Biochemical and functional analysis of the ubiquitin binding properties of the NF-κB regulator NEMO

Dissertation
Zur Erlangung des Doktorgrades
der Naturwissenschaften

vorgelegt beim Fachbereich 14
Biochemie, Chemie und Pharmazie
der Johann Wolfgang Goethe-Universität
in Frankfurt am Main

von
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aus Blaubeuren

Frankfurt am Main 2012

(D30)

Vom Fachbereich Biochemie, Chemie und Pharmazie
der Johann Wolfgang Goethe-Universität als Dissertation angenommen

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Datum der Disputation

Zusammenfassung

Einleitung

Posttranslationale Modifikationen, z. B. Phosphorylierung, regulieren wesentliche Eigenschaften von Proteinen, wie die Lokalisation, Konformation, Aktivität, Stabilität und Interaktionsfähigkeit. Eine besondere Form der Proteinmodifikation stellt die Ubiquitylierung dar. Dabei wird Ubiquitin, ein 76 Aminosäure großes Protein, mit seinem C-Terminus kovalent an ein Lysin oder den N-Terminus eines Substratproteins gebunden. Durch Ubiquitylierung von Proteinen werden verschiedene zelluläre Prozesse, wie Signalübertragung, Reparatur von DNA-Schäden, Zellteilung, Vesikeltransport und auch Proteinabbau reguliert.

Die Ubiquitylierung wird durch eine geordnete Abfolge von drei verschiedenen Enzymen katalysiert. Zunächst aktiviert ein E1 Enzym, mittels einer C-terminalen Adenylierung, freies Monoubiquitin, das dann über eine Thioesterbindung an ein Cystein des E1 Enzyms gebunden wird. Anschließend wird das Ubiquitin auf eines von ca. 35 Ubiquitin konjugierenden E2 Enzymen übertragen und schließlich mit Hilfe einer, von ca. 600, E3-ligasen an ein Substratprotein gebunden.

Ubiquitin hat die besondere Eigenschaft verschiedene Ketten zu bilden, indem ein Ubiquitinmolekül an eines der sieben Lysine (K6, K11, K27, K29, K33, K48, K63) oder den N-Terminus eines anderen Ubiquitinmoleküls gebunden wird. Somit kann Ubiquitylierung in verschiedenen Formen vorkommen, wobei diese unterschiedliche Funktionen besitzen. Die bekannteste Auswirkung der Ubiquitylierung ist der regulierte Abbau des modifizierten Proteins durch das Proteasom. Dies wird hauptsächlich durch K48-verknüpfte Ubiquitinketten verursacht. Es wurden jedoch bereits alle anderen möglichen Formen von Ubiquitinketten in Zellen entdeckt, wobei einigen dieser Kettentypen spezifische Funktionen zugewiesen werden konnten. K63-verknüpfte Ubiquitinketten spielen z. B. eine wichtige Rolle bei der Reparatur beschädigter DNA oder auch bei der Übertragung von zellulären Signalen, die zur Aktivierung des Transkriptionsfaktors NF-κB führen.

Die zellulären Auswirkungen der Ubiquitylierung werden in den meisten Fällen durch Ubiquitinrezeptoren vermittelt, die eine oder mehrere Ubiquitinbindedomäne (UBD) besitzen. Bisher wurden mehr als 20 verschiedene UBD-Typen identifiziert. Die meisten UBDs binden Ubiquitin an einer hydrophoben Stelle im Bereich der Aminosäure I44. Einige UBDs können nur bestimmte Ubiquitinketten binden, was zu einer besseren Regulierbarkeit der Auswirkungen der Ubiquitylierung führt. Solche spezialisierten UBDs interagieren meistens an zwei Stellen mit den Ubiquitinketten, die sie selektiv binden können. In diesen Fällen sind die Interaktionsstellen nur in den jeweiligen Kettentypen, die mit der selektiven UBD interagieren können so zueinander orientiert, dass es zu einer Bindung mit der selektiven UBD kommen kann.

Das Protein NEMO ist ein Ubiquitinrezeptor, dessen UBD, die UBAN (UBD in ABIN proteins and NEMO), selektiv bestimmte Ubiquitinketten bindet. NEMO spielt eine zentrale Rolle bei der Aktivierung der Transkriptionsfaktorfamilie NF-κB, indem es den IKK-Kinasekomplex reguliert. Dieser Kinasekomplex sorgt durch die Phosphorylierung des NF-κB-Inhibitors IκBα für dessen proteasomalen Abbau, wodurch NF-κB aktiviert wird. NF-κB kann durch viele extra- und intrazelluläre Signale aktiviert werden, wozu auch die Aktivierung des TNF-Rezeptors (TNFR) durch das Zytokin TNFα zählt. Am aktivierten TNFR werden viele Proteine durch verschiedene Ubiquitinketten modifiziert. NEMO bindet Ubiquitin über seine UBAN und diese Interaktion ist absolut notwendig, um NF-κB zu aktivieren. Einige Studien kamen zu dem Schluss, dass die gezielte Bindung von NEMO an K63-verknüpfte Ubiquitinketten ausschlaggebend für die Aktivierung von IKK ist. Jedoch spielen lineare Ubiquitinketten, die über den N-Terminus verknüpft sind, auch eine wichtige Rolle bei der Aktivierung von NF-κB und außerdem hat die UBAN von NEMO hat eine sehr hohe Affinität zu linearen Ubiquitinketten.

Aus vorherigen Studien wurde nicht ganz ersichtlich, welche Ubiquitinkettentypen NEMO bevorzugt bindet und welche dieser Interaktionen relevant für die Aktivierung von NF-κB ist.

Ein Ziel dieser Arbeit war daher eine detaillierte Analyse der Ubiquitinbindungspreferenz von NEMO. Da die meisten Studien zur Interaktion von NEMO mit Ubiquitin entweder mit einzelnen NEMO-Domänen oder mit Zelllysat durchgeführt wurden, konnte bisher keine klare Aussage über die Ubiquitinbindungspreferenz von NEMO gemacht werden. In dieser Arbeit wurde bakteriell aufgereinigtes NEMO verwendet, um die Interaktionen mit verschiedenen Ubiquitinketten unterschiedlicher Länge im Detail zu untersuchen. Durch die Rekonstitution von NEMO knock-out Zellen mit NEMO-Mutanten, die kein Ubiquitin mehr binden können, wurde dann analysiert, welche Rolle diese Interaktionen bei der Aktivierung von NF-κB durch den TNF-Rezeptor spielen.

