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Ubiquitylation and deubiquitylation in the regulation of the transcription factor NF- κ B activation

Konstantinos Poalas

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Ubiquitylation and deubiquitylation in the regulation
of the transcription factor NF- κ B activation

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*στον Πατέρα μου Φόρη, που μου έμαθε ελευθερία
στην Μάνα μου Μαρία, που μου έμαθε αφοσίωση
στον Αδερφό μου Δημήτρη, που μαζί μάθαμε να μοιραζόμαστε
στη Λάϊστα, που με κρατάει Άνθρωπο*

to my Father, who taught me freedom

to my Mother, who taught me dedication

to my Brother, with whom we learnt to share

to Laïsta that keeps me Human

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Abbreviations

A20	TNFAIP3: Tumor necrosis factor, alpha-induced protein 3
AP-1	Activator protein 1
ARD	Ankyrin repeat domain
BCL10	B-cell CLL/lymphoma 10
BCR	B-cell receptor
CARD	Caspase recruitment domain
CARMA1	CARD and MAGUK scaffold protein 1
CBM	CARMA1-MALT1-BCL10 complex
CD40	Cluster of differentiation 40
cIAP	cellular Inhibitor of apoptosis
CK1 α	Casein Kinase 1 α
CYLD	Cylindromatosis
DLBCL	Diffuse large B-cell lymphoma
DUB	Deubiquitinylase
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
HECT	Homologous to the E6-AP Carboxyl Terminus
IFN	Interferon
IKK	I κ B kinase
IL	Interleukin
IRAK	Interleukin-1 receptor-associated kinase
I κ B	Inhibitor of NF- κ B
JNK	JUN N-terminal kinase
LPS	Lipopolysaccharide
LUBAC	Linear ubiquitin chain assembly complex
MAGUK	Membrane-associated guanylate kinase
MALT1	Mucosa associated lymphoid tissue lymphoma translocation gene 1
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
MEKK	Mitogen-activated protein kinase kinase
MTDH	Metadherin
MYD88	Myeloid differentiation primary response protein 88
NEMO	NF- κ B essential modulator
NF- κ B	Nuclear factor of immunoglobulin κ -light chain enhancer in activated B-cells
NFAT	Nuclear factor of activated T-cells
NIK	NF- κ B inducing kinase
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerisation domain
NUTs	NF- κ B ubiquitinylated transmitters
OTU	Ovarian tumour domain
PKC	Protein kinase C
PTM	Post-translational modification

RHD	REL-homology domain
RING	Really interesting new gene
RIP	Receptor-interacting protein kinase
RLR	RIG-I-like receptor
RNF	RING-finger protein
RPS3	Ribosomal protein S3
SCF	SKP1-CUL1-F-box-protein
TAB	TAK1-binding protein
TAK1	TGF β -activated kinase 1
TCR	T-cell receptor
TLR	Toll-like receptor
TNFR	Tumour necrosis factor receptor
TNF α	Tumour necrosis factor α
TRAF	TNF-receptor-associated factor
UBD	Ubiquitin-binding domain
USP	Ubiquitin-specific protease
Wnt	Wingless integration
β -TrCP	beta-transducing repeat containing E3 ubiquitin protein ligase

Abstract

Large signalosome assembly is a prerequisite for NF- κ B signaling upon engagement of various immunoreceptors. Adaptor proteins containing protein-protein interaction domains oligomerise in response to such stimuli in order to propagate signaling. Each immunoreceptor uses distinct adaptors, as well as common ones, to achieve that. The main characteristic shared by these proteins is their ability to undergo poly-ubiquitylation in a non-degradative manner, leading to optimal NF- κ B activation. In this work, we aimed to identify novel deubiquitylating enzymes that control ubiquitylation status. That is how USP34 came up to be a negative regulator of NF- κ B signaling in TCR-activated Jurkat cells, a T lymphocyte cell line. Our data suggest a model whereby USP34 prevents excessive NF- κ B activation by acting rather late, directly or indirectly on the NF- κ B:I κ B α dimers, downstream of IKK, altering transcription factor DNA binding affinity. In parallel, studies of the endocellular membrane microenvironment that hosts mature signalosomes in response to TCR-, TNFR- and CD40 ligation led to the identification of an ER-residing protein, Metadherin (MTDH), which seems to globally integrate signaling before forwarding it to downstream pathway components able to activate IKK.

Résumé

L'activation de la signalisation NF- κ B par de nombreux immunorécepteurs met en jeu un large signalosome. Afin de propager cette signalisation en réponse à différents stimuli, l'oligomérisation d'adaptateurs pourvus de domaines d'interaction protéine-protéine est nécessaire. Alors que certains adaptateurs sont communs d'autres sont spécifiques à un immunorécepteur donné. Une des principales caractéristiques partagées par toutes ses protéines est leur capacité à être poly-ubiquitylé de façon non-dégradative afin d'aboutir à une activation optimale de NF- κ B. Ce projet avait pour objectif d'identifier de nouvelles déubiquitylases impliquées dans la signalisation NF- κ B. C'est ainsi que nous avons identifié USP34 comme étant un régulateur négatif de la signalisation NF- κ B induite par le TCR dans des cellules Jurkats, une lignée de lymphocytes T immortalisés. Nos données suggèrent un modèle dans lequel USP34 permet d'éviter l'activation excessive de NF- κ B, en agissant directement ou indirectement sur les dimères NF- κ B/I κ B α , en aval d'IKK, et en modulant l'affinité du facteur de transcription pour l'ADN. Parallèlement, l'étude du microenvironnement des membranes endocellulaires responsables du recrutement des signalosomes formés en réponse à l'activation du TCR, du TNFR et du CD40 a permis l'identification d'une protéine - clé de la signalisation NF- κ B, la MTDH. Cette protéine du RE s'est révélée être un relais déterminant pour l'activation d'IKK et donc la propagation du signal NF- κ B.



nce upon a time there were many little primitive cells living in the vast oceans of planet Earth. Their life was not that peaceful, as they had to compete with each other day and night for the uptake of the limiting amount of nutrients in order to survive in a hostile and frightful environment.

As time was flowing some of them made up new ways to use more rapidly and efficiently the available nutrients or get a safe sanctuary to get protected from the tremendous ancient face of the planet, giving them a selective advantage in survival. Life continued silently and unnoticed, soon all of them had developed different ways to earn their living. Little by little, step by step they even managed to change their whole environment which has turned much more friendly.

At that moment they decided to get organised in smaller or larger societies living altogether and cooperating for their common good and well-being. Nothing has been the same ever since, as this was the initial event for the creation of multicellular organisms and the onset of the exponential increase of biodiversity permitting to living organisms spread around all heights and depths of the planet, resulting in all the living things we can (or still cannot) see around us today.

Multicellularity could only be achieved after satisfying some very essential needs such as regulated cell replication and growth, programmed cell death, cell-cell and cell-matrix adhesion, regulated developmental processes, cell type specialisation and alloreactivity-immunity. These adaptations could offer to that organism ideal advantage for survival. Markedly the NF- κ B transcription factor is implicated in regulation of all these processes by controlling proliferation, antiapoptotic protein synthesis, adhesion molecules expression, development driving, cell specialisation and most commonly regulation of inflammation and immune response. Immunity in a multicellular organism consists of many defence mechanisms against rapidly growing, invading pathogens that can disrupt the organism's integrity leading to a potentially lethal infectious disease. It also offers protection against noxious chemicals, radiation and other threats to normal function. The first line of defence against environmental danger consists of innate immune response mechanisms, highly conserved amongst species from the most primitive metazoans to the fruit flies and mammals. Adaptive immune response found in higher vertebrates together with innate immune response are both critical for survival and are essentially regulated by NF- κ B [1, 2].

I. Historic overview

It has been 27 years since the first publication in 1986 (Figure 1), identifying the Nuclear Factor of immunoglobulin κ -light chain enhancer in activated B-cells (NF- κ B) as a transcription factor present in the nuclei of mature B-lymphocytes and plasma cells, able to bind on the enhancer sequence 5'-GGGACTTCC-3' of this gene [3].

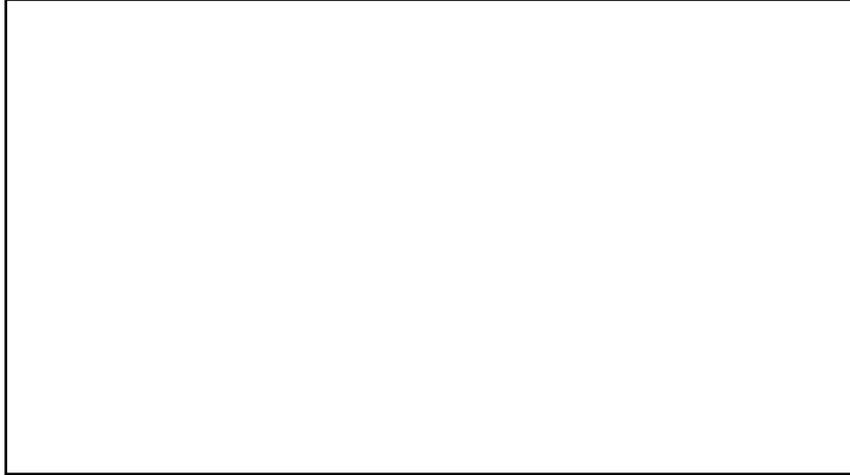


Figure 1: First publication on NF- κ B in 1986, in Cell journal by Ranjan Sen and David Baltimore. Bottom right: Schematic representation of the essential 475bp AluI-AluI fragment containing the κ enhancer as defined by Picard and Schaffner (1984)... [3].

The initial goal of that project was to understand the mechanisms of enhancer function and their role in the activation of tissue-specific genes. The Immunoglobulin (Ig) enhancers were the first found to be tissue-specific with the Ig gene expression to be governed by three types of tissue-specific sequences. These sequences (promoter, enhancer, an extra intragenic sequence in the case of μ heavy chain gene) share an octameric motif (ATTTGCAT) located at a characteristic distance of all sequenced variable region genes. The fact that a nuclear factor had been found to interact with that sequence and the definition of enhancer sequences in viruses that potentiate transcription from a variety of promoters had already given rise to the idea of the existence of *trans*-acting factors that recognise these *cis*-regulatory DNA elements to regulate gene transcription. That is how this group tried to identify nuclear factors interacting with Ig μ and κ enhancer sequences by using an electrophoretic mobility shift assay (EMSA).

Initially, studies on the κ enhancer interactions with nuclear extracts deriving from different types of mouse cells showed that nucleoprotein complexes were only present in B lymphoid cells. Further tests on B-cell lines have been performed in nuclear extracts deriving from different developmental stages such as *pre-B cell lines* (HAFTL, 38B9, 70Z, PD), mouse (WEHI 231, AJ9) and human (EW) *B-cell lines*, mouse (SP2-0, MPC11) and human (KR12, 8226) *plasma cell lines* and *T-cell lines*. This nucleoprotein complex was named NF- κ B, as it was only detected in the B-cell lines and the plasma-cell lines revealing not only tissue-specificity in B lymphoid lineage but also stage-specificity [3]. The theory was broadened right away as nucleoprotein complex could also be detected in nuclear extracts of pre-B cells (70Z/3) upon lipopolysaccharide (LPS) stimulation and non-lymphoid cells (HeLa) upon phorbol ester stimulation. Furthermore, translation inhibitors could not prevent this induction, even partially promoted it, showing that gene transcription is activated by post-translational modifications of pre-existing precursor molecules [4].

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Soon after NF- κ B discovery, in 1988 it became clear through a series of studies including EMSA experiments that NF- κ B is located in the cytosolic fraction of unstimulated cells in a form where DNA-binding activity can be activated by dissociating agents. In phorbol ester stimulated cells, NF- κ B activity is almost quantitatively recovered in the nuclear fraction. Taken together with the previous observations, it was proposed that NF- κ B binding activity could be due to covalent modifications or conformational changes or release from an inhibitor. The latter idea, being more compatible with existing data, was further expanded proposing either that an inhibitor could cover DNA-binding site and nuclear translocation signal of NF- κ B or maintain it in a low-DNA-affinity and hidden-nuclear-localisation-signal conformation [5].

Further experiments indicated the presence of this inhibitor in cytosolic fractions treated with a dissociating agent and depleted of NF- κ B, in a gel filtration elution fraction able to block nucleoprotein complex formation detected by EMSA. They have further shown that the Inhibitor of NF- κ B (I κ B) is a protein that can form a stoichiometric complex with NF- κ B and convert it into an inactive form in a reversible, saturable and specific reaction. Function of Hormone Receptors, and more specifically Glucocorticoid Receptor, was already known at that time. After substrates previously bound by inhibitors are released, translocation into the nucleus and gene transcription follows. Thus the proposed molecular mechanism for NF- κ B activation, was that of “inducible gene expression by which a transcription factor:inhibitor complex is dissociated by the action of TPA (phorbol ester 12-O-tetradecanoylphorbol 13-acetate) presumably through the activation of PKC (Protein Kinase C). The dissociation event results in activation and apparent nuclear translocation of the transcription factor. It would appear that I κ B is the target for the TPA-induced dissociation reaction” [6].

Figure 2: Milestones in I κ B research. The timeline represents a selection of major advances in the understanding of the molecular functions of I κ B family proteins. [7]

PKC activation by phorbol esters was already known, so it was hypothesised that I κ B undergoes phosphorylation in order to dissociate from NF- κ B. This event would somehow permit nuclear translocation, bringing together the information reflecting the cytoplasmic activation state of PKC and possibly of other signaling systems. Several studies demonstrated I κ B phosphorylation but all efforts to identify the responsible kinase, as PKC was not the one, were in vain. This “ghost” protein mediating this absolutely necessary modification was only revealed after intensive research, several years later. In 1996, it was proposed that phosphorylation is performed by a 700 kDa multisubunit kinase [8]. In 1997, several papers at

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the same time described a kinase subunit able to phosphorylate I κ B α upon stimulation with TNF α and IL-1. This previously characterised protein (CHUK) was renamed as the I κ B Kinase α (IKK α) or IKK1. Practically at the same time, IKK β or IKK2 was described as another catalytic subunit of the IKK signalosome, phosphorylating I κ Bs upon TNF α stimulation [9-13]. A third component of the IKK complex was also characterised as IKK γ or NEMO (NF- κ B essential modulator) as it could interact with IKK α and IKK β and is needed for activation of the complex upon various stimuli [14, 15].

Current model of NF- κ B activation proposes that upon phosphorylation-induced degradation of I κ B, liberated NF- κ B can translocate into the nucleus. There it bind on specific DNA sequences (cis elements) of 9-10 nucleotides, called the κ B sites, according to the pattern 5'-GGGRNNYYCC-3' (where R is a purine, Y a pyrimidine and N can be any nucleotide) to initiate transcription. No other transcription factor has ever attracted more experimental attention, counting more than 40.000 published articles (Figure 3). Today NF- κ B is synonymous with immunity and inflammation, development and apoptosis, survival and proliferation.



Figure 3: 25 years of NF- κ B literature [16]

II. Why to study NF- κ B?

II.1. Latency - Rapidity

What was astonishing in the first place (and still is) about NF- κ B is the fact that it is a pre-existing system, retained in a latent state. All basic components, already present and poised for induction in the cell's surface, cytoplasm and nucleus, await the right stimulus to initiate a response. A cascade of aggregation events and enzymatically catalysed biochemical reactions, involving many types of post-translational modifications, takes place during signal transmission, thus offering the privilege of instantaneous response, winning precious time that could be fatal for a cell waiting for protein synthesis to be completed [2].

II.2. Induction by various stimuli

Another fact concerning NF- κ B transcription factor, is the large variety of potential inducers (more than 150 [17]), recognised by receptors (either cell-surface or endocellular ones) that mediate bridging of the environmental conditions with the nucleus. As such have been characterised some pathogen sensors and some proinflammatory cytokines (e.g. IL-1 (Interleukin-1) which acts on macrophages and fibroblasts), that also introduce feedback loops in the system. Antigens are a different category of inducers, in the case of B- and T-cells, and glutamate, in the case of neurons. DNA-damage caused by ionising irradiation or genotoxic chemical compounds also participate in NF- κ B induction [1, 2]. All these inducers generate various and complex transduction pathways, that often crosstalk with each other, finally converging, independently of the origin, in the regulation of NF- κ B:I κ B complex stability and localisation.

II.3. Activation of innumerable targets

Nowadays, several κ B binding sites have been detected in a plethora of genes promoters and enhancers (more than 150 [17, 18] out of the approximately 20.000 encoded by the human genome), responsible for synthesis of proteins implicated in various fundamental cell processes including antimicrobial peptides, inflammatory cytokines, chemokines, interferons, acute phase response proteins, cell adhesion molecules, growth factors, stress-response proteins, anti-apoptotic proteins and viral proteins.

Cytokines are the messenger molecules starring in immune response regulation, contributing in promotion of proliferation and differentiation of specific cells (e.g. macrophages, granulocytes, T-cells, B-cells), acute phase response induction in inflammation and antiviral protection of healthy cells [2]. They are mainly produced by and secreted from the immune system cells, either to act locally in an autocrine way, introducing positive feedback loops, paracrine manner, activating neighbouring cells, or globally in an endocrine way.

Overall NF- κ B controls events at multiple levels. First it ensures constant availability of pathway components like NF- κ B and I κ B proteins, so that the pathway can be readily responsive at any moment (κ B sites are present in the promoters of such genes). In addition, NF- κ B controls activation of the signal-originating cell as well as activation and intercellular

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communication with neighbouring cells through cytokines and other far away located cells through chemokines. Secreted NF- κ B-dependent products of activated cells can further drive third target cells, through additional NF- κ B-dependent processes, to code for proteins necessary for migration and adhesion in the spot of the signal origin. When all kinds of activated cells are present in the source of signal induction, they all start a party of NF- κ B-activating and NF- κ B-guided events in order to produce molecules necessary for the initiation and control of the inflammatory response at the beginning, and resolution at the end of the alarm.

II.4. Resolution

An additional factor one has to keep in mind is that inflammation, as mentioned earlier, is the most essential part of the immunological response. The series of events is perfectly orchestrated and coordinated by the immune system cells whose communication depends on protein production from NF- κ B-controlled genes. Inflammation is the only way to repel and eliminate pathogens, viruses, genetically or physically damaged cells etc. and it can only be successful if it is accurate in space and time. A too much localised or delayed immune reaction is as dangerous and critical for tissue health as an extensive or prolonged one. The latter is highly undesirable as it may damage nearby tissues risking their integrity. So, inflammation must be self-limiting and self-resolving. Proinflammatory effects are also accompanied by anti-inflammatory ones. Consequently, successful response does not only lie in its onset but also in the elegant ability to precisely control it at all stages till resolution. Thus, the ability to self-deteriorate is of great importance when potential danger has disappeared. Setting the system in the default condition follows, where NF- κ B-dependent genes are only expressed in basal levels. The importance of this mechanism is underscored by the fact that the same transcription factor drives the expression of both inflammation initiation and resolution genes. Furthermore, inflammation is important not only for clearing intruders but also for the turnover and repair of damaged tissues [1].

II.5. NF- κ B and diseases

II.5.a. Inflammation-related diseases

Chronic inflammation, autoimmunity, immunodeficiency and allergies are pathological conditions characterised by NF- κ B malregulation. Many systemic syndromes and diseases have been characterised as such, symptomatology of which varies from mild signs during early stages of development to more severe forms as it they advance. There are examples affecting all tissues and organs. [18]

Tissue/organ/system	Disease
Intestine	Inflammatory Bowel
Pancreas	Diabetes Mellitus type I
Bones and joints	Rheumatoid Arthritis
Skin	Psoriasis
Cardiovascular	Atherosclerosis
Muscles	Muscular Dystrophy, Asthma
Central nervous	Alzheimer's, Multiple Sclerosis
Ectoderm-derived	Incontinentia Pigmenti
CD4 ⁺ T-cells	HIV

II.5.b. Cancerogenesis

Indications for the oncogenic action of NF- κ B in human had already appeared since the dawn of its discovery, as a result of the homology of c-Rel protein with the retroviral v-Rel oncogene causing cancer in avian lymphoid cells (avian reticuloendotheliosis viral oncogene homolog) [18-22]. Somatic mutations, genomic amplifications and deletions, chromosomal translocations lead to constitutively active NF- κ B in a variety of haematological malignancies and solid tumours (Table 1). These kinds of malfunctions are often followed by constitutive signaling originating from receptors and pathway components or permanent inactivation of inhibitory proteins involved in the signaling cascades. In human, lymphomas are usually caused by malignant B-lymphocytes (90%) at various differentiation stages and less often (10%) derive from T-lymphocytes. Classification is mainly based on patient's symptoms and phenotype. Some of these diseases (e.g. ABC DLBCL) are addicted to constitutive, aberrant NF- κ B activity in order to survive [23-25].

Human cancers that have been linked to constitutive NF- κ B activation

Various haematological malignancies and solid tumours that exhibit constitutive nuclear factor κ B (NF- κ B) activation are listed below:

Haematological malignancies. Multiple myeloma (1% of all cancers and more than 10% of total haematological malignancies), mantle cell lymphoma, MALT lymphoma, diffuse large B-cell lymphoma (DLBCL) (25% of all lymphoma cases) [subcategorised in germinal center B-cell-like (GCB) DLBCL, activated B-cell-like (ABC) DLBCL and primary mediastinal B-cell lymphoma (PMBL)], Hodgkin's lymphoma, myelodysplastic syndrome, adult T-cell leukaemia (HTLV-1), acute lymphocytic leukaemia, acute myeloid leukaemia, chronic lymphocytic leukaemia and chronic myeloid leukaemia.

Solid tumours. Breast, cervical, prostate, renal, lung, colon, liver, pancreatic, oesophageal, gastric, laryngeal, thyroid, parathyroid, bladder and ovarian cancers. Also, melanoma, cylindroma, squamous cell carcinoma (skin, and head and neck), oral carcinoma, endometrial carcinoma, retinoblastoma and astrocytoma/glioblastoma.

Table 1: Human cancers that have been linked to constitutive NF- κ B activation. (Adapted from [23] with information from [1, 18, 25, 26])

Even more crowded is the group of malignancies where activated NF- κ B is largely detected in the cells nuclei, pointing out the effect of either mutations that affect components of upstream signaling pathways of the malignant cells, or the presence of proliferation promoting factors in a tumor microenvironment. In other words carcinogenesis can occur by two mechanisms. First, by the multistage, mutation accumulating process, involving the classical sequence of tumor initiation and tumor progression events in the case of chemical-caused tumorigenesis. Second, it can be facilitated through chronic tumor-promoting inflammation by creating a microenvironment that allows cells with cancer-causing mutations to thrive, before any tumor initiation events. These events are actually believed to be more widespread, as chronic inflammation can create a microenvironment favourising transformation of healthy cells, angiogenesis to feed the malignancy and tumor cell growth and invasion [1, 18, 23].

II.5.c. Medical treatment

Chronic inflammation diseases and cancers are currently treated with a wide range of pharmaceutical substances that mainly block inflammatory events, or the proliferative activity of cells through inhibition of NF- κ B activating signaling pathways. Glucocorticosteroids have been used as immunosuppressive and anti-inflammatory drugs, since they can induce I κ B

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synthesis. Non-steroidal anti-inflammatory drugs like aspirin have also been used to prevent I κ B degradation and extensive NF- κ B activation. The same approach is followed by the use of naturally occurring or synthetic proteasome inhibitors like lactacystin, peptide aldehydes and boronic acid peptides. Genotoxic agents and irradiation are also used in order to introduce genetic damage in rapidly proliferating cancer cells, thus promoting their elimination by apoptosis. Other examples are anti-TNF or anti-IL1 therapy for chronic inflammation diseases [1, 27].

Most anticancer approaches though have a small therapeutic index and do not discriminate between malignant and normal cells. The ones mentioned above, have a broad range of action, affecting essentially all rapidly growing cell populations. Cancer cells are not the only type of rapidly growing cells that need active NF- κ B, since stem cells of the skin epithelium, the intestine epithelium and the bone marrow, self-renew and proliferate in a continuous way to maintain homeostasis. Inevitably, agents that affect cell proliferating capacity in a general manner and block the pleiotropic effects of NF- κ B also cause serious side effects on healthy cells, not to mention that resistance and subsequent relapse of the disease is highly possible. Furthermore all pathological conditions described earlier are multifactorial, meaning practically that similar phenotypes and disease classification do not derive from the same genetic and molecular background, making each patient a unique case. Cancer is not one but many different diseases. In addition this type of drugs do not act in a well-known way and they can affect many different processes in cells. Very often, combinatorial drug therapy is provided so as to equilibrate the undesirable and harmful side effects caused [18, 23].

During the last few years, new approaches and therapies are being applied, focusing on more specific stages of action in signal transduction pathways. IKK-specific, I κ B phosphorylation and proteasome-specific inhibitors are clinically tested. The use of inhibitors specific for TCR pathway-restricted molecules that only affect adaptive immunity is also being tested [24]. The advantage of these approaches lies in the fact that they have a more specific way of action as they specifically target intermediate factors and signal transducers participating in the NF- κ B activation pathway. Thus they can sensitise cancer cells to standard chemotherapy-induced death. New class of compounds acting as multitarget drugs has attracted clinical attention, acting on diverse regulatory pathways that are essential for proliferation and survival of cancer cells [HSP90 (Heat shock protein 90) blockers and HDAC (Histone deacetylases) and ubiquitin-proteasome system inhibitors] [1]. Like that, non-specific effects in signaling can be reduced. New antitumoral therapies also focus on targeting the inflammatory components of the tumour rather than the malignant cells (as they undergo mutations rapidly and develop drug resistance) depriving it from proliferating signals [1, 23]. Side effects on healthy cells still come up though.

That makes an absolute necessity the direction towards an individualised, molecular-targeting treatment adapted to each patient's needs in the future. NF- κ B-based therapies against cancer or chronic inflammation conditions will require molecular profiling to determine disease subtype, presence of genetic lesions in NF- κ B regulators and NF- κ B activity in the pathway. Expression of biomarkers and bioinformatic analysis of this kind of information, would offer the possibility to predict if a patient can benefit and respond to NF- κ B inhibition treatment. For this goal many signaling pathways need to be further illuminated in order to have a better understanding and a deeper insight, so as to design an accurate and specific treatment with maximal efficiency and minimal side effects [23, 24]. A characteristic example of targeted therapy is MALT1. MALT1 is necessary for CARMA1-BCL10-MALT1 complex formation (see chapter IV.4.b) and signal propagation. Chromosomal translocations of this gene have been found in several ABC-DLBCL patients (55% of MALT-type lymphomas), leading to constitutive activation of NF- κ B and subsequent increased

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proliferation rate and resistance to apoptosis. These lymphomas are often specifically addicted to MALT1 cleavage activity, as MALT1 can cleave and neutralise signal-inhibitory factors. The benefits from targeting MALT1 as anti-cancer therapy lay in the fact that it is the only paracaspase that cleaves after arginine, unlike caspases that cleave after aspartate. Its implication in NF- κ B signaling only through antigen receptors (BCR-TCR) renders it an ideal target. So far, screening for small molecule inhibitors have revealed some very good candidates (MI-2, phenothiazine derivatives) able to kill or sensitise ABC-DLBCL cells due to their high specificity, pharmacokinetic properties and manageable toxicity [25, 28].

III. NF- κ B core components and activators

III.1. REL family

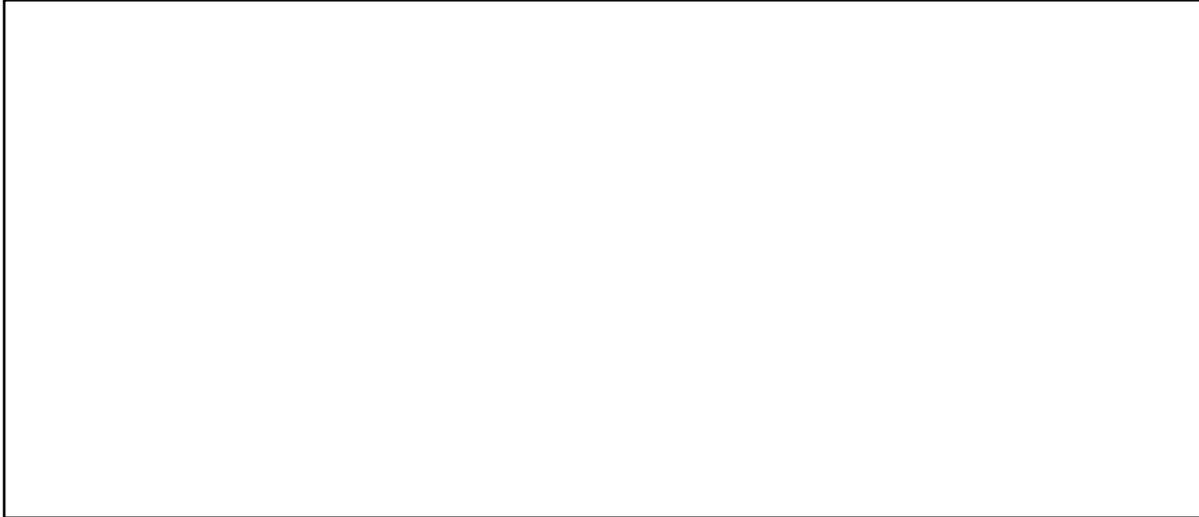


Figure 4: Mammalian NF- κ B/REL family members. All mammalian REL-related proteins - RELA/p65, c-REL, RELB, p105, p50, p100 and p52 - contain REL homology domains (RHDs). The amino-terminal portion of the RHD is responsible for both backbone and sequence-specific contacts within the major groove of the κ B enhancer. The carboxy-terminal portion of the RHD mediates dimerization with other NF- κ B/REL-family members and forms the site for physical docking to the I κ Bs. Only RELA, c-REL and RELB contain carboxy-terminal transactivation domains (TADs). The p105 and p100 proteins contain ankyrin repeats (indicated by pink circles), as well as glycine-rich regions (GRRs). The GRRs are important for co-translational processing of p105 to p50 and post-translational processing of p100 to p52. Phosphorylation of RELA at serines (S)276, S311, S529 and/or S536 is required for optimal NF- κ B transcriptional activity. Acetylation of RELA at lysines (K)122, K123, K218, K221 and K310 regulates distinct functions of NF- κ B, including DNA binding, I κ B α association and RELA-mediated transactivation. The leucine zipper (LZ) of RELB is required for transactivation by RELB [29].

The NF- κ B family of **eukaryotic transcription factors** consists of five members in mammals: RELA or p65 or NF- κ B3, RELB, c-REL, NF- κ B1 (p50 and the precursor molecule p105), NF- κ B2 (p52 and the precursor protein p100) [Figure 4]. RELA and NF- κ B1 are ubiquitously expressed, NF- κ B2 expression is restricted to stomach epithelium and areas of haemopoietic organs [30], RELB expression is restricted to dendritic cells and lymphoid tissue [31] and c-REL expression is generally restricted to haematopoietic cells [27, 32, 33]. Active form of the transcription factors is their combination in hetero-dimeric or homo-dimeric complexes, each one with distinct functions. RELB is an exception, as it cannot form homodimers *in vivo* and fails to form heterodimers with either RELA or c-REL [16] (see table 2). These proteins share a highly conserved 300-amino-acid Rel Homology Domain (RHD) on the amino terminus, previously named NRD (NF- κ B/Rel/Dorsal). This domain is responsible for dimerisation, DNA-binding as well as I κ B interaction [34]. Within the RHD lies a Nuclear Localisation Signal (NLS) motif (a conserved cluster of positively charged amino acids), responsible for translocation into the nucleus. Additionally, RELA, RELB and c-REL possess a transactivation domain (TAD), on the carboxy terminus, that guides strong transcriptional activation in target genes [16, 27, 35, 36]. p50 and p52, that lack the TAD, can only promote transcriptional activation by interacting with Rel or even non-Rel proteins that do contain this domain. Otherwise, in the form of homodimers, they function as transcriptional repressors either by binding constitutively on κ B DNA, limiting access to other transcription factors, or by antagonising transcriptionally active complexes for binding.

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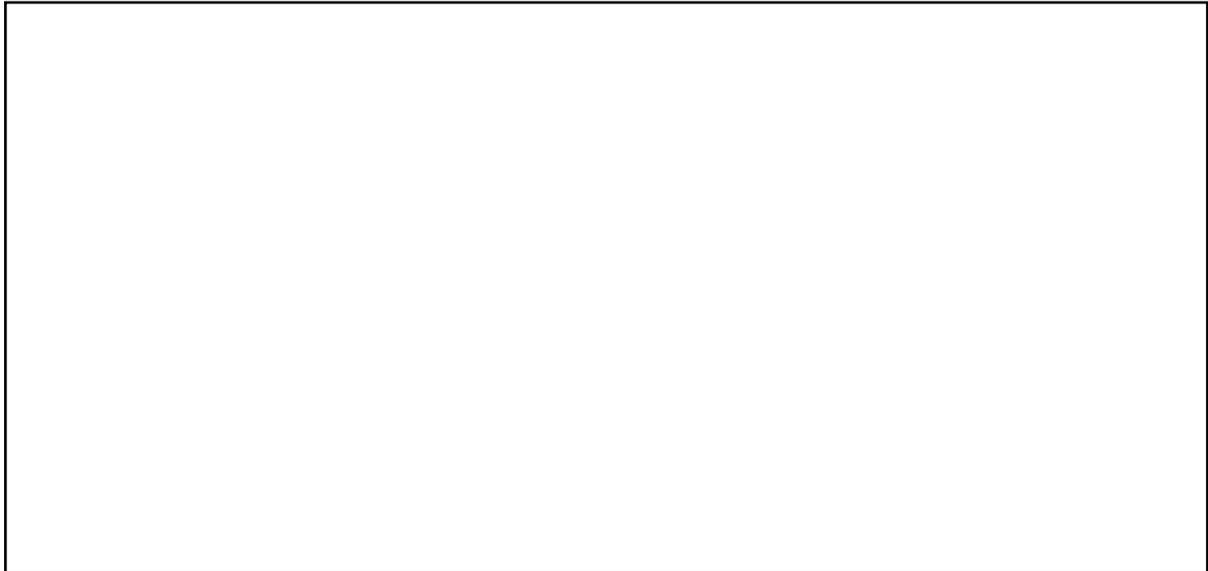


Table 2: NF- κ B dimer combinations and functions [29]

Dimers transfer into the nucleus through the importins system (importin $\alpha 3$ and $\alpha 4$) and out of it by the exportin protein XPO1 (CRM1 homolog) [37-39]. Each dimer complex binds on DNA with different specificity, affinity and activation potential, resulting from different threedimensional structure and stereochemistry of the dimers interacting with the respective DNA sequences [18, 36] (Figure 5). In other words, specificity derives from the structure of amino acid side chains of the transcription factor dimers contacting DNA bases of the κ B site [40]. The most abundant and stable NF- κ B dimer in cells is p65:p50 [36]. Homologs of Rel genes such as Dif, Dorsal and Relish or v-Rel oncogene also exist in other organisms (Drosophila or chicken virus respectively). A system similar to Dorsal/Dif even exists in plants upregulating pathogenesis resistance proteins. Nuclear Factor of Activated T cells (NFAT) is the closest relative of the NF- κ B transcription factors as it contains a Rel-homology domain [36, 41].

Figure 5: NF- κ B:DNA complex structure [40]

The role of NF- κ B in physiological processes in the organism has been extensively studied by the generation of knockout (KO) mice for each one of the Rel-family members. RELA-KO mice die at day 16 of gestation due to massive liver degeneration driven by extensive apoptosis in embryo's liver cells. This shows a critical role in the developmental stage either

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due to apoptosis promotion or because of lack of proliferating factors. Surprisingly *RELA*^{-/-} fetal liver stem cells were able to reconstitute haematopoietic lineages in lethally irradiated mice, meaning that *RELA* is not needed for development and differentiation of blood cells. *RELB* KO mice grow normally until 10 days postnatally, when lethal multi-organ inflammation develops. KO of the other REL proteins permits normal development but causes defective lymphocyte activation and other malfunctions [33, 42-47] (further details are available on Table 3). Generating double KO mice, which in general results in more severe phenotypes, reveals **redundant and complementary action** of the NF- κ B proteins. The detection of genetic mutations of the NF- κ B subunits in cancer, also outline their important role in cancerogenesis, as indicated in Table 4. Taken together, these data outline the important role of NF- κ B in many biological events such as development and immune response [18, 27, 36].

