiquitination process or decrease the stability and expression of RIPK2. Simultaneously, XIAP appears not to undergo significant ubiquitination, or its expression/ubiquitination levels were too low to be detected with the employed method.

These insights provide a foundation for further understanding the role of ubiquitination in regulating cellular signaling pathways, particularly the function of the ubiquitin-proteasome system and its potential regulatory mechanisms.

4 Discussion

This study delves into the intricate roles of NEMO, RIPK2, and XIAP within the NF-κB signaling pathway, with a focus on their interactions and post-translational modifications, particularly ubiquitination. NEMO, acting as the regulatory subunit of the IKK complex, is pivotal for the activation of NF-κB upon various stimuli, including genotoxic damage. Our findings reinforce the significance of ubiquitination, facilitated by XIAP, in the activation of RIPK2, which in turn influences the ubiquitination of NEMO and subsequent activation of NF-κB. This process underscores the complexity of cellular signaling mechanisms that govern immune responses and inflammation.

Through the application of immunoprecipitation and the use of λ-Phosphatase and USP2, our study highlights the critical nature of phosphorylation and ubiquitination in modulating the stability and signaling roles of these proteins. Specifically, USP2's role in trimming ubiquitin chains elucidates the enhanced clarity and quantification of RIPK2 and XIAP in Western blot analyses, by eliminating the variability introduced by ubiquitination. This approach has allowed for a more nuanced understanding of the interactions between NEMO, RIPK2, and XIAP, revealing the dynamic balance of post-translational modifications in regulating NF-κB signaling.

The observed patterns of RIPK2 and XIAP expression, alongside their modifications, suggest a complex regulatory network. The modulatory effects of specific treatments on these proteins' expression levels point to a sophisticated orchestration of cellular signaling pathways, particularly highlighting the pivotal

role of ubiquitination in shaping the outcomes of NOD signaling.The results from the His-tagged and HA-tagged ubiquitin pull-down assays provide valuable contrasts in understanding protein ubiquitination and stability. The His-tag assay suggested that L18-MDP might enhance the ubiquitination or expression of RIPK2, while various inhibitors potentially decrease its stability or expression. Conversely, the HA-tagged assay indicated a general stability of ubiquitin proteins, with specific treatments affecting the detectability and stability of RIPK2 and XIAP post-deubiquitination.

The absence of significant traces of XIAP in both assays underlines the protein's low ubiquitination levels or expression under the tested conditions, suggesting its regulation might be less dependent on ubiquitination compared to RIPK2. Moreover, the differential effects observed with USP2 treatment across the assays underscore the enzyme's role in enhancing protein stability and detectability, particularly through deubiquitination.

The significance of this research lies in its nuanced exploration of the regulatory mechanisms of critical signaling proteins and the identification of post-translational modifications as key modulators. By illuminating the specific conditions under which RIPK2 and XIAP are stabilized or degraded, this study provides a framework for future interventions aiming to control NF-κB signaling. The interpretations presented here are supported by rigorous analysis and are consistent with the growing body of evidence underscoring the role of ubiquitination in cellular signaling.

In conclusion, the findings presented here not only enhance my comprehension of immune signaling pathways but also suggest potential nodes for therapeutic intervention, offering hope for the treatment of chronic inflammatory conditions and immune-related disorders.

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