Ergebnisse

Um *In vitro* Bindungsstudien zwischen NEMO und verschiedenen Ubiquitinketten durchführen zu können, wurden K48- und K63-verknüpfte Ubiquitinketten zunächst enzymatisch hergestellt. Dazu wurde Monoubiquitin mit einem E1-aktivierendem und den Kettentypen spezifischen E2-Enzymen inkubiert. Die so synthetisierten Ketten wurden durch Kationenaustausch ihrer Größe nach getrennt, wodurch aufgereinigte Ubiquitinketten mit definierter Länge und Verknüpfung erhalten wurden.

Um die Ubiquitinbindungspreferenz von NEMO zu untersuchen, wurden dann zunächst linear-, K48- und K63-verknüpfte tetra-Ubiquitinketten mit verschiedenen bakteriell aufgereinigten NEMO-Domänen, sowie mit NEMO in voller Länge, inkubiert und die Bindungen mittels pull-down und Western blot Analysen untersucht. Dabei wurde festgestellt, dass die UBAN von NEMO sehr stark mit linearem tetra-Ubiquitin interagiert, während die Bindung an K48- und K63-verknüpfte tetra-Ubiquitinketten sehr schwach ist. Die UBAN befindet sich im C-terminalen Bereich von NEMO und ganz am Ende des C-Terminus ist eine Zinkfingerdomäne von der eine Studie zeigte, dass sie schwach an Ubiquitin binden kann. Jedoch konnte diese Bindung bei pull-down Untersuchungen in der vorliegenden Arbeit nicht bestätigt werden. Der Zinkfinger und die UBAN zusammen indes banden auch K48- und K63-verknüpfte tetra-Ubiquitinketten,

jedoch um einiges schwächer als lineares tetra-Ubiquitin. Interessanterweise interagierte NEMO in voller Länge hauptsächlich mit linearem-, jedoch nur schwach mit K48- und K63-verknüpftem tetra-Ubiquitin.

Da am TNFR verschiedene Ubiquitinketten zur gleichen Zeit vorhanden sind, stellte sich die Frage, welchen Ubiquitinkettentyp NEMO bevorzugt bindet, wenn es auf unterschiedliche Kettentypen trifft. Um dies zu untersuchen, wurde bakteriell aufgereinigtes und an MBP gebundenes NEMO mit verschiedenen Ubiquitinketten unterschiedlicher Länge inkubiert. Dabei zeigte sich, dass NEMO, unabhängig von Kettenlänge oder Kettentyp, immer bevorzugt mit linear verknüpften Ubiquitinketten interagierte. Da in vorherigen Studien beobachtet wurde, dass NEMO hauptsächlich mit K63-verknüpften Ketten interagiert, und da lineare und K63-verknüpfte Ketten strukturell sehr ähnlich sind, wurde bei diesen kompetitiven Experimenten ein Hauptaugenmerk auf den Vergleich zwischen diesen beiden Kettentypen gelegt. In diesen Versuchen stellte sich heraus, dass NEMO auch dann bevorzugt mit linearen Ketten interagierte, wenn diese gemeinsam mit sehr langen K63-verknüpften Ubiquitinetten mit NEMO inkubiert wurden. Bei einer semiquantitativen Analyse zeigte sich, dass NEMO mindestens 50 mal stärker an lineares als an K63-verknüpftes Ubiquitin bindet, da bereits bei Zugabe von 20 ng linear verknüpften Ubiquitinketten eine ähnlich starke Bindung zu sehen war, wie bei 1 µg K63-verknüpften Ubiquitinketten.

Eine Kristallstruktur, die in einer Kooperation im Labor von Prof. Wakatsuki bestimmt wurde, zeigt, dass die NEMO-UBAN mit linearem di-Ubiquitin auf eine besondere Art interagiert, indem die UBAN die hydrophobe I44-Region eines der beiden Ubiquitinmoleküle, sowie die Verbindungsregion zwischen den beiden Ubiquitinmolekülen bindet. Im Gegensatz dazu interagiert K63-verknüpftes di-Ubiquitin nur mit der hydrophoben Region um I44 mit der UBAN, was anhand einer anderen Kristallstruktur gezeigt werden konnte. Ausgehend von der Kristallstruktur des Komplexes aus der UBAN und linearem di-Ubiquitin, wurden Aminosäuren von NEMO mutiert, die mit der Verbindungsregion von Ubiquitin interagieren, um dadurch die Relevanz dieser Region für die Bindung an lineares Ubiquitin zu überprüfen. Interaktionsstudien mit diesen Mutanten zeigten dann, dass die Bindung an lineares Ubiquitin durch diese Mutationen tatsächlich