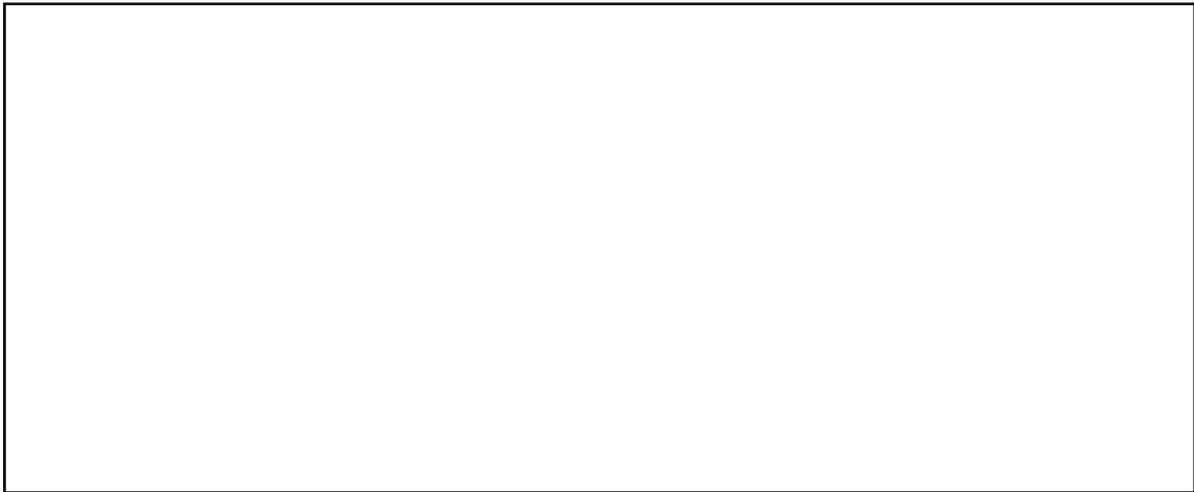


Table 3: Phenotype of NF- κ B KO mice [27]

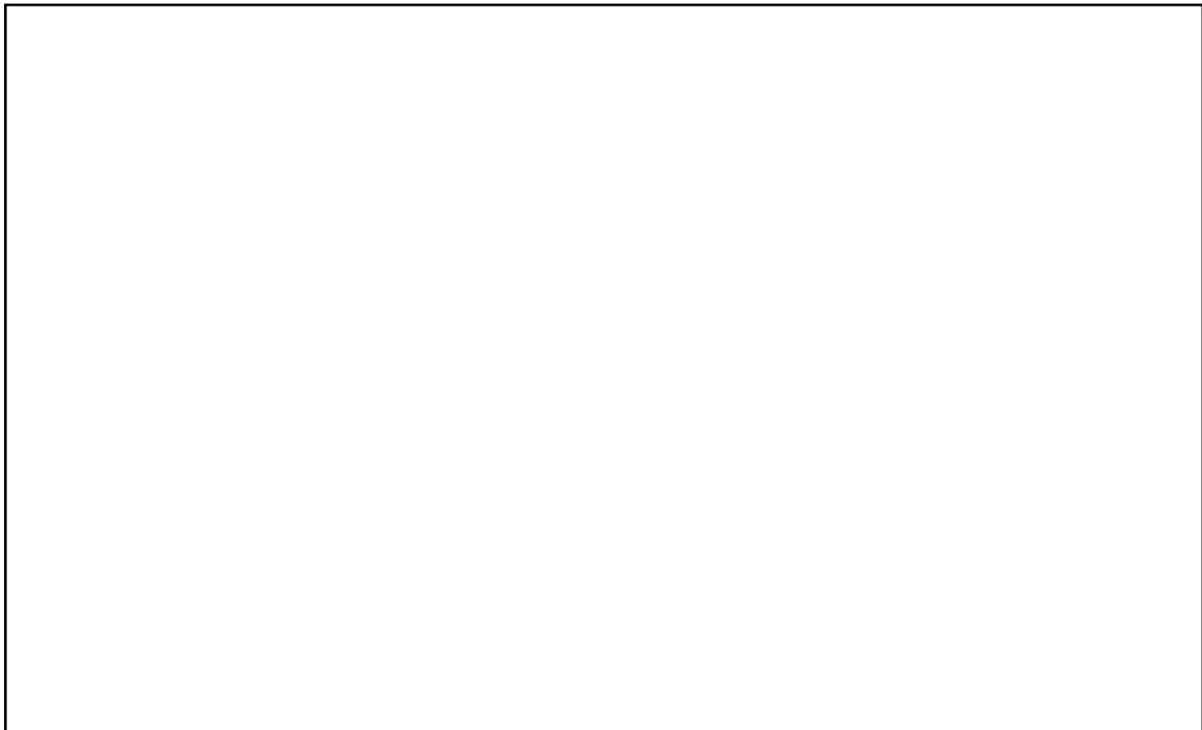


Table 4: NF- κ B subunit mutations and cancer. [48]

III.2. I κ B family

According to the classical model, potentially active NF- κ B dimers are sequestered in the cytoplasm in complexes with the I κ B family proteins that cover their NLS motif, thus preventing nuclear localisation and DNA binding. As research has moved forward on the field and new, quite heterogeneous, roles of these proteins have been revealed, a more precise characterisation is that of **NF- κ B co-factors or chaperones** that promote formation of otherwise unstable NF- κ B-dimers in either the cytoplasmic or the nuclear compartment of the cells. I κ Bs can influence co-activator recruitment on the NF- κ B:DNA complexes, altering transcriptional intensity, or mediate cross-talk between NF- κ B and other signal transduction pathways. They are even able of removing DNA-bound NF- κ B, thus terminating the transcriptional response [7, 16, 49]. NF- κ B-free I κ B molecules are quite unstable themselves as their half-life is very short, of approximately 10 minutes for I κ B α , so complex formation with their respective partners is a matter of their own stability [7, 50, 51].

Figure 6: I κ B family members. Domain organization and structural motifs found in human I κ B proteins are depicted. The illustrated I κ B β protein represents the human isoform-1. The UniProt database (<http://www.uniprot.org>) was used as a reference for protein sequence information of all I κ B family members. [7]

Typical or professional proteins of this group are I κ B α , I κ B β and I κ B ϵ , which are ubiquitously expressed with increases in certain tissues. There are also other members in this family, called atypical, such as I κ B ζ , BCL-3 etc whose expression is low in various types of cells but is induced upon stimulation [5, 6, 52-59]. The family's common characteristic is the presence of multiple ankyrin-repeat domains (ARD) mediating protein-protein interactions (Figure 6). The precursor molecules p105 and p100 also possess these domains, together with the RHD, functioning both as inhibitors and activators of transcription [7, 36].

Activation of NF- κ B takes place once dimers dissociate from the I κ Bs. The onset is phosphorylation of the inhibitors on serine residues located in generally conserved patterns named "destruction boxes" or "degron motifs" (-DSGXXS-) [60, 61]. This step permits recognition of the phosphorylated substrate by ubiquitinating systems, leading to supplemental ubiquitin tagging of the I κ Bs for further digestive processing or destruction by the proteasome. Deliberated transcription factor dimers can then translocate into the nucleus and regulate gene expression [7, 16, 62].

III.2.a. Typical/professional IκBs: IκBα, IκBβ, IκBε

The first and best characterised NF-κB inhibitor is IκBα. It preferentially associates to p65:p50 dimers both in the cytoplasm, preventing them from translocating into the nucleus, and also in the nucleus, displacing them from DNA and exporting them out of it thanks to a nuclear export signal (NES) it possesses (Figure 7). Actually it has been shown that the IκBα:p65:p50 complex can shuttle constantly between cytoplasm and nucleus. This dynamic state favours cytoplasmic localisation in resting conditions. Upon certain types of stimulation, NF-κB-bound IκBα undergoes rapid phosphorylation on serines 32 and 36 in the degron motif. IκBα phosphorylation is a necessary but not enough event to release NF-κB. IκBα must further be ubiquitinated and degraded by the proteasome in order to achieve activation. The phosphorylated form is then recognised by βTrCP and processed by the SCF complexes of E3 ligases where polyubiquitin chains are conjugated on this substrate [63-65]. Proteasomal degradation of IκBα follows, consequently releasing p65:p50 to translocate into the nucleus, bind on DNA and promote transcription of target genes. The sequence of events is IκBα phosphorylation→IκBα degradation→NF-κB release and not IκBα phosphorylation→NF-κB release→IκBα degradation [66-69]. Impressively, NF-κB drives transcription and neo-synthesis of its own inhibitor, as several κB sites are present in the promoter of the NFKBIA gene (coding for IκBα protein), introducing a negative feedback loop in the system [7, 16]. This loop allows close control and adjustment of response intensity, termination of transcriptional activity and system reset to default conditions. This critical point highlights the necessity of continuous control of inflammatory events as mentioned earlier, since the fragile equilibrium of inflammation benefits and dangers has to be maintained to avoid extensive damage in tissues [7].

Figure 7: NF-κB:IκBα complex structure. PEST: proline, glutamic acid, serine, threonine rich region, responsible for NF-κB binding and inhibiting DNA binding, ARD: Ankyrin repeat domain [40]

While IκBε (discovered in 1997 [53-55]) seems to act in a similar way to IκBα, IκBβ (discovered in 1990 [52]) acts as a cytoplasmic inhibitor only in a constitutively phosphorylated state. It then undergoes signal-induced phosphorylation (in the two serine residues of the destruction box) and degradation, like IκBα. At the same time, *de novo* synthesised hypophosphorylated form of IκBβ acts as an activator by migrating to the nucleus and stabilising DNA-bound NF-κB dimers and protecting them from IκBα, thus augmenting late transcription of certain genes [7, 16, 70, 71]. All three typical IκB family members have

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similar properties and their manner of action follows the same principles. On the contrary, induction, degradation and re-synthesis kinetics vary and partner preference (i.e. NF- κ B dimers) differs, as shown in Table 5 [72].

Table 5: I κ Bs functions and properties [7]

Several KO mice have been generated in order to study the role of the I κ B proteins. I κ B α KO pups are born normally but die a few days postnatally due to abnormal development. They display extensive granulopoiesis and skin defects, possibly because of rapid differentiation of skin basal layer cells. Thymocytes and splenocytes have significantly elevated nuclear NF- κ B levels in contrast to brain and fibroblasts where NF- κ B levels are only slightly elevated. NF- κ B activation also leads to up-regulation of only a part of target genes [73]. These events are attenuated in I κ B α /p50 double KO mice, suggesting that constitutive nuclear NF- κ B is the source of abnormalities in the I κ B α KO mice. Mouse embryonic fibroblasts (MEFs) do not need I κ B α for signal-dependent activation of NF- κ B, but it is absolutely required in order to down-regulate NF- κ B after activation [74]. Tables 6 and 7 include additional information for the phenotypes of the various I κ B KO mice, outlining their important roles. Triple knockdown (KD) experiments of the typical I κ B family members in MEFs have shown that the dynamic NF- κ B shuttling equilibrium (i.e. distribution in the two compartments, favouring the cytosolic) is generally not affected, probably because of the inhibitory action of p100 and p105 that compensate for the absence of I κ B α , I κ B β and I κ B ϵ . Basal DNA-binding activity is increased but there is no induction upon stimulation compared to wild type cells [75]. This means that professional I κ Bs are essential for preventing NF- κ B:DNA complex formation under resting conditions, as NF- κ B:I κ B complexes shuttle constantly between nucleus and cytoplasm. Furthermore these proteins are essential for stimulus responsiveness as in their absence, association of NF- κ B with other cellular factors prevents DNA binding. So the existence of typical I κ Bs allows on one hand inhibition of basal NF- κ B DNA-binding activity and on the other hand renders NF- κ B responsive to certain external stimuli [7, 16, 75].

Table 6: Phenotype of I κ Bs KO mice [7]



Table 7: Phenotype of I κ Bs KO mice. MEFs: Mouse embryonic fibroblasts, Δ C: carboxy-terminal deletion [27]

III.2.b. Atypical I κ Bs

Additional I κ B family members also participate in regulation of transcriptional activity of NF- κ B. BCL-3 can function both as transcriptional repressor and activator (as it possesses a transactivation domain) depending on its localisation and post-translational modifications (PTMs). I κ B ζ is an inducible transcription activator and I κ BNS is an inducible transcription repressor. All together they adjust and participate in the fine-tuning of transcriptional response. Complexity of the system at the level of I κ Bs can be understood by the data of Table 7, where the different members of this family can be induced rapidly or slowly by NF- κ B, be or not its targets, preferably bind certain type of dimers against others, be distributed in different proportions in cytoplasm and nucleus, function in an activating or inhibiting manner [7].

III.3. Bifunctional members NF- κ B1 and NF- κ B2

NFKB1 gene codes for the p105 protein that undergoes constitutive processing to p50 by the proteasome, at co-translational and post-translational level. Full-length protein contains both RHD and ARD motifs. A glycine-rich region of p105 serves as a stop signal for

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proteolysis, permitting release of the p50 fragment from the proteasome, which only contains the RHD motif [76-80]. The fate and final role of the synthesised protein depends on the pool of binding partners available at a given time in cells. One possibility is that the p105 acts as a classical I κ B by binding to existing NF- κ B dimers, while constitutive processing is inhibited. Another possibility is that, in presence of free NF- κ B subunits, p65 for example, formation of p105:p65 dimers is favoured, leading to proteasomal processing that gives rise to p50:p65 dimers. p105 can also participate in formation of higher-order complexes (e.g. p105:p65:p105:p65) [16, 81]. Obviously the distinct role of p105/p50 adds an additional regulation layer on the overall mechanism. As shown in the triple KD experiment mentioned above, in the absence of professional I κ Bs, p105 concentration in the cell is increased, probably due to association with NF- κ B dimers in order to prevent overactivity and extensive gene expression. The ability of the same protein to act in opposite ways is remarkable.

NFKB2 gene encodes the p100 protein, that also contains both RHD and ARD motifs. Similar to p105, it can act as a typical I κ B maintaining principally cytosolic localisation of the NF- κ Bs or it can undergo proteasomal processing to give rise to the p52 that contains the RHD and is able to bind on DNA. NFKB2 can be induced by certain types of stimulation, usually different from those regulating stability of professional I κ Bs. Both p105 and p52 can dimerise with any of the NF- κ B subunits and particularly with RELB, contributing to their stabilisation. The RELB:p52 dimer can promote late transcription as it is resistant to I κ B α 's inhibitory action. In certain cases RELB can repress gene transcription [7, 16, 82]. To conclude, the fate of both p105 and p100 depends on the pool of NF- κ B subunits present in the cell.

III.4. IKKs – Canonical and non-canonical activation pathways

As mentioned above, I κ B degradation can only be performed after it has been phosphorylated. Proteins responsible for this activity are the I κ B Kinases (IKKs). Several groups have described the IKK as a 550-900 kDa complex consisting of at least three different subunits. IKK α and IKK β are the subunits with the catalytic activity since they possess a Kinase Domain (KD) [8-15]. IKK γ or NEMO (NF- κ B essential modulator) is the regulatory subunit of the complex (Figure 8). The exact stoichiometry of the complex is unknown but IKK α :IKK β heterodimers and NEMO homodimers are thought to exist in a proportion of 1:1:2 (IKK α ₁:IKK β ₁:NEMO₂), together with other not yet characterised partners [16, 83, 84].

Figure 8: Members of the IKK complex. The IKK complex consists of IKK α (IKK1 or CHUK), IKK β (IKK2), and NEMO (IKK γ). Relevant domains of each protein are indicated. (LZ) leucine zipper domain, (SDD) scaffolding and dimerization domain, (ULD) ubiquitin-like domain, (Z) zinc finger domain, (CC) coiled-coil domain, (NBD) NEMO-binding domain, (α) α -helical domain, (IBD/DimD) IKK-binding domain/dimerization domain, (MOD/UBD) minimal oligomerization domain/ubiquitin-binding domain [16]

IKK α and IKK β also contain a scaffold/dimerisation domain (SDD), an Ubiquitin-like domain (ULD) and a NEMO Binding Domain (NBD). NEMO contains a C-terminal minimal

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oligomerisation domain (MOD) and a N-terminal dimerisation domain (DimD) together with a N-terminal IKK binding domain (IBD) and 2 Ubiquitin binding domains (UBD) [Figure 8]. These domains mediate interaction with IKK α and β and also allow formation of NEMO dimers, trimers, tetramers etc. Each domain offers distinct characteristics to each subunit and permits complex assembly and activation, which can be regulated in a positive or negative way through phosphorylation of NBD of IKK α and β or phosphorylation of DimD-IBD of NEMO respectively [16, 83, 85, 86].

Dimerisation of the catalytic subunits is necessary for inducible activation. IKK activity can be induced by phosphorylation of certain residues in both IKK α and IKK β and more specifically on Ser176 and Ser180 or Ser177 and Ser181 respectively. Organisation in high-order multi-IKK structures upon stimulation may provide the conditions to undergo trans auto-phosphorylation and auto-activation. At the same time aggregation of various molecules also increases proximity with IKK kinases (IKK-K). The only known IKK kinases (IKK-K) are the TGF β -activated kinase 1 (TAK1) and the NF- κ B Inducing Kinase (NIK) that upon certain conditions can phosphorylate and activate IKK complex subunits. According to signal origin, activity of the IKK complex can be modulated in different ways. Certain signals involve direct trans auto- or TAK1-mediated phosphorylation and activation of IKKs, in order to successfully transduce the signal through IKK β -dependent phosphorylation of I κ B α [87]. These pathways are called **canonical or classical**, and NEMO participation is necessary. **Alternative or non-canonical** activation can occur by stimuli that lead to trans-auto- or NIK-mediated phosphorylation and activation of IKK α and further IKK α -mediated phosphorylation of p100 when in complex with RELB. NEMO does not participate in the alternative pathway, which is usually working with slower kinetics. Some signals induce both the canonical and noncanonical pathways

NEMO gene locus lies in the X chromosome and several gene mutations or deletions have been linked with genetic disorders in human, while gene KO in mice leads to lethality due to massive apoptosis in the liver, as observed in the case of IKK β or RELA KO mice. In the case of Incontinentia Pigmenti syndrome where NEMO is mutated, female heterozygous individuals develop skin lesions with hyper-proliferation and increased apoptosis of keratinocytes and abnormal hair, teeth and central nervous system (CNS) development and function, while male foetuses die in uterus [18, 88]. 85% of the patients produce a truncated form of NEMO devoid of activity. Several other genomic abnormalities of the NEMO gene locus have been identified in X-linked ectodermal dysplasia with immunodeficiency syndrome [88, 89]. More details for the phenotypes of mice lacking the IKK genes are available in the following table.



Table 8: Phenotype of IKK KO mice [27]

III.4.a. TAK1 and canonical pathway

In addition to IKK trans auto-phosphorylation and auto-activation, TAK1 is also able to phosphorylate IKK. TAK1 is implicated in the canonical NF- κ B activating pathways, at the level they converge to IKK activation. TAK1 is a member of the MAPKKK family proteins and is needed for proper activation and survival of T and B cells by inducing nuclear localisation of NF- κ B and activation of MAPK pathways. Mice lacking TAK1 die during gestation due to abnormal development of the neural tube (Table 9). Embryonic fibroblasts derived from TAK1-null mice exhibit dramatically impaired NF- κ B and JNK activation through TNFR, IL-1R and TLR signaling. In another model of inducible KO mice, massive apoptotic cell death was observed in haematopoietic cells in the bone marrow, spleen, thymus and hepatocytes leading to lethality a few days after KO induction [90, 91]. It has also been suggested that, similarly to NEMO, ubiquitinylation of TAK1 is required for its full activity [92, 93].

Complexes consisting of TAK1 and TAB (TAK1 binding) adaptor proteins need to be formed so as to promote TAK1 activity. TAK1 is constitutively bound on TAB1. Upon stimulation an additional subunit, TAB2 or the homologous TAB3, becomes part of the complex in order to acquire full kinase activity. TAB1-deficient mice embryos die at later stages of gestation due to abnormal cardiovascular and lung morphogenesis whereas TAB2-deficient embryos die earlier because of liver degeneration and apoptosis (Table 9). IKK activation and NF- κ B or JNK activity upon stimulation are not affected in cells lacking TAB1 or TAB2. Another study though has shown that simultaneous silencing of TAB2 and TAB3 is able to inhibit IKK and JNK activation, supporting perhaps abundant roles [90].

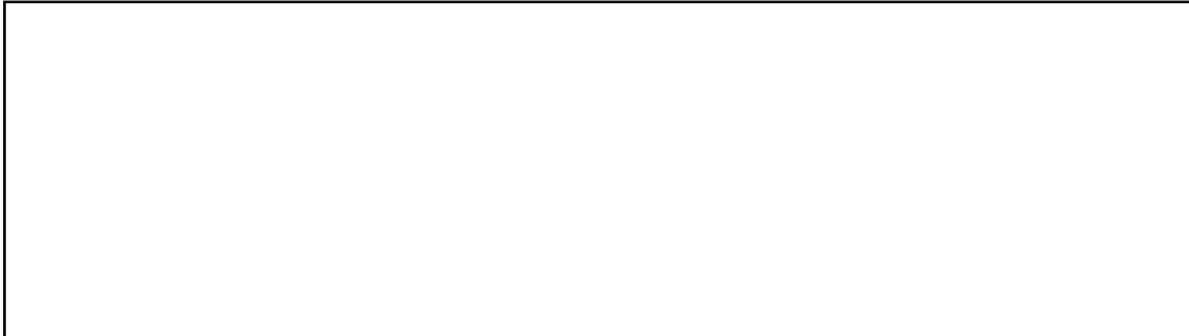


Table 9: Phenotype of TAK1, TAB1, TAB2 KO mice [90]

These events outline the importance of TAK1 and TAB proteins during development but also point out unique functions of the subunits, independent from each other, as embryos do not develop the same phenotypic characteristics. This is further supported by the fact that only TAK1 plays a direct and vital role in NF- κ B signaling [90].

Sixteen years since the discovery of the IKKs have not been sufficient to answer questions concerning their activation, inactivation and substrate specificity. What is sure, and has been proposed in many publications, is that the assembly of large complexes plays a key role in NF- κ B activity regulation, especially in the canonical pathways [16, 83].

III.4.b. NIK and noncanonical pathway

Historically, NIK was the first IKK-K to be characterised. Soon it was shown that it is implicated in noncanonical NF- κ B-activating pathways, by phosphorylating the p100 subunit. For more details concerning the mechanism see chapter IV.6.

IV. Ubiquitin-dependent regulation of NF- κ B

IV.1. Ubiquitylation, general concepts

Ubiquitin is, as the name states, a ubiquitously expressed small protein of 76 amino acids long. High degree of evolutionary conservation among species, along with the fact of co-transcription/co-translation with ribosomal proteins, outlines its importance and has attracted researchers attention for more than 30 years (Figure 9). Ubiquitin is encoded by four genes in mammals. Ubiquitylation is a post-translational modification (PTM) through which ubiquitin can be covalently attached on target proteins, altering their properties and functions. Approximately 80% of all cellular proteins are targeted by ubiquitin. It contributes in regulation of events like protein half-life and degradation, DNA repair, endocytosis, autophagy, subcellular distribution and trafficking, transcription, signal transduction, immunity, inflammation and virtually every cellular process [16, 89, 94-96].

Figure 9: Timeline of the discovery of the Ubiquitin-Proteasome system (UPS). The diagram shows sum of the pivotal discoveries and milestones that historically lead to the identification of the UPS intracellular protein degradation. At present, several diseases related to protein misfolding and defect in protein degradation have been identified, and specific inhibitors of the UPS are now in clinical trials [97].

The machinery capable of tagging proteins with ubiquitin, through a multistep procedure, consists of three types of enzymes. The E1 enzyme activates Ubiquitin in an ATP-dependent manner. Activated Ubiquitin is then forwarded to the E2 conjugating enzyme (or UBC-ubiquitin conjugating) and the E3 ligation enzyme (Figure 10). In humans there are 2 E1 enzymes, around 40 E2 and 600-700 E3 ligases. Concerning the latter, they are subdivided in two categories, the around 30 HECT (Homologous to the E6-AP Carboxyl Terminus) E3s and the more abundant (>600) RING (Really Interesting New Gene) E3s. The difference lies in the mechanism of catalysis for isopeptide bond formation between ubiquitin and the acceptor protein. The active site of HECT E3 ligases forms an intermediate with ubiquitin before covalent binding to the target (Figure 11, left panel). On the other hand, RING E3s mostly act as scaffolds, mediating direct ubiquitin transfer from E2 conjugating enzyme to substrate (Figure 11, right panel) [16, 89, 95, 96].

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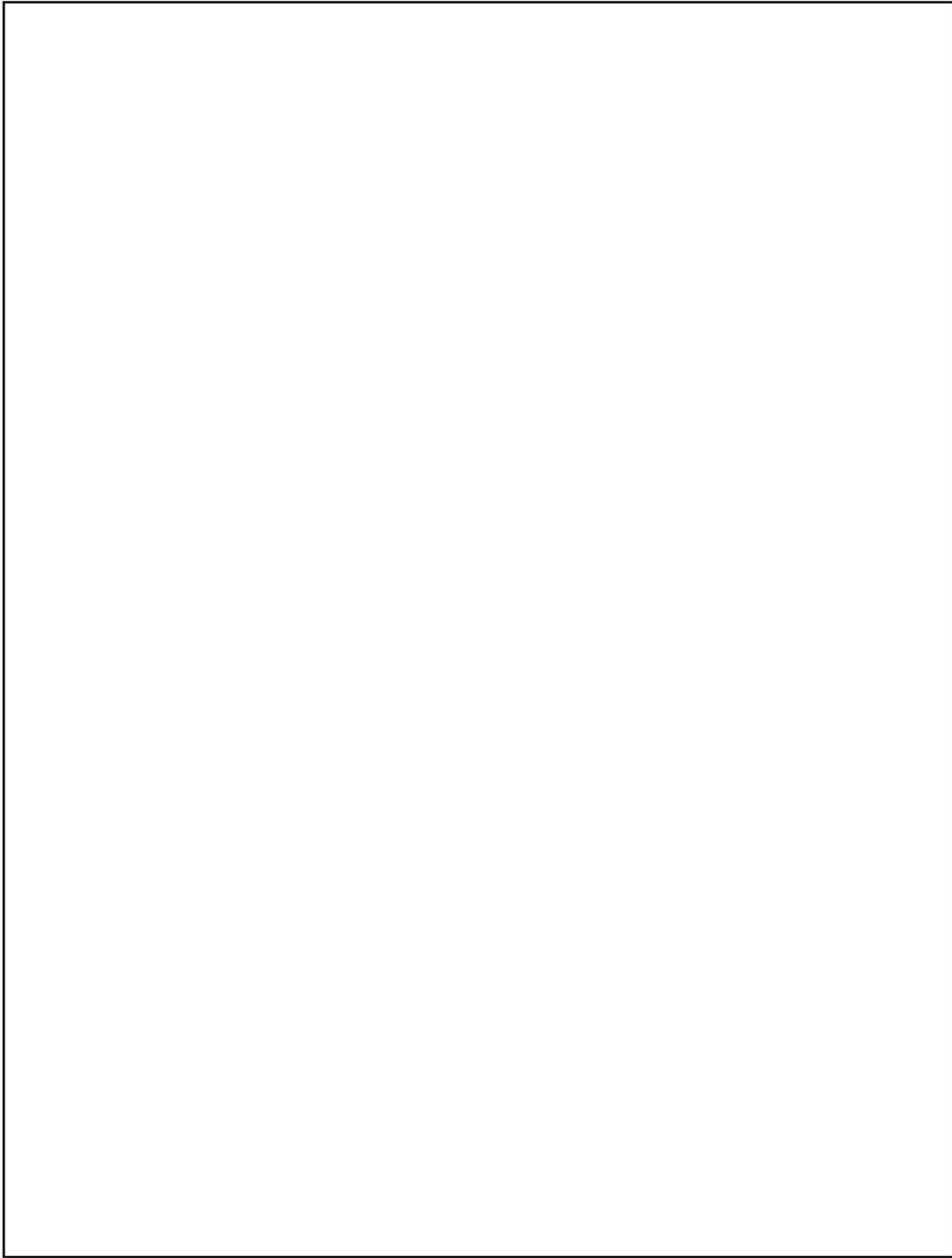


Figure 10: Ubiquitylation series of reactions [96]

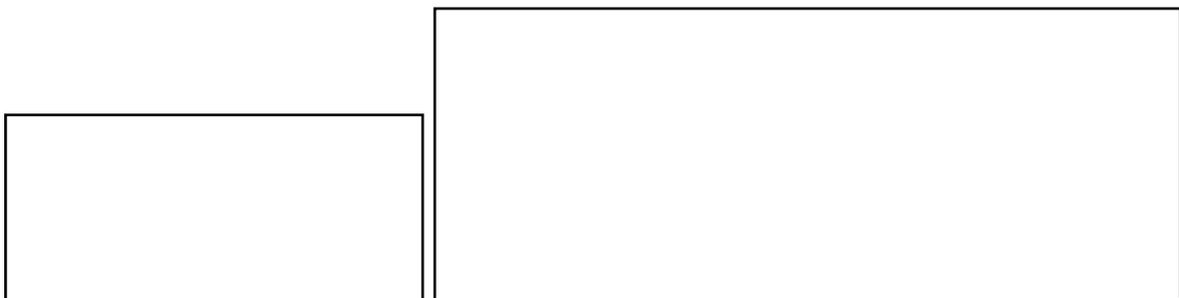


Figure 11: HECT E3 ligase (left) and RING E3 ligases (right). Ub: Ubiquitin (yellow), E2 conjugating enzyme (green), HECT domain (light blue), RING domain (red) [96]

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Isopeptide bond formation takes place between the C-terminal glycine residues (G76) of Ubiquitin and the ϵ -amino group of the target proteins lysine. Additional nucleophilic amino acids have been described to work as ubiquitin acceptors, including threonine, serine and cysteine. Substrates can either be mono-ubiquitinated (conjugation of a single ubiquitin molecule) once or multiple times (multi-mono-ubiquitination), or poly-ubiquitinated. In the last case, long chains are sequentially formed either on the substrate-attached Ubiquitin or on E2-E3 enzymes and further transferred to substrates en bloc [16, 89, 95, 96, 98].

Ubiquitin contains 7 lysine (Lys or K) residues that can serve as ubiquitin acceptors themselves, for chain formation (see figure 10). N-terminal methionine (Met or M) acts also as an acceptor. As a result there are 8 different types of poly-ubiquitin chains according to the acceptor site: K6, K11, K27, K29, K33, K48, K63 and M1 or linear or head-to-tail chains. Each type has a different impact on the fate of the protein, for example K48 or K11 chains usually tag proteins for proteasomal degradation whereas K63-tagged proteins or linear (M1) ubiquitin conjugates are implicated in non-proteolytic functions, usually acting as scaffolds for signaling complexes to assemble [16, 89, 95, 96, 99-102]. Heterotypic and mixed chain types also exist, their roles though remain poorly understood.

Free Ubiquitin molecules adopt a structure of a mainly polar globular surface with a large hydrophobic area centered on the Leu8-Ile44-Val70 residues, conformation though changes in in the form of ubiquitin chains. For example K48 chains adopt a closed conformation in solution, where the hydrophobic patch is buried within the protein, but the structure remains rather dynamic. On the other hand, K63 and linear chains have an extended conformation and hydrophobic surfaces are fully exposed. Thus, it is clear that different linkages between ubiquitin moieties alter molecular topology and probably chain properties, dictating signaling outcome [89].

While the E3 ligases guide target specificity, it is the E2 enzymes that dictate the type of polyubiquitin chain that will be assembled. Furthermore, RING E3s can be present and active as monomers, homodimers, heterodimers or multi-subunit complexes. The latter case further increases target range and specificity as shown by the example of the tripartite SCF E3 ligases that count up to 69 different complexes in mammals [95, 96]. Of note, SCF ^{β TrCP} is the E3 ligase complex that ubiquitinates I κ B for degradation upon stimulation (see figure 11, right panel). Very often the E3 ligases are organised in complexes together with E2 enzymes resulting in high enzymatic processivity. Some proteins even possess both E2 and E3 activities in the same molecule.

Various motifs called Ubiquitin Binding Domains (UBD) are present in proteins and mediate interactions with ubiquitin-tagged substrates, in the same way phosphorylated substrates can be recognised by the respective motifs. A large variety of UBDS exists and are classified in approximately 20 families, according to their three-dimensional structure. Most of them bind on the hydrophobic patch of ubiquitin around Ile44 but they also recognise adjacent surfaces, altering specificity. Certain UBDS are responsible for recognition of mono-ubiquitinated substrates whereas others preferentially bind long chains and can even discriminate linkage type. Some of them for example preferentially associate to K48 ubiquitin chains, others to K63 type, linear etc [89].

Binding affinity of individual domains for ubiquitin monomers is generally very low (which is logical considering the high concentrations in cells). For this reason various strategies have been developed to augment the binding capacity of ubiquitinated proteins to the respective receptor proteins. A single ubiquitin molecule can be recognised by a complex of 2 or more UBDS combined together (1ub by >1ubd). One ubiquitin can equally be recognised by a single UBD (1ub by 1ubd). Two or more ubiquitin molecules can also

interact with a single UBD (>1 ub by 1ubd). By keeping in mind that any combination of UBDs can occur in a protein and that any association of such proteins with one another is possible, it becomes clear that recognition and interaction with ubiquitinated substrates can occur with extremely high avidity [89].

Interactions can be further enhanced by the presence of many mono-ubiquitin modifications, or by formation of long polyubiquitin chains on a protein, or even by oligomerisation of poly-ubiquitinated proteins. Combining at the same time the synergistic strength of bond affinities together with similarly grouped together ubiquitin molecules in the form of polymers, results in a quite complex organisation level of the machinery involving ubiquitin-tags and ubiquitin-receptors [89].

As mentioned earlier, the type of poly-ubiquitin chain attached to a protein dictates their fate. Processing and degradation of ubiquitin-tagged proteins is mediated by the proteasome, a large multi-subunit complex. Chain length and type of linkage play an important role in the process, as short chains or type K63 ones do not contain the appropriate signal for degradation. K63 or linear poly-ubiquitin chains though are indispensable for some signal transduction pathways and especially those responsible for activation of the transcription factor NF- κ B [89].

IV.2. Ubiquitylation at the IKK and IKK-K level

All signal transduction pathways include cascades of substrate phosphorylation and aggregation events. In the case of NF- κ B activation, ubiquitylation plays an additional key role [8]. Not only IKK members, i.e. NEMO, undergo ubiquitylation, but also various adaptor molecules acting upstream of IKK in NF- κ B-activating pathways (and whose effects converge on its phosphorylation-dependent activation) [103]. The implication of ubiquitin receptors is also indispensable in the regulation of NF- κ B. The IKK-K TAK1 and even NEMO interact with ubiquitinated proteins in order to promote phosphorylation and induction of IKK β enzymatic activity [104].

IV.2.a. Ubiquitylation and NEMO

NEMO undergoes poly-ubiquitylation in a K63 manner in response to various stimuli like TCR engagement or NOD2 and TLR binding on bacterial products [86]. The E3 ligase is not yet known, it is speculated though to be TRAF6. There is controversial data concerning the role of NEMO ubiquitylation in activation of NF- κ B. A paper of 2004 shows that NEMO needs to undergo K63-type multiubiquitylation on K399 when mimicking TCR stimulation, in order to fully activate NF- κ B since lysine to arginine mutants show lower activity. So this event is important during antigen stimulation, probably to mediate recruitment of upstream regulators, like IKK-Kinases, or to induce IKK autophosphorylation [105]. In contrast, a publication of 2008 opposes this model. Lysine 392 to arginine mutation in murine NEMO (equivalent of K399 in human) was introduced into mouse germline and NEMO-K392R mice were generated, in which K63-linked ubiquitylation of NEMO was disrupted. These mice have no apparent defect in antigen receptor signaling. However, primary macrophages and dendritic cells exhibit weakened cytokine production responses to TLR (Toll-like receptor) agonists. Of note, no skin disorders or immunodeficiency developed as in the case of NEMO mutations in humans. NF- κ B and MAPK signaling are largely unaffected in cells from these mice despite the decreased levels of cytokine production upon

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LPS stimulation. Thus, the proposed role of K63 ubiquitylation of NEMO is that of a rheostat for TLR-dependent responses in innate immunity, that does not go through NF- κ B activation [106].

At the same time, NEMO undergoes a newly characterised modification, that of linear ubiquitylation by the LUBAC (linear ubiquitin chain assembly complex) in response to cytokines (TNF α) and some bacteria [107]. LUBAC consists of three subunits: the E3 ligases RNF31 (RING Finger Protein 31, also called HOIP) and RBCK1 (also known as HOIL-1), and Sharpin [108]. At present time, RNF31 is the only E3 ligase known to catalyse linear ubiquitylation. Some studies support that in Sharpin-deficient cells LUBAC is unstable failing to activate IKK and this defect causes cell death that further triggers inflammatory diseases [109-111]. However others reported normal or even hyperactivated cells without the LUBAC. The exact role of NEMO ubiquitylation is not clear and needs further investigation, especially when considering that on the same lysine residues both linkage type ubiquitin chains can be attached [112-114].

Figure 12: NEMO mutations in human pathology.