verhindert wurden, jedoch konnten längere K63-Ketten – wahrscheinlich durch eine weitere Interaktionsstelle – immer noch schwach an NEMO binden.
Da somit NEMO-Mutanten identifiziert wurden, die zwar noch an längere K63-verknüpfte, aber nicht mehr an lineare Ubiquitinketten binden können, konnte der Einfluss der Bindung von NEMO an lineare Ubiquitinketten auf die Aktivierung von NF-κB *in vivo* getestet werden. Dazu wurden MEF (mouse embryonic fibroblast)-Zellen, die kein NEMO enthalten, mit Wildtyp NEMO oder den NEMO-Mutanten rekonstituiert und anschließend mit TNFα behandelt. Dabei zeigte sich, dass NEMO-Mutanten, die keine linearen Ubiquitinketten binden können, nicht in der Lage sind NF-κB vollständig zu aktivieren. Zusätzlich zum NF-κB-Signalweg, aktiviert der TNFR auch den Apoptosesignalweg, wobei dieser durch NEMO und NF-κB unterdrückt wird. Interessanterweise starben Zellen mit NEMO-Mutanten, die kein lineares Ubiquitin binden können jedoch an TNFα induzierter Apoptose, während Zellen die Wildtyp NEMO exprimierten überlebten. Dies zeigte, dass die Interaktion zwischen NEMO und linearem Ubiquitin nötig ist, um NF-κB und andere Signalwege am TNFR vollständig zu aktivieren. Um zu überprüfen, ob eine stärkere Bindung von NEMO an K63-verknüpfte Ubiquitinketten die Aktivierung von NF-κB ermöglicht, wurden NEMO-Chimären erzeugt, bei denen der Zinkfinger durch die NZF (Npl4 zinc finger)-Domäne von TAB2 ersetzt wurde, die spezifisch an K63-verknüpfte Ubiquitinketten bindet. *In vitro* Interaktionsstudien zeigten dann, dass die NEMO-NZF-Chimären eine ähnlich hohe Affinität zu K63-verknüpften Ubiquitinketten hatten, wie NEMO mit einer intakten UBAN zu linearen Ubiquitinketten. NEMO knock-out MEFs wurden anschließend mit NEMO-NZF-Chimären, bei denen die UBAN mutiert war, rekonstituiert und die Zellen wurden mit TNFα behandelt. Dabei zeigte sich, dass NEMO-NZF-Chimären nicht in der Lage waren NF-κB vollständig zu aktivieren. Jedoch konnte NF-κB teilweise aktiviert werden, was darauf hindeutet, dass die Bindung von NEMO und linearen Ubiquitinketten zwar nötig ist, um NF-κB vollständig zu aktivieren, aber die Bindung an K63-verknüpfte Ketten auch ein gewisses NF-κB Aktivierungspotential besitzt.

Diskussion

In dieser Arbeit konnte gezeigten werden, dass NEMO ein selektiver Ubiquitinrezeptor für lineare Ubiquitinketten ist. Anhand der Kristallstruktur des Komplexes aus der UBAN und linearem di-Ubiquitin wird ersichtlich, dass lineares di-Ubiquitin an zwei Stellen, über die Verknüpfungsregion und über die hydrophobe Region um I44, an die UBAN bindet. Diese Bindung ist bei K63-verknüpftem Ubiquitin nicht möglich, was eine Kristallstruktur des Komplexes aus der UBAN und K63-verknüpftem di-Ubiquitin zeigt. Diese spezielle Interaktion zwischen der UBAN und linear verknüpftem Ubiquitin stellt wohl die molekulare Grundlage für die hohe Bindungsselektivität von NEMO gegenüber diesem Ubiquitinkettentyp dar. Interessanterweise können längere, über Lysine verknüpfte, Ubiquitinketten ebenso die UBAN und NEMO binden, vermutlich über eine zusätzliche Bindungsstelle. Sobald aber gleichzeitig lineare Ubiquitinketten vorhanden sind, bindet NEMO ausschließlich diesen Kettentyp.
Diese Ubiquitinbindungspreferenz hat Auswirkungen auf die Funktion von NEMO. NEMO, das keine linearen Ubiquitinketten binden kann, ist nicht fähig TNFα-induziertes NF-κB vollständig zu aktivieren, sowie diese Zellen vor Apoptose zu schützen. Wie wichtig lineare Ubiquitinketten am TNFR sind zeigen auch Studien, bei denen LUBAC, die E3-Ligase, die diesen Kettentyp synthetisiert, entfernt wurde und dadurch NF-κB nicht mehr durch TNFα aktiviert werden konnte. Für die Aktivierung von NF-κB ist der Kinasekomplex IKK verantwortlich, wobei NEMO (auch IKKγ genannt) die regulatorische Unter-einheit darstellt. Möglicherweise wird durch die spezifische Bindung von NEMO an lineare Ubiquitinketten eine hohe Konzentration an IKK-Komplexen verursacht, die es diesen ermöglicht sich gegenseitig durch Phosphorylierung zu aktivieren. Möglich ist auch, dass die gezielte Bindung von NEMO an lineare Ubiquitinketten eine Konformationsänderung im IKK-Komplex verursacht, welche diesen aktiviert. Zusammenfassend konnte gezeigt werden, dass nur die spezifische Bindung von NEMO an lineare Ubiquitinketten und nicht die schwächere Bindung an K63-verknüpfte Ketten, eine vollständige und effiziente Aktivierung von NF-κB und anderer Prozessen am TNFR gewährleistet.

1. Introduction

1.1 The ubiquitin system

Posttranslational modification (PTM) is an important mechanism to regulate various properties of a protein[1]. These properties modulated by PTMs include a proteins localisation, conformation, stability, activity and interaction with other proteins. One important type of PTM is the covalent attachment of a functional group to an amino acid. Probably the most intensively studied PTM is phosphorylation, where a phosphate gets attached to a serine, threonine or tyrosine residue. Analogous to phosphorylation proteins also get modified by a group of small proteins from the ubiquitin family. Ubiquitin itself consists of 76 amino acids and protein ubiquitylation plays an important role in almost every aspect of cellular physiology[2]. Ubiquitin was discovered as a critical component of an energy dependent protein degradation process in the early 1980s by Aaron Ciechanover, Avram Hershko and Irwin Rose who were awarded the Nobel Prize in chemistry in 2004 for their findings related to the ubiquitin system[3]. Ubiquitin usually gets ligated to a lysine of a substrate protein through a cascade of enzymatic reactions[2, 4, 5]. In contrast to most of the other protein modifications, ubiquitin can form chains on a substrate by attaching a ubiquitin molecule to an amino acid of another ubiquitin molecule. This polyubiquitylation is mainly known for its role in the regulation of protein degradation[4]. However, in the last years it became evident that protein ubiquitylation can also function as a signal regulating a plethora of cellular events, ranging from protein quality control over DNA-damage and several signalling pathways to cellular proliferation[2, 6].

Ubiquitin has a very stable globular structure comprised of an α-helix surrounded by five β-strand folds. It is the founding member of a family of proteins that have a ubiquitin-like structure and most of them can also get covalently attached to other proteins (or in some cases phospholipids), which can alter the function, localisation, conformation and stability of their substrates[5]. So far, 17 members of the family of ubiquitin-like proteins (UBLs) have been discovered (SUMO-1/-2/-3, Nedd8, FAT10, URM1, USM1 ISG15, GABARAP and GABARAPL-1/-2/-3, LC3A/B/C and ATG12)[7]. The evolutionary

success of the ubiquitin fold is additionally reflected by the existence of ubiquitin-like domains (ULDs) in many proteins, where they mediate important functions such as protein interactions[8-10].

The relevance of ubiquitin is also manifested by its vast abundance in all eukaryotic cells. Its sequence is highly conserved throughout the eukaryotic kingdom, in which the human ubiquitin differs only in three amino acids from the sequence in *Saccharomyces cerevisiae*[11]. In order to ensure a proper ubiquitin supply of the cell the human genome encodes 14 copies of the ubiquitin sequence, distributed over four genes[12]. The genes UBB and UBC have 3 and 9 ubiquitin copies respectively, where the ubiquitin sequences are directly connected, leading to the translation of ubiquitin chains linked in a head-to-tail fashion. The two other ubiquitin genes, UBA52 and RPS27A, are linked to ribosomal subunit proteins and translated as fusion proteins. After translation, single ubiquitin molecules are cleaved by specific isopeptidases to form the cellular pool of free monoubiquitin[13, 14].

1.1.1 Modification of proteins by different forms of ubiquitylation

Proteins can get modified by ubiquitin in many different ways, which gives this modification a great variety of possible outcomes depending on the type of ubiquitylation[2, 15]. Some proteins are monoubiquitylated where only one ubiquitin is attached to a certain lysine residue. This form of ubiquitylation is for instance involved in the regulation of receptor sorting or trans-lesion DNA-polymerisation[16, 17]. A special form of monoubiquitylation is called multi-monoubiquitylation where more than one lysine in a protein is modified with a ubiquitin molecule.

The most abundant type of ubiquitylation however comes in form of polyubiquitin chains. Ubiquitin has seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) and each can be the target of another ubiquitin molecule[2, 15, 18] (Figure 1A). Additionally, in recent years it was discovered that also the N-terminal methionine of ubiquitin can be a target of ubiquitin conjugation, forming linear ubiquitin chains[19]. This sums up to eight different linkage types that can possibly be formed by a diubiquitin (diUb) unit in a

ubiquitin chain. Each linkage type leads to a different structure of diUb, which can result in different functions[20, 21] (Figure 1B). Additionally, each chain type has a unique chemical environment surrounding its linker region, which gives every linkage an individual recognition site for protein interactions. Due to these aforementioned differences, each chain type can be considered as a distinct PTM. Adding even more complexity to the ubiquitin system is the fact, that ubiquitin can form chains of two to a yet unknown number of moieties *in vivo* and the length of the chain might also influence the physiological outcome. For example does the degradation of ubiquitylated proteins depend on the modification of at least four ubiquitin moieties for an efficient removal by the proteasome[22, 23]. For many other functions controlled by ubiquitylation however, the role of the chain length is still not clear.

Most cellular functions mediated by ubiquitylation depend on the recognition by ubiquitin receptors, which are proteins with ubiquitin binding domains (UBDs)[24] (see chapter 1.1.3). These proteins "read" the ubiquitin signal and translate it into a physiological response. Many UBDs, which selectively bind to di- or polyubiquitin chains, are able to specifically recognize the conformation or the special linkage area that exists only in a certain type of chain. A very important surface of ubiquitin for the interaction with other proteins is the hydrophobic area surrounding I44[24] (Figure 1B). Depending on the linkage and thus on the conformation, the two I44 patches of a diUb-molecule can be positioned differently in relation to each other. The structures of several diUb-types have been solved and this shows that diUbs linked via K6, K11 and K48 have a rather compact structure enabling the two moieties to interact with each other, while K63 and linearly linked diUb are rather elongated without intramolecular interactions (Figure 1B). However, recently it has been reported that linear diUb can also have a more compact structure under certain circumstances[21]. This indicates, that the conformation of diUbs is somewhat flexible giving them the possibility to interact with different surfaces. Although some types of linkages share a more compact conformation, their I44 patches can still be positioned differently and thus can also mediate different interactions and physiological outcomes.