Mutations generating Incontinentia Pigmenti (IP) (red), Ectodermal Dysplasia with immunodeficiency (EDA-ID) (blue) and immunodeficiencies without EDA (ID) (green) are indicated. To simplify the figure, frameshift mutations are not presented. Basically, those mutations located before the proline-rich (PR) domain of NEMO (indicated by a black bar near the ZF) generate IP, whereas those located after PR generate EDA-ID. Frameshift mutations located inside PR tend to generate IP when the number of extra amino acid (aa) that is added is large and EDA-ID when it is limited. Δ 37 represents a

mutation that produces a protein lacking the first 37 aa. Δ (IP) represents the most frequent mutation found in IP that is due to a genomic rearrangement of the NEMO locus. Δ 271–276 and Δ exon 9 are caused by splicing mutations. The X420W mutation changes the stop codon to a Trp codon, also adding 27 aa, and is responsible for the OL-EDA-ID (EDA-ID with Osteopetrosis and Lymphedema) syndrome. CC, coiled coil; LZ, leucine-zipper; ZF, zinc finger. 'C' and 'H' labels indicate cysteine and histidine residues that tetra-coordinate the zinc in the ZF [115].

NEMO contains two UBDs, able to bind linear but also K63 and K11 polyubiquitin chains. Mutations in such domains are found in multiple pathological conditions, indicating the importance of ubiquitin sensing in NF- κ B activating pathways (Figure 12) [88, 89]. In experiments performed on a fragment of NEMO containing the one UBD, it was shown that it preferentially recognises linear di-ubiquitin chains to K63 ones with very high affinity. In contrast, structural studies performed on the other UBD (carboxy-terminal ZF domain) showed that this motif has no preference for chains of any linkage type. Whatismore, full length NEMO, containing both UBDs, interacts stronger with K63-linked chains, most likely because of cooperative binding, and binds longer K63-linked chains with increased affinity. NEMO thus may act as a multifunctional ubiquitin receptor recognising either short linear or longer K63-, K11-linked ubiquitin chains. Linkage type specificity may depend on local concentration of ubiquitin chains conjugated to substrates in defined subcellular compartments, a matter that needs further investigation [89, 112, 113].

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Although there is no concrete explanation of the above events for the moment, the dual role of NEMO in NF- κ B pathways is undeniable. Especially when considering that alterations of its ubiquitin-modification capacity (of either linear or K63 ubiquitylation or recognition of poly-ubiquitin chain types) leads to severe signaling malfunctions and pathological phenotypes [112].

IV.2.b. Ubiquitylation and TAB:TAK1 complex

Similarly to NEMO, the adaptor proteins TAB2 and TAB3 contain UBDs that recognise and facilitate binding on K63-linked poly-ubiquitin chains. Upon stimulation TAK1:TAB1:TAB2/3 complex can bind on a variety of ubiquitylated substrates, deriving by various receptors engagement through signal transduction pathways. This anchoring event leads to enzymatic activation of TAK1 which is then able to process IKK β phosphorylation and IKK-complex-mediated activation of the NF- κ B transcription factors [116]. Overall the current model proposes that TAK1 acts upstream of IKK maybe by branching signal transduction from upstream complexes to NF- κ B and MAPK pathways [104]. Interestingly, TAK1 also seems to integrate signals originating from various canonical NF- κ B activating pathways in order to activate IKK β , whereas non-canonical i.e. through NIK-IKK α , are TAK1-independent [90, 116].

So far, the importance of ubiquitylation in the regulation of the NF- κ B activation pathways is evident at many levels. K48-linked poly-ubiquitylation of I κ B α is necessary for proteasomal degradation in order to promote transcriptional activation. In the non-canonical pathway, K48 ubiquitylation of p100 by NIK-activated IKK α is necessary in order to undergo proteasomal processing for the production of active p52:RELB dimers. In the canonical pathways TAK1-activated IKK β and NEMO are indispensable. NEMO acts as a receptor of K63 and linear ubiquitin chains in the complex with IKK α/β together with the TAB subunits that act as K63-linked ubiquitin receptors, complexed with TAK1. Indeniably ubiquitin sensing is necessary to achieve activation and signal transduction.

Each signaling pathway, originating from distinct signal sensors that launch a series of events, passes through ubiquitylation of various adaptor molecules in order to finally activate NF- κ B transcriptional activity. These adaptors act as ubiquitylated signal transmitters as this modification can be recognised by central regulators (NEMO, TAB2/3) mediating maximal IKK activity and subsequent NF- κ B activation. There are some specific and some common adaptors in each pathway that undergo ubiquitylation. There have been several models proposed in order to explain these observations.

IV.3. Role of Signalosomes

As described above, signaling in most canonical NF- κ B-activating pathways proceeds through aggregation and oligomerisation of a series of adapter proteins possessing protein-protein interaction domains, for the formation of oligomers, such as CARDs (Caspase Recruitment Domains), TRAFs (TNF Receptor Associated Factor), DDs (death domains), BIRs (baculoviral inhibition of apoptosis protein repeats), RHIMs (RIP homotypic interaction motifs) etc. Conformational changes of distinct receptors, triggered by binding of the respective ligand, facilitate distinct as well as common adapter proteins' access, providing links to downstream components. In addition to signalosome formation, these subunits are ubiquitin-tagged in a way that does not condemn proteins for proteasomal degradation but

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rather promotes signal transmission to activate NF- κ B by bridging otherwise diffuse and remote proteins. Various models are currently proposed to explain how these events finally converge in regulation of oligomerisation-mediated, phosphorylation-dependent IKK activation status [16, 117, 118].

1-NEMO ubiquitinylation or ubiquitin binding of upstream poly-ubiquitinated adaptors might function in the same way as phosphorylation i.e. induce conformational changes in the IKK complex leading to IKK β phosphorylation and activation.

2-Induced proximity model can explain how IKK phosphorylation occurs, as scaffolds of many ubiquitinated proteins grouped together can serve as recruiting platforms for even more proteins containing UBDs. The TAK1 complex, through TAB2/3, and the IKK complex, through NEMO, may be found close enough to allow IKK phosphorylation by TAK1 and trans-autophosphorylation of IKK subunits by neighbouring IKK complexes.

3-Alternatively, non-degradative K63 poly-ubiquitinylation might increase signalosome stability either by preventing K48 ubiquitinylation and degradation of intermediate molecules or by physically stabilising and fixing complexes through “cross-linking” of UBD-containing proteins.

4-Yet another poorly explored hypothesis is that non-degradative ubiquitinylation can be separated from but necessary for signaling to occur. Ubiquitin linkages other than K48 could target signalosomes components, rendering them recognisable by appropriate chaperones in order to disassemble these multiprotein complexes and reset signaling pathways to default conditions. This could guarantee immediate disposal of the subunits in case of reappearance of the stimulating signal [16].

Research at the present stage cannot clearly exclude any of these models or even the possibility that all can be true. Many questions await to be answered.

IV.4. Ubiquitinylation during antigen receptor-mediated NF- κ B activation

IV.4.a. Overview of early TCR signaling

T-cell receptor (TCR) is a plasma membrane protein complex consisting of various dimers of non-polymorphic CD3 proteins together with a single heterodimer of the variable TCR α and β chains. The latter are responsible for recognition of linearised antigenic peptides bound on major histocompatibility complex (MHC) of antigen presenting cells (APCs), whereas CD3 subunits mediate interactions with intracellular proteins. Upon antigen presentation, TCR aggregation and conformational alterations initiate signal transduction. CD3 proteins possess cytoplasmic tails containing ITAMs (immunoreceptor tyrosine-based activation motifs) [119]. These motifs undergo phosphorylation on tyrosine residues, serving as docking sites able to recruit cytosolic PTKs (protein tyrosine kinases) like ZAP70 [120]. PTKs may further phosphorylate a spectrum of substrates leading to downstream signals that when appropriately integrated, together with signals from co-receptors, can lead to activation of T lymphocytes (Figure13). Following TCR engagement and phosphorylation cascades, PLC γ 1 (Phospholipase C γ 1) is found in the proximal signaling complex where it undergoes activating phosphorylation. PLC γ 1 can then hydrolyse its substrate, producing the second messenger molecules IP $_3$ (inositol triphosphate) and DAG (diacylglycerol), each with distinct functions in signal transduction. The second messenger IP $_3$ induces enrichment of the cytoplasm with another second messenger, Ca $^{2+}$. IP $_3$ -induced ion channels opening of the endoplasmic reticulum leads to Ca $^{2+}$ release in the cytoplasm. Emptying endoplasmic reticulum stores further triggers a sustained influx of extracellular Ca $^{2+}$ through plasma

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membrane channels [121]. Increased cytoplasmic concentration of Ca^{2+} results in activation of calcium-sensitive proteins that regulate activity of various transcription factors such as NFAT (nuclear factor of activated T cells) family [122]. NFAT translocates into the nucleus where it can form complexes with a variety of other transcription factors, usually AP-1 (activator protein-1), resulting in differential gene expression profiles and functions (Figure 13) [123].

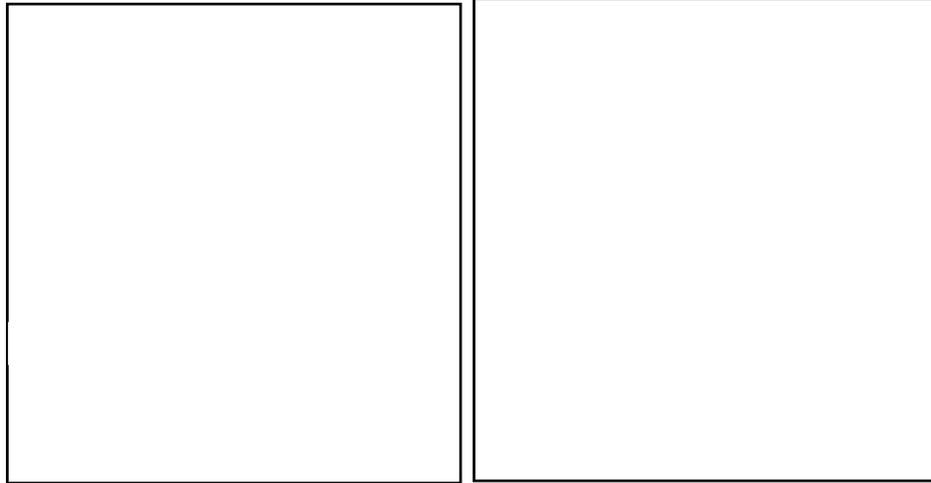


Figure 13: Early tyrosine phosphorylation events in response to TCR ligation (left) and schematic overview of TCR-originating signalling pathways (right). Engagement of TCRs and costimulatory CD28 receptors promote signalling cascades of kinases and adaptor proteins (left panel and yellow). They trigger pathways resulting in the activation of the transcription factors NFATc (red), NF- κ B (green) and AP-1 (blue). These transcription factors cooperate with each other during the activation of several genes, e.g. IL-2. DAG: diacylglycerol; IP3: inositol-1,4,5-trisphosphate; PIP2: phosphatidylinositol-4,5-bisphosphate; PKC θ : protein kinase C theta; PLC γ : phospholipase C gamma. [123, 124]

Second messenger DAG leads to activation of two major pathways through recruitment of the Ser/Thr kinase PKC θ (Protein Kinase C θ) to the plasma membrane. In that location it can initiate Ras-dependent MAPK phosphorylation cascades, leading to activation of the AP-1 transcription factors [125]. PKC θ activation also leads to the activation of the NF- κ B transcription factors by promoting assembly of high-order signaling complexes that activate IKK [126]. Despite the high specificity and affinity between TCR and the antigenic peptide bound on the MHC of APCs, the signal is not enough to achieve maximal stimulation of T cells. It would rather cause anergy, a state in which T cells are refractory to restimulation. Costimulatory signals mediated by co-receptors are also needed. Magnitude of response is considerably augmented in the case of CD28 co-ligation. This latter event promotes expression of genes favouring survival, maximises transcriptional activity of NFAT, enhances Ca^{2+} flux and subsequently all downstream events etc [127]. In laboratory conditions, the use antibodies against CD3 and CD28 can activate T cells. For a long time it has been known that phorbol esters, like PMA, together with ionophores, like ionomycin, activate PKC θ , causing Ca^{2+} release and mimicking TCR activation effects. In addition, TCR signaling initiates a program of cytoskeletal rearrangements that results in polarisation and activation of T lymphocytes. Affinity and avidity of integrins for their ligands also increase as a result of TCR-mediated biochemical events [123].

IV.4.b. TCR and canonical NF- κ B signaling

So after TCR engagement, parallel proximal signaling cascades, implicating kinase activation and integration of adaptors and phospholipases, converge in the generation of

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second messengers that activate PKC [123]. Whereas early signals are transduced by successive tyrosine phosphorylation events, after PLC γ 1 activation and IP $_3$ and DAG production, signal-propagation strategies change. Signaling events like serine/threonine phosphorylation and lysine ubiquitinylation take place downstream of PKC, promoting complex formation that, in the case of NF- κ B activation, culminate in IKK complex activation. According to the current model, activated PKC θ phosphorylates the protein CARMA1 [caspase recruitment domain (CARD) and membrane-associated guanylate kinase (MAGUK)-containing scaffold protein 1] (Figure 14a) [128, 129].



Figure 14: Model for protein-kinase-C-mediated CARMA1 activation. **a** | A fraction of cellular CARMA1 (caspase-recruitment domain (CARD)–membrane-associated guanylate kinase (MAGUK) protein 1) resides in lipid rafts, probably recruited by interactions between the SH3 (SRC-homology 3) domain of CARMA1 and an unknown protein(s). Domains of CARMA1 that are required to trigger the oligomerization of downstream components – such as the coiled-coil (CC), CARD and possibly guanylate kinase (GUK) domain – are inaccessible to their binding partners. Protein kinase C- β (PKC β) or PKC θ activated by antigen-receptor signals phosphorylate specific serine residues in the PKC-regulated domain (PRD) of CARMA1. **b** | The phosphorylation of CARMA1 (indicated by red stars) destabilizes inhibitory intramolecular interactions, allowing binding interactions with downstream molecules such as B-cell lymphoma 10 (BCL10), and CC-driven oligomerization of CARMA1. **c** | Oligomerization of CARMA1 recruits additional CARMA1 molecules from the cytosol or membrane. The oligomerization of CARMA1 triggers a downstream oligomerization cascade (through BCL10) that is necessary for IKK (inhibitor of nuclear factor- κ B (I κ B) kinase) activation. **d** | Further amplification of the antigen-receptor signal might be gained by a higher-order multimerization of CARMA1 proteins by intermolecular MAGUK interactions – comprised of PDZ (PSD95, DLGA and ZO1 homology), SH3 and GUK domains – as described for other MAGUK proteins [130].

CARMA1 is a multi-domain scaffold protein (Table 10), specifically expressed in lymphocytes, a fraction of which is constitutively localised in plasma membrane lipid rafts (regions of specific constitution) [131]. After its phosphorylation, CARMA1 undergoes conformational changes that permit interaction with other proteins through the CARD motif,

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as well as oligomerisation with additional CARMA1 units, which are further recruited at the membrane layer through the coiled-coil (CC) domain (Figure 14b-c). The GUK domain modulates homotypic interactions, subsequently promoting multimerisation of CARMA1 oligomers so as to shape higher-order signaling complexes (Figure 14d). Lymphocytes from CARMA1-deficient mice are unable to proliferate or activate IKK complex or JNK through TCR engagement, outlining its importance in the signaling pathway [132-134]. Mutations in CARMA1 have been identified in ABC DLBCL patients, in some cases in exons coding for the coiled-coil domain. Mutations in this domain promote constitutive NF- κ B activation in lymphoma cell lines and enhanced activity upon antigen receptor engagement [135]. In similar cases, gain-of-function mutations of CARMA1 were reported to lead to constitutive NF- κ B activation in B-cells likely due to spontaneous oligomerisation [136].

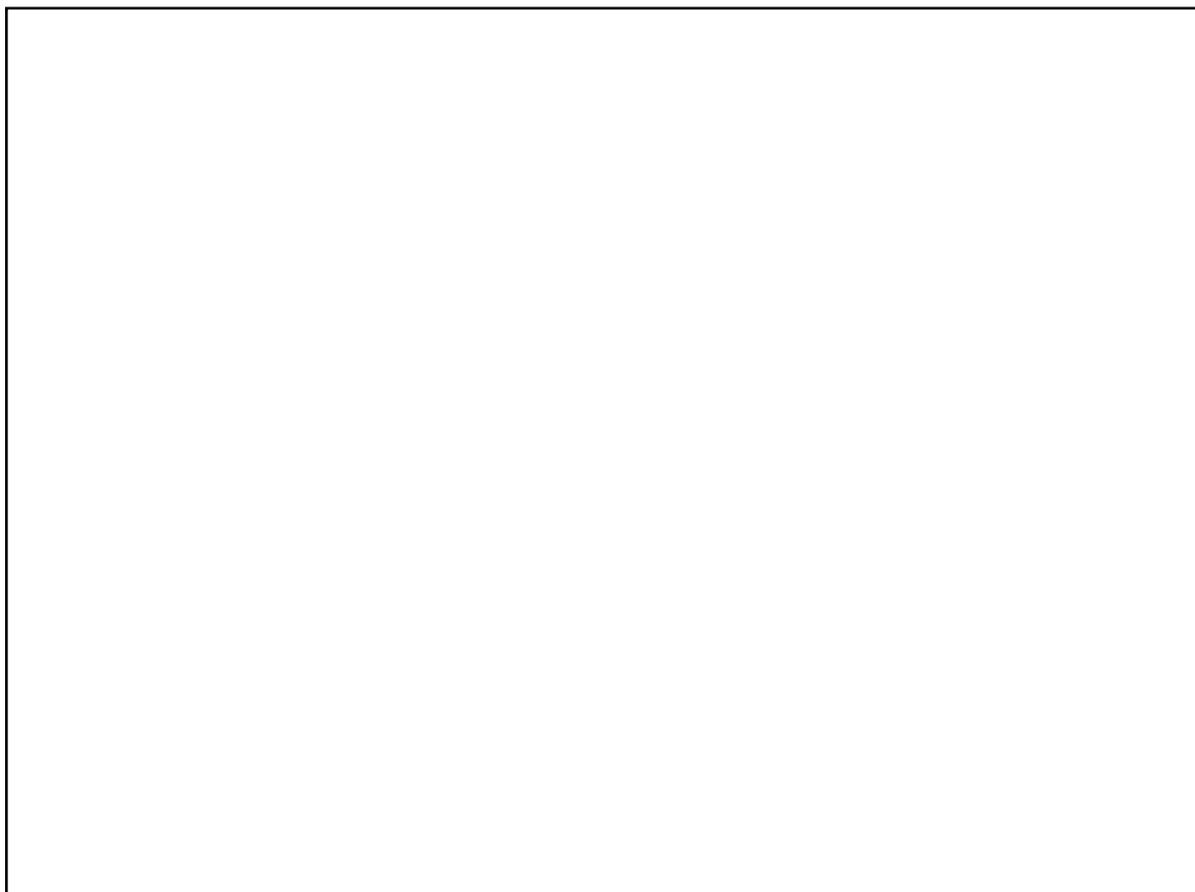


Table 10: The structural domains of some of the CARMA1-signalosome components are shown, together with known functional data relating to these domains. aa, amino acids; BCL-10, B-cell lymphoma 10; CARD, caspase-recruitment domain; CARMA1, CARD-membrane-associated guanylate kinase (MAGUK) protein 1; CC, coiled-coil; CUE, cue-1 homologous; DD, death domain; GUK, guanylate kinase; Ig, immunoglobulin; IKK, inhibitor of nuclear factor- κ B (I κ B) kinase; LZ, leucine zipper; MALT1, mucosa-associated-lymphoid-tissue lymphoma-translocation gene 1; NUB, NEMO (IKK γ) ubiquitin binding; PDZ, PSD95, DLGA and ZO1 homology; PRD, protein-kinase-C-regulated domain; RING, really interesting new gene; SH3, SRC-homology 3; S/T kinase, serine/threonine kinase; S/T rich, serine/threonine rich; TAB, TAK1-binding protein; TAK1, transforming growth factor- β (TGF β)-activated kinase 1; TRAF, tumour-necrosis factor receptor (TNFR)-associated factor; ZnF, zinc finger. [130]

CARMA1 structural reconfiguration and oligomerisation renders its protein-protein interaction domains accessible to downstream binding partners such as BCL10 (B-cell CLL/lymphoma 10) and MALT1 (mucosa-associated-lymphoid-tissue lymphoma-translocation gene 1), forming the CARMA1:BCL10:MALT1 (CBM) complex, a big signalosome that promotes IKK activation (Figure 15) [137, 138]. Additional proteins are also recruited on this complex such as CK1 α (Casein Kinase 1 α), TRAF2 (TNF receptor-associated factor 2), TRAF6, TAK1 and NEMO, which are also key effector molecules

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regulating signal transduction. Ectopic expression of these proteins leads to their spontaneous oligomerisation, mimicking the configuration they normally acquire upon stimulation. Therefore this is sufficient to cause IKK activation in the absence of inducers [130, 139]. Studies have shown that CARMA1 also participates in an intrinsic feedback control mechanism that counteracts and finally attenuates excessive NF- κ B activation. This is achieved because receptor-activated CARMA1 undergoes K48-linked poly-ubiquitylation and further proteasomal degradation [140].

BCL10 is a small CARD-containing protein (Table 10). After stimulation BCL10 can interact with CARMA1 through their CARD motifs. BCL10 oligomerisation then mediates signal transmission towards the IKK complex [137]. Overexpression of BCL10 has been detected in many MALT lymphomas, where constitutive NF- κ B activity is observed. Bcl10 KO mice either die during gestation, due to impaired apoptosis and neural tube malformation, or are born with severe immunodeficiency. Bcl10^{-/-} lymphocytes are unable to activate NF- κ B in response to antigen receptor engagement even if early phosphotyrosine induction, MAPK activation, AP-1 activation and calcium flux are normal. Another study in Bcl10-deficient mice showed that Bcl10 is necessary for B cell maturation and differentiation, as well as function. Marginal zone B cells from these mice were unable to fully proliferate or activate NF- κ B in response to LPS stimulation [92, 116, 130, 137, 141].

Upon direct or TCR/CD28-induced PKC activation, BCL10 undergoes ubiquitylation, followed by degradation. This is not observed in the case of TNF α or LPS stimulation. It was initially reported that BCL10 degradation was not affected by proteasome inhibitors but was rather accompanied by transient localisation to lysosomes for degradation signal termination [142]. This negative feedback loop was proposed to be due to phosphorylation of BCL10 by activated IKK, which leads to β -TrCP-dependent ubiquitylation of BCL10. In contrast another study showed that proteolysis is proteasome-dependent [143]. More recently, BCL10 poly-ubiquitylation has been found to be of the K63-type, a modification that promotes interaction with ubiquitin receptors like NEMO or TABs 2 and 3 [92, 116, 130, 144-146]. Lysines 31 and 63 of BCL10 have been identified as ubiquitin-acceptors, since mutation of these residues did not affect CBM complex assembly, but did prevent its ubiquitylation, NEMO recruitment and NF- κ B activation [145]. These models keep evolving and new data is added. For example, it was shown that TCR-induced K63 poly-ubiquitylation of BCL10 causes association with an autophagy factor, required for both NF- κ B signaling initiation at first, and gradual autophagic degradation later on, in order to attenuate the signal. The proposed role of this activity is to protect cells from excessive NF- κ B activation and negative consequences such as cellular senescence in primary effector T cells, as it is only observed in these cells and not in naive ones [146].

In resting cells, BCL10 is present in the form of heterodimers in complex with the paracaspase MALT1 through its immunoglobulin domains (Table 10). As the name states, this latter is a gene commonly translocated in MALT lymphomas. Lymphocytes from Malt1 KO mice present defective proliferation and IKK activation upon TCR ligation, revealing its key role in the NF- κ B signal transduction pathway [138, 147, 148]. Following TCR stimulation, MALT1 undergoes K63 poly-ubiquitylation via TRAF6. It is recruited together with BCL10 on the CARMA1 scaffold, resulting in the formation of the CBM complex, enabling signal transmission [92, 116, 130, 149].

The CBM complex is also thought to recruit TRAF2 and TRAF6 oligomers through MALT1, as well as RIP1, contributing to maximal signalosome activity (Table 10, Figure 15). These adaptors are also used in NF- κ B-activating pathways of the innate immunity. Many theories exist on how TRAFs promote this function. TRAFs contain coiled-coil domains that mediate homotypic and heterotypic protein-protein interactions. They also possess RING

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finger domains suggesting an E3 ligase function. TRAF2 and 6 have been shown to undergo themselves K63-linked poly-ubiquitylation (is this a meaningless coincidence?) the latter being auto-ubiquitylated. The role of TRAF2 and TRAF5 in TNFR-mediated NF- κ B activation is well established, since double-KO cells are defective in IKK activation. The same is true for TRAF6 in IL-1R and TLR signaling towards IKK activation. TRAF6 knock down in other cells mildly impairs IKK activation following TCR ligation, whereas the double knock down of TRAF2 and TRAF6 causes a more severe impairment. These findings confirm the cooperation between innate and adaptive immunity key molecules and strategies in order to activate NF- κ B [16, 116, 117, 130].

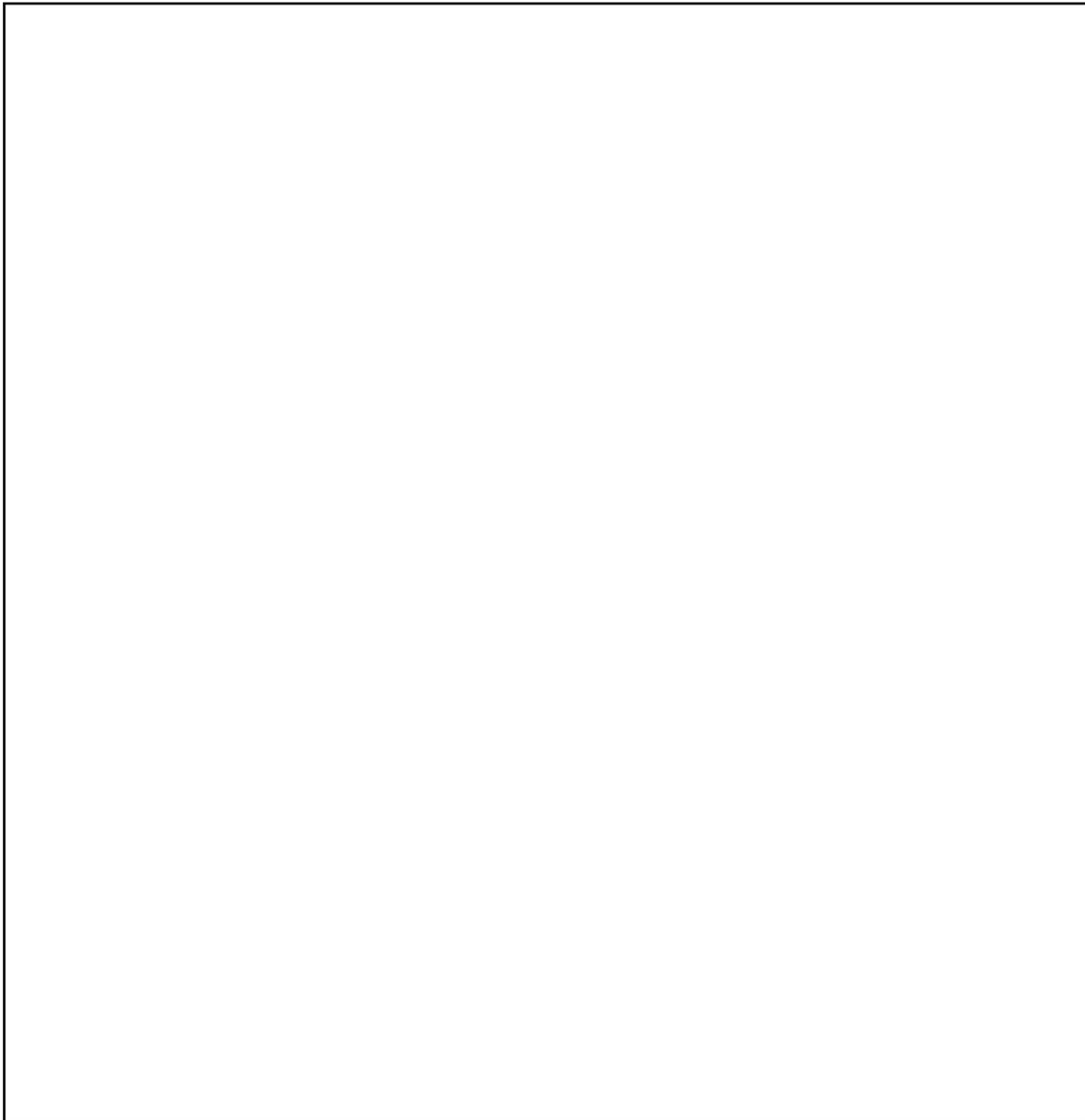


Figure 15: Model of the CARMA1-signalosome oligomerization cascade and IKK activation. Activated, trimerized CARMA1 (caspase-recruitment domain (CARD)-membrane-associated guanylate kinase (MAGUK) protein 1) binds BCL10 (B-cell lymphoma 10), which in turn binds MALT1 (mucosa-associated-lymphoid-tissue lymphoma-translocation gene 1). This positions the TRAF (tumour-necrosis factor (TNF)-receptor-associated factor)-binding sites of MALT1 into a configuration mimicking that of three TNF receptors bound to the pseudotrimeric TNF ligand. Interaction with the trimerized MALT1 activates TRAF6 (**a**) and TRAF2 (**b**), perhaps by stabilizing TRAF trimerization or by inducing a conformational shift of existing TRAF trimers. TRAF6 and TRAF2 contribute equally to IKK (inhibitor of NF- κ B ($\text{I}\kappa\text{B}$) kinase) activation in T cells. TRAF6 catalyses auto-K63-linked polyubiquitylation (**a**), whereas activated TRAF2 targets RIP1 (receptor-interacting protein 1) for K63-linked polyubiquitylation (**b**). The resulting K63-linked polyubiquitin moieties in both pathways probably recruit the ubiquitin-binding proteins IKK γ and TAB2 (TAK1-binding protein 2) or TAB3 of the TAK1

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(transforming-growth-factor- β -activated kinase 1) complex. Recruitment of IKK γ to TRAF6 or RIP1 could trigger K63-linked polyubiquitylation of IKK γ (which is required for IKK γ activation). Binding of the TAK1 complex to K63-linked polyubiquitylated IKK γ would place TAK1 in direct proximity to its substrate, IKK β . Activation of TAK1 by intermolecular and intramolecular autophosphorylation would then allow its phosphorylation of IKK β and thus, subsequently, I κ B phosphorylation (not shown). **c** | TRAF2 trimerization is mediated by parallel coiled-coil (CC)-domain interactions. The TRAF-homology domain forms a trefoil of β -sheets that bind to upstream TRAF-binding sites. **d** | The IKK γ trimer is formed of a 6-helix bundle composed of anti-parallel α -helices made up of the CC2 and leucine-zipper (LZ) domains of each IKK γ molecule (shown in cross section). The IKK γ (NEMO) ubiquitin-binding (NUB) domain is localized to one face of the IKK γ trimer. At the other face of the trimer are the N- and C-termini; the N-terminus contains a binding site for IKK α and IKK β , and the C-terminus consists of a zinc finger (ZnF) that is K63-linked polyubiquitylated downstream of MALT1. UBC13, ubiquitin-conjugating enzyme 13; UEV1A, ubiquitinconjugating enzyme E2 variant 1A; RING, really interesting new gene domain [130].

The model of oligomerisation and assembly of ubiquitylated adaptors for the formation of signalosomes is observed in all canonical NF- κ B activating pathways. **BCR** (B-cell receptor) is the membrane-bound form of immunoglobulins, or most commonly antibodies, able to recognise the epitopes of soluble antigens in their three-dimensional conformation. In the case of BCR engagement, the pathway is highly similar to that of TCR with the difference that PLC γ 2 is responsible for second messengers' production, to activate PKC β that will phosphorylate CARMA1 [16, 130].

The following schema summarises the cascade of TCR-originating (similar to those of BCR) events and depicts the most important molecules implicated in NF- κ B activation.

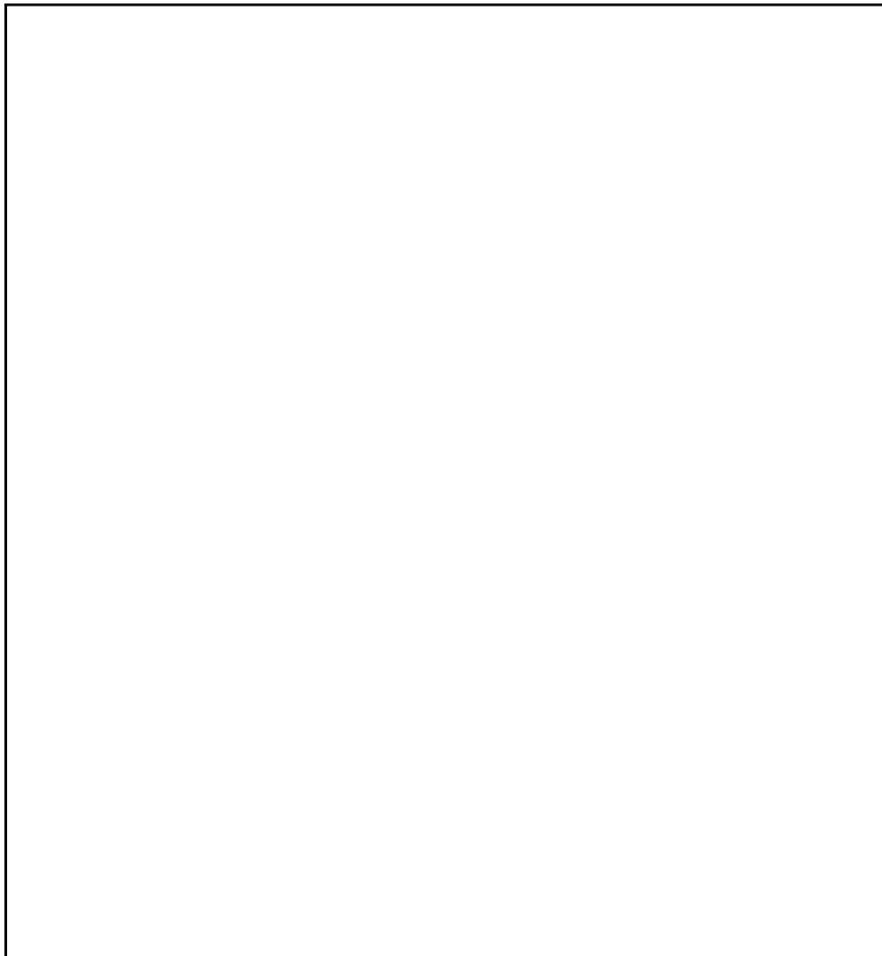


Figure 16: Overview of the TCR-NF- κ B pathway. Lys-48-linked ubiquitin chains are shown in red, and Lys-63-linked ubiquitin chains are shown in green. [150]

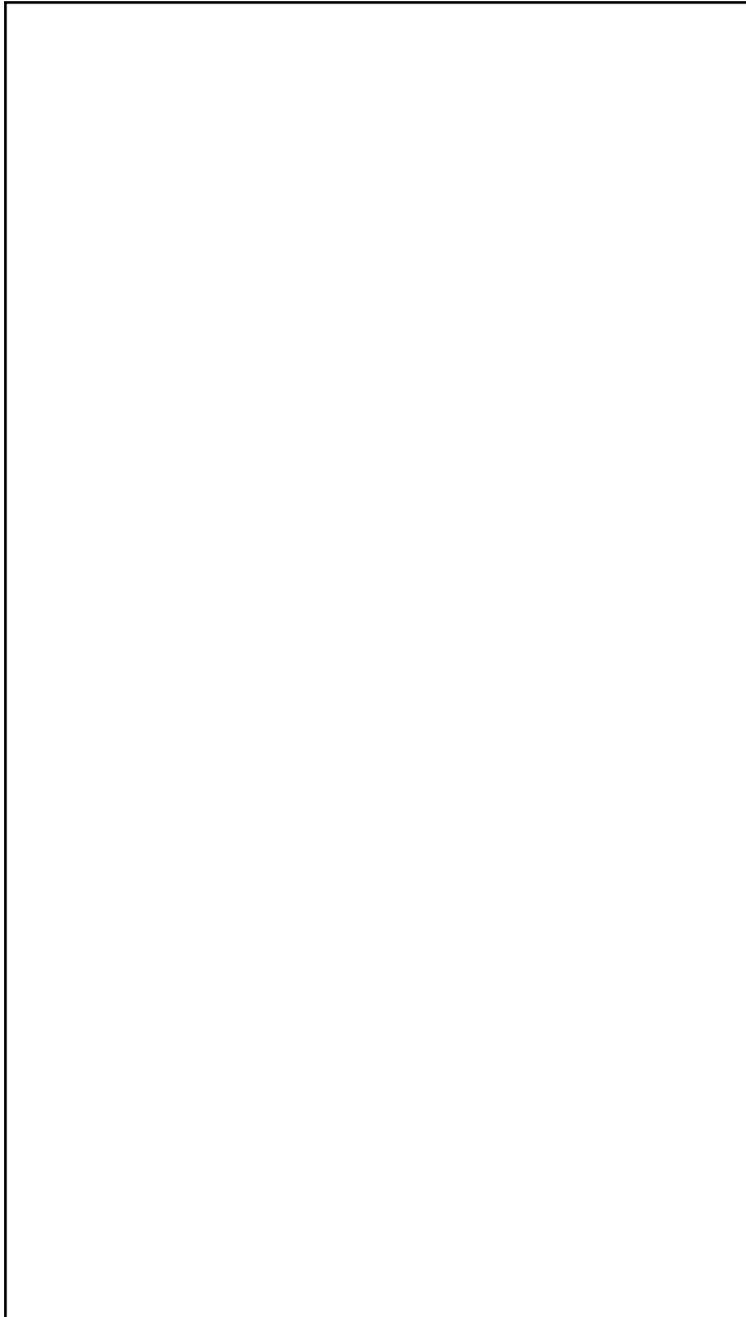
IV.5. Other canonical NF- κ B pathways

Figure 17: NF- κ B-activating stimuli and composition of the respective IKK-activating complexes. In response to ligand stimulation, receptors that activate NF- κ B recruit intracellular proteins to initiate signal transduction. (A) TNFR1 recruits TRADD, RIP1, TRAF2, cIAP1 and 2, and LUBAC (which comprises SHARPIN, HOIP and HOIL1). Formation of ubiquitin chains allows the TAB-TAK and IKK complexes to be recruited. (B) The CD40-associated IKK-activating complex consists of TRAF3, TRAF2, cIAPs, LUBAC and the TAB-TAK and IKK complexes. (C) Stimulation of IL1R or TLRs induces recruitment of MYD88 and the kinases IRAK1 and IRAK4. IRAK4-mediated phosphorylation of IRAK1 causes dissociation of IRAK1 from the receptor and formation of a secondary complex that contains IRAK1, TRAF6 and Pellino and the TAB-TAK and IKK complexes. (D) Following TCR engagement, activated protein kinase C (PKC) phosphorylates CARMA1. A complex composed of CARMA1, BCL10, MALT1, TRAF2 and TRAF6 is assembled, which recruits the TAB-TAK and IKK complexes. (E) Binding of bacterial peptidoglycans to NOD1 or NOD2 results in the assembly of a complex consisting of RIP2, TRAF6, TRAF2, cIAP, XIAP and the TAB-TAK and IKK complexes. (F) TRIM25-mediated ubiquitylation of RIG-I allows association with MAVS and recruitment of TRAF6, TRAF2 and the TAB-TAK and IKK complexes. (G) DNA double-strand breaks (DSBs) induced by ionizing radiation are sensed by poly(ADP-ribose) polymerase 1 (PARP1) and ATM. This triggers formation of a nuclear complex, phosphorylation and sumoylation of NEMO and the export of ATM to the cytoplasm. Here, a complex containing TRAF6, cIAP and the TAB-TAK and IKK complexes is assembled. (H) DSBs induced by chemotherapeutic agents are sensed as in G, but lead to coupled nuclear export of ATM and NEMO and to the formation of an XIAP- and

ELKS-containing complex which mediates IKK activation. K63-linked ubiquitylation is shown in blue, with linear chains in red. Green circles marked 'P' represent phosphorylation, sumoylation is indicated by purple circles marked 'S' and yellow circles in G and H indicate ADP-ribosylation. PIAS γ , protein inhibitor of activated STAT protein gamma; IR, ionizing irradiation [92].

Upon TNF α binding to the TNFR (Figure 17A), a huge signalosome is assembled at the level of the receptor, consisting of various scaffolding and adaptor proteins. cIAP1 and 2 (cell inhibitors of apoptosis) are the ubiquitylated transmitters restricted to this canonical pathway, associating with RIP1 and TRAF2 that are common to other NF- κ B activating pathways. These proteins undergo K63-linked poly-ubiquitylation promoting TAK1 and IKK activation. LUBAC is recruited as well, to mediate linear ubiquitylation of RIP1 and

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NEMO. RIP1 has also been mentioned to undergo K11 and K48 ubiquitinylation but their roles remain unclear [92, 95, 113, 116, 117].

In the case of IL-1R or TLRs (Figure 17C), MYD88 serves as adaptor for TRAF6 and IRAK1 that undergo K63 ubiquitinylation for successful signal transmission and TAK1 and IKK β activation. RIP1 also participates in a similar way upon stimulation of TLR3 or TLR4. These receptors belong to the same family and they function as sensors of cytokine IL-1 and extracellular pathogen-associated molecular patterns of bacterial, viral or fungal origin respectively [92, 95, 113, 116, 117].

RLRs (RIG-I-like receptors) are cytosolic receptors that recognise viral RNA present in infected cells and promote activation of NF- κ B and of IRF3 (via IKK ϵ and TBK1) that mediate transcriptional induction of type I interferons (IFN I). Similarly, RIG-I (retinoic acid-inducible gene-I) undergoes K63 ubiquitinylation in order to activate TAK1 and IKK β (Figure 17F). RIP1 is also important in this pathway. [92, 95, 113, 116, 117].

NLRs (NOD-like receptors) (Figure 17E) are a large family of evolutionarily conserved intracellular receptors that detect bacterial peptidoglycans or toxins in the cytoplasm. Engaged NOD2 (nucleotide-binding oligomerisation domain 2 protein) recruits RIP2 and they both undergo K63 ubiquitinylation. Together with K63-ubiquitinated TRAF6 they further activate TAK1 complex, IKK complex (through IKK β phosphorylation) and subsequently NF- κ B [92, 95, 113, 116, 117].

IKK can also be activated in the case of DNA damage (Figure 17G and H) through a series of SUMOylation and ubiquitinylation of fractions of the regulatory subunit (NEMO) lying in the nucleus, followed by its nuclear export, binding and activation of the catalytic subunits (IKK α and β). RIP1 is also implicated in this process [92, 95, 113, 116, 117].

Additional information concerning the KO of key molecules of the NF- κ B pathways in mice and their phenotypes are summarised in the following table.

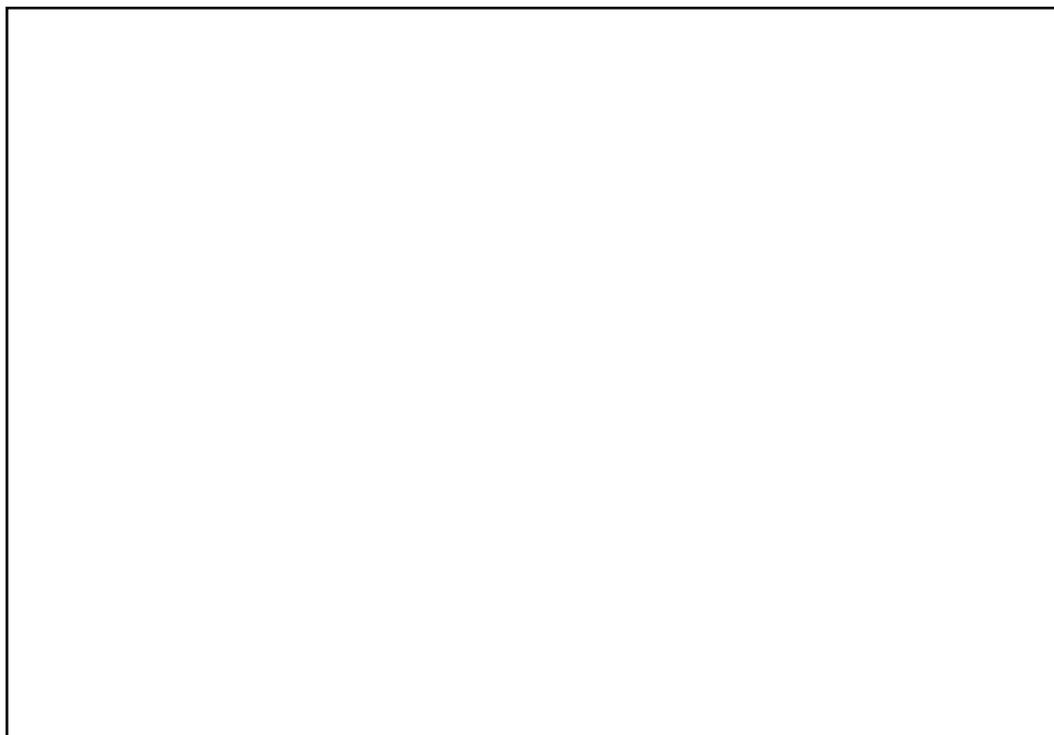
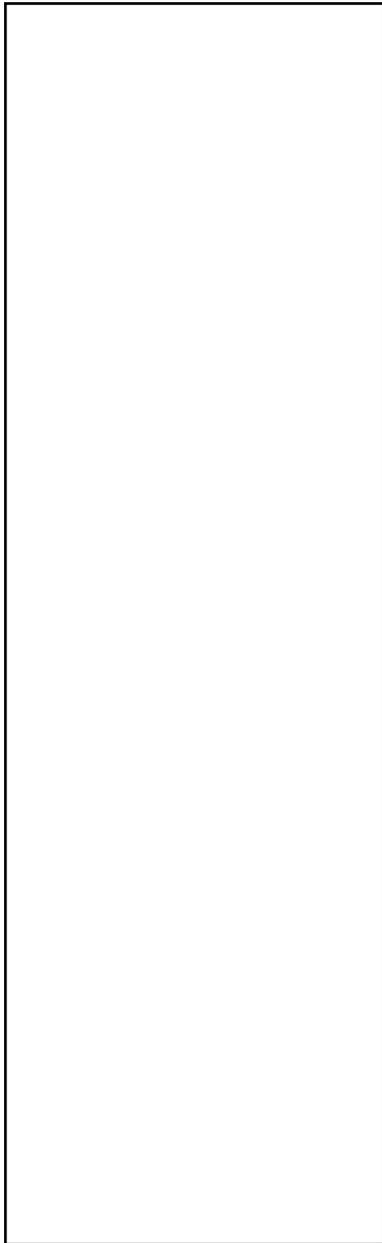


Table 11: Phenotype in KO mice of various components of NF- κ B signaling pathways [27]

IV.6. Noncanonical NF- κ B pathways



Activation of NF- κ B through the noncanonical pathway is also dependent on ubiquitinylation but in another context. The current model proposes that NIK in resting cells forms a complex with TRAF2, TRAF3 and cIAP1/2. This interaction leads to the constitutive TRAF3-dependent K48 polyubiquitinylation of NIK and its subsequent proteasomal degradation [152]. For example, upon engagement of receptors like CD40 in B-cells, both canonical (Figure 17B) and noncanonical NF- κ B pathways are activated (Figure 18). The complex consisting of NIK:TRAF2:TRAF3:cIAP1/2 is recruited to the receptor [153] and this event promotes displacement and release of NIK from the complex. Alternatively, this conformation provokes TRAF2-mediated activation of cIAP1/2 through K63 ubiquitinylation that further mediates ubiquitinylation of type K48 of TRAF3 leading to its degradation. After NIK is released and rescued from degradation, it can phosphorylate and activate IKK α [16, 82, 95, 117, 154].

IKK α then mediates phosphorylation of p100. This modification promotes recognition of p100 by the E3 ligase complex SCF ^{β TrCP} that ubiquitinylates its substrate in a K48-linked manner. Thus p100 is taken to the proteasome where it undergoes processing, producing finally the p52 NF- κ B component that can dimerise with RELB, translocate into the nucleus and regulate gene expression. Noncanonical pathways are activated and resolved with slower kinetics than the canonical ones [16, 82, 95, 117, 154-157].

Figure 18: Canonical and noncanonical activation of NF- κ B. Upon CD40 stimulation, TRAF2/5 targets cIAP1/2 for K63-linked polyubiquitination, which then mediate K48-linked polyubiquitination of TRAF3, which targets it for proteasomal degradation. This prevents the constitutive degradation of NIK by TRAF3, allowing NIK-mediated IKK α activation and IKK α -mediated phosphorylation of p100. This leads to partial processing of p100 into the p52 NF- κ B subunit. The noncanonical NF- κ B dimer p52/Rel-B then translocates to the nucleus. [151]

V. DUBs in termination of NF- κ B signaling

Ubiquitylation is a reversible reaction that is performed by the Deubiquitylating enzymes (DUBs), which hydrolyse the isopeptide bond between ubiquitin molecules in chains or between ubiquitin and targeted proteins. This process participates in the maturation of ubiquitin molecules (as they are cotranslated with ribosomal proteins in a premature form), ubiquitin disassembly, recycling and homeostasis. DUBs participate in various biological events such as cell cycle control, DNA repair, histone modification and chromatin remodelling, receptor degradation and recycling, endocytosis, secretion, signaling, regulation of transcription and RNA processing. DUBs can mediate editing of ubiquitin conjugates in order to alter or terminate signal transduction and change protein fate either by sending them to the proteasome or by saving them from degradation [158, 159].

Almost 100 DUBs are encoded in the human genome and have been classified into 6 families (Figure 19), according to the structure of their catalytic domain (Figure 20) and consequently the mechanism through which they catalyse deconjugation of ubiquitin. The largest DUB family in humans consisting of about 60 members is that of the Ubiquitin-Specific Peptidases (USPs), which contain highly conserved USP domains. There are also 4 members in the UCH family (ubiquitin carboxy-terminal hydrolase), 14 OTUs members (ovarian tumour proteases), 4 MJDs (Machado-Joseph disease protein domain proteases), 7 MCPIPs (monocyte chemotactic protein-induced protein), 4 JAMMs (JAB1/MPN/Mov37 metalloproteases). All of them are cysteine proteases apart from the last group that belongs to the catalytic class of zinc-metalloproteases [89, 159, 160].

Figure 19: Classification of human DUBs. Human DUBs are classified into six families represented by different colors: USPs, UCHs, OTUs, MJDs, JAMMs and MCPIPs. The catalytic core domain of each individual enzyme is indicated in plain light red if the DUB is active and stripped if inactive. Additional common domains are also shown in different colors. Proteins are represented with their N termini oriented towards the center of the circle. The length of each DUB corresponds to the size of the protein in amino acids. USPs, ubiquitin-specific proteases; UCHs, ubiquitin carboxy-terminal hydrolases; OTUs, ovarian-tumor proteases; MJDs, Machado–Joseph disease protein domain proteases; JAMMs, JAMM/MPN domain-associated metalloproteases; MCPIPs, monocyte chemotactic protein-induced proteins [159].

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The exact mechanisms that determine selectivity of each DUB family for a certain ubiquitin-linkage have not been elucidated yet. Structural and functional analyses indicate that USPs and OTUs recognise both K48 and K63-linked poly-ubiquitin chains or even K11-linked ones. JAMMs and MJDs are K63 specific [159]. It is known that specificity is altered, depending on the partners and adaptor proteins that participate in substrate recognition. Specificity is determined by the recognition mechanism of both the target protein and the attached ubiquitin moiety. Subcellular localisation and partner interaction also contribute to target selection [95, 160].



Figure 20: Structures of the catalytic domains of subclasses of Ub-Specific Proteases (Yellow) with Ub (Blue). Structures show the remarkable variability in secondary structure between the DUB classes. Catalytic centers are shown as Van der Waals spheres (carbon, gray; nitrogen, blue; oxygen, red; sulfur, orange; zinc, purple) and have been aligned for easy comparison [160].

In general DUBs are synthesised as active enzymes but they only assume the appropriate conformation when bound to ubiquitin. Their availability and activity is tightly regulated by various mechanisms acting at different levels:

1-Transcriptional control (Figure 21, left panel).

2-PTMs: as many proteins, several DUBs can undergo phosphorylation, ubiquitylation, proteolytic cleavage, modifications by reactive oxygen or nitrogen species etc (Figure 21, right panel).

3-Changes in subcellular localisation facilitate interaction with specific substrates.

4-DUBs interactions with other domains, proteins and co-factors lead to allosteric conformation changes (Figure 21, right panel). Many of them contain UBDs that mediate activity and specificity. Others contain ubiquitin-like domains within their catalytic region controlling auto-inhibition. Their regulation and specificity though largely depends on association with other subunits forming higher-order macromolecular complexes as in the case of the proteasome [159, 160].

Figure 21: DUBs regulation. DUB activity is regulated at various levels, including transcription (left panel), degradation, and binding to stimulatory or inhibitory cofactors (right panel). The exact mechanism whereby these co-factors regulate DUB activity is unknown but may occur at multiple levels (for example, phosphorylation, subcellular localization), stimulating conformational changes or conferring specificity [160].

Ubiquitylation/deubiquitylation reactions are usually coupled, permitting rapid and fine adjustment of signaling events involving ubiquitin conjugation and deconjugation, which outline once more the dynamic and reversible nature of this kind of PTMs. For example, a DUB can dimerise with an E3 ligase in order to antagonise and counteract its possibly auto-ubiquitylating activity, preventing excessive degradation or signaling events (Figure 22, left panel). In other cases, proximity between E3 and DUB can offer simultaneous regulation through ubiquitylation of the substrate together with its respective deubiquitylase to adjust and control substrate's destiny (Figure 22, middle panel). Furthermore DUB-E3 association

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can offer specificity on the DUB through E3 ligase binding on the respective target (Figure 22, right panel). In the case of the OTU protease A20, ubiquitin ligase and deubiquitylating activity are even hosted on the same polypeptide, permitting A20 to edit ubiquitin linkage type of its substrates from one type to another (see next chapter). Like most enzyme-substrate interactions, DUB-target interactions are generally weak and transient, possibly justifying why such methods that promote binding have evolved [159, 160].

Figure 22: Processivity – DUB/E3 interactions. DUBs and E3 are often found in a complex together. These interactions, which occur between USP7 and HDM2, for example, serve to reverse E3-mediated autoubiquitination (left panel) or allow the E3 to regulate the target and its DUB simultaneously as in the case of USP20 and pVHL (middle panel). Alternatively, DUB/E3 interactions confer

specificity to the DUB, as in the case of Ubp2 and Rsp5 (right panel). E3 Ub-ligase and Ub-protease activity is indicated with black arrows and red arrows, respectively. Ub conjugated to E2 is not shown for clarity [160].

V.1. DUBs and NF- κ B

So far two DUBs have been extensively studied as they were proven to intervene at different levels of the NF- κ B-activating pathways. Both proteins have been implicated in several genetic disorders and pathological conditions like cancer. Those are A20 or TNFAIP3 (TNF α -induced protein 3), an OTU family member, and CYLD (familial cylindromatosis), a USP family member, description of which will follow. Other DUBs implicated in NF- κ B regulation have also been studied but in less extent, examples of some will be also given.

V.1.a. A20

A20 or TNFAIP3 is a cytoplasmic protein, ubiquitously expressed in low levels and can be rapidly induced in response to various NF- κ B activating stimuli. On the contrary, in resting thymocytes and peripheral T lymphocytes, it is highly expressed but is downregulated in response to TCR ligation. Thus its protein levels can be both regulated in the transcriptional and post-translational level. It is made of various domains such as the OTU domain, responsible for ubiquitin deconjugation, UBD, for ubiquitin attachment, and even a domain that permits interaction with E2 enzymes, so as to function as an E3 ligase, promoting ubiquitylation. There are also domains that mediate interactions with proteins that have been mentioned earlier, like TRAF2, TRAF6 and RIP1 (Figure 23) [95, 161].



Figure 23: Biochemical characteristics of A20 protein function. The de-ubiquitylating (DUB) activity of A20 is mediated by the catalytic cysteine at position 103 (C103) within the OTU domain. A20 also contains seven zinc fingers (ZFs), which

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mediate its E3 ubiquitin ligase activity (via ZF4) and its ubiquitin-binding activity. Indeed, A20 binds to ubiquitylated E2 enzymes such as UBCH5A via ZF4–ZF7, to K63-linked polyubiquitin chains via ZF4 and to linear polyubiquitin chains via ZF7. A20 also interacts with substrates such as receptor-interacting protein 1 (RIP1) via ZF1–ZF3 (with ZF1 being crucial for binding), with E3 enzymes such as TNFR-associated factor 6 (TRAF6) via the OTU domain, and with ubiquitin-binding proteins such as TAX1-binding protein 1 (TAX1BP1), I κ B kinase- γ (IKK γ), A20-binding inhibitor of NF- κ B activation 1 (ABIN1) and ABIN2 via the ZF domain. Some of these latter interactions may occur through the mutual binding of A20 and the interacting protein to ubiquitin chains. The regions that mediate the interaction of A20 with the E3 enzymes RING-finger protein 11 (RNF11) and ITCH, as well as with itself, have not been clearly defined. In addition to their other functions, the C103 and ZF4 motifs have been shown to support the degradation of E2 enzymes. A20 also undergoes post-translational modifications; for example, a site of A20 phosphorylation by IKK β is indicated. Human A20 is cleaved by the paracaspase MALT1 at the site indicated by an asterisk. The site at which mouse A20 is cleaved has not been precisely determined, but the region where it is cleaved is indicated by a bracket. [161]

A20-deficient mice die perinatally due to multi-organ inflammation showing an important role of A20 in the inflammatory process [162]. Those mice could be rescued when treated with broad-spectrum antibiotics and revealed hypersensitivity of TLR signaling in response to intestinal flora in the absence of A20 [163]. MEFs derived from A20^{-/-} mice exhibit persistent TNF α and IL-1-induced NF- κ B activity due to prolonged IKK activation and I κ B α degradation. Further studies performed in conditionally KO mice in a lineage-specific and temporally controlled way have confirmed initial findings showing immune defects in some cases [161]. Human genetic studies have shown a correlation between single nucleotide polymorphisms (SNPs) of the TNFAIP3 gene and multiple inflammatory and autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis, psoriasis etc (Figure 24) [164]. This has also been shown in mice expressing reduced A20 levels that develop spontaneous inflammation, dictating a protective-inhibitory action. Polymorphisms in the A20 gene have been accused of promoting oncogenic transformation and malignancies development like glioma or lymphomas, as 18% of B-cell lymphomas (ABC-DLBCL, MALT and Hodgkin's) possess biallelic mutations of TNFAIP3 gene [95, 161, 165].

Figure 24: Polymorphisms and mutations in TNFAIP3 gene and related diseases. The figure shows a schematic of the TNFAIP3 gene locus, which encodes A20. Exons encoding the amino-terminal OTU domain are shown in orange and exons encoding the carboxy-terminal zinc fingers (ZFs) of A20 are shown in green. The C103, ZF4 and ZF7 motifs are highlighted. Non-coding exons, including AT-rich sequences at the end of exon 9, are shown in brown. Human germline single-nucleotide polymorphisms (SNPs) in the TNFAIP3 locus that are associated with autoimmune and autoinflammatory diseases are indicated (labelled with their reference SNP (rs) numbers), as are somatic mutations that have been identified in the coding exons of TNFAIP3 in human B cell lymphomas [161].

At the molecular level, A20 seems to control cell death as well as a variety of signal transduction pathways, the best studied being the NF- κ B pathway. The domains that this protein possesses render it the ability to interact with ubiquitylated substrates and remove or add ubiquitin moieties. NF- κ B is negatively regulated by A20 either by K63-linked ubiquitin

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removal and degradation tagging of signal mediators (Figure 25A) or by disrupting complexes of E2 UBCs with E3s that positively regulate the pathway like TRAFs (Figure 25B) [166]. Of note, A20 gene is a NF- κ B target, so it part of a negative feedback loop [95, 161]. Furthermore, it has been shown that A20 can directly impair IKK activation in a non-catalytic fashion, by sensing ubiquitin chains. It can specifically recruit NEMO and this event is sufficient to block IKK phosphorylation by TAK1 [167].

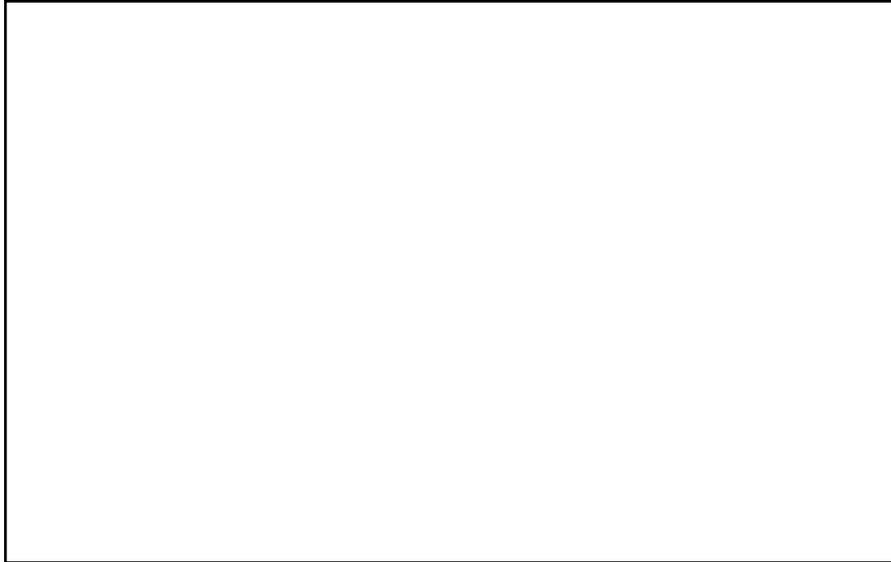


Figure 25: Mechanisms of A20 inhibition of NF- κ B.

(A) The ubiquitin-editing function of A20. In response to TNF stimulation, A20 expression is induced and inhibits NF- κ B in a negative feedback loop in a two-step manner. (1) A20 first hydrolyzes K63-linked polyubiquitin chains on RIP1 in an OTU-dependent manner to inhibit IKK and NF- κ B signaling. (2) A20 then conjugates K48-linked polyubiquitin chains onto RIP1 to trigger its proteasomal degradation. **(B)** Disruption of E2:E3

ubiquitin enzyme complexes by A20. The E3 ligase TRAF6 inducibly interacts with the E2 enzymes Ubc13 and UbcH5c upon IL-1R / TLR4 stimulation. (1) A20 interacts with Ubc13, UbcH5c, and TRAF6 and disrupts the binding between TRAF6 and the E2 enzymes. (2) A20 then conjugates K48-linked polyubiquitin chains on Ubc13 (and UbcH5c) to trigger its proteasomal degradation. [93]

Upon TNF α stimulation, A20 binds to K63-ubiquitinated RIP1 and removes the chain. RIP1 is not further able to recruit signal promoting mediators like TAB:TAK1 and NEMO:IKK, so signal is attenuated. Surprisingly, not only does it remove K63-linked chains, but also mediates K48-ubiquitinylation of RIP1 and promote its degradation, contributing thus in definite signal termination (Figure 25A) [168-170]. In the case of TRAF2, TRAF6 and cIAPs, their interaction with their respective E2 enzyme is disrupted by A20, preventing substrate ubiquitinylation. Regarding TRAF2, A20 also promotes its lysosomal degradation and as far as TRAF6, A20 can mediate its K63-tagged deubiquitinylation, in IL-1R and TLR activated pathways, in a quite different manner as it mediates entire chain cleavage rather than step-wise ubiquitin dissociation [166, 171-173]. Of note, A20 preferentially processes unanchored K48-linked poly-ubiquitin chains, but not K63-linked ones, to mono-ubiquitin. RIP2 and TBK1 (TANK-binding kinase 1) are also deubiquitinated by A20 in NLR-originating signalling [174]. Of note, NF- κ B signaling in response to TCR, BCR and CD40 stimulation is also controlled by A20. As A20 is expressed in high basal levels in lymphocytes, in contrast to other cell types, it is downregulated upon TCR engagement to allow proper signal transmission. A20 can remove poly-ubiquitin chains from MALT1 to inhibit interactions with downstream components of the pathway, whereas MALT1 cleaves recruited A20 to impair its inhibitory action [175, 176]. Balance between these counteracting and mutually exclusive events can guide the outcome of the response. The exact mechanisms through which all these events are regulated remain ill defined and are a subject of further research studies [93, 95, 161].

The activity and the specificity of A20 are regulated through interaction with various proteins forming the large macromolecular “A20 ubiquitin-editing complex” (Figure 26). **ABIN1** (A20-binding inhibitor of NF- κ B 1) for example, is thought to act as an A20-adaptor,

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altering its substrate choice and action and mainly inhibit TRAF3-dependent signals. Of note, ABIN1 contains a UBD highly similar to that of NEMO and bridges A20 with the latter. A20 activity can also be regulated via activatory phosphorylation by IKK β that leads to less NF- κ B activity, introducing an additional negative feedback loop in the system [93, 95, 161]. More partners-members of the “A20 ubiquitin-editing complex” have been characterised, as TAX1-binding protein 1 (**TAX1BP1**) for example, which is essential for NF- κ B and JNK signaling termination upon TNF α , IL-1 and LPS stimulation. TAX1BP1-deficient mice die during gestation and MEFs show elevated and persistent IKK and JNK activation upon the above stimuli, due to enhanced ubiquitinylation of RIP1 and TRAF6. This occurs because of the inability of A20 to recruit RIP1 and deubiquitinylate TRAF6 [177]. **ITCH** is another E3 ligase, member of the editing complex needed for termination of TNFR signaling by mediating A20 recruitment and inactivation of RIP1 [178]. Similarly, **RNF11** knockdown leads to persistent NF- κ B and JNK signaling in response to TNF α and LPS treatment. The explanation lies in the fact that RNF11 interacts with A20 and TAX1BP1 in a stimulus-dependent manner, in order to negatively regulate RIP1 and TRAF6 ubiquitinylation upon TNF and LPS stimulation respectively [179].

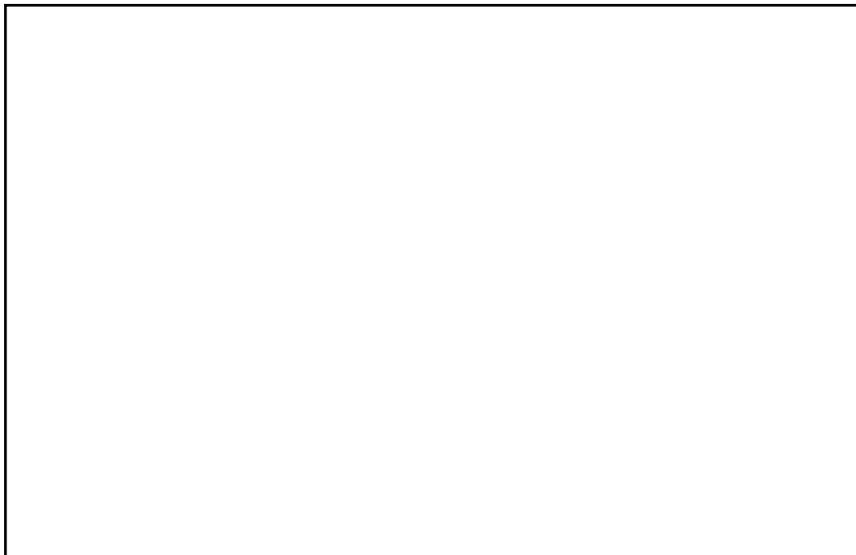


Figure 26: Activation and assembly of the A20 ubiquitin-editing complex. TNF stimulation activates NF- κ B and induces A20 expression as part of a negative feedback loop. IKK α phosphorylates TAX1BP1, which nucleates the A20 ubiquitin-editing complex and is required for interactions between TAX1BP1, Itch, RNF11 and A20. The A20 ubiquitin-editing complex inhibits RIP1 K63-linked polyubiquitination to terminate NF- κ B signaling downstream of TNFR1 [93].

V.1.b. CYLD

CYLD is an ubiquitously expressed USP family member and a well-characterised regulator of NF- κ B signaling pathways as well as antiviral signaling, MAPK pathways, proximal TCR signaling, cell cycle and calcium signaling. It is the product of a unique tumour suppressor gene in humans, mice, flies and worms that was first found in 1995 to be mutated in a rare genetic disease called Cylindromatosis. In 2003, three groups described CYLD association with NF- κ B activating pathways, showing that it interacts with NEMO. CYLD is able to negatively regulate the activation of NF- κ B following stimulation of various receptors such as nerve growth factor receptors (EDAR, XEDAR), TNF superfamily receptors (TNFR, CD40, LMP1), and Toll/IL-1 superfamily receptors. Regulation of NF- κ B activity by CYLD is achieved by removing non-K48-linked polyubiquitin chains from TRAF2 adaptor and to a lesser extent TRAF6, thus attenuating signal transmission. CYLD truncations in cylindromatosis result in reduced enzymatic activity, which is related to pathology. CYLD KD augments basal and inducible NF- κ B activity and renders resistance to apoptosis, demonstrating a possible role in oncogenesis [180-184].

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Figure 27: CYLD mutations in human pathology. The mutations of CYLD reported in the literature are indicated together with the various structural/functional domains of CYLD. Frameshift mutations are represented by a thick arrow, nonsense mutations are represented by a broken arrow and splicing mutations are indicated by a thin arrow. The two identified missense mutations are also indicated. Mutations found in **cyldromatosis** and **MFT** are shown in **black** and **red**, respectively. CAP-gly domains (CAP) have been described as microtubule-binding domains, but no evidence for CYLD interacting with the cytoskeleton has yet been provided. Instead, the third CAP-gly domain of CYLD represents the binding site for NEMO. A PXQXS/T-like motif that allows interaction with TRAF-2 is indicated between the 2nd and 3rd CAP domains. A phosphorylation site cluster also lies within this region (not indicated). ‘C’ and ‘H’ boxes are subdomains of the USP catalytic domain that contain the active cysteine and histidine residues (adapted from [115] with info from [95]).



Individuals with Cyldromatosis suffer from benign tumours in hairy parts of the body as hair producing follicles and associated glands are sensitive to transformation events. These inactivating *CYLD* gene mutations lead to excessive NF-κB activation and, as a consequence, an increased proliferation rate (Figure 27) [185]. Another genetic disease called Multiple Familial Trichoepithelioma (MTF) gives rise to similar phenotypes and was found to be also dependent on mutations of the same genetic locus [186]. Inactivating mutations have also been detected in several cancers as well, such as T-cell acute lymphoblastic leukemia (T-ALL), melanoma, colon, lung and hepatocellular carcinoma etc. *CYLD* gene is frequently mutated in multiple myeloma, and quite rarely in B-cell lymphomas, unlike A20 mutations [94, 95, 115]. The following table shows the physiology of *Cyld* genetic models, confirming its important role in immunity and NF-κB activation.

Genetic model	Phenotype
<i>Cyld</i> ^{-/-} Drosophila	shorter lifespans
<i>Cyld</i> ^{Δ9/Δ9} mice	die postnatally because of respiratory problems
<i>Cyld</i> ^{-/-} mice	susceptibility to chemically-induced skin tumours, sterile males due to testicular atrophy (NF-κB activation and induction of anti-apoptotic protein Bcl2)
<i>Cyld</i> ^{-/-} T-cells	fewer CD4 ⁺ and CD8 ⁺ (defect in proximal TCR signaling), impaired development of NKT, spontaneous activation of peripheral T lymphocytes
<i>Cyld</i> ^{-/-} Keratinocytes	elevated Cyclin D1 expression and higher proliferation rates
<i>Cyld</i> ^{-/-} B-cells	spontaneous activation and inflammation (constitutive NF-κB activation)
<i>Cyld</i> ^{-/-} Macrophages	hypersensitivity to TLR, anti-CD40 and TNF (enhanced NF-κB activation and proinflammatory cytokine production)
<i>Cyld</i> ^{-/-} Dendritic Cells	hyperactive phenotype (augmented NF-κB activation)
<i>Cyld</i> ^{-/-} Osteoclasts	abberant differentiation causing severe osteoporosis

Table 12: Phenotype of *Cyld* genetic models. Δ9: mutant lacking exon9 that encodes for the catalytic domain [93, 94].

The amount of CYLD in cells is regulated both at the transcriptional, post-transcriptional and the post-translational level. Since TNFα and microbes can trigger CYLD expression, it is considered as an NF-κB-dependent event, introducing an auto-regulatory feedback mechanism through TRAF2 and TRAF6 deubiquitylation. This permits tight control of NF-κB activity and prevents extensive tissue damage from uncontrolled inflammation reactions. Apart from recruitment of activators and repressors on the promoter region, epigenetic controls including DNA methylation and histone deacetylation also alter CYLD mRNA levels

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in many pathological conditions [94]. Of note, eight different alternative splicing variants have been discovered in human to date and five in *Drosophila*. Deubiquitinating activity and specificity of CYLD also depends on post-translational modifications and protein-protein interactions, as in the case of A20. IKK α and β in the NF- κ B pathway and IKK ϵ in the interferon pathways (following TLR, NLR or RLR activation) can phosphorylate CYLD in order to permit accumulation of ubiquitinated TRAF2 and signal amplification. Regulation of the level of CYLD protein through ubiquitination-dependent proteasomal degradation has been reported in many studies [93, 94].