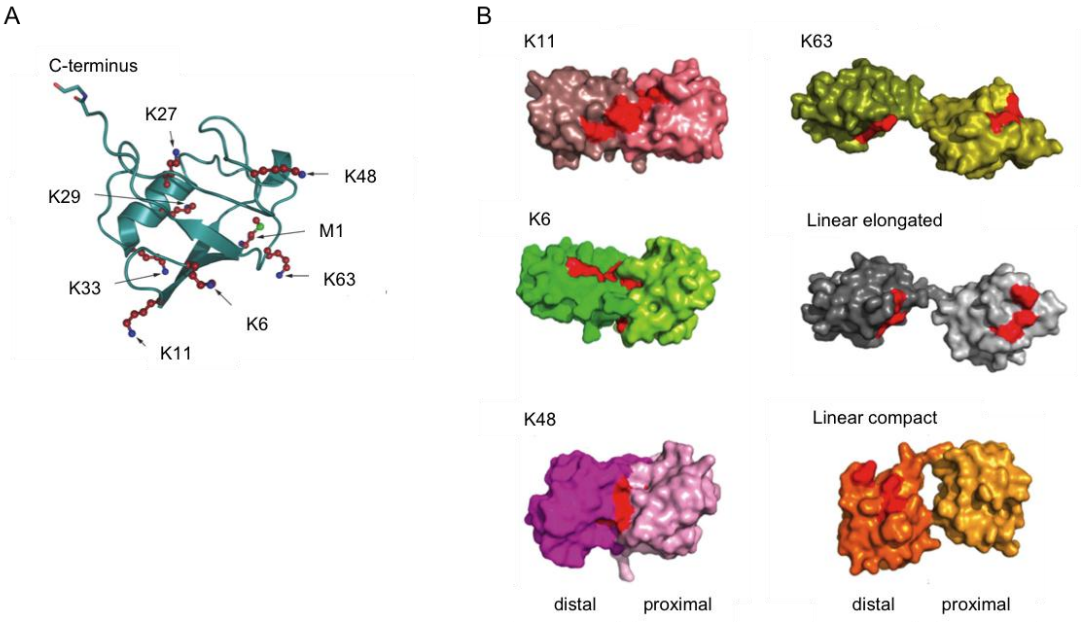


Figure 1: Structure of ubiquitin and different diUb-molecules. A) Ubiquitin has a compact globular structure with a characteristic β-grasp fold. The C-terminus of one ubiquitin can get attached to one of the lysine residues (red), or to the N-terminal methionine of another ubiquitin. K63 is very close to M1 enabling similar structure in K63- and M1- linked diUbs (compare K63 and linear elongated structures in B. B) diUb molecules with different linkages have distinct structures. K6-, K11- and K48-linked diUbs have a compact conformation where the hydrophobic I44 patches (red) facilitate intramolecular interaction, while K63-linked diUb is elongated. Linear diUb can have a compact structure but can also be elongated and the elongated linear diUb-conformation is similar to K63-linked diUb. The proximal ubiquitin is the one that would be attached to a lysine residue of a substrate and thus has a free C-terminus in the diUb-molecule. Fig. 1A is modified from[25]. Structure: PDB code 1UBQ,[26]. Fig. 2A is modified from[21]. Structures: K11 (PDB code 3nob,[27]), K6 (PDB code 2xk5,[28]), K48 (PDB 1aar,[29]), K63 (PDB code 2jf5,[20]), linear elongated (PDB code 2w9n,[20]), linear compact (PDB code 3axc,[21]).

1.1.2 The ubiquitin conjugation machinery

An elaborate system has evolved in order to covalently attach ubiquitin and other UBLs to a substrate. A cascade of enzymatic reactions leads from the ATP-dependent activation of ubiquitin to its ligation to, usually, the ε-amino group of a lysine of a target protein. These ubiquitin conjugation steps are catalysed by the activating enzymes E1, a conjugating enzyme E2 and a ligating enzyme E3[5] (Figure 2).

Ubiquitin and other UBLs are first activated by E1 enzymes[7]. At present eight E1 enzymes are known and most of them activate only one type of UBL (e. g. Uba2/SAE1-heterodimer activates SUMO-1/-2/-3 and Uba7 activates ISG15). Until recently it was thought that only Uba1 is able to activate ubiquitin, but now it has been discovered that also Uba6 is an activator of this most abundant member of the UBL family. Uba6 can additionally activate FAT10, a UBL that consists of two ULDs. The first step in the ubiquitylation process is the only one that needs ATP as a source of energy. In order to activate ubiquitin the E1 first has to adenylate it at its C-terminus[5] (Figure 2). The next step leads to a covalent thioester linkage between the catalytic cysteine of the E1 and the C-terminus of ubiquitin[7].

After the activation of ubiquitin by the E1 enzyme, it gets transferred to an E2 ubiquitin-conjugating enzyme[30, 31]. In this step ubiquitin gets covalently attached to the cysteine in the catalytic centre of the E2 to form a thioester. A characteristic feature of many UBLs is that they only have one or two E2s that catalyse their conjugation. Ubc9 e.g. is specific for SUMO-proteins while NEDD8 has its cognate E2s Ubc12 and NCE2[31]. In contrast, around 35 E2s are able to accept ubiquitin from an E1 enzyme. This much higher E2 enzyme diversity for ubiquitin reflects its various functions in all aspects of cellular physiology as well as its large number of substrates[32]. Such variety of E2 enzymes can give the ubiquitin system a layer of regulation in two ways. First, ubiquitin E2 enzymes combine only with a subset of E3 ligases to ubiquitylate a substrate[30, 31]. Since the substrate specificity for ubiquitylation is determined by the E3 enzymes (see below), E3 activation and thus substrate ubiquitylation can specifically be regulated on the level of E2s. The second regulatory process controlled by E2s is achieved by the ability of some E2s to define the linkage type of a chain, which can cause distinct physiological outcomes[2].

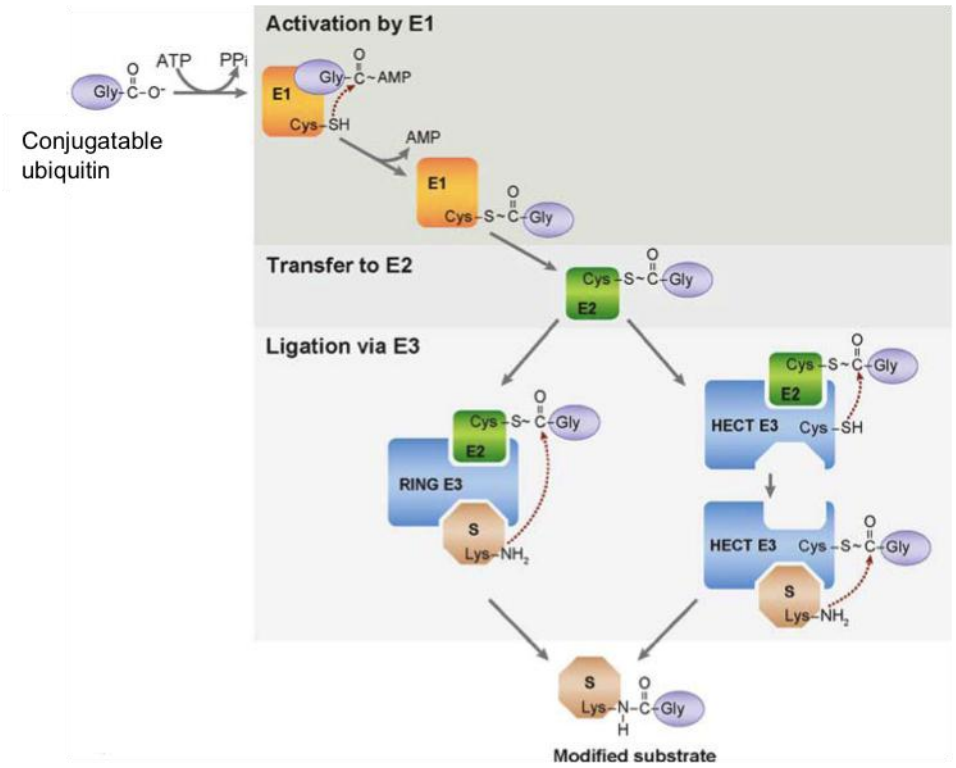


Figure 2: The ubiquitin conjugation pathway. In the first step, catalysed by the E1 enzyme, ubiquitin gets adenylated, which is the reaction that needs ATP. This leads to formation of a thioester bond of the ubiquitin C-terminus with the E1. Next ubiquitin is transferred to an E2 enzyme also by forming a thioester bond. RING E3 ligases transfer ubiquitin directly from the E2 to a substrate, while the E3 facilitates the interaction. HECT E3-ligases on the other hand first bind the ubiquitin covalently and then transfer it to a substrate. (Modified from (Kerscher et al, 2006))

The final step in the ubiquitylation process is the ligation of ubiquitin to its substrate. In this step ubiquitin gets covalently attached, usually, to the ε-amino-group of a lysine residue. However, it is also possible that ubiquitin gets bound to the amino group of a methionine at the N-terminus of a target protein, which might also be another ubiquitin molecule[19, 33, 34]. Normally this ligation process is catalysed by an E3 enzyme, although there are a few examples known where proteins can get monoubiquitylated by transferring ubiquitin to a target protein only with the assistance of an E2[35]. For some UBLs their respective E2 is normally sufficient for the transfer to a substrate. SUMO-UBLs e.g. have with Ubc9 only one cognate E2 and only in certain cases the SUMO-conjugation is assisted by an E3 enzyme[36]. A molecular explanation for this difference in

ubiquitylation and SUMOylation is that SUMOylation only takes place at a defined amino acid motif in the substrate. This motif is a recognition site for the Ubc9, giving SUMOylation its specificity and leaving SUMOylation without need for an E3. The substrate selection of ubiquitylation on the other hand is executed by a set of approximately 600 E3 protein ligases, which all can have different substrate recognition sites, making it difficult to predict a lysine residue as target for ubiquitylation[37, 38]. The existence of this set of different E3 ligases is an advantage in terms of the regulation of ubiquitylation. This way ubiquitylation can get regulated on the level of E3 activation, leading to ubiquitin modification only of the specific pool of proteins that are substrates for a certain E3.
There are two major groups of E3-ligases: the HECT-family and the RING-domain containing E3-ligases, which will be discussed in the following sections.

1.1.2.1 HECT-domain containing E3-ligases

Around 30 E3-ligases with a HECT-domain are encoded in the human genome[37]. The HECT-domain comprises around 350 amino acids and is always located at the C-terminus of the E3-ligase. The N-termini of HECT-type E3-ligases on the other hand are diverse and important for substrate selection. HECT-type E3-ligases, in contrast to RING-domain E3s, have a conserved cysteine residue to which the C-terminus of ubiquitin gets covalently attached, forming an intermediate thioester bond (Figure 2). The N-terminus of the HECT domain binds the E2-enzyme, while the C-terminus contains the cysteine to which the ubiquitin gets attached[39]. The ubiquitin then gets transferred to a lysine residue of a substrate, which can lead to the formation of different types of ubiquitin chains. The mechanism for the chain formation can differ among the HECT-ligases. The HECT-E3-ligase E6AP e.g. synthesises K48-linked ubiquitin chains on its conserved cysteine, while the ligase KIAA10 can produce K48- and K29-linked ubiquitin chains as free entities[40, 41]. Itch, an E3-ligase important for innate immunity, on the other hand produces preferentially K63-linked ubiquitin chains[42]. The ubiquitin chain type specificity can be independent of the E2 but can be regulated by the HECT-domain alone, as 60 amino acids at the C-terminus

of the HECT domain determine the chain type specificity of Rsp5, a *S. cerevisiae* HECT-type E3-ligase, producing preferentially K63-linked ubiquitin chains[43].

1.1.2.2 RING-domain containing E3-ligases

RING-domain E3-ligases are by far the more abundant type, comprising around 600 putative members, accounting for approximately 95% of the human E3-ligases[38]. A typical RING-domain coordinates two zinc atoms and forms a rather globular structure important for protein interaction. The U-box domain is a variant of the RING-domain that has a similar structure but lacks the zinc binding residues. Instead, structural integrity is conveyed by hydrogen bonding, facilitated through conserved charged and polar residues[44]. Ring-domain E3-ligases can function as monomers, homo- or heterodimers or as multi-subunit complexes. In contrast to HECT-type E3-ligases, ubiquitin does not get covalently attached to the RING-finger protein. Instead, the RING-domain binds the ubiquitin loaded E2-enzyme, to bring the thioester bond of the ubiquitin-E2 linkage in close proximity to the target lysine of the substrate, which is bound to another site of the E3.

There are principally two different kinds of RING-domain containing proteins. Some bind as monomers or dimers directly the E2 and the substrate as e.g. Cbl or IAP-proteins[45-47]. The other group of RING-E3s is the family of cullin-RING-ligases (CRL), which are composed of multisubunit complexes containing many interchangeable subunits[48]. CRLs usually consist of the scaffolding cullin-protein, which can interact with a RING-domain containing adapter protein that is responsible for E2-binding. Substrate specificity is reached by another adapter protein that binds to the cullin and to the substrate that gets ubiquitylated. An interesting feature of CRLs is that they have to get modified with the UBL NEDD8 on the cullin subunit. This leads to a conformational change, bringing the E2-ubiquitin thioester in close proximity to the lysine residue of the substrate[49].

Recently, a third mechanism, how E3-ligases transfer ubiquitin to a substrate was discovered. Members of a subfamily of RING-containing E3 ligases harbour two RING domains separated by a domain in between and are thus called RBR

(Ring Between Ring) E3s[50]. So far 15 members of this protein family have been shown to be encoded by the human genome. The first RING domain of RBRs has high sequence and structural similarity to other RING domains, which is the reason why it has been hypothesized that these E3-ligases use the same mechanism as other RING-E3 ligases to catalyse the ubiquitin transfer to a substrate. However, Klevit *et al*. could show that in some RBRs the second RING domain contains a cysteine that covalently binds ubiquitin before the ubiquitin gets transferred to a lysine[51, 52]. This mechanism resembles rather that of HECT-domain E3s making RBR-proteins in a way a hybrid of RING and HECT E3-ligases.