Excessive activation of TAK1 and its downstream targets (IKK-NF- κ B, JNK-AP-1, NFAT), can be prevented by CYLD as it selectively removes K63-linked poly-ubiquitin chains from several proteins. Concerning the NF- κ B pathways, CYLD-dependent deconjugation of K63-type polyubiquitin chains from TRAF2, TRAF6, RIP1 and NEMO, attenuates IKK activity and signal transmission. Removal of K48-linked chains from TRAF2 preventing its degradation has also been reported. In mature T lymphocytes, CYLD restrains IKK and NF- κ B activation in the TCR pathway by removing K63-linked chains from TAK1 [187, 188]. It also cleaves both K48 and K63-linked ubiquitin chains lck kinase, linking it with its substrate ZAP70 (proximal TCR signaling). Interestingly, it affects an atypical NF- κ B-activating pathway by preventing BCL3 (an I κ B protein) ubiquitinylation. K63 polyubiquitinylation of BCL3 leads to its nuclear translocation and complex formation with p50 or p52 dimers that activate cyclin D1 expression to promote cell cycle G1 to S phase transition and subsequently cell proliferation [189]. This is the case in many tumour cell lines where CYLD activity is downregulated and there is an excessive production of cyclin D1 [93-95, 112, 159, 190].

Similarly to A20, CYLD requires adaptor molecules to interact and deubiquitinate TRAF6 in diverse NF- κ B activating pathways. One example is p62 protein. In murine neuronal cells deficient in p62, an accumulation of insoluble ubiquitinated proteins and more specifically K63-ubiquitinated tropomyosin-related kinase A (TrkA) receptor was observed. This was attributed in part to diminished activity of CYLD. It seems that p62 acts as an adapter, permitting interaction of CYLD with TRAF6 in order to form a complex of an active CYLD fraction, so as to remove the K63-linked polyubiquitin chains from TrkA and attenuate NF- κ B activity. Thus p62 has an equilibrating role, regulating the counteracting effects of the CYLD:p62:TRAF6 complex in order to balance the turnover of K63-tagged proteins as in the case of TrkA [191, 192]. Optineurin is another example of a protein-partner of CYLD that alters its activity. Optineurin normally competes with NEMO for binding to ubiquitinated RIP in order to prevent NF- κ B activation and it was known to interact with CYLD. A mutant though (H486R, common event in glaucomas) is unable to inhibit TNF α -dependent induction of NF- κ B. In experiments where Optineurin was mutated or silenced, CYLD was no more capable of inhibiting NF- κ B signaling and showed drastically reduced interaction with ubiquitinated RIP, whose level was increased. So Optineurin mediates interaction with ubiquitinated RIP facilitating its deubiquitinylation, subsequently reducing NF- κ B activity [193].

V.2. Other DUBs implicated in NF- κ B regulation

Although CYLD and A20 have some common substrates, there is little functional redundancy between them. CYLD is most important in basal NF- κ B activity control whereas A20 plays a limiting role towards inducibly activated NF- κ B. Several DUBs have been reported that, in a lesser extent, may play a role at various levels of NF- κ B regulation. These

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DUBs are Cezanne, hydrolysing K11-linked polyubiquitin chains of RIP1 [194, 195], USP11 targeting RIP1 and I κ B α [196], USP15 removing I κ B α ubiquitin tags [197], USP7 targeting TRAF6, NEMO or p65 [198, 199], MCPIP1 targeting TRAF2, TRAF6 and RIP1 etc [93, 95, 159, 200]. USP9X has recently been described as a positive regulator of the TCR signaling pathway, as in vivo KD attenuates T-cell proliferation. It interacts with and removes ubiquitin chains from Bcl10 facilitating association with Carma1 and CBM complex formation [201]. What should be kept in mind is that all DUBs have still not been identified. A novel DUB called OTULIN or gumby has been recently identified as mediating dissociation of linear poly-ubiquitin chains formed by the LUBAC. It interacts with components of the Wnt pathway and RNF31 of NF- κ B pathways, altering NEMO association with RIP1 and linear ubiquitylation of RIP2 [202-204].

VI. Project goals

NF- κ B is a transcription factor that has been extensively studied throughout the last decades. Diverse stimuli lead to activation of NF- κ B through various signal transduction pathways, all converging in regulation of IKK activity. Active IKK can phosphorylate I κ B, which is degraded, so that NF- κ B can be released from inhibition and can translocate into the nucleus to initiate gene transcription. Expression of a broad range of target-genes, which are implicated in many fundamental processes like cell proliferation and immune response, is controlled by this transcription factor. As a consequence NF- κ B has also been implicated in various pathological conditions like cancer, due to dysregulation of its activity.

Despite the diversity of activators, all pathways use similar basic principles. Upon induction, adaptor molecules oligomerise and organise in large signalosomes through specific protein-protein interacting domains. These molecules need to undergo ubiquitinylation in a non-degradative manner, forming large polyubiquitin chains that facilitate aggregation of additional complexes able to further transmit the signal. Such events are highly regulated in order to mediate responses of accurate intensity and duration. Signalosome assembly is controlled at various levels including spatiotemporal availability of the components and their status of activation. Moreover, Ubiquitin conjugation is also strictly controlled by the E3 ligases and the DUBs that mediate ubiquitinylation and deubiquitinylation respectively.

Knowing the importance of ubiquitinylation in signal transduction pathways that activate NF- κ B, this project was planned in order to identify and characterise additional DUBs implicated in regulation of signaling outcome and intensity upon TCR ligation in the human T-cell line Jurkat. A siRNA library screening was performed with sequences targeting most of the deubiquitinating enzymes encoded by the human genome. The impact of gene silencing on the TCR-dependent NF- κ B pathway was measured by luciferase assays, after transfecting Jurkat cells with the respective siRNA. In parallel, identification of additional proteins that participate in signal transduction and impact NF- κ B activity through a mass spectrometry analysis contributed to the identification of additional NF- κ B partners. We tried to answer fundamental questions like how are ubiquitinated molecules regulated in the pathway, how they are recycled or set to default conditions, by which proteins, and at which level. Also we tried to understand how membrane-associated clusters transmit the signal to cytoplasmic components, how is this regulated and by which kind of proteins.

The major goal of these studies was to get a better insight in regulatory mechanisms of NF- κ B activation at the molecular level. Research is an investment for the future and the knowledge acquired may be used to provide patients with the appropriate treatment, adapted to the molecular background of their disease, in order to increase therapeutic efficiency and minimise undesired reactions. Newly identified pathway components may serve as potential anti-tumoural targets with these advantages.

RESULTS

RESULTS

Article: *Negative regulation of NF- κ B signaling in T lymphocytes by USP34*

We have screened 98 deubiquitinating enzymes encoded by the human genome in order to identify new proteases implicated in TCR-dependent NF- κ B signaling regulation. To achieve this, we used Jurkat E6.1 human T-cell line, transfected with siRNAs from a library with two sequences targeting each candidate molecule. By using a NF- κ B-responsive, firefly luciferase-coding vector, we were able to evaluate NF- κ B activity, by a luciferase assay, in response to stimulation with PMA/ionomycin agents and anti-CD3/anti-CD28 antibodies. Cells were lysed and after substrate addition, luminescence values were obtained. Values were normalised with a second plasmid stably and independently coding for Renilla luciferase.

Silencing of the protein called Ubiquitin-specific protease 34 (USP34) was found to increase NF- κ B activity upon TCR stimulation. USP34 is a non-nuclear, non-organellar protein (as we have shown by performing fractionation experiments) of molecular weight of 404 kDa, with only one characterised domain, the catalytic. Cells were transfected with an additional siRNA sequence in order to verify successful and specific silencing and lysates were analysed by immunoblot (IB). Confirmation of the importance of this discovery came by additional knockdown experiments, where NF- κ B targets were also upregulated upon stimulation, compared to non-targeting siRNA transfected cells. NFKBIA, IL-2 and TNF-R mRNA levels were increased, as revealed by RT-PCR (reverse transcription-polymerase chain reaction). The same holds true for IL-2 protein production and secretion, as measured in cell culture supernatants by ELISA (enzyme-linked immunosorbent assay). Cells were then transfected with a construct coding for the catalytic domain of USP34, stimulated and resubmitted to a luciferase assay. The reverse experiment had indeed the reverse result, meaning that overexpressed USP34 catalytic domain is enough to dampen NF- κ B activity upon TCR stimulation.

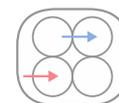
After determining the role of USP34 in the negative control of NF- κ B activity, we tried to get an insight in signaling events at the molecular level. Proximal signaling events like global tyrosine phosphorylation, assembly of CBM complex and ubiquitinylation of the adaptors BCL10 and MALT1 were unaltered by the silencing of USP34. No interaction with core components of the NF- κ B pathway was observed by co-IP. In addition, parallel signaling diverging from TCR towards activation of the NFAT and MAPK-dependent AP-1 transcription factors was mainly unaffected as assessed by IB (phospho-ERK profile) and luciferase assay respectively. Nuclear extracts from unstimulated and stimulated USP34-silenced cells were analysed by IB and were compared to extracts from non-targeting siRNA transfected cells and were also subjected to non-radioactive EMSA (electrophoresis mobility shift assay). This experiment revealed a similar pattern of nuclear distribution of the p65 NF- κ B component [also visualised by immunofluorescence experiment (not shown)] in terms of time and quantity, but with altered affinity for κ B DNA, as in USP34-silenced cells this was enhanced. It was also observed in cytoplasmic fractions and in whole cell lysates that I κ B α resynthesis after degradation was attenuated and delayed in USP34-silenced cells.

This led us to test if the same events are observed upon stimulation by TNF α and genotoxic stress induction (additional NF- κ B-activating signals converging with the TCR-originating one, at the TAB:TAK/IKK level). Luciferase assay again showed a two-fold increase in NF- κ B activity in USP34-silenced cells upon treatment. TNF α -stimulated cell lysates were also subjected to IB. The same attenuated and delayed I κ B α resynthesis pattern was observed in USP34-KD cells. Since the effect on I κ B α resynthesis and NF- κ B activity is shared in these pathways, we directly assessed IKK activity (the first common step of these pathways) by verifying IKK α/β phosphorylation status, as this reflects its activity. This was

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unaffected in non-targeting compared to USP34-targeting siRNA transfected cells after PMA/ionomycin and TNF α stimulation, if not attenuated in the case of PMA/ionomycin treatment, possibly due to a negative feedback loop resulting from NF- κ B overactivation.

Overall, the strictly cytoplasmic and large USP34 protein was shown to negatively regulate specifically NF- κ B signaling, in response to TCR and other stimuli, by acting downstream of IKK, stabilising resynthesised I κ B α and suppressing overt NF- κ B (p65) DNA affinity.



SHORT REPORT

Open Access

Negative regulation of NF- κ B signaling in T lymphocytes by the ubiquitin-specific protease USP34

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Abstract

Background: NF- κ B is a master gene regulator involved in plethora of biological processes, including lymphocyte activation and proliferation. Reversible ubiquitinylation of key adaptors is required to convey the optimal activation of NF- κ B. However the deubiquitylases (DUBs), which catalyze the removal of these post-translational modifications and participate to reset the system to basal level following T-Cell receptor (TCR) engagement continue to be elucidated.

Findings: Here, we performed an unbiased siRNA library screen targeting the DUBs encoded by the human genome to uncover new regulators of TCR-mediated NF- κ B activation. We present evidence that knockdown of Ubiquitin-Specific Protease 34 (USP34) selectively enhanced NF- κ B activation driven by TCR engagement, similarly to siRNA against the well-characterized DUB cylindromatosis (CYLD). From a molecular standpoint, USP34 silencing spared upstream signaling but led to a more pronounced degradation of the NF- κ B inhibitor I κ B α , and culminated with an increased DNA binding activity of the transcription factor.

Conclusions: Collectively, our data unveils USP34 as a new player involved in the fine-tuning of NF- κ B upon TCR stimulation.

Keywords: DUBs, NF- κ B, Ubiquitinylation, T-Cell receptor

Findings

Nuclear factor- κ B (NF- κ B) transcription factors initiate transcription of genes essential for mounting an adequate immune response [1]. Ubiquitously expressed NF- κ B heterodimers of Rel family proteins are normally sequestered in the cytosol of the cells by Inhibitors of NF- κ B (I κ Bs) proteins [2]. In lymphocytes, the ligation of antigen receptors assembles the so-called CBM complex that consists of the scaffold CARMA1 and the heterodimer BCL10/MALT1 [3]. The CBM microenvironment drives oligomerized BCL10 and MALT1 to undergo K63-linked non-degradative ubiquitinylation [4-7]. This authorizes the recruitment and activation of the I κ B kinase (IKK) complex that comprises two catalytic subunits (IKK α and

IKK β) and a regulatory subunit (NEMO, also called IKK γ) [8]. IKK phosphorylation of I κ Bs precipitates their K48-linked ubiquitinylation and proteasomal elimination, and thereby allows NF- κ B to translocate to the nucleus where it binds DNA and initiates transcription [8]. NF- κ B-dependent neosynthesis of I κ Bs subsequently drives NF- κ B to shuttle back to the cytosol [1]. Although reversible ubiquitinylation processes are central for T-cell receptor-(TCR)-mediated NF- κ B activation, the deubiquitylases (DUBs) in charge of trimming these poly-ubiquitin chains to ensure optimal signaling, as well as to reset the system to basal levels remain poorly defined [9]. Thus far, two DUBs, namely cylindromatosis (CYLD) and A20 (also known as TNFAIP3), were demonstrated to negatively regulate antigen receptor signaling [9,10]. Herein, we provide evidence that Ubiquitin-Specific Protease 34 (USP34) also contributes to the fine-tuning of NF- κ B upon TCR engagement.

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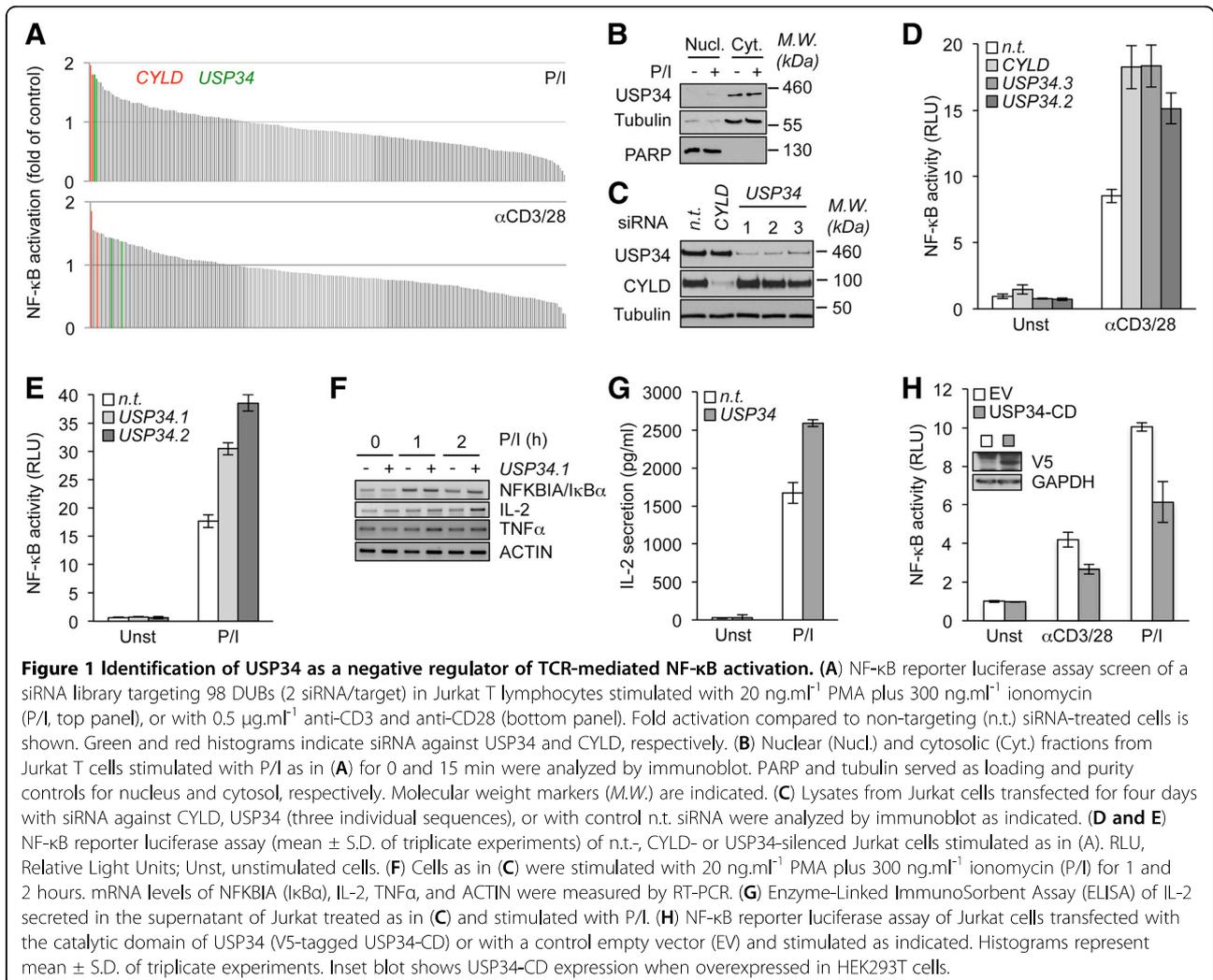
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To identify additional negative regulators of TCR-mediated NF- κ B activation, we conducted a siRNA library screen against 98 DUBs through a gene reporter luciferase assay in Jurkat T cells stimulated with either anti-CD3 and anti-CD28 antibodies or PMA plus ionomycin to mimic TCR engagement (Figure 1A and Additional files 1 and 2). As expected, CYLD silencing led to an enhanced NF- κ B activity upon TCR stimulation (Figure 1A). Furthermore, this screening also uncovered siRNA sequences specific for USP34 that potentiated NF- κ B activation with a similar magnitude to CYLD siRNA (Figure 1A). USP34 encompasses a 404 kDa protein with a central catalytic domain [11]. However, little is known about this DUB, albeit it was previously linked to the Wnt developmental signaling pathway [12]. Subcellular fractionation experiments showed that USP34 was essentially distributed in the cytosol of cells regardless of TCR stimulation, and was notably absent from the nucleus and organelles (Figure 1B and Additional file 3A). We next verified by immunoblot that CYLD and USP34 endogenous levels

were efficiently decreased by their respective siRNA sequences (Figure 1C). Of note, an additional siRNA duplex specific for USP34 was also included to reinforce our initial findings (named sequence 3). Consistent with the primary screening, NF- κ B reporter activity was similarly boosted upon TCR stimulation in USP34- and CYLD-silenced Jurkat when compared to control non-targeting siRNA transfected cells (Figure 1D and E). As a consequence, the levels of the NF- κ B targets NFKBIA (I κ B α), interleukin-2 (IL-2) and TNF α , as measured by RT-PCR were increased in USP34-knocked down cells (Figure 1F). Accordingly, downstream IL-2 secretion was enhanced in supernatants of USP34-silenced cells (Figure 1G). Finally, ectopic expression of a plasmid encoding for the catalytic domain of USP34 (USP34-CD [13]) markedly dampened TCR-mediated NF- κ B activity (Figure 1H). Because USP34-CD is a large segment (383 amino acids), it is possible that in addition to the catalytic domain, it also comprises a domain required for the binding to its partners to regulate NF- κ B in lymphocytes. Collectively,



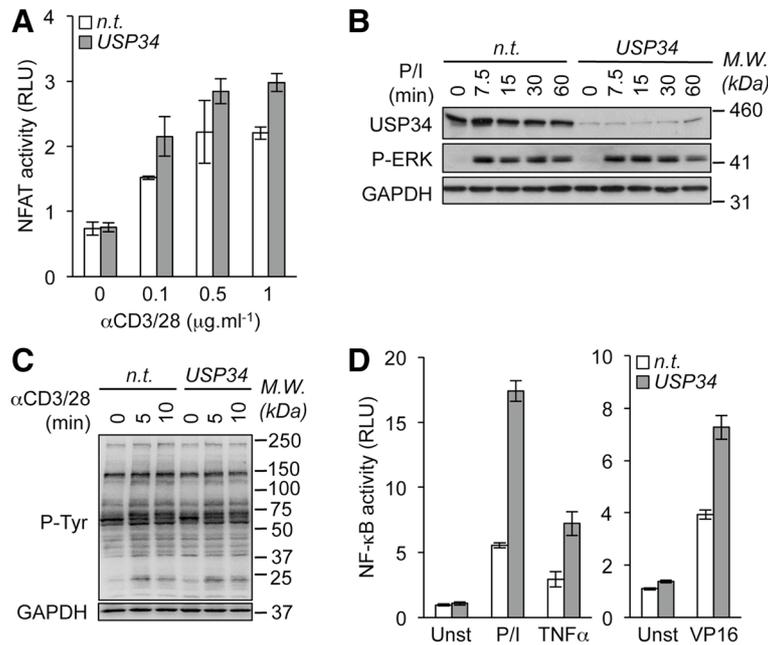


Figure 2 Knockdown of USP34 selectively potentiates NF-κB activation. (A) NFAT reporter Luciferase assay (mean ± S.D. of triplicate experiments) of Jurkat cells transfected with control non-targeting (n.t.) or USP34 siRNA and stimulated with 0, 0.1, 0.5, and 1 μg.ml⁻¹ anti-CD3 and anti-CD28 antibodies (αCD3/28). RLU, Relative Light Units. (B) n.t.- and USP34-silenced Jurkat cells were stimulated with 20 ng.ml⁻¹ PMA plus 300 ng.ml⁻¹ ionomycin (P/I) for 0, 7.5, 15, 30 and 60 min. Cell extracts were prepared and analyzed by immunoblot as indicated. Molecular weight markers (M.W.) are shown. (C) Cells as in (B) were stimulated with 1 μg.ml⁻¹ anti-CD3 and anti-CD28 antibodies. Cell extracts were prepared and general tyrosine phosphorylation pattern (P-Tyr) was evaluated by immunoblot. GAPDH served as a loading control. (D) NF-κB reporter Luciferase assay of Jurkat cells transfected as in (A) and stimulated with 10 ng.ml⁻¹ PMA plus 300 ng.ml⁻¹ ionomycin (P/I), with 10 ng.ml⁻¹ TNFα, or with 40 μM etoposide (VP16). Shown is the mean ± S.D. of triplicate experiments. Unst, unstimulated.

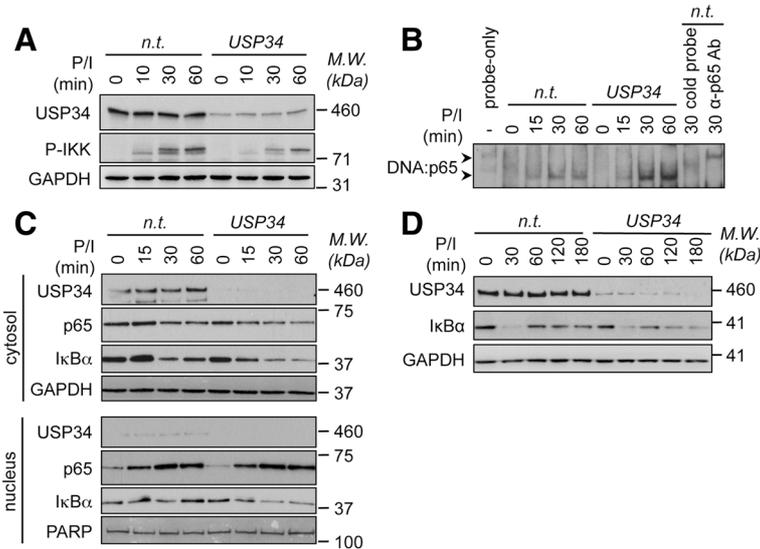


Figure 3 Silencing of USP34 enhances IκBα degradation and NF-κB binding to DNA. Jurkat cells were transfected with non-targeting- (n.t.) or USP34-targeting siRNA for 72-96 h. (A) Lysates from cells stimulated with 40 ng.ml⁻¹ PMA plus 300 ng.ml⁻¹ ionomycin (P/I) for 0, 10, 30 and 60 min were analyzed by immunoblot as indicated. M.W., molecular weight markers. (B) NF-κB binding to DNA was assessed on nuclear extracts from cells stimulated as in (A) for the indicated time by non-radioactive electrophoretic mobility shift assays (EMSA) using biotin-labeled κB element DNA sequence. Note that DNA:p65 complexes were chased away with a cold probe, and were shifted when a p65 antibody was added. (C) Nuclear and cytoplasmic extracts from cells stimulated as in (A) were analyzed by immunoblot as indicated. GAPDH and PARP served as loading controls. (D) Lysates from cells stimulated as in (A) were assessed by immunoblot as indicated.

our data suggest that USP34 is a cytosolic protein, which functions as a negative regulator of NF- κ B upon TCR engagement.

In addition to NF- κ B, TCR ligation kindles various signaling pathways including Nuclear factor of activated T-cells (NFAT) and the Mitogen-activated protein kinase (MAPK) Extracellular signal-regulated kinases (ERK) [14]. Gene reporter assays showed only modest increase in NFAT activation in USP34-silenced when compared to control cells (Figure 2A). Furthermore, ERK phosphorylation occurred normally without USP34 (Figure 2B). Keeping with this, no overt change in the general pattern of tyrosine phosphorylation was observed upon TCR stimulation, further arguing against a general impairment of TCR signaling in the absence of USP34 (Figure 2C). We next investigated whether USP34 also curtailed NF- κ B activity emanating from TCR-autonomous signaling triggers. To this end, USP34-silenced Jurkat cells were stimulated with the cytokine TNF α or with the genotoxic stress agent etoposide that functions via an unconventional ATM/PIASy/Sumoylated-NEMO axis [15]. Paralleling the situation with TCR, knocking down USP34 markedly increased NF- κ B in cells treated with TNF α or etoposide (Figure 2D). Supporting previous studies with CYLD-deficient cells [10,16], CYLD silencing in Jurkat cells also increased TNF α - and etoposide-mediated NF- κ B activation (Additional file 4). Combined, these results indicate that USP34 shares some functional similarities with CYLD and selectively targets the NF- κ B signaling pathway.

To gain insights on the signaling basis for the exacerbated NF- κ B activity in USP34-depleted cells, we first examined BCL10 and MALT1 ubiquitylation status since it governs the strength of TCR-mediated NF- κ B activation [4-6]. BCL10 ubiquitylation, which can be assessed in fractions enriched with heavy membranes [17], remained unchanged without USP34 (Additional file 3A). Moreover, pull-down of CK1 α to precipitate the CBM complex [7,17], showed similar amounts of ubiquitylated MALT1 bound in both control- and USP34-siRNA transfected cells (Additional file 3B). Keeping with this, BCL10 association to CARMA1 occurred normally without USP34 (Additional file 3C). We finally evaluated the impact of USP34 on the phosphorylation of IKK, which reflects its activation [8]. IKK phosphorylation was not exacerbated in those cells, and rather appeared slightly decreased (Figure 3A, and Additional file 3D). Although puzzling, this might result from a feedback loop triggered by enhanced NF- κ B. We next assessed directly NF- κ B DNA binding activity. Consistent with the gene reporter assays, more active NF- κ B-DNA complexes were detected in nuclear extracts from TCR stimulated cells when USP34 was silenced (Figure 3B). Although no obvious difference in NF- κ B subunit p65 translocation into the nucleus was detected, the degradation of the primary NF- κ B inhibitor

I κ B α was more pronounced in cytosolic fractions from USP34-silenced cells when compared to control cells (Figure 3C). Accordingly, I κ B α degradation was also prolonged or more dramatic in lysates from cells transfected with USP34 siRNA even on longer time points up to 3 hours (Figure 3D). Hence, our results suggest that USP34 likely functions downstream of the CBM-IKK nexus to enhance NF- κ B activation.

Almost 100 DUBs were identified in the human genome and yet, only a few have been ascribed a function [11]. As for TCR signaling, the well studied A20 and CYLD thwart NF- κ B at different levels [10]. CYLD targets and inhibits the ubiquitin-dependent IKK β kinase TAK1 and therefore prevents aberrant lymphocyte activation [18,19], while A20 dampens NF- κ B activity by trimming K63-ubiquitin chains attached to MALT1 [20,21]. Our study now unveils USP34 as an additional negative regulator of NF- κ B in lymphocytes. How USP34 tempers NF- κ B activity remains unclear. In contrast to CYLD and A20, which target apical signaling [18,20], USP34 rather seems to function downstream of the IKK complex. Given that USP34 does not bind to the NF- κ B core components (our unpublished results), we favor a model in which USP34 impacts on the activity of a cytosolic co-activator to ensure I κ B α fine-tuning [22,23]. Alternatively, USP34 might also intervene in other checkpoints to control NF- κ B signal outcome and intensity such as post-translational modifications, nuclear shift, or DNA three-dimensional structure [22,24,25]. Nevertheless, our data illustrates how various layers of control cooperate to ensure the fine-tuning of NF- κ B following engagement of the TCR.

Additional files

Additional file 1: Methods description.

Additional file 2: Design of the siRNA library screen.

Additional file 3: BCL10 and MALT1 ubiquitylation, CBM assembly, and IKK phosphorylation in USP34-silenced cells.

Additional file 4: CYLD and USP34 silencing enhanced etoposide- and TNF α -mediated NF- κ B activation.

Abbreviations

CYLD: Cyldromatosis; DUBs: Deubiquitinylases; ERK: Extracellular signal-regulated kinases; I κ Bs: Inhibitors of NF- κ B; IKK: I κ Bs kinase; MAPK: Mitogen-activated protein kinase; NFAT: Nuclear factor of activated T-cells; NF- κ B: Nuclear factor- κ B; PMA: phorbol 12-myristate 13-acetate; TCR: T-Cell receptor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KP designed and conducted most experiments, analyzed the data and wrote the manuscript. EMH, NC, SMD, HML, CL, and CA designed and conducted experiments, and analyzed the data. EMH and JG helped drafting the manuscript. JG and AV analyzed the data. NB conceived the project, analyzed the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

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RESULTS

Additional File 1: Methods description

Cell culture and reagents

Jurkat T lymphocytes (clone E6.1) and human embryonic kidney 293T cells (HEK293T) were obtained from the ATCC. Cells were stimulated with 0.1-1 $\mu\text{g.ml}^{-1}$ of anti-CD3 and anti-CD28 antibodies (BD Biosciences), or with 10-40 ng.ml^{-1} phorbol 12-myristate 13-acetate (PMA, Sigma) plus 300 ng.ml^{-1} ionomycin (Calbiochem). $\text{TNF}\alpha$ (R&D) and etoposide (VP16, Sigma) were also used. Plasmid encoding the catalytic domain of USP34 was kindly provided by V. Quesada and C. Lopez-Otin [1], and was subcloned in a V5-backbone (Invitrogen, Life technologies).

siRNA library and transfections

siRNA library targeting 98 human DUBs (two oligoribonucleotide duplexes per target, based on [2]) was purchased from Sigma (see Additional File 2). Jurkat cells were transfected by electroporation with a BTX ECM 830 apparatus (BTX, Harvard Apparatus). siRNA sequences used were: USP34.1, 5'-GGAUCUAGCAAUGAGGUUA[dT][dT]-3' (Sigma); USP34.2, 5'-GAUCUUAGGGCUGAAGUAA[dT][dT]-3' (Sigma); USP34.3, 5'-GGCAAGACAUUUGGCUGACUGUAUU-3' (Invitrogen); CYLD, 5'-GAACAGAUUCCACUCUUUA[dT][dT]-3' (Sigma). Negative controls were from Sigma and Invitrogen.

Luciferase assays

Firefly luciferase constructs downstream of κB or NFAT-responsive elements were co-transfected with renilla luciferase pRL-TK (Int-) plasmid (Promega). Luciferase activities were analyzed by measuring light emission using the Dual-Luciferase Kit (Promega), with firefly

RESULTS

luminescence units normalized to renilla firefly luciferase luminescence units (BMG microplate reader).

Enzyme-linked ImmunoSorbent Assay (ELISA)

Human IL-2 production and release was determined after overnight stimulation, in the culture supernatants by an enzyme-linked immunosorbent assay (R&D).

Reverse Transcription-Polymerase Chain Reactions (RT-PCR)

RT-PCR were carried out as previously described [3]. Briefly, equal amounts of RNA (Qiagen RNeasy mini kit) were reverse transcribed (Superscript III, Invitrogen), and cDNA were then amplified (RedTaq ready mix, Sigma). Primers used were: NFKBIA, 5'-GCAAATCCTGACCTGGTGT -3' and 3'- GCTCGTCCTCTGTGAACTCC -5'; TNF α , 5'-TCCTTCAGACACCCTCAACC -3' and 3'- AGCCCCAGTTTGAATTCTT -5'; IL-2, 5'-ACCTCAACTCCTGCCACAAT -3' and 3'- GCCTTCTTGGGCATGTAAAA -5'; β -actin, 5'-AGCACTGTGTTGGCGTACAG -3' and 3'- GGACTTCGAGCAAGAGATGG -5'.

Immunoblots and immunoprecipitation

Stimuli were washed out twice with PBS 1X and cell pellets were lysed at 4°C with 50 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% Igepal, supplemented with Complete protease inhibitors (Roche). 20 nM calyculin A was added to the lysis buffer when IKK phosphorylation was assessed. Lysates were cleared by centrifugation at 9,000g at 4°C and total protein concentration was determined with a micro BCA kit (Thermo scientific). 5-20 μ g were resolved in 5-8% Tris-Acetate SDS-PAGE gels (Invitrogen) or 5-20% Tris-Glycine gels

RESULTS

according to company's instructions and electrotransferred onto nitrocellulose membranes (Amersham). Antibodies to USP34 were from Bethyl laboratories. Antibodies against BCL10 (A-6), CK1 α (C-19), CYLD (H-6), LAMP2 (H4B4), MALT1 (B-12), NF- κ B p65 (C-20 and F-6), and tubulin (TU-02) were from Santa Cruz Biotechnology. Antibodies against CARMA1 (1D12), I κ B α , phosphorylated-I κ B α (5A5), phosphorylated IKK $\alpha\beta$ (16A6), and phosphorylated ERK1/2 (E10) were purchased from Cell Signaling Technology. Antibodies against GAPDH and V5 tag (Sigma), phosphorylated tyrosine (4G10, Millipore), and PARP (BD biosciences) were also used. HRP-conjugated secondary antibodies were purchased from Jackson. Immobilon chemiluminescent substrate (Millipore) was used for protein detection with autoradiography films. Immunoprecipitations were carried out as previously described [4]. Briefly, post-centrifugation lysates were precleared with protein G-sepharose (Roche) for 30 min prior to 1-2h incubation with antibodies and fresh protein G-sepharose at 4°C. After four washes, immunocomplexes were denatured and resolved by SDS-PAGE.

Electrophoretic Mobility Shift Assay (EMSA) and subcellular fractionation

5-10.10⁶ cells were washed with PBS1X and placed for 5 min at 4°C in 187.5 μ l Buffer A (10 mM HEPES pH 7.4, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM Na₃VO₄, 1 mM NaF, and Complete proteases inhibitors). Plasma membranes were solubilized by adding 12.5 μ l of 10% Igepal (Sigma) for 5 min at 4°C. Nuclei were pelleted with a 600g centrifugation and washed twice with buffer A. Nuclear proteins were then extracted with 30 μ l Buffer C (20 mM HEPES pH 7.4, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM Na₃VO₄, 1 mM NaF, and Complete proteases inhibitors) under rocking conditions. Nuclear extracts were cleared by centrifugation at 13,000g. EMSA to detect NF- κ B:DNA complex formation was

RESULTS

performed by using a non-radioactive kit with biotin-labeled or unlabeled DNA sequences (5'-AGTTGAGGGGACTTTCCCAGGC-3') containing κ B element (Affymetrix). Jurkat nuclear extracts were treated according to manufacturers protocol. Biodyne B nylon membranes (Thermo scientific) were used for the electrotransfer. Subcellular fractionation experiments were performed as previously described [5]. In brief, cells were mechanically permeabilized with 27G^{1/2} syringe (Becton Dickinson) in 20 mM HEPES pH 7.9, 1.5 mM MgCl₂, 60 mM KCl, and protease inhibitors. Nuclei were eliminated by a 1,000g centrifugation, and supernatants were further spun at 10,000g to yield crude heavy membranes pellets. Cytosolic fractions were obtained after an additional 25,000g centrifugation.