A special RBR containing E3 that uses this conjugation mechanism is the linear ubiquitin chain assembling complex (LUBAC)[19, 53]. LUBAC consists of the RBR containing proteins HOIL-1L, HOIP and SHARPIN (LUBAC can be composed of a HOIP/HOIL/SHARPIN-trimer or a HOIP/HOIL- or HOIP/SHARPIN-dimer)[54-56]. This E3-complex specifically synthesises linear ubiquitin chains regardless of which E2 provides the ubiquitin, resembling rather the chain type determining mechanism of HECT E3s[19].

1.1.3 Ubiquitin-binding domains: Ubiquitin signal deciphering devices

Most of the cellular events that are regulated by ubiquitylation are executed by so called ubiquitin receptors[24]. These proteins have the ability to bind to ubiquitin, which can have various outcomes such as activation, translocation or accumulation of the modified protein. Ubiquitin receptors have distinct domains, which facilitate the ubiquitin binding and are thus called ubiquitin-binding domains (UBDs).

Table 1: Ubiquitin binding domains

UBD	Ubiquitin-binding specificity	Representative protein	Function
α- helical			
UIM	MonoUb, K63, K48	S5a/Rpn10, Vps27, STAM, Epsins, Rap80	Degradation, endocytosis, MVB biogenesis, DNA repair
MIU (IUIM)	K48-, K63-linked chains,	Rabex-5	Endocytosis
DUIM	2 x MonoUb	Hrs	MVB biogenesis
UBM	MonoUb	Pol-i, Rev-1	DNA damage tolerance
UBA	MonoUb, K63-, K48-linked chains	Rad23/hHR23A, Dsk2, p62	Proteasome targeting; kinase regulation; autophagy
CUE	MonoUb	Vps9, TAB2, TAB3	Endocytosis, kinase regulation
GAT	MonoUb	GGA3	MVB biogenesis
VHS	MonoUb	STAM, GGA3	MVB biogenesis
UBAN	Linearly linked chains	NEMO, ABIN1-3, optineurin	NF-κB signalling
Zinc finger			
UBZ	MonoUb, K63-, K48-linked chains	Pol-h; Pol-k, Tax1BP1, NEMO	DNA damage tolerance, NF-κB-signalling
NZF	MonoUb	Npl4, Vps36, TAB2, TAB3	ERAD, MVB biogenesis, kinase regulation, TNFR-signalling
Znf_UBP (PAZ)	MonoUb, K63, K48	USP5/IsoT; HDAC6	Proteasome function, aggresome/autophagy
ZnF_A20	K48-. K63- linearly linked chains,	Rabex-5, A20	Endocytosis, kinase regulation
PH domain			
PRU	MonoUb, K48-linked chains	Rpn13	Proteasome function
GLUE	MonoUb	Eap45	MVB biogenesis
Ubc-like			
UEV	MonoUb	Uev1/Mms2	DNA repair, MVB biogenesis, kinase regulation
Ubc	MonoUb	UbcH5C	Ubiquitin transfer
Others			
SH3	MonoUb	Sla1/CIN85	Endocytosis
PFU	MonoUb	Doa1/Ufd3	ERAD
jab1/MPN	MonoUb	Prp8	RNA splicing

1.1.3.1 Types of UBDs

All UBDs described so far contain regular secondary structures[24]. This is in contrast to sites that interact with other UBLs, like SUMO- or ATG8-proteins, where a short motif of around four hydrophobic amino acids, flanked by a stretch of negatively charged residues, is sufficient for a targeted interaction[57, 58]. UBDs can be found in a multitude of proteins, which are involved in all kinds of cellular processes. Considering the high functional diversity of ubiquitin interaction, as well as the many different kinds of ubiquitin signals, it is not surprising that various distinct domains have evolved to facilitate ubiquitin interaction with high accuracy and specificity[24]. Around 20 families of UBDs are known so far and according to their structure they can get categorized into different groups (Table 1). The majority of the UBDs consist of one or more α-helices. Another large structural group are UBDs that have a zinc finger fold. The UBDs of E2s consist of a beta-sheet and at least two UBD-families have a PH-domain structure[24].

1.1.3.2 UBD-interaction with monoUb

The surface of ubiquitin consists of rather polar residues. An area surrounding I44 however contains mainly hydrophobic residues, including L8 and V70[2]. This hydrophobic patch is the interaction surface for most UBDs and in many cases the interaction is lost when I44 is mutated. Exceptions are for instance the ZnF-A20 of Rabex-5, which mainly interacts with D58, or the ZnF-UBP of the deubiquitylase (DUB) IsoT, which mainly interacts with the C-terminal part of ubiquitin[24]. This unconventional interaction of the ZnF-UBP of IsoT ensures that it only cleaves unanchored ubiquitin chains[59].

1.1.3.3 Avid interactions increase the affinity of ubiquitin receptors towards ubiquitin

The affinity of most UBDs to monoubiquitin is rather low, with Kds in high μM ranges[24, 60]. This fact leads to the question how ubiquitin receptors can efficiently interact with ubiquitylated substrates to reliably propagate a cellular

event. In order to overcome this problem, certain mechanisms have evolved to increase the affinity of ubiquitin receptors and ubiquitylated proteins[24, 60, 61]. These mechanisms can be grouped in three different forms of avid interactions. First, a ubiquitin receptor has, additionally to its UBD, another interaction site with the ubiquitylated protein. Second, a UBD has two or more interaction surfaces for ubiquitin and can thus bind two moieties at the same time. Third, a ubiquitin receptor can contain more than one UBD, which can be used for ubiquitin binding. A variant of this mechanism is the dimer- or multimerisation of UBD-containing proteins, which brings many UBDs together to interact with an ubiquitylated protein. UBDs that can interact with more than one ubiquitin molecule can either bind to multiple monoubiquitin or to several moieties in a polyubiquitin chain. This kind of interaction however can also cluster two monoubiquitylated proteins, which e.g. are integrated in a membrane[60].