Additional References

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4

RESULTS

Additional File 2: Design of the siRNA library

Name	RefSeq ID	siRNA ID or sense siRNA
BAP1	NM_004656	SASI_Hs01_00105395
		SASI_Hs01_00105396
UCH-L1	NM_004181	SASI_Hs01_00178415
		SASI_Hs01_00178416
UCH-L3	NM_006002	SASI_Hs01_00200423
		SASI_Hs01_00200424
UCH-L5, UCH37	NM_015984	SASI_Hs01_00142742
		SASI_Hs01_00142743
USP1	NM_001017415	SASI_Hs01_00157121
		SASI_Hs01_00157122
USP2	NM_171997	SASI_Hs01_00174976
		SASI_Hs02_00367754
USP3	NM_006537	SASI_Hs01_00023593
		SASI_Hs01_00023595
USP4, UNPH	NM_199443	SASI_Hs01_00028215
		SASI_Hs01_00028216
USP5	NM_003481	SASI_Hs01_00184929
		SASI_Hs01_00184930
USP6, TRE-2, TRE17	NM_004505	SASI_Hs02_00337765
		SASI_Hs02_00337766
USP7, HAUSP	NM_003470	SASI_Hs01_00079539
		SASI_Hs01_00079540
USP8, UBPY	NM_005154	SASI_Hs02_00339089
		SASI_Hs01_00136039
USP9X, FAF-X	NM_001039590	SASI_Hs01_00031439
		SASI_Hs02_00308597
USP9Y, FAF-Y	NM_004654	SASI_Hs01_00081909
		SASI_Hs02_00338066
USP10	NM_005153	SASI_Hs01_00213007
		SASI_Hs01_00213008
USP11	NM_004651	SASI_Hs01_00148685
		SASI_Hs01_00148686
USP12, UBH1	NM_182488	SASI_Hs01_00167303
		SASI_Hs01_00167305
USP13, ISOT-3	NM_003940	SASI_Hs01_00108438
		SASI_Hs01_00108439
USP14, ISOT	NM_005151	SASI_Hs01_00016087
		SASI_Hs01_00016089
USP15	NM_006313	SASI_Hs01_00059894
		SASI_Hs01_00059895
USP16, UBPM	NM_001001992	SASI_Hs01_00121496
		SASI_Hs02_00341586
USP17L3	XM_001720764 1 s	CACGUUAACUUUACACACUdTdT
		CUAUCAUUGCGGUCUUUGUdTdT
		GCAAUAUCCUGAGUGCCUUdTdT
		GUUGUCACGACGGACAUAUdTdT
		GCAACAAACUUGCCAAGAAAdTdT
		GGAAGAUGUCCAUGAAUUUdTdT
		GACAUUACUUCUCUUAUGUdTdT
		CUCAAGAAGGCCAGUGGUAdTdT
		CACCUUAGACCACUGGAAAdTdT
		GAGAUUCUCCGAUGUCGCAdTdT
USP18, UBP43	NM_017414	SASI_Hs01_00221412

RESULTS

		SASI_Hs01_00221413
USP19	NM_006677	SASI_Hs01_00130241 SASI_Hs01_00130242
USP20, VDU1	NM_001008563	SASI_Hs01_00099343 SASI_Hs01_00099344
USP21, USP23	NM_001014443	SASI_Hs01_00177787 SASI_Hs01_00177788
USP22	XM_042698	SASI_Hs01_00276309 SASI_Hs01_00276310
USP24	XM_934749	SASI_Hs01_00301651 SASI_Hs01_00301652
USP25	NM_013396	SASI_Hs02_00344762 SASI_Hs01_00191397
USP26	NM_031907	SASI_Hs01_00043149 SASI_Hs01_00043150
USP28	NM_020886	SASI_Hs01_00077918 SASI_Hs01_00077919
USP29	NM_020903	SASI_Hs01_00032719 SASI_Hs01_00032720
USP30	NM_032663	SASI_Hs01_00155679 SASI_Hs01_00155680
USP31	NM_020718	SASI_Hs01_00020641 SASI_Hs01_00020642
USP32	NM_032582	SASI_Hs01_00086230 SASI_Hs01_00086231
USP33, VDU2	NM_201626	SASI_Hs01_00142382 SASI_Hs01_00142383
USP34	NM_014709	SASI_Hs01_00089169 SASI_Hs02_00346129
USP35	XM_935137	SASI_Hs01_00291572 SASI_Hs01_00291573
USP36	NM_025090	SASI_Hs02_00357787 SASI_Hs01_00194136
USP37	NM_020935	SASI_Hs01_00018875 SASI_Hs02_00354323
USP38	NM_032557	SASI_Hs01_00189218 SASI_Hs02_00360065
USP39, SAD1	NM_006590	SASI_Hs01_00034674 SASI_Hs01_00034675
USP40	NM_018218	SASI_Hs01_00145783 SASI_Hs02_00351527
USP41	XM_036729	SASI_Hs02_00482814 SASI_Hs02_00482815
USP42	XM_932196	SASI_Hs01_00248233 SASI_Hs01_00248234
USP43	XM_934221	SASI_Hs01_00274671 SASI_Hs01_00274672
USP44	NM_032147	SASI_Hs02_00359381 SASI_Hs01_00075742
USP45	XM_931124	SASI_Hs01_00265784 SASI_Hs01_00265785
USP46	NM_022832	SASI_Hs01_00080807 SASI_Hs01_00080808
USP47	NM_017944	SASI_Hs01_00112225 SASI_Hs01_00112227
USP48	NM_032236	SASI_Hs01_00077498 SASI_Hs01_00077499
USP49	NM_018561	SASI_Hs01_00055585

RESULTS

		SASI_Hs02_00352112
USP50	NM_203494	SASI_Hs02_00373416 SASI_Hs02_00373417
USP51	NM_201286	SASI_Hs02_00372924 SASI_Hs02_00372925
USP52	NM_014871	SASI_Hs01_00133440 SASI_Hs01_00133442
USP54	NM_152586	SASI_Hs02_00366408 SASI_Hs02_00366409
USPL1	NM_005800	SASI_Hs02_00340306 SASI_Hs01_00128479
CYLD	NM_015247	SASI_Hs01_00177192 SASI_Hs01_00177191
DUB-3	NM_201402	SASI_Hs02_00372940 SASI_Hs02_00372941
Ataxin-3	NM_030660	SASI_Hs02_00358236 SASI_Hs02_00358237
Ataxin-3-like	XM_045705	SASI_Hs01_00259290 SASI_Hs01_00259291
Josephin-1	NM_014876	SASI_Hs02_00346483 SASI_Hs01_00072064
Josephin-2	NM_138334	SASI_Hs01_00128488 SASI_Hs01_00128489
TAF1D	NM_024116	SASI_Hs01_00119698 SASI_Hs01_00119700
TNFAIP3, A20	NM_006290	SASI_Hs01_00033556 SASI_Hs01_00033557
ZA20D1, Cezanne-1	NM_020205	SASI_Hs01_00089029 SASI_Hs01_00089030
OTUD7i, Cezanne-2	NM_130901	SASI_Hs01_00055782 SASI_Hs01_00055783
OTUD4, HIN-1	NM_017493	SASI_Hs02_00372731 SASI_Hs02_00372732
OTUD6A, HIN-6	NM_207320	SASI_Hs01_00018704 SASI_Hs01_00018705
OTUD2, YOD1	NM_018566	SASI_Hs01_00052848 SASI_Hs01_00052849
OTUD6B	NM_016023	SASI_Hs01_00222466 SASI_Hs02_00348712
OTUD5	NM_017602	SASI_Hs01_00052828 SASI_Hs01_00052829
OTUB1, Otubain-1	NM_017670	SASI_Hs02_00378146 SASI_Hs02_00378147
OTUB2, Otubain-2	NM_023112	SASI_Hs01_00010701 SASI_Hs01_00010702
OTUD1	XR_040065	SASI_Hs02_00515603 SASI_Hs02_00515604
ZRANB1, TRABID	NM_017580	SASI_Hs01_00155779 SASI_Hs02_00350242
VCPIP1, VCIP135	NM_025054	SASI_Hs02_00357704 SASI_Hs01_00165636
STAMPB, AMSH	NM_006463	SASI_Hs01_00051759 SASI_Hs01_00051760
STAMBPL1, AMSH-like	NM_020799	SASI_Hs01_00241436 SASI_Hs01_00241437
BRCC3i, BRCC36, BRCC3	NM_001018055	SASI_Hs02_00356685 SASI_Hs01_00193368
COPS5, CSN5, JAB1, SGN5	NM_006837	SASI_Hs02_00342404

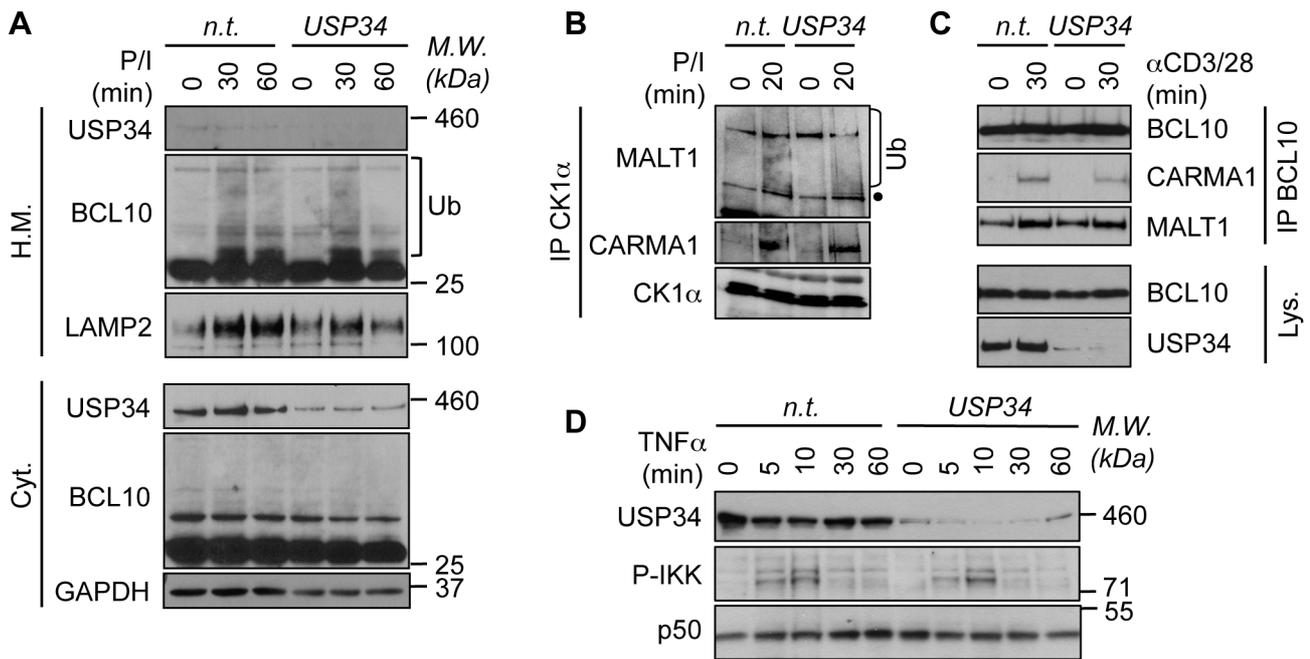
RESULTS

		SASI_Hs01_00209042
COPS6, CSN6, SGN6	NM_006833	SASI_Hs02_00342398 SASI_Hs01_00117938
EIF3S3, eIF-3-gamma	NM_003756	SASI_Hs01_00072290 SASI_Hs01_00072291
EIF3S5, eIF-epsilon	NM_003754	SASI_Hs01_00246686 SASI_Hs02_00336223
MYSM1	XM_055481	SASI_Hs01_00255872 SASI_Hs01_00255873
MPND	NM_032868	SASI_Hs01_00162447 SASI_Hs01_00162448
PSMD14, POH1, RPN11	NM_005805	SASI_Hs02_00340316 SASI_Hs01_00024446
PRPF8, PRP8	NM_006445	SASI_Hs01_00015918 SASI_Hs01_00015919
PSMD7, RPN8	NM_002811	SASI_Hs02_00334491 SASI_Hs02_00334492
USP17L5	XM_001130437	SASI_Hs02_00387302 SASI_Hs02_00387303
USP17L1P	XR_040280	SASI_Hs02_00517496 SASI_Hs02_00517497
IFP38	NG_005632 s	CCGAUGACUUUGAGACCAUdTdT
USP17L3	XM_001720764	CACGUUAACUUUACACACUdTdT CUAUCAUUGCGGUCUUUGUdTdT GCAAUAUCCUGAGUGCCUdTdT GUUGUCACGACGGACAUAUdTdT GCAACAAACUUGCCAAGAAdTdT GGAAGAUGUCCAUGAAUUUdTdT GACAUUACUUCUCUUAUGUdTdT CUCAAGAAGGCCAGUGGUAdTdT CACCUUAGACCACUGGAAAdTdT GAGAUUCUCCGAUGUCGCAdTdT
USP17L4	XM_001720370	CACGUUAACUUUACACACUdTdT CUAUCAUUGCGGUCUUUGUdTdT GCAAUAUCCUGAGUGCCUdTdT GUUGUCACGACGGAUUAUAdTdT GCAACAAACUUGCCAAGAAdTdT CACCUUAGACCACUGGAAAdTdT GGAAGAUGUCCAUGAAUUUdTdT CUCAAGAAGGCCAGUGGUAdTdT GAGAUUCUCCGAUGUCGCAdTdT CGGAUUAUACUUCUCUUAUdTdT
USP17L6	NR_027279	CUGGACAUCGCCUGGAUAdTdT CCAAGGUCCUCAUCCUUGUdTdT GAGAUUCUCCGAUGUCACAdTdT GCCUAUCAUUGUGGUGUUUdTdT GCUUCCUUGCAGUGCCUGAdTdT GGAAGAUGCCCAUGAAUUUdTdT
USP17L7	XM_001723172	GAGAUUCUCCGAUGUCACAdTdT GCGUAUUGGAGAUCUAAAAdTdT CACCUUAGACCACUGGAAAdTdT CUCUCAACGUGUCAUCUdTdT CUCAUUCUUGUAUUGAAGAdTdT CAAAGAACUCAUUGGAGAdTdT GACGUUAACUUUACCCACUdTdT CAUGAAUUUCUCAUGUUUAdTdT CAUGUUCUGUACUAUGCAAAdTdT CUCAAGAAGGCCAGUGGUAdTdT

RESULTS

USP17L8	XM_001720762	GAGAUUCUGCGAUGUCACAdTdT GUUGUCACAACGGAUUUAdTdT CUAUCCUUGCGGUCUUUGUdTdT
DUSP19	AB038770	GAAUUUUAUUGAAGAAGCAAdTdT GUUGCAUAUGGAGUUGAAAdTdT CCAACAUCCUGUCUUUUUdTdT GACUUUACAUAUAAGAGCAAdTdT GCUUCCUCAGUGACUUUAdTdT GACUCAUAUUCUUAUGUUdTdT

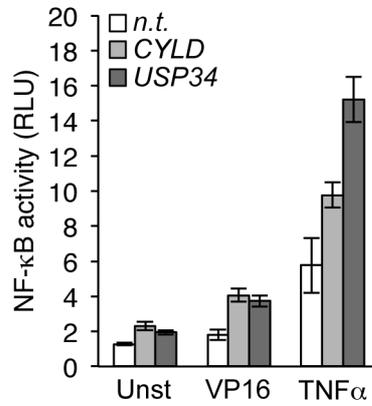
RESULTS



Additional File 3: BCL10 and MALT1 ubiquitinylation, CBM assembly, and IKK phosphorylation in USP34-silenced cells.

(A) Non-targeting siRNA- (n.t.) or USP34-silenced Jurkat cells were stimulated with 20 ng.ml⁻¹ PMA plus 300 ng.ml⁻¹ ionomycin (P/I). Cells were mechanically permeabilized, and crude heavy membrane (H.M.) and cytosolic (Cyt.) fractions were prepared. Immunoblots were performed as indicated. LAMP2 and GAPDH are markers for H.M. and Cyt. fractions, respectively. M.W., molecular weight markers. (B and C) Cells as in (A) were stimulated with P/I (B) or with 1 μg.ml⁻¹ anti-CD3 and anti-CD28 (C) as indicated. Lysates were prepared and immunoprecipitated (IP) with antibodies against CK1α (B) or BCL10 (C). Immunoblots were performed as indicated. Lys, lysates. (D) Lysates from n.t.- or USP34-silenced cells stimulated with 10 ng.ml⁻¹ TNFα were examined as indicated by immunoblot. p50 served as a loading control.

RESULTS

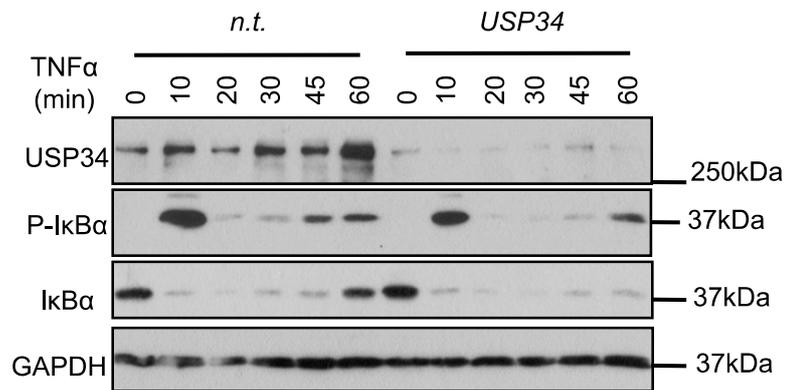


Additional File 4: CYLD and USP34 silencing enhanced etoposide- and TNF α -mediated NF- κ B activation.

NF- κ B reporter luciferase assay (mean \pm S.D.) of non targeting siRNA (n.t.)-, CYLD- or USP34-silenced Jurkat cells stimulated with 40 nM etoposide (VP16), or with 10 ng.ml⁻¹ TNF α . Unst, unstimulated; RLU, relative light units.

RESULTS

Unpublished data



Phosphorylation and degradation of IκBα in USP34-silenced Jurkat cells upon TNFα stimulation.

Jurkat cells were transfected with non-targeting (n.t.) and USP34-targeting siRNA sequences and were then stimulated with 10 ng/ml TNFα for the time shown. Cell extracts were prepared and analysed by immunoblot as indicated. Molecular weight figures on the right.

RESULTS

Article: *The Endoplasmic Reticulum Acts as a Platform for Ubiquitylated Components of Nuclear Factor κ B Signaling*

Distinct adaptor molecules, following similar activation principles, need to oligomerise to form high-order complexes and undergo ubiquitylation in order to propagate signaling in response to various NF- κ B-activating stimuli. Such proteins are RIP1, TRAF2, BCL10, RIP2, IRAK1 in TNFR-, CD40-, TCR-, NOD2-, TLR4-originating pathways respectively. We use the term NUTs (NF- κ B Ubiquitylated Transmitters) to describe these key adaptors. It has been shown that some signalosomes tend to crystallise on organelles' surface. By performing differential centrifugations of mechanically lysed cells in isotonic buffer, we isolated heavy membrane (HM) fractions in an effort to isolate those signalosomes in order to study their structure, composition and formation mechanisms and potentially identify new molecules implicated in NF- κ B pathways.

TNF α -stimulated HEK293T (kidney) cells, CD40L-stimulated BL41 (B lymphocytes), and PMA/ionomycin-stimulated Jurkat E6.1 (T lymphocytes) were submitted to subcellular fractionation and IB analysis. Ubiquitylated NUTs were only after stimulation detected in the HM fraction but not in the cytosolic one. Disruption of CBM complex assembly (by CARMA1 or MALT1 KD) prevented poly-ubiquitylation of BCL10, whereas downstream interventions (NEMO-silencing) did not alter ubiquitylation profile. Overexpression of ubiquitylation-prone, wild type BCL10, led to enrichment of the HM fraction with the ubiquitylated form, whereas when ubiquitylation-resistant, lysine-mutated BCL10 was used, this event was prevented. This suggests that NUTs ubiquitylation is a pre-requisite for their HM accumulation. Similar results were obtained in MDP-treated (muramyl dipeptide, a bacterial wall peptidoglycan), Poly(I:C)-treated (Polyinosinic-polycytidylic acid, a synthetic double strand RNA analog), and LPS-treated HEK293T cells, where RIP2, RIP1, IRAK1 ubiquitylated species respectively (through NLR, TLR3 and TLR4 signaling) were detected in HM fraction.

Further analysis was needed to identify the organelles recruiting NUTs, as HM fractions contain several membrane compartments. Additional centrifugation and gradient density centrifugation steps led to stepwise exclusion of plasma membrane contamination, lysosome and mitochondria, with the help of organelle specific protein-markers, suggesting that inducible NUT accumulation takes place in the ER. Trypsin treatment of HM fraction eliminated this phenomenon as shown by IB, indicating NUT accumulation in the cytosolic side of the ER.

In order to verify the presence of the molecular information necessary for signal propagation, a cell-free experimental system was used. In an ATP-supplemented *in vitro* reaction, I κ B α phosphorylation induction (thus IKK activation) in cytosolic fractions from unstimulated cells was assessed in the presence of HM fractions from HEK293T, BL41 and Jurkat cells stimulated with TNF α , CD40L and anti-CD3/anti-CD28 respectively. Activated HM fractions were able to induce I κ B α phosphorylation of non-activated cytosolic fractions in a dose-dependent manner. This effect was lost in trypsin-treated HM fractions. Experiments with ER-enriched fractions from activated cells confirmed the ER implication in signal transmission process.

Further research led to the identification of the transmembrane, ER-residing protein Metadherin (MTDH), through an IP experiment of BCL10 and RIP1 in stimulated Jurkat and HEK293T cell lysates followed by mass spectrometry proteomic analysis. As shown by IF experiments, MTDH is located in the ER and its localisation is not affected by TNF α or

RESULTS

PMA/ionomycin stimulation. Co-IP experiments of RIP1 and BCL10, together with TRAF2 confirmed modest but reproducible MTDH interaction with these proteins, in a signal inducible manner. The reverse IP, that of MTDH, confirmed interaction with ubiquitinated species of RIP1 in response to TNF α stimulation in HeLa cells. MTDH was also shown to preferentially bind synthetic K63-linked polyubiquitin chains *in vitro* to K48-linked ones. The domain mediating these interactions lies in the region between the 200th and the 300th aa. So ubiquitinated NUTs can be sensed and recruited by MTDH at the level of ER in response to cytokine stimulation and TCR engagement, possibly via K63-type of ubiquitin chains.

The implication of MTDH in NF- κ B activity was then tested by RNA interference experiments. *MTDH*-silenced, TNF α -stimulated HEK293T cells were used for NF- κ B luciferase assay. Diminished NF- κ B activity was observed, compared to cells transfected with non-silencing siRNA. This was confirmed by the reduced amounts of IL-8 secretion, as detected by ELISA. The significance of these findings lie in the fact that MTDH KD causes a decrease of NF- κ B activity, similar to that of KD of essential molecules of the pathway like RIP1 and TRAF2. Nuclear translocation of the RELA subunit is impaired and I κ B α phosphorylation and degradation is attenuated whereas MAPK/ERK pathway is unaffected. The same holds true for HeLa cells stably expressing shRNA against a non-coding region of MTDH mRNA, stimulated with TNF α . This defect was partly restored by introducing a construct coding for MTDH in these cells. In BL41 cells stimulated with CD40L the effect of MTDH KD on NF- κ B activity was the same. IB analysis has shown that JNK pathway was also impaired whereas non-canonical NF- κ B pathway events, like p100 to p52 processing or degradation of TRAF2 and TRAF6, were not affected.

The effect of *MTDH*-silencing was the same even in Jurkat cells in response to PMA/ionomycin stimulation. Phosphorylated Tyrosine pattern, a marker of TCR proximal signaling upon engagement, and calcium flux were not altered in the absence of MTDH meaning that early events and later signal divergence are not affected. These results were also acquired in primary T lymphocytes where less proliferation and reduced activation marker CD25 expression was observed in response to TCR stimulation. Further experiments testing the effect of MTDH KD in initial experiments (stimulation with MDP, Poly(I:C), LPS, Sentaivirus) has again confirmed and fortified the hypothesis of a rather broad effect of MTDH on signal transduction and upregulation of NF- κ B activity.

ER morphology and function was not altered in the absence of MTDH and NF- κ B activating ER-stress sensors KD did not have any impact on NF- κ B activation, revealing that no general defects of the ER occur in the absence of MTDH. By revisiting the cell-free system, it was shown that HM fraction from activated *MTDH*-silenced cells was not able anymore to drive I κ B α phosphorylation in cytoplasmic fraction from unstimulated cells. Accumulation of NUTs ubiquitinated species on the HM fraction was also disrupted even if their ability to undergo ubiquitinylation was not affected.

Overall, it becomes clear that the MTDH-dependent ER participation is necessary for assembly of ubiquitinated adaptors, the NUTs, in order to propagate signaling of a broad range of NF- κ B activators.

The Endoplasmic Reticulum Acts as a Platform for Ubiquitylated Components of Nuclear Factor κ B Signaling

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RESULTS

A. USP34

Many parameters remain ill defined and several questions unanswered regarding USP34. Its rather unusual way of action regulating I κ B α stability and NF- κ B nuclear activity from the cytoplasm remain intriguing.

A.1. USP34 is a highly conserved protein involved in NF- κ B signaling

Ubiquitin-specific protease 34 or ubiquitin carboxy-terminal hydrolase 34 or KIAA0729 or KIAA0570 are the various names given to USP34, an impressively large protein encoded by the genetic locus 2p15 of 283260 base pair long. This gene product pre-mRNA undergoes splicing of 65 exons to form the mature mRNA molecule of 11326 bp (NM_014709.3). The coding region consists of 10640 bp, which is translated to a protein (NP_055524.3) of 3546 amino acids (i.e. 404 kDa). The only characterised domain of USP34 so far is the UCH (Ubiquitin carboxy-terminal hydrolase, aa 1891-2236) catalytic segment, which belongs to the cysteine peptidase family C19. It is classified as a USP deubiquitinylase according to the structure of this domain. USP34 is the largest between the other USPs and all deubiquitinating enzymes in general. Human USP34 is 99,8% identical to chimpanzee Usp34 (JAA43377.1) and 96,5% identical to mouse Usp34 (NP_001177330.2), as obtained by pairwise multiple sequence alignment of the protein sequences using protein BLAST algorithm with the default parameters. The catalytic domain of human USP34 (346aa) is 100% identical (!) to fragment of the *in silico* translated CG5794-RF transcript of the *Usp34* gene in *Drosophila*, as revealed by pairwise alignment performed as above. These facts reveal high conservation of USP34 among species, suggesting an important role.

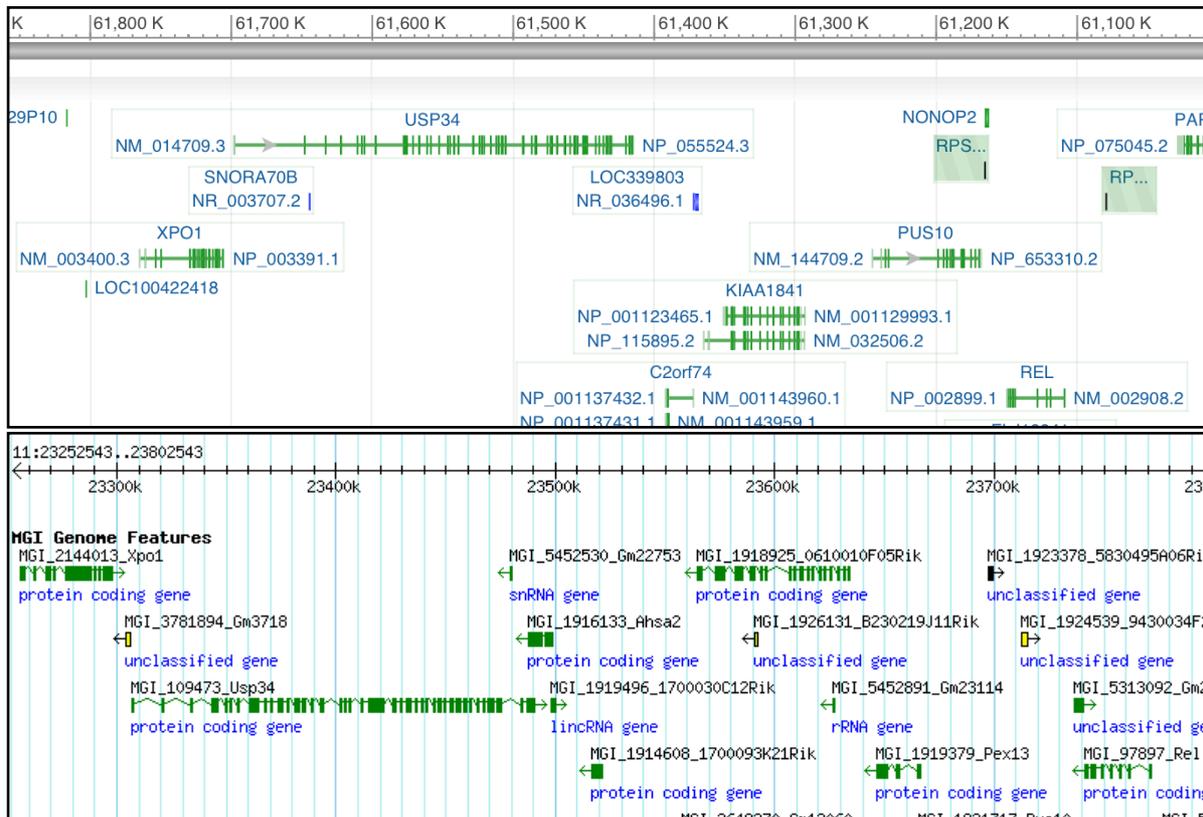


Figure 28: *USP34* genetic locus on human chromosome 2 (top) and on mouse chromosome 11 (bottom). Neighbouring genes like *XPO1* and *REL* are also depicted (National Center for Biotechnology Information - www.ncbi.nlm.nih.gov/gene and Mouse Genome Informatics - www.informatics.jax.org).

So far only a few papers exist describing USP34 and its roles. It was discovered and characterised as a deubiquitinating enzyme in 2003, together with 21 novel USP enzymes, by screening the human genome databases with the BLAST algorithm looking for DNA sequences encoding novel putative USPs, similar to previously described family members. USP34 expression was tested in brain where USP34 transcripts could be amplified by RT-PCR. Enzymatic activity was assayed after cloning and expression of catalytic domain or full-length proteins to verify which of them were functional. USP34 catalytic domain did demonstrate enzymatic activity (Figure 29) [205].

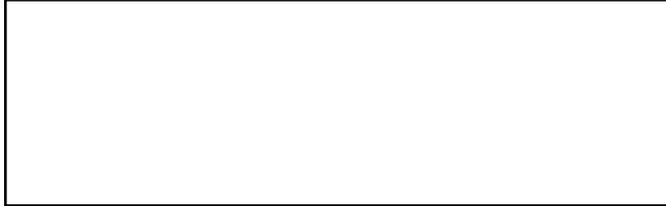


Figure 29: Enzymatic analysis of the identified human USPs. For each experiment, XL1-Blue cells expressing a GST-USP fusion protein and Ub-M- β -gal were lysed and subjected to immunoblotting with an anti-gal antibody. The arrows indicate non-processed recombinant Ub-M- β -gal (a), USP-processed recombinant β -gal (b), and endogenous β -gal (c). The result of each experiment is indicated below the corresponding lane. (+), active; (-), inactive. [205]

gal (c). The result of each experiment is indicated below the corresponding lane. (+), active; (-), inactive. [205]

Since, USP34 has been reported to interact with the protein AXIN1 by a study aiming to the identification of novel components of AXIN1 and AXIN2 complexes by mass spectrometry analysis (AXIN destabilises β -catenin when in the cytoplasm and promotes DNA-binding when in the nucleus). This group proposed that USP34 silencing enhances AXIN1 degradation and β -catenin-mediated transcription [206]. It has also been proposed that USP34 undergoes phosphorylation in an irradiation-inducible and ATM/ATR-dependent manner, functioning as a potential G1 checkpoint regulator [207]. Another group has also reported USP34 implication in regulation of TGF β /BMP signaling by controlling R-Smad protein levels through ubiquitinylation [208].

Our results now demonstrate that **USP34 also exerts a negative role in NF- κ B activation**, as witnessed by increased binding to DNA, gene reporter activity, NF- κ B target genes mRNA and cytokines secretion when USP34 is silenced and cells are TCR-stimulated [209]. Cloning and further overexpression of full-length USP34 protein was not attempted given its size. To circumvent this issue, we subcloned the catalytic domain into a eukaryote expression vector. The main goal was to test if USP34 overexpression would have the opposite effect (i.e. diminished NF- κ B activity upon stimulation) and if the catalytic domain alone would be sufficient. High expression of USP34 catalytic domain (CD) in cells is indeed sufficient to attenuate TCR-induced NF- κ B signaling. Apparently the 346aa long CD (which is quite long for a single motif region) not only is it functional but it is also able to interact with its cognate substrate, modifying finally NF- κ B signaling. Whether signal attenuation is due to an excessive amount of the CD or to the absence of an activity-modulating domain (via a PTM or interaction with an additional regulatory molecule) is unclear and needs further investigation.

A.2. USP34 selectively governs NF- κ B activation

As mentioned earlier, CYLD impacts proximal phosphorylation events in T lymphocytes, by interacting with Lck, deubiquitinating it and promoting interaction with its downstream substrate ZAP70 [190]. By contrast, no overt changes in phospho-tyrosine profiles were observed in USP34-silenced Jurkats, in response to TCR stimulation, as shown in Figure 30. **Hence, Tyrosine phosphorylation pattern is not affected by USP34.**



Figure 30: P-Tyr pattern in CYLD^{-/-} thymocytes upon stimulation with anti-CD3/anti-CD28. Arrows indicate reduced phosphorylation. [190]

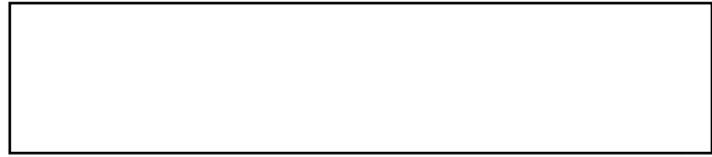


Figure 31: Example of NFAT/NF- κ B binding sites. The core of the NFAT binding site is shown in bold. [41]

In addition to NF- κ B, TCR signaling also diverges toward MAPK (mitogen activated protein kinase) pathways involving p38, JNK, Fos, ERK, in order to adjust and regulate the activity of AP-1 or other transcription factors [123]. CYLD also impacts activation of those pathways, modifying activity of JNK and ERK [190, 210]. Again, this is not the case in USP34-KD cells, as this does not affect ERK phosphorylation pattern upon stimulation and it only slightly increases NFAT activity, as detected by luciferase assay. This contrasted with T cells from CYLD-deficient mice, which exhibit hyperactive NFAT responses [211]. As for the slight increase after USP34 KD, NFAT contains a DNA binding domain with strong structural similarity to the DNA binding domain of NF- κ B, i.e. RHD, so we can imagine that when the latter is overactivated, it may also interact with the NFAT-specific promoters, technically leading to enhanced luminescence emission. A typical example of dual recognition by the two families of transcription factors is the κ 3 element of the TNF α promoter, which binds NFAT dimers as well as certain REL-containing ones *in vitro*. The same holds true for the p50/RELA-specific promoter of the HIV-1 (human immunodeficiency virus) long terminal repeat that also supports weak NFAT binding (figure 31) [41]. In conclusion, we show that USP34 has a **NF- κ B-specific effect** upon TCR stimulation.

A.3. USP34 functions downstream or in parallel of the CBM-IKK nexus

As extensively discussed in the introduction part, assembly of the CBM (CARMA1:BCL10:MALT1) complex is a key element for propagation of the signal towards the IKK complex. Cooperation-communication between the two signalosomes is mediated by oligomerisation and poly-ubiquitinylation of several adaptor molecules and their further identification by the various UBDs [212]. As mentioned before, DUBs like A20 and CYLD do intervene and do mediate regulation of these steps. As USP34 is another DUB, we tested whether these events are affected. CBM assembly, as shown by CK1 α and BCL10 co-IP experiments, occurs normally upon stimulation, regardless of USP34 presence. Ubiquitinylation pattern of MALT1, as assessed by MALT1 IP, immunoblotted with anti-Ubiquitin, is also unaffected during stimulation, passing from a non-ubiquitinylated form into an ubiquitinylated one, with the same intensity. Non-degradative ubiquitinylation and deubiquitinylation of BCL10 upon TCR stimulation occurs normally [144], with or without USP34, as detected in heavy membrane cell fractions enriched with the ubiquitinylated form of BCL10. Co-IP experiments (data not shown) did not reveal any interaction of USP34 neither constitutive nor inducible with key elements of the pathway like CARMA1, MALT1, BCL10, TAK1, IKK β , RELA, I κ B α , newly characterised LUBAC (RNF31, Sharpin, RBCK1), TRAF2, TRAF6, GSK3 β (common kinase with the Wnt- β -catenin pathway). It is clear though that formation of transient structures cannot be easily detected by co-IP. Only

CK1 α was found to constitutively and strongly interact with USP34. However, since CBM assembles normally in the absence of USP34, we infer that USP34 binds a different pool of CK1 α , which is not regulating CBM [139]. Overall we have shown that **USP34 acts downstream of the CBM complex level and does not interact with core components of the pathway.**

Although surprising at first glance, no difference in the translocation rates of the RELA NF- κ B subunit was detected in terms of time and quantity during stimulation. That is how we inferred that the “quality” of NF- κ B is altered in the absence of USP34. This was indeed the case as resulted by non-radioactive EMSA analysis of the nuclear extracts. Stimulation of USP34-silenced cells leads to enhanced affinity of NF- κ B to DNA, and specifically that of the RELA subunit. Another observation made was the retarded and less intense reappearance of I κ B α in cytosolic fractions after stimulation, also obvious in whole cell lysates. About 30 minutes after stimulation of Jurkat, we get maximum elimination of I κ B α (due to phosphorylation, K48-polyubiquitylation and proteasomal degradation) and later an increase of protein levels under normal conditions. NFKBIA is a target gene of NF- κ B so transcriptional activation of this gene rapidly increases its mRNA levels, as part of a negative feedback loop (mentioned earlier). As shown in paper figure 1F, mRNA levels indeed increase after PMA/ionomycin stimulation and further increase in cells lacking USP34. So the fact that NFKBIA mRNA levels are extensively present in these cells is not compatible with delayed and low neo-synthesised I κ B α protein levels. Somehow **USP34 alters NF- κ B DNA binding activity and modulates newly synthesised I κ B α stability upon TCR stimulation.**

A.4. USP34 ensures NF- κ B fine-tuning downstream of various immunoreceptors



Figure 32: Signal convergence at the TAK1-IKK level [116]

One important question was “is this effect restricted to TCR-dependent NF- κ B activation, or does it occur in other activating pathways”. The fact that AgR-specific CBM complex was not affected made this idea look very attractive. NF- κ B activity was measured by luciferase assay in response to different factors like TNF α stimulation or induction of genotoxic stress with the topo-isomerase inhibitor agent etoposide (VP16). Enhanced NF- κ B activity was again observed in USP34-KD cells compared to non-targeting siRNA transfected ones. The same effect on neo-synthesised I κ B α was observed in lysates

of TNF α -stimulated cells. All these pathways converge in the regulation of the IKK activity (Figure 32) that phosphorylates I κ B α , the onset event leading to its proteasomal elimination. As mentioned earlier IKK activity can be regulated by its phosphorylation status either due to auto- or TAK1-dependent phosphorylation. Thus phosphorylated form of the IKKs would reflect catalytic activity. As TAB:TAK1 and IKK complexes are the first molecules shared in these pathways, we verified the IKK phosphorylation status by IB in cells stimulated with PMA/ionomycin and TNF α , in the presence or absence of USP34. Clearly the hypothesis of enhanced-prolonged I κ B α degradation due to overactivated IKK collapsed, as IKK phospho-status was the same with or without USP34. In fact, upon PMA/ionomycin stimulation, IKK phosphorylation was attenuated in the absence of USP34, probably as a secondary effect of

NF- κ B overactivation, as part of an auto-inhibitory mechanism to fine-tune signal intensity. Thus we consider that **USP34 acts downstream of the IKK level** to regulate NF- κ B activity, a mechanism shared by TCR-, TNFR- and DNA damage-induced signaling.

Keeping in mind that USP34 is strictly cytoplasmic it seems that whatever the implication in regulation of the pathway, it must take place before translocation of NF- κ B into the nucleus. After the IKK complexes and before nuclear translocation of NF- κ B, the only well-characterised complexes so far are those of the NF- κ B dimers with the various I κ Bs. Thus, it is highly possible that **USP34 primarily regulates cytoplasmic NF- κ B conformation and secondarily I κ B α stability**, directly or most probably indirectly, through a third intermediate molecule. There are several parameters that could modulate transcription factor activity, analysis of which will follow.

A.5. A working Model for USP34 in the regulation of NF- κ B

Transcription is a fundamental biologic process that is finely regulated. Several classes of molecules are needed for successful transcription initiation including (1) transcription factors that are able to bind on DNA sequences of gene promoters and enhancers, (2) proteins that associate with and regulate transcription factor activity called coactivators or corepressors, (3) proteins that architecturally support and facilitate recruitment of these proteins on DNA molecules, and (4) basal transcription apparatus constituted by RNA polymerase II and general transcription factors that recognise general patterns that are common in all promoter sequences (e.g. TATA-box). Supramolecular complexes constituted of these components that can function and initiate transcription are called **enhanceosomes** (see example in next figure) [213, 214].

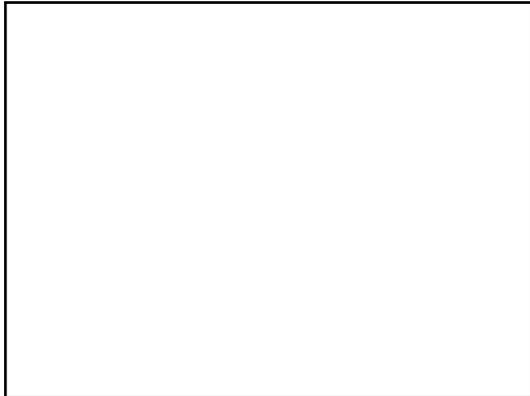


Figure 33: Model of the TNF α -induced E-selectin enhancer. After induction by cytokine, heterodimers of NF- κ B bind to the promoter, which is constitutively occupied by an ATF-2/c-Jun heterodimer. In parallel with nuclear accumulation of NF- κ B, ATF-2 and c-Jun are phosphorylated by p38 kinase and c-Jun N-terminal kinase, respectively. The binding of HMG I(Y) at multiple sites increases the binding of NF- κ B and bends DNA in a way that facilitates the formation of a higher-order complex. The transcriptional activators make extensive protein–protein contacts with the coactivator and the basal complex. Indicated are interactions between the transactivation region of p65 and the N- and C-terminal regions of CBP, an association between c-Jun and the coactivator, and interactions between TFIIB and CBP. Collectively, these events place multiple transcriptional activators in a favorable architecture to compete for the coactivator that is

present in limiting amounts. Because RNA polymerase II is constitutively associated with CBP/p300, binding of the coactivator to the E-selectin enhancer may also efficiently recruit the polymerase. [213]

Enhanceosome constitution depends on many parameters, like DNA sequence and its methylation-acetylation status, combination of diverse transcription factors and their PTMs, coactivators and corepressors association and their PTMs respectively are only a few (!) examples. So what would happen to this sensitive-equilibrium complex if one of these parameters changed? That is where our model of USP34 regulation is based. Several PTMs and association of various partners have been reported for the NF- κ B transcription factors, thus altering its activity by different ways. A PTM of NF- κ B might directly alter DNA affinity or indirectly partner recruitment, leading to different transcriptional potential. Reversly, a PTM of partners might directly affect association with the transcription factor and indirectly NF- κ B transcriptional capacity (Table 13). Only for the RELA subunit the PTMs reported so far include phosphorylation on at least 8 residues (T254, S276, S311, T435, S468, T505, S529, S536), acetylation on at least 5 residues (K122, K123, K218, K221, K310),

methylation on at least 3 residues (K37, K218, K221), ubiquitinylation, SUMOylation, poly(ADP-ribo)sylation (PARsylation), S-Nitrosylation etc [16, 27, 29, 48, 83, 214-216].

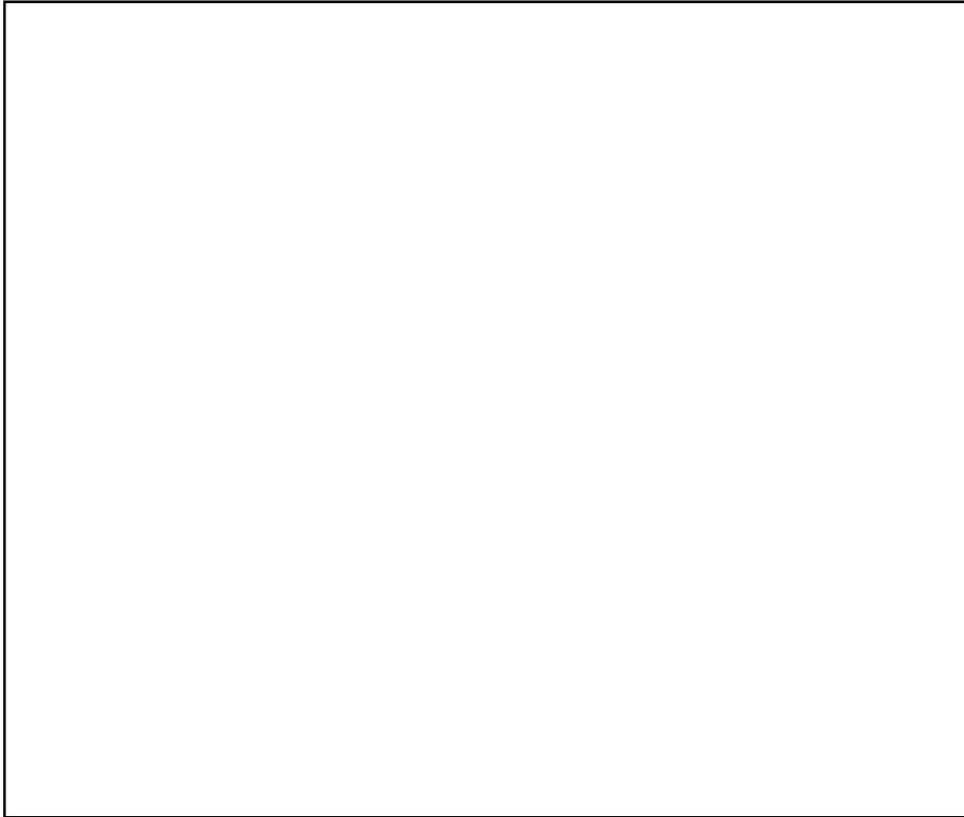


Table 13: PTMs of the NF-κB family members that regulate binding activity. [215]

A PTM on a NF-κB subunit can cause a conformational change that directly favours or blocks κB DNA binding. Instead or additionally this conformational change may indirectly alter interactions with other molecules necessary for NF-κB activity. Those molecules could either be their well-known partners, IκBs, or any other interacting protein like coactivators and corepressors or heterologous transcription factors (Figure 34). Serine 536 phosphorylation of RELA, for example, catalysed by various kinases, weakens the interaction with newly synthesised IκBα, leading to a dramatic increase of RELA nuclear export further prolongating κB DNA access. The same event takes place upon inducible acetylation of RELA on lysines 218 and 221. By having this in mind, together with the fact that free IκBα is very unstable with a half-life of less than 10 minutes in contrast with its great stability when complexed with NF-κB, we can hypothesise that a USP34-dictated PTM on NF-κB can regulate IκB binding ability, and subsequently transcription duration and intensity. Alternatively and/or additionally, a PTM can alter the recruitment capacity of associating factors (e.g. coactivators like CBP or other transcription factors like c-Jun) necessary for enhanceosome formation and transcription initiation (Table 14). Additional experiments will be needed to identify USP34's partners and targets and mouse models will reveal an *in vivo* effect in mammals. A screening for mutations on cancer patients might even reveal an unexpected correlation with the disease.

USP34 KD effect	Active	Passive
NF-κB-direct	Increase DNA binding affinity	Prevent IκB/corepressor recruitment or enhanceosome stabilisation
NF-κB-indirect	Increase coactivator-mediated DNA binding (e.g. RPS3)	Prevent IκB/corepressor recruitment or enhanceosome stabilisation

Table 14: Effect of USP34 silencing and their potential cause

An interesting point is that native NF- κ B of nuclear extracts (>200 kDa) has more than a hundred times higher DNA affinity compared to p65:p50 reconstituted heterodimers from purified monomers (115 kDa). This means that there are additional factors attached to NF- κ B, critically contributing to transcriptional activity. The NF- κ B complex consists of REL and synergistically interacting non-REL subunits (Table 15). This is the case of RPS3. Despite nuclear localisation of p65, RPS3 silencing in cells results in failed recruitment on gene regulatory sites and transcription induction [217]. In contrast with CBP, which is not incorporated into the DNA binding complex, RPS3 physically associates with p65, p50, and I κ B α in resting cells and specifically translocates into the nucleus in response to TCR and TNF stimulation [215].

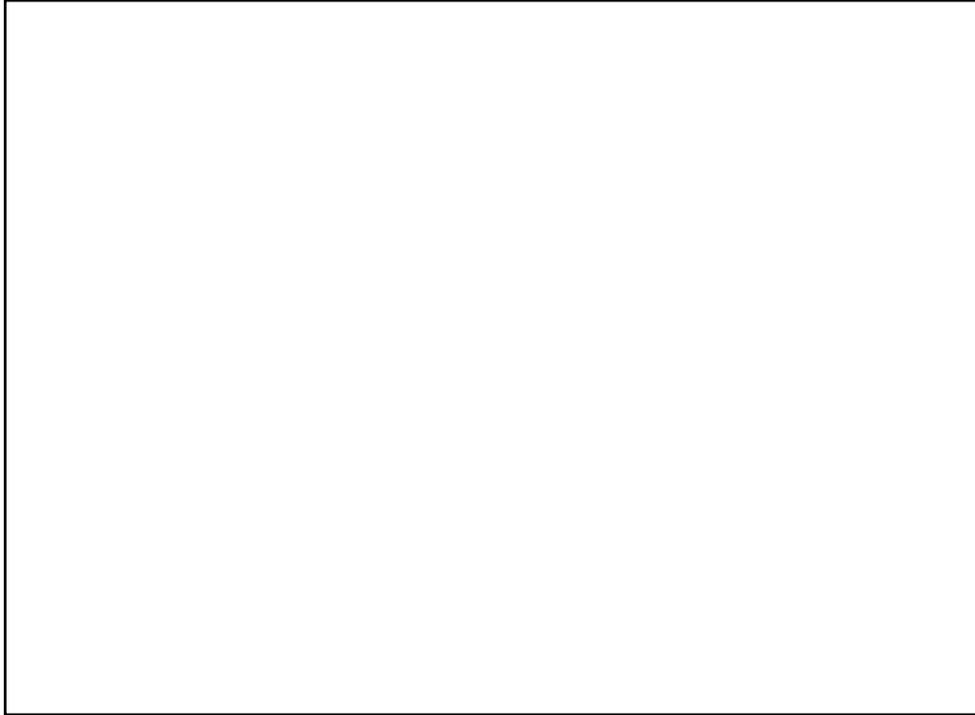


Table 15: NF- κ B-associating proteins that regulate DNA binding activity. [215]

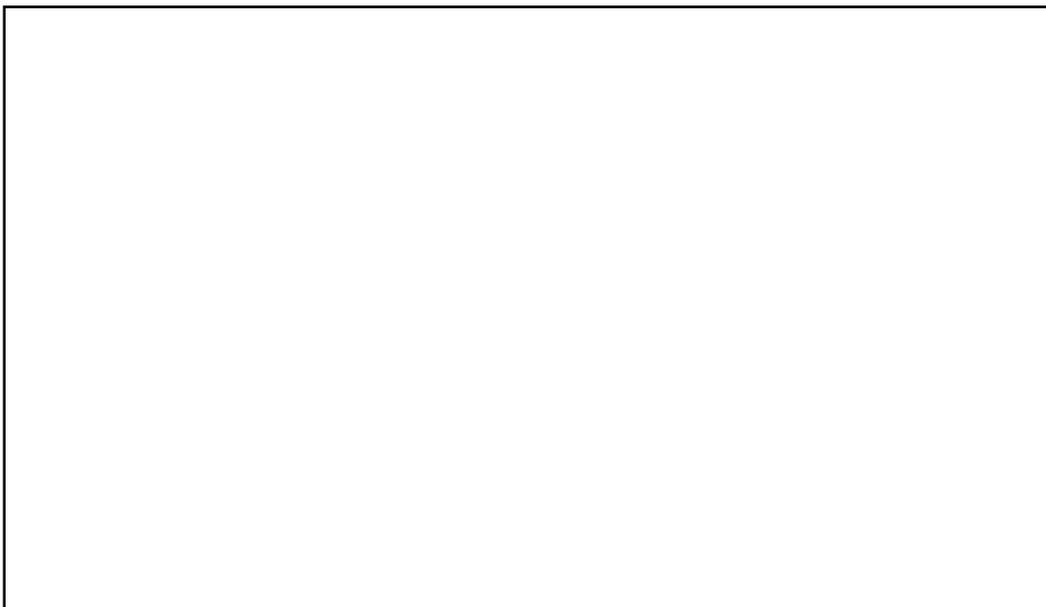


Figure 34: Crosstalk mechanisms involving NF- κ B subunits. The transcriptional activity of NF- κ B subunits is subject to regulation via a variety of PTMs, including phosphorylation, acetylation and methylation. As PTMs have the potential to

modulate the interaction of NF- κ B with coactivators, corepressors and I κ B proteins, as well as the binding of NF- κ B to cooperatively functioning, heterologous transcription factors, they represent a major determinant of selectivity in the induced gene expression signature and are thought to be critical for integration of non-NF- κ B pathways and contextual tailoring of the transcriptional response. The formation of NF- κ B-containing enhanceosomes at the promoters of target genes requires cooperative action between transcription factors, which facilitates both the integration and regulation of non-NF- κ B pathways. NF- κ B activity also affects heterologous pathways, such as the Jnk and p53 pathways, through transcriptional regulation of signaling pathway components. Gadd45 β , growth-arrest and DNA damage-inducible protein; MnSod, manganese superoxide dismutase; Fhc, ferritin heavy chain; XIAP, X-linked inhibitor of apoptosis protein. [214]

A.6. A role for USP34 in *Drosophila*?

Remarkably, basic innate immunity signaling pathways are conserved among species in terms of structure and function. Signal transduction initiating from TLRs, NLRs, IL-1R as pathogen sensors together with signal transducers and amplifiers such as IRAK, MYD88, TRAF, IKK and REL transcriptional regulators are even present in early metazoans, sponges, sea anemones and jelly fish. Of note, RLR family originated much later and probably exists only in vertebrates [1]. Two well-characterised NF- κ B-like signaling pathways in *Drosophila* show great similarities in terms of organisation with mammalian pathways and are necessary for immune responses against pathogens by promoting production of humoral antimicrobial peptides.

IMD (immune deficiency) pathway mainly functions in immune defenses maintaining *Drosophila* homeostasis against Gram-negative bacterial infection. Bacterial peptidoglycans bind on the appropriate receptors and scaffold protein IMD is recruited. IMD is a death domain (DD)-containing protein (homologous to mammalian RIP1), which mediates recruitment of other DD-containing proteins. Two complexes can be formed, one recruiting Dredd (Caspase-8 homolog) and another one recruiting the E3 ligase Iap2 (like TNFR complexes I and II). IMD:Iap2 complex induces *Drosophila* TAB2:TAK1 (dTAB2:dTAK1) activation that further activates *Drosophila* IKK complex consisting of Ird5 (immune response deficient 5) and Kenny or Key, IKK β and NEMO homologs respectively. Subsequently Dredd and Key phosphorylate the NF- κ B-like factor Relish that undergoes ubiquitinylation and proteasomal processing, similar to its mammalian homologs p105 and p100. Truncated-activated Relish (Rel-68) translocates into the nucleus promoting production of several antimicrobial peptides (Figure 35). IMD pathway also bifurcates to the *Drosophila* JNK pathway [218-220].

Toll pathway (homolog of human Toll-like receptors TLR) on the other hand responds to Gram-positive bacteria or fungi and it is involved in innate immunity and developmental processes. In the presence of these pathogens, ligand of Toll receptor is produced rapidly leading to its activation. dMYD88 adaptor is recruited via TIR (TLR/IL-1R) domains. As a result, dMYD88-Tube-Pelle heterotrimeric complex is formed through DD-mediated interactions. Assembly of this complex is necessary for phosphorylation (possibly by Pelle) of the I κ B-like protein Cactus and its subsequent proteasomal degradation. This last event is the onset for release of the NF- κ B-like transcription factors Dorsal and/or Dif (Dorsal-related immunity factor) and their translocation into the nucleus to initiate transcription of genes rendering antimicrobial protection (Figure 35) [218-220].

As in humans, ubiquitin-related factors are important for negative regulation of IMD signaling including dCYLD, dUSP36, dSCF E3 ligase complex etc. *Drosophila* CYLD specifically binds Key and is able to down-regulate antimicrobial peptides gene expression. dUSP36 prevents constitutive IMD-dependent signal activation through its catalytic activity, acting upstream or at the level of IMD. In a PhD study focusing on identification of additional

Drosophila DUBs implicated in NF- κ B regulation, a dsRNA library was created targeting 23 DUBs in *Drosophila* genome that possess a close human homolog. Immune competent S2 cells were transfected with the respective sequences and NF- κ B activity was measured in the presence or absence of immune challenge. Out of this screening, *Drosophila* USP34 (*Dmel/CG5794*) was found to negatively regulate IMD and Toll pathways, as *dUsp34* silencing led to overactive NF- κ B either with or without stimulation. dUSP34 was proposed to act downstream of IMD. Inducible KD was performed in adult flies in order to study the effect *in vivo*. IMD pathway activation was observed in the absence of immune challenge, showing that dUSP34 is necessary in order to prevent its constitutive activation, whereas Toll pathway was unaffected under the same conditions. Reversly, overexpression of the gene *in vivo* led to increased fly sensitivity to both Gram-negative and Gram-positive bacterial infection. Subcloning of catalytic domain and ectopic expression in bacteria failed as no catalytic activity was observed. Local gene inactivation was also performed so as to study the effect of *dUsp34*-silencing in wing development, where slight malformations were observed [218]. Although *Drosophila* NF- κ B pathways are not as well characterised as the human pathways, it is highly possible that **USP34 acts in diverse NF- κ B pathways in an evolutionarily conserved manner, targeting common downstream molecules.**



Figure 35: **Parallelisation of NF- κ B pathways in mammals and *Drosophila*.** Genes encoding additional homologs of the mammalian TLR/IL-1R (Toll) and TNFR (PGRP-LC) pathways exist in flies: these include homologs of RIP1 (IMD), MYD88 (dMYD88), FADD (dFADD), TRAF2 and TRAF6, which are components of receptor-associated complexes in mammals, homologs of TAB2 and TAK1 (dTAB2 and

dTAK1), which regulates IKK β and NEMO homologs (Ird5 and Key respectively). Some of these putative *Drosophila* genes function in the Toll and IMD pathways. Abbreviations: ANK, ankyrin domain; DD, death domain; DED, death effector domain; FADD, Fas-associated death-domain-containing protein; PGRP-LC, Peptidoglycan recognition proteins; IRAK, IL-1R-associated kinase; Rel, REL homology domain; RIP, receptor-interacting protein; TIR, Toll/IL-1R domain; TRADD, TNFR-associated death-domain-containing protein; TRAF, TNFR-associated factor. (adapted from [219] with [220])

B. MTDH

Signalosome formation upon stimulation occurs through several steps that culminate in NF- κ B activation. Adaptor proteins containing certain protein-protein interacting domains are oligomerised and recruited close to the ignition site to further organise in high-order complexes able of propagating signaling and activate IKK and downstream components (Figure 18). Ubiquitinylation is of major importance as analysed above. How apical

signalosomes finally achieve cytoplasmic NF- κ B activation remains poorly understood. A two-step model has been proposed, in which signalosomes undergo maturation after their formation and are subsequently released in the cytosol to transfer and transmit signals (Figure 36) [153]. Our work suggests that signaling hubs released from their immunoreceptors crystallise at the surface of the ER, and that this organelle functions as a gateway to ensure NF- κ B fine-tuning. Upon TNF α , CD40 and TCR stimulation (the latter being the part where I contributed in this work), ubiquitinated RIP1, TRAF2 and BCL10 respectively were recruited on heavy membranes, free from other signalosome components such as TRADD and cIAP1 in the case of TNFR or cIAP1 in the case of CD40, which strictly remain in the cytosolic fractions. The same holds true for RIP2, as immature signalosome components stay in the cytosolic fraction (i.e. NOD2). This, along with previous observations, suggests that **signalosomes mature**, are liberated from the membranes, and **only certain information-containing/information-transmitting molecules (i.e. NUTs) are recruited on organelles**. However, the notable absence of IKK or NF- κ B in ER fractions suggests that these complexes likely further evolve to propagate signalling. Our work therefore urges us to reevaluate the initial two-steps model and unveils the spatio-temporal plasticity of NF- κ B signalosomes.

What drives the dissociation of these complexes from their initial receptor remains elusive. The release of the IKK-MEKK1-activating complex from CD40 relies on TRAF3 proteasomal degradation [153]. However, we found that pre-treatment with the proteasome inhibitor MG132 does not preclude BCL10 ubiquitinylation at the ER, suggesting that alternative mechanisms exist. In that view, IKK β was shown to phosphorylate BCL10 within the CBM, causing destabilisation of the complex [221]. Hence, future works will be aimed to define how apical complexes are released from their receptors.

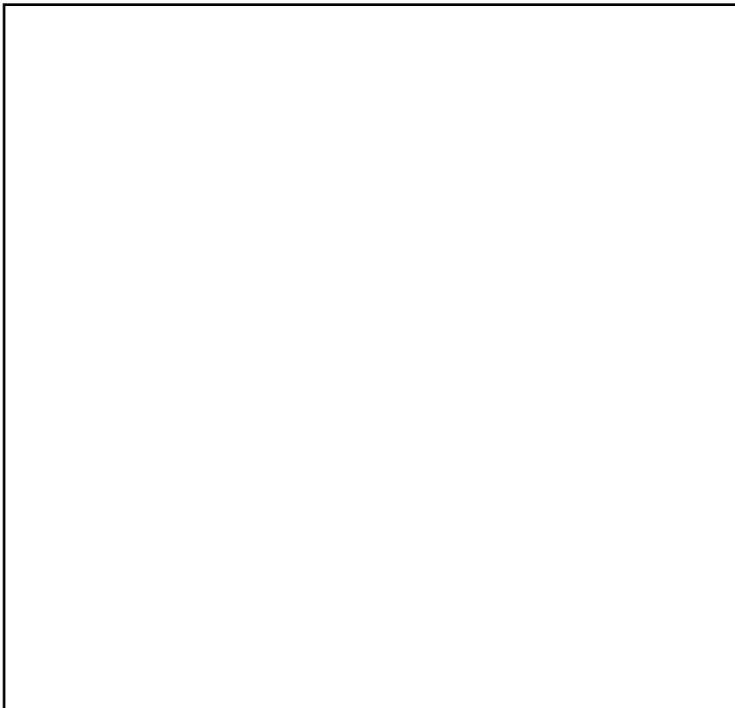


Figure 36: A two-stage signaling mechanism. (a) In nonstimulated B cells, only CD40 is membrane-associated. (b) Receptor engagement induces trimerization and recruitment of TRAF2, TRAF3, c-IAP1/2, and Ubc13. (c) Next to be recruited into this complex are IKK γ and MEKK1. Interactions between IKK γ and MEKK1 and K63-linked polyubiquitin chains catalyzed by TRAF2 and Ubc13 stabilize the complex. (d) c-IAP1/2 catalyze K48-linked polyubiquitination of TRAF3 whose proteasomal (26S) degradation results in translocation of the receptor-assembled signaling complex into the cytosol where MEKK1 is activated and in turn activates downstream components of its signaling module. [153]

Mass spectrometry identified MTDH as an ER component involved in recruiting the ubiquitinated NUTs. MTDH possesses one transmembrane span at the amino-terminal side and most of the protein is oriented towards the cytosol [222]. Although initially characterized as an ER resident, MTDH was later found on cell surface, or moving from the ER to the

nucleus in response to TNF α stimulation. In our hands, MTDH is constantly located at the ER. Discrepancies can be explained by the fact that previous studies have mostly used overexpression approaches, possibly forcing the protein to localise elsewhere from its native environment. MTDH overexpression has been reported in various cancers and despite promoting metastasis and rendering resistance to chemotherapy, it has never attracted attention as a treatment target. Ectopic MTDH has been linked to aberrant activation of NF- κ B, ERK, p38, PI3K/Akt and autophagy. We now report that endogenous MTDH participates in innate and adaptive NF- κ B signalling. Interaction experiments together with the KD experiments showed that it is **part of the signal transduction mechanism through recognition of K63-ubiquitylated substrates**. *In vivo* models will reveal whether compensatory mechanisms exist and if MTDH-targeting therapy could be applied in cancer patients to dampen NF- κ B.

We found no overt impact of MTDH knockdown on some unrelated ER functions, suggesting that MTDH does not participate to the ER homeostasis. In that view, growing evidences show that the ER beds various other biological processes, as speculated by the presence of proteins with ER-unrelated functions. This is for example the case for the ER multi-pass protein STING, which governs type I IFN production in reponse to pathogen-derived cytoplasmic DNA. Hence, MTDH and STING can be considered as a new class of ER proteins, not involved in ER-related functions, but rather in collecting and converting apical signals into biological responses.

The exact mode of function of MTDH remains unclear. It is believed that signals from the plasma membrane towards intracellular components, organellar or cytoplasmic, are transmitted in a contact-dependent manner. ER is a dynamic structure and, as is the case of T lymphocytes, it is able to get close to the plasma membrane to regulate the Ca²⁺ flux, mediated by contact of the ER protein STIM1 and the plasma membrane, channel protein Orai1. Since the ER is found in extreme proximity to other cell compartments (only 10-25 nm away from the plasma membrane for example), we favor a model in which MTDH collects ubiquitylated NUTs to increase their local concentration and authorize NF- κ B activation. ER can be the bridge that permits contact between the various plasma membrane receptors and MTDH. MTDH can bind ubiquitin chains, despite the absence of a typical UBD. Motifs able to interact with ubiquitin and ubiquitylated substrates are still to be discovered [223].

What could be the benefit of signal integration at intermediate sites (ER in specific) during the cascade of reactions through additional energy- and time-consuming steps and not promote direct activation of downstream pathway components? This may offer an additional checkpoint in signal transduction regulation. It can smoothen spontaneous activation of the receptors preventing erroneous NF- κ B signaling, which can be dangerous for cells and tissues. Similar to the need of costimulatory molecules (e.g. CD28 costimulation additionally to TCR), a need to accumulate and centralise activated molecules can guarantee reponse induction only if stimulation is of a given intensity, in an “all-or-nothing” mechanism. ER centralisation can also act as an additional control point contributing to fine-tuning of signaling outcome. Another question is what happens to the ubiquitylated NUTs and how the response is mediated. There are about 50 E3 ligases that possess transmembrane domains possibly able of modifying membrane-bound proteins. We can consider that there are also transmembrane DUBs implicated in signal termination and setting of the system at default conditions, as inhibition of the proteasome does not alter ubiquitylation and deubiquitylation pattern of BCL10. Further research is needed to illuminate all these signal transduction steps.

C. Last words

Overall, this project contributes in the scientific community to go one step further towards deeper understanding of another aspect of the multi-level regulation in signal transduction pathways and specifically those activating NF- κ B. It also highlights the complexity of these systems and reinforces the belief of tight regulation and fine-tuning, selected by nature throughout centuries of Life evolution. Immune responses can be as beneficial as dangerous. The proof for this lies in the fact that several cancers and inflammatory diseases originate from or result in NF- κ B malregulation. Treatment of those diseases at a molecular level, specifically targeting the branch of the pathway that has gone out of control, can reduce side-effects of widely used therapies and also sensitise malignant cells to cytotoxic drugs in combinatorial therapies. Therapeutic efficacy of proteasome inhibitors for treatment of multiple myeloma or mantle cell lymphoma points the ubiquitin-proteasome system as an attractive target for development of new anticancer strategies [159].

In the case of DUBs with a positive regulatory role on NF- κ B, their excessive activity can lead to persistent inflammation or enhanced proliferation rate. Reducing their enzymatic activity or availability in cells could counteract this effect. The use of inhibitors, specifically targeting active sites of those enzymes, could block their activity. Additionally, agents intervening at the level of extracellular factors, signal transduction pathways or nuclear factors in the mechanisms controlling their synthesis and regulation, could reverse their effects [159].

In the case of DUBs acting as negative regulators of NF- κ B (tumour protective DUBs like CYLD, A20, potentially USP34), new therapeutic approaches could be introduced aiming to upregulate their activity, possibly lost during tumour progression. At the transcriptional level, agents promoting DNA demethylation could counteract promoter hypermethylation that silences tumor-suppressor genes in cancers, as in the case of A20 in several lymphomas. In other pathological cases, expression of anti-tumour/anti-inflammatory genes is maintained at the same levels as under healthy conditions due to the presence of repressors. Inhibiting repressor expression could induce a more strict control in the system. For example, reduced CYLD expression in basal cell carcinomas was reversed by downregulating its repressor (Snail). Similarly, elimination of factors as kinases, phosphatases or binding partners that inhibit tumour protective action of a DUB, could also alter cancerogenic phenotype. Another approach would be synthetic lethality induction. This is a condition between two genes in which loss of one gene does not affect cell viability, whereas loss of both results in cell death. So targeting a DUB gene that is synthetic lethal to a cancer-relevant mutation on malignant cells should specifically eliminate cancer cells without affecting the healthy ones [159].

As for MTDH and ER, we could benefit from the fact that it plays a role of a control centre for NF- κ B activating pathways in order to achieve optimal signaling. Targeting MTDH or ER, without affecting its physiology, could dampen NF- κ B signaling in cancers where it is overactivated. Additional studies are needed because the same role of central regulation could lead to undesired side effects.

For the development of any kind of treatment, well understanding of signaling pathways regulation, crosstalk and correlation at the molecular level, is a prerequisite step. This can permit accurate prediction of the effect of an approach in the system, avoiding as much as possible side effects. Deubiquitylating enzymes can offer this possibility, but many challenges remain. Further understanding of their way of action and answering to the upcoming questions can serve this purpose.

Participation of the Cell Polarity Protein PALS1 to T-Cell Receptor-Mediated NF- κ B Activation

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Abstract

Background: Beside their established function in shaping cell architecture, some cell polarity proteins were proposed to participate to lymphocyte migration, homing, scanning, as well as activation following antigen receptor stimulation. Although PALS1 is a central component of the cell polarity network, its expression and function in lymphocytes remains unknown. Here we investigated whether PALS1 is present in T cells and whether it contributes to T Cell-Receptor (TCR)-mediated activation.

Methodology/Principal Findings: By combining RT-PCR and immunoblot assays, we found that PALS1 is constitutively expressed in human T lymphocytes as well as in Jurkat T cells. siRNA-based knockdown of PALS1 hampered TCR-induced activation and optimal proliferation of lymphocyte. We further provide evidence that PALS1 depletion selectively hindered TCR-driven activation of the transcription factor NF- κ B.

Conclusions: The cell polarity protein PALS1 is expressed in T lymphocytes and participates to the optimal activation of NF- κ B following TCR stimulation.

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Introduction

Establishment and maintenance of cell polarity is chiefly orchestrated by a tightly regulated interplay between three multi-protein complexes: i) Scribble (SCRIB)/Discs Large (Dlgh1)/Lethal giant larvae (Lgl) complex, ii) partitioning-defective (PAR) 3 and PAR6/ atypical protein kinase C (aPKC) complex, and iii) Crumbs (CRB)/ Protein Associated with Lin Seven 1 (PALS1)/ PALS1-associated tight junction protein (PATJ) complex [1,2]. However, each complex is not exclusive, as PAR6 links PALS1 to PAR3/PAR6/aPKC [3]. In T lymphocytes, cell polarity proteins were shown to partition the leading edge from the uropod at the cell rear, and therefore participate to cell migration, homing, and scanning [4,5,6]. In addition, SCRIB and Dlgh1 are transiently recruited to the nascent immunological synapse formed with an antigen-presenting-cell (APC) [4]. Their depletion in lymphocytes has been associated with an alteration of antigen receptor-mediated activation [7,8,9,10].

The adaptor PALS1 is crucial for cellular architecture as it maintains the apico-basal polarity in epithelial cells and authorizes indirect interactions between CRB and PATJ [11,12]. Interestingly, Dlgh1 and PALS1 share a COOH-terminal part composed of a PSD-95/Dlg/ZO-1 (PDZ) domain followed by an SH3 domain adjacent to an inactive Guanylate kinase (GK) homology region [2]. This unique sequence of PDZ/SH3/GK defines the so-called

membrane-associated guanylate kinase (MAGUK) proteins family, a group of molecules that serve as scaffolds to organize multi-protein signalosomes through their protein-protein interaction domains [13]. For example, the MAGUK-containing CARMA1 emerges as a central regulator of lymphocytes activation and proliferation downstream of antigen receptor stimulation [14]. Indeed, CARMA1 operates as scaffold to recruit the heterodimer BCL10/MALT1 (CBM complex), a key step for conveying NF- κ B signaling [14,15,16]. In addition to its established role in polarity, Dlgh1 was shown to modulate lymphocyte proliferation upon T-cell receptor ligation, possibly through p38 recruitment or via the transcription factor NF-AT [9,10,17,18].

Although the MAGUK PALS1 plays a central role in the establishment of cell polarity, its contribution to lymphocyte activation remains elusive [8]. Here we show that PALS1 mRNA and protein is expressed in human lymphocytes. Furthermore, knocking down of PALS1 with small interfering RNAs (siRNAs) led to a decreased proliferation of human T lymphocytes, resulting from a reduced activation of the transcription factor NF- κ B.

Results and Discussion

PALS1 expression in T lymphocytes

Although several cell polarity proteins have been characterized in lymphocytes [4,5], PALS1 expression in T cells remains to be

determined [8]. To address this question, we first performed RT-PCR analysis on resting human CD3⁺ T cells and Jurkat lymphocytes extracts, and detected mRNA for PALS1 (Figure 1A). These mRNA were efficiently translated into protein, as antibodies against PALS1 detected a band, which was absent from PALS1-siRNA transfected primary T lymphocytes lysates (Figure 1B). Similar results were obtained with Jurkat T cells (Figure 1B). Of note, PALS1 levels remained unchanged in cells stimulated with antibodies to CD3 and CD28, or with PMA and ionomycin (Figure 1C). We next investigated PALS1 subcellular location by confocal microscopy. In contrast to epithelial cells where it accumulate to tight junctions [12], PALS1 did not reach membrane domains and remains essentially cytosolic with punctuate structures. Additional staining revealed

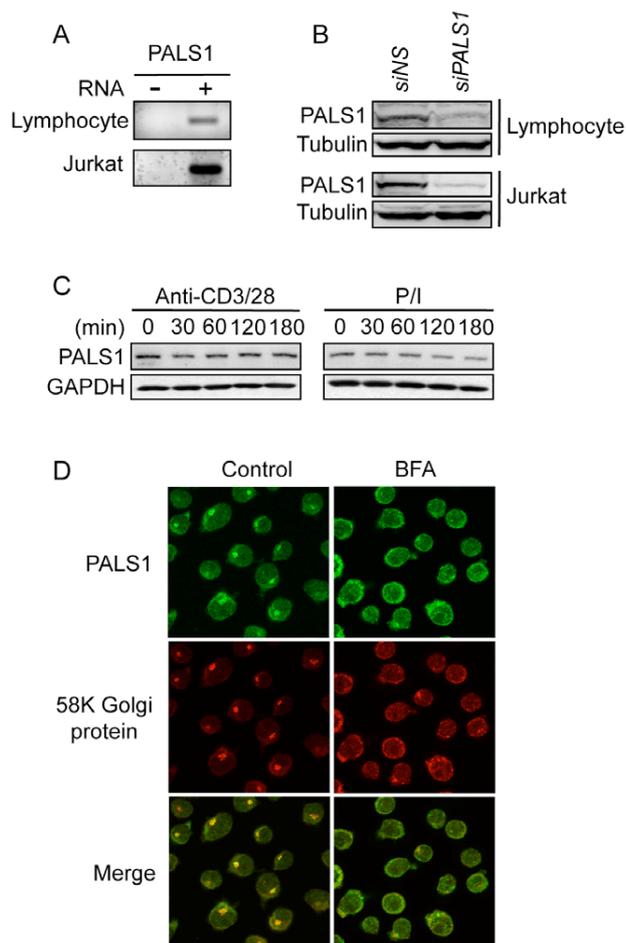


Figure 1. Expression of the cell polarity protein PALS1 in human T lymphocytes. **A**, Analysis of PALS1 mRNA in primary human T lymphocytes, and Jurkat cell line by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). **B**, Immunoblot analysis of PALS1 in cell lysates from human T lymphocytes and from Jurkat cells. The specificity of PALS1 antibodies was validated with lysates from cells transfected with siRNA against PALS1. Tubulin was used as a loading control. **C**, Immunoblot analysis of cell extracts from Jurkat cells stimulated as indicated with 1 $\mu\text{g}\cdot\text{ml}^{-1}$ anti-CD3 and anti-CD28, or with 40 $\text{ng}\cdot\text{ml}^{-1}$ PMA together with 300 $\text{ng}\cdot\text{ml}^{-1}$ ionomycin (P/I). GAPDH served as a loading control. **D**, Confocal microscopy pictures of PALS1 and 58K Golgi protein in Jurkat T lymphocytes either untreated or incubated with 10 $\mu\text{g}\cdot\text{ml}^{-1}$ Brefeldin A (BFA). doi:10.1371/journal.pone.0018159.g001

that these structures coalesced with the Golgi apparatus (Figure 1D and Figure S1). Accordingly, Brefeldin A-triggered disassembly of the Golgi apparatus also disrupted PALS1 punctuate structures (Figure 1D). This is reminiscent of PALS1 relocation to the Golgi apparatus in cells infected with SARS coronavirus [19]. Last, we observed that TCR-mediated stimulation only promoted a discrete redistribution of PALS1 within the cytosol of Jurkat cells (Figure S1). Altogether, our results suggest that similarly to Dlg1, SCRIB, CRB3, and PKC ζ [4,5], the cell polarity protein PALS1 is expressed in lymphocytes at both mRNA and protein level.

Requirement of PALS1 for optimal T cell activation and proliferation

Because SCRIB and Dlg1 were proposed to modulate lymphocyte proliferation [7,8,9,10], we evaluated whether PALS1 might also participate to T cell activation. To this end, peripheral blood lymphocytes (PBL) were purified on Ficoll-isopaque gradients. Primary human T cells were nucleofected for three days with siRNA targeting PALS1, prior stimulation with anti-CD3 and anti-CD28 antibodies. PALS1 knockdown led to a significant decrease in TCR-mediated induction of the activation markers CD69 and CD25 on cell surface (Figure 2A, B). This was accompanied by a reduction in Carboxyfluorescein Succinimyl Ester (CFSE) dilution, which reflects cell proliferation (Figure 2C). Collectively, these data suggest that PALS1 participates to the optimal lymphocyte activation and subsequent proliferation upon TCR stimulation.

Role of PALS1 in TCR-mediated signaling

To further explore how PALS1 impacts on lymphocyte proliferation, early signaling pathways emanating from the TCR were examined in Jurkat cells transfected with PALS1 siRNA. We did not detect major alteration in the general pattern of tyrosine phosphorylation, or mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinases (ERK) 1/2 phosphorylation upon TCR stimulation (Figure 3A). Only a slight but consistent increase in TCR-mediated phosphorylation of p38 was noted (Figure 3A). Moreover, CD3-induced calcium mobilization was largely normal in PALS1-knockdown Jurkat cells (Figure 3B).

We next analyzed TCR-mediated activation of NF-AT and NF- κ B transcription factors. siRNA-treated Jurkat T cells were co-transfected with firefly luciferase constructs driven by NF-AT or NF- κ B binding sequences and with a renilla luciferase control. PALS1 knockdown had only a marginal effect on NF-AT activity following stimulation with PMA and ionomycin, or with antibodies to CD3 and CD28 (Figure 3C and Figure S2). In sharp contrast, NF- κ B activity was significantly reduced without PALS1 (Figure 3D). Interestingly, tumor necrosis factor- α (TNF α)-induced NF- κ B activation remained essentially unaffected, underscoring the selective involvement of PALS1 in the TCR-NF- κ B pathway (Figure S3). Altogether, our data unveiled an unexpected role for PALS1 in TCR-mediated NF- κ B activation.

PALS1 participates to the optimal activation of NF- κ B upon TCR stimulation

To gain insights on how PALS1 modulate NF- κ B, we first investigated the transcription factor binding ability by electrophoretic mobility shift assay (EMSA). Less NF- κ B bound to its specific probe in nuclei extracts from PALS1-siRNA transfected cells following TCR stimulation (Figure 4A). As expected, Oct-1 binding remained unchanged without PALS1. Consistent with a diminished NF- κ B activity, both the phosphorylation and

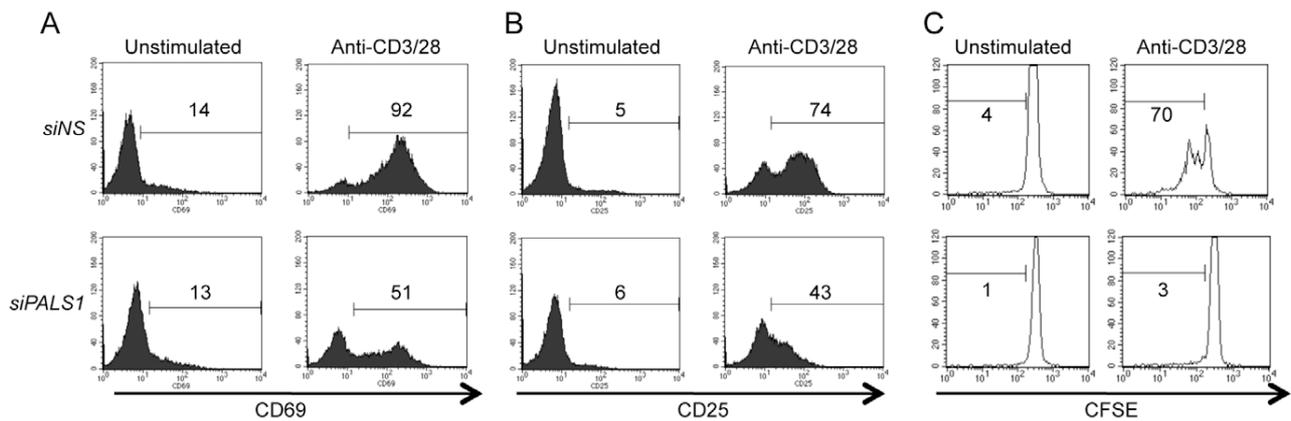


Figure 2. PALS1 requirement for optimal activation and proliferation in lymphocytes. **A and B,** Human peripheral blood T lymphocytes were transfected with siRNA for *PALS1* or nonspecific (*NS*) siRNA. Three days later, cells were stimulated with $1 \mu\text{g}\cdot\text{ml}^{-1}$ anti-CD3 and anti-CD28. CD69 and CD25 induction at the cell surface were examined 6 hours and 16 hours post-stimulation, respectively. **C,** Cells as in (A) were loaded with Carboxyfluorescein Succinimidyl Ester (CFSE), and stimulated with $1 \mu\text{g}\cdot\text{ml}^{-1}$ anti-CD3 and anti-CD28 for 72 hours. The percentage of CD69- and CD25-positive cells, and of dividing cells, is shown. These data are representative of four independent experiments. doi:10.1371/journal.pone.0018159.g002

subsequent proteasomal degradation of NF- κ B inhibitor, I κ B α , were severely decreased in the absence of PALS1 (Figure 4B). Because TCR-induced NF- κ B activation relies on the assembly of the CBM complex [15], BCL10 was immunoprecipitated from nonspecific (*NS*-) and *PALS1*-siRNA transfected Jurkat cells. MALT1, which forms a heterodimer with BCL10, coprecipitated with BCL10 regardless of stimulation. Although PALS1 was not found bound to BCL10, its absence diminished CARMA1 recruitment (Figure 4C, and data not shown). Hence, our data suggest that PALS1 participates to the optimal translocation and activation of NF- κ B upon TCR stimulation, possibly by favoring the CBM assembly.

Role of PALS1-associated proteins during TCR-mediated NF- κ B

Since PALS1 nucleates a ternary complex containing CRB3 and PATJ, and further binds PAR6 to maintain cell polarity [3,20,21], their contribution to TCR-mediated NF- κ B was evaluated. Similarly to PALS1, mRNA for PATJ, CRB3, PAR6, were efficiently detected by RT-PCR (Figure 5A). The same hold true for the unrelated cell polarity protein SCRIB (Figure 5A). siRNA-based knockdown of PALS1 and CRB3 significantly decreased NF- κ B activation in cells treated by antibodies against CD3 and CD28, or with a mixture of PMA and ionomycin. Although less dramatic, similar results were observed with PAR6 or PATJ knockdown. By contrast, NF- κ B was normally activated in the absence of SCRIB (Figure 5B). In agreement, I κ B α phosphorylation was diminished in lysates from CRB3-depleted cells, and to a lesser extent from PATJ- or PAR6-siRNA transfected cells, and not from SCRIB-depleted cells. Again, ERK phosphorylation occurred normally (Figure 5C, D, E, and F). Altogether, our data suggest that PALS1 implication in the TCR-NF- κ B pathway is inextricably linked to its cell polarity partners.

In summary, our data show that the cell polarity protein PALS1 is expressed in lymphocytes and contributes to their optimal activation. Although Dlg1 and SCRIB were proposed to modulate NF-AT or p38 [9,10,17,18] and NF-AT [7] respectively, a distinct scenario likely occurs for PALS1. Our results support a model in which PALS1 participates to NF- κ B activation, upstream of I κ B α phosphorylation and degradation. However, how precisely PALS1 modulates NF- κ B remains unclear. Because MAGUK function as scaffold units to organize and integrate multi-molecular

signaling complexes [13], it is tempting to speculate that PALS1 nucleates its own signalosome. For example, CARMA1 anchors a >900 kDa complex including the heterodimer BCL10/MALT1 [22], and Dlg1 was reported to bind to Lck, Zap70, Wasp [17], and p38 [10]. In our hands, PALS1 did not integrate the CBM, but its absence reduced CARMA1 binding to BCL10. It will therefore be interesting to identify PALS1 partners in the context of lymphocyte activation. In line with this, CRB3, PATJ and PAR6, which all bound PALS1 to maintain cell polarity [2], also participate to NF- κ B signaling upon TCR ligation in lymphocytes, and might therefore complex with PALS1 in lymphocytes. Altogether, our results strengthen the unexpected function of cell polarity proteins in lymphocyte proliferation [7,8,9,10], and unveil an original role for PALS1 during TCR-mediated NF- κ B activation.

Materials and Methods

Cell culture and reagents

Jurkat T cells E6.1 were purchased from ATCC. CD3⁺ human T lymphocytes from healthy donors (Etablissement Francais du Sang) were isolated with the MidiMacs system (Miltenyi Biotec). Cells were activated with a mixture of soluble anti-CD3 ϵ (HIT3 α , BD Biosciences) and anti-CD28 (BD Biosciences), or with 20–40 ng \cdot ml⁻¹ phorbol 12-myristate 13-acetate (PMA, Sigma) and 300 ng \cdot ml⁻¹ ionomycin (Calbiochem). Carboxyfluorescein Succinimidyl Ester (CFSE) and Brefeldin A were purchased from Sigma, and the calcium-sensitive dye Fluo-4 was from Invitrogen.

Cell lysates preparation, immunoprecipitations, and immunoblots

Cells were washed twice with PBS 1X and lysed with 50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% Igepal, 2 mM EDTA, supplemented with complete protease inhibitors (Roche). Lysates were cleared by a centrifugation at 10,000g at 4°C, and protein concentration determined (micro BCA kit, Pierce). Samples were resolved on 5–20% SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham). For Immunoprecipitations, samples were precleared with protein G-sepharose beads (Roche) for 30 min prior to overnight incubation with antibodies and additional protein G-sepharose beads at 4°C, as previously

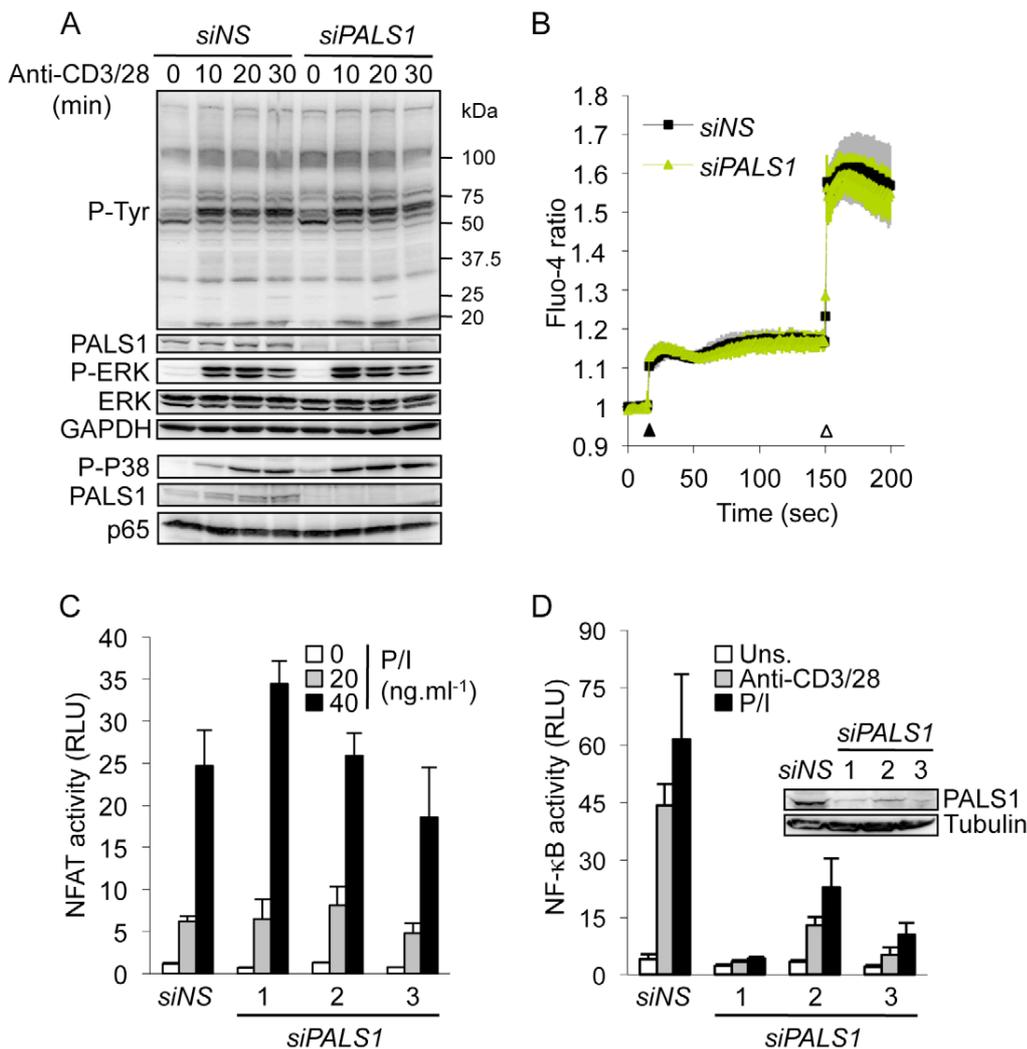


Figure 3. Role of PALS1 on early TCR-mediated signaling. **A**, Jurkat cells were transfected with nonspecific (*NS*) and *PALS1*-siRNA, and left three days prior stimulation with $1 \mu\text{g}\cdot\text{ml}^{-1}$ anti-CD3 and anti-CD28 for 0, 10, 20, and 30 min. Cell lysates were prepared and immunoblots were performed as indicated. GAPDH, and p65 served as loading controls. Five other experiments provided same results. **B**, *NS*- and *PALS1*-siRNA transfected Jurkat cells were loaded with the calcium-sensitive dye Fluo-4 and stimulated with $1 \mu\text{g}\cdot\text{ml}^{-1}$ anti-CD3 (closed symbol), or with $1 \mu\text{g}\cdot\text{ml}^{-1}$ ionomycin (open symbol). Shown is the mean \pm s.d. of triplicate measurements (one out of two independent experiments). **C**, **D**, Jurkat lymphocytes were transfected with *NS*- or with three individual siRNA sequences targeting *PALS1*. After three days, cells were co-transfected with siRNA and with NF-AT or NF- κ B firefly luciferase reporter gene together with a control Renilla plasmid for an additional 24 hours. Cells were then stimulated with 20 or 40 $\text{ng}\cdot\text{ml}^{-1}$ PMA and 300 $\text{ng}\cdot\text{ml}^{-1}$ ionomycin (P/I), or 0.5 $\mu\text{g}\cdot\text{ml}^{-1}$ anti-CD3 and anti-CD28. Histograms represent the mean \pm s.d. of triplicate experiments. RLU, relative light units. The inset immunoblot shows the level of *PALS1* knockdown. Data shown are representative of at least five independent experiments.
doi:10.1371/journal.pone.0018159.g003

described [23]. Antibodies to BCL10 (A-6), I κ B α (C-21), MALT1 (B-12), Tubulin (TU-02), *PALS1* (H-250), SCRIB (C-6), PAR6 (G-9) and p65 (C-20) were purchased from Santa Cruz. Phospho-specific antibodies against I κ B α , ERK, p38, and antibodies to CARMA1 and to ERK were from Cell Signaling Technologies. Anti-phosphorylated Tyrosine (4G10, Millipore), anti-GAPDH (Sigma), and Immobilon (Millipore) chemiluminescent substrates were also used.

Luciferase assays

Firefly luciferase constructs downstream of promoters for NF- κ B or NF-AT were co-transfected with renilla luciferase pRL-TK (Int) plasmid (Promega). Luciferase activities were analyzed using the Dual-Luciferase Kit (Promega), with firefly fluorescence units

normalized to renilla luciferase fluorescence units (BMG microplate reader).

siRNA and transfections

All siRNA used were from Invitrogen (Stealth). *PALS1*.1, 5'-CCAAGGAAACAGUAAUCCAUGUAAA-3'; *PALS1*.2, 5'-GAGGAGAUUCUUAACCUAUGAGGAAA-3'; *PALS1*.3, 5'-CAGAACAAUGGCCACUACUUUGAU-3'; CRB3, 5'-CCAUCACUGCUAUAUCGUGGUCUU-3'; PATJ, 5'-GCAUGAAU-UUCUGACUCCUAGAUG-3'; SCRIB, 5'-UGGGAGGCAA-CGAUCUGGAAGUGCU-3'; PAR6 5'-GAGCGGGUCCAG-GAAUCUUAUCU-3'. Jurkat cells were transfected by electroporation with a BTX ECM 830 apparatus (BTX, Harvard Apparatus), as previously described [16]. For primary cells, PBL

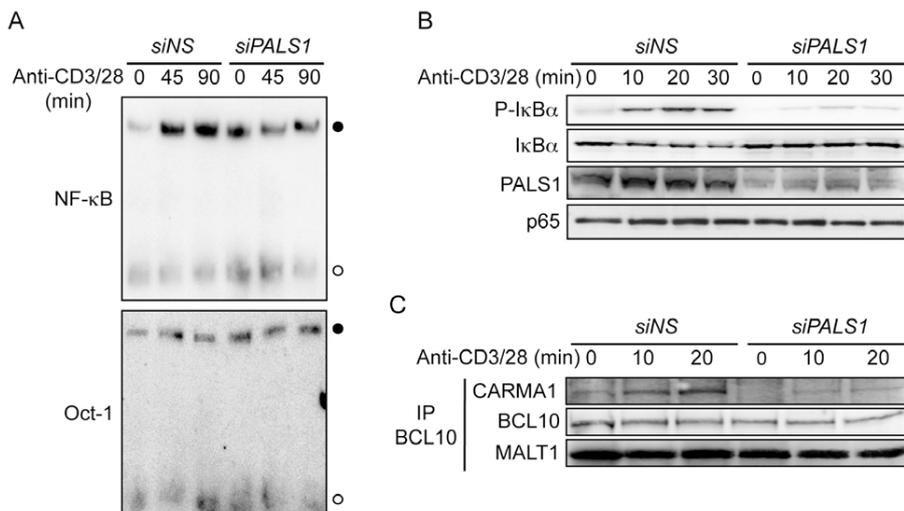


Figure 4. Involvement of PALS1 in TCR-mediated activation of NF- κ B. **A**, Jurkat cells were transfected with nonspecific (NS)- and PALS1-siRNA. Three days later, cells were stimulated with $1 \mu\text{g}\cdot\text{ml}^{-1}$ anti-CD3 and anti-CD28 for 0, 45, and 90 min. Nuclear extracts were prepared to analyze the binding of NF- κ B and Oct-1 by electrophoretic mobility shift assays (EMSA) with specific probes (closed circles). Free probe is also indicated (open circles). **B**, Cells as in (A) were stimulated with $1 \mu\text{g}\cdot\text{ml}^{-1}$ anti-CD3 and anti-CD28 for 0, 10, 20, and 30 min. Cell extracts were prepared and immunoblots were performed as indicated. **C**, Cells as in (A) were stimulated with $1 \mu\text{g}\cdot\text{ml}^{-1}$ anti-CD3 and anti-CD28 for 0, 10, 20 min. BCL10 was immunoprecipitated (IP) from cell lysates, and the binding of CARMA1, and MALT1 was assessed by immunoblot. Data shown are representative of at least three independent experiments.
doi:10.1371/journal.pone.0018159.g004

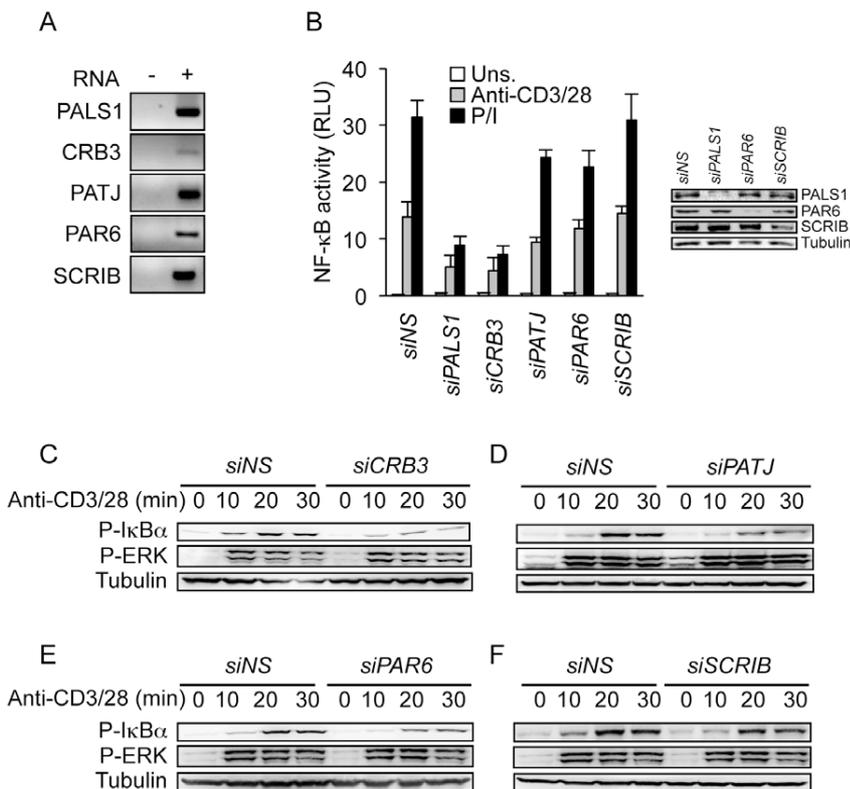


Figure 5. Role of PALS1 cell polarity partners in NF- κ B signaling. **A**, Expression of cell polarity proteins PALS1, CRB3, PATJ, PAR6, and SCRIB by RT-PCR in Jurkat T lymphocytes. **B**, Jurkat were transfected with nonspecific (NS)-, CRB3-, PAR6-, PATJ- and SCRIB-siRNA. After three days, cells were then co-transfected with siRNA and with NF- κ B firefly luciferase reporter gene together with a control Renilla plasmid. 24 hours later, cells were stimulated with $0.5 \mu\text{g}\cdot\text{ml}^{-1}$ anti-CD3/CD28 or with $20 \text{ ng}\cdot\text{ml}^{-1}$ PMA and $300 \text{ ng}\cdot\text{ml}^{-1}$ ionomycin (P/I). Shown is the mean \pm s.d. of triplicate experiments. RLU, relative light units. **C–F**, Immunoblots as indicated of NS-, CRB3-, PAR6-, PATJ- and SCRIB-siRNA transfected Jurkat cells stimulated with $1 \mu\text{g}\cdot\text{ml}^{-1}$ anti-CD3 and anti-CD28 for 0, 10, 20, and 30 min. Data shown are representative of three independent experiments.
doi:10.1371/journal.pone.0018159.g005

were purified from blood on Ficoll-isopaque gradients. PBL were nucleofected with the Nucleofactor system and T cell solution (Amaxa, program U14), and left for three days in culture medium prior treatment.

Nuclear protein extraction and electrophoretic mobility shift assay (EMSA)

4 μ g of nuclear extracts from Jurkat cells were examined for NF- κ B- and Oct1-binding activity by electromobility shift assay (Panomics kit). Samples were resolved on a 6% native polyacrylamide DNA retardation gel in 0.5X TBE buffer and analyzed using a FUJI LA4000 system.

Reverse transcriptase PCR

2 μ g of total RNA from purified human blood T cells or Jurkat were used for the RT-PCR reactions (RNeasy and OneStep kits, Qiagen). Primers were designed as follows: PALS1: Forward (F), 5'-CTCCTTCATGCAACAGACCA-3' and Backward (B), 5'-CACTTTTACTGGCCACGAT-3'; CRB3: F, 5'-CACCTGCTCCTCGCTACTG-3' and B, 5'-CACTGTTTTGCCTT-CATCCA-3'; PATJ: F, 5'-CAACGAGCATCCTGACTGAA-3' and B, 5'-GGCGTGGTTGTGAGGACTAT-3'; PAR6: F, 5'-GTTGCCAACAGCCATAACCT-3' and B, 5'-CAGGTCACTGCTGTCATCGT-3'; SCRIB: F, 5'-CGCAAGGACA-CACCTCACTA-3' and B, 5'-CCTCCTCCTGAGGAC-TACCC-3'.

Confocal microscopy

Cells were left for 10 min on poly-lysine coated slides (Thermo Scientific) prior fixation with PBS1X containing 4% paraformaldehyde. For TCR crosslinking experiments, cells were incubated with 5 μ g.ml⁻¹ anti-CD3 at 4°C for 15 min. After two washes, cells were incubated with 5 μ g.ml⁻¹ of goat anti-mouse (Jackson) for 20 min either at 4°C or 37°C. To disassemble Golgi apparatus, cells were treated with 10 μ g.ml⁻¹ Brefeldin A for 60 min. Samples were permeabilized with 0.05% Triton-X100 in PBS1X for 5 min, and non-specific sites blocked with 10% FCS in PBS1X. Antibodies used were: PALS1 (Millipore), 58K Golgi (Abcam), Alexa-488 conjugated goat anti-rabbit IgG or Alexa-594 conjugated goat anti-mouse IgG (Invitrogen). Samples were analyzed using a Leica confocal microscope SP6.

Cell surface staining

Cells were incubated for 30 min at 4°C with FITC- and PE-conjugated antibodies against CD25 and CD69 (ImmunoTools)

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and the respective isotype controls in PBS containing 0.5% BSA. After one wash with ice-cold PBS-BSA, cells were analyzed by flow cytometry with a FACSCalibur (BD Biosciences).

Supporting Information

Figure S1 Impact of stimulation on PALS1 subcellular location. **A**, Jurkat were stimulated 30 min with 40 ng.ml⁻¹ PMA and 300 ng.ml⁻¹ ionomycin. Shown are confocal microscopy pictures of PALS1 and 58K golgi protein. **B**, CD3 was crosslinked at the plasma membrane of Jurkat cells either at 4 or 37°C for 20 min. Micrographs show double staining for CD3 and PALS1. (EPS)

Figure S2 PALS1 is dispensable for TCR-mediated NF-AT activation. Jurkat lymphocytes were transfected with NS- or with PALS1-siRNA. After three days, cells were co-transfected with siRNA and with NF-AT firefly luciferase reporter gene together with a control Renilla plasmid for an additional 24 hours. Cells were then stimulated with 20 ng.ml⁻¹ PMA and 300 ng.ml⁻¹ ionomycin (P/I), or with 1 μ g.ml⁻¹ anti-CD3 and anti-CD28. Histograms represent the mean \pm s.d. of triplicate experiments. RLU, relative light units. (EPS)

Figure S3 Role of PALS1 cell polarity partners on TNF α -induced NF- κ B activation. **A**, Jurkat were transfected with nonspecific (NS)-, PALS1-, CRB3-, PAR6-, and PATJ-siRNA for three days. Cells were then co-transfected with siRNA and with NF- κ B firefly luciferase reporter gene together with a control Renilla plasmid. 24 hours later, cells were stimulated with 10 ng.ml⁻¹ TNF α for 6 hours. Shown is the mean \pm s.d. of triplicate experiments. RLU, relative light units. (EPS)

Acknowledgments

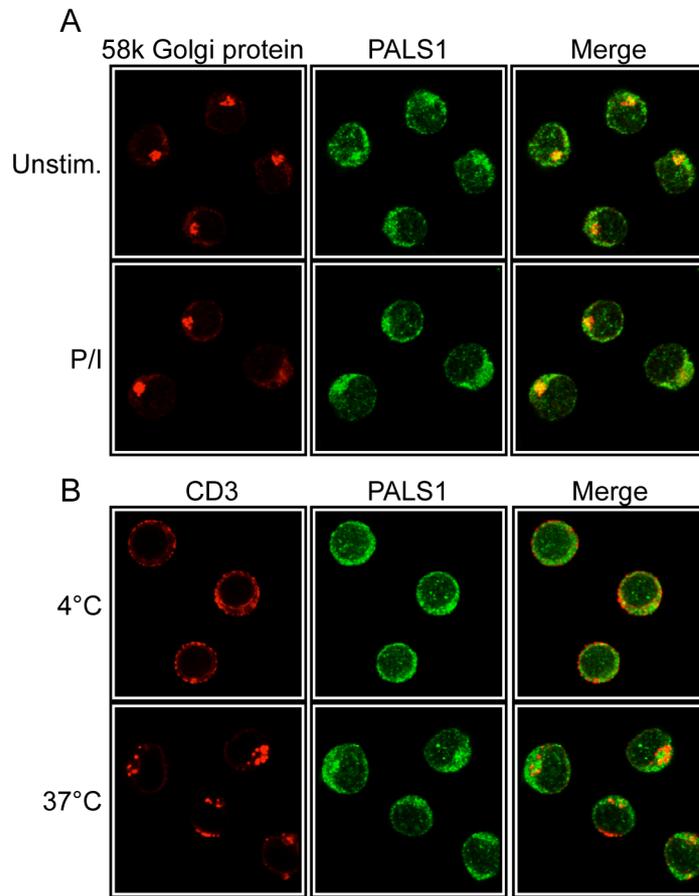
We thank D. Arnoult and J. Gavard for helpful discussions and critical reading of the manuscript.

Author Contributions

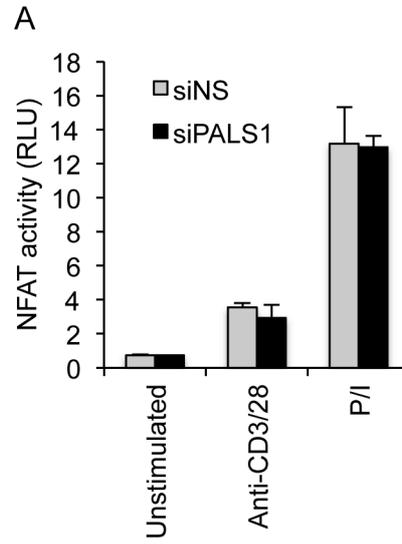
Conceived and designed the experiments: GC NB. Performed the experiments: GC CD KP EH NB. Analyzed the data: GC CD KP EH AV NB. Wrote the paper: GC NB.

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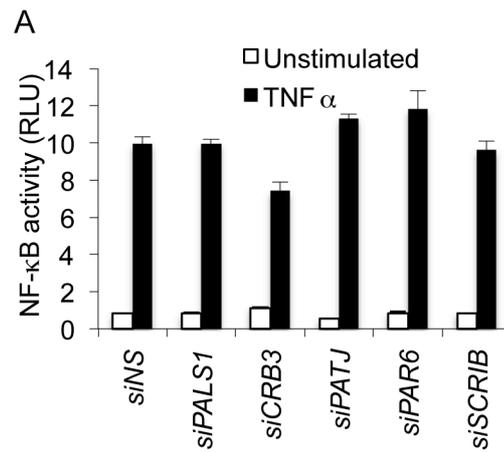
Supplementary 1



Supplementary 2



Supplementary 3



Caspase-3 triggers a TPCK-sensitive protease pathway leading to degradation of the BH3-only protein puma

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