1.1.3.4 Multivalent recognition of di-or polyUb enables chain type selectivity

As already discussed, a multivalent interaction between a UBD and a ubiquitin chain can lead to higher affinity[24]. Moreover, it can also be responsible for a selective binding to distinct types of ubiquitin chains. This selective interaction is important for the targeted recognition of a certain chain type conveying a distinct signal. This signal, which should mediate a certain cellular process, is integrated in the structure of the chain type (Figure 1B). Some ubiquitin receptors, e.g. ones responsible for targeting ubiquitylated proteins to the proteasome for their degradation, preferentially interact with K48-linked ubiquitin chains, which is the most important ubiquitin signal for protein degradation[62]. The proteasomal receptor RAD23A has a UBA-domain that binds around four times stronger to K48- than to K63-linked ubiquitin chains and around 70 times stronger to K48-linked chains than to monoubiquitin[63]. A UBD-binding preference for a certain chain type can be either due to a characteristic positioning of the ubiquitin moieties or due to the difference in the linker region (Figure 1 and Figure 3). The difference in the linker region results from the different ubiquitin surfaces around each lysine to which the C-terminus of the

next ubiquitin is attached to. Since most UBDs interact with the I44 patch, the orientation of this surface area from two ubiquitins in a chain is an important factor for the determination of the binding of either a UBD with two interaction surfaces or a protein with two UBDs. Thus the proper binding of a receptor to a ubiquitin chain relies on the potential orientation of two or more defined interaction sites with the UBD(s)[24].

The co-crystal structure of RAD23A-UBA2 with K48-linked diUb thus explains the preferential interaction with this chain type[64]. The UBA2 of RAD23A contacts the I44 patches of both K48-linked ubiquitin moieties, which wrap around the UBD. K48-linked diUb has a compact conformation, where the I44-patches interact with each other (Figure 1B). Since the conformation of ubiquitin and also polyubiquitin fluctuates[21, 61], the UBA2 could slip in during a more open state, which causes a relatively strong binding. This cannot be accomplished with rather elongated diUbs such as linear or K63-linked chains.

Another example how the positioning of the I44-patch in different ubiquitin chains types determines UBD-interaction specificity is the selective interaction between RAP80 and K63-linked ubiquitin chains[65] (Figure 3A). RAP80 has two UIMs in a tandem, enabling and restricting it to interact only with K63-linked ubiquitin chains. The structural basis for this selectivity is the distance of the two UIMs, determined by the length of the linker. This ensures that each UIM can interact with I44 of K63-linked diUb but not with diUb of other linkages such as linear- or K48-linked diUb. The specific interaction of RAP80 with K63-linked ubiquitin chains is important to target DNA-damage repair proteins to the sites of DNA-damage, which is labelled by this type of chains[66].

As described above RAP80 utilises two UIMs to accomplish specific binding to K63-linked ubiquitin chains. The adaptor protein TAB2 on the other hand uses a different mechanism, where only one UBD, its NZF, ensures binding specificity to K63-linked ubiquitin chains[67, 68] (Figure 3A). The co-crystal structure of the TAB2-NZF with K63-linked diUb shows, that it interacts with both ubiquitin moieties via their I44-patch. This interaction is only possible, when the two I44 patches are positioned properly, which is only assured by the K63-linkage.

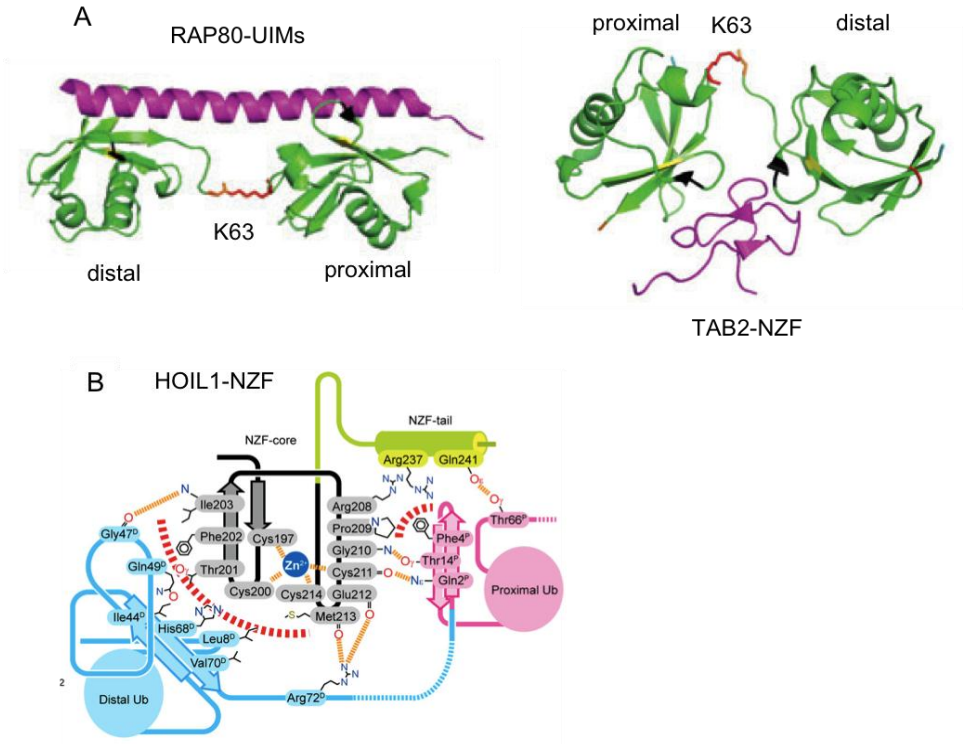


Figure 3: Ubiquitin chain type selectivity of different ubiquitin receptors. A) Two RAP80 UIMs (pink) in tandem bind to the I44 patches of two K63-linked ubiquitin moieties (green) and the length of the UIM-linker determines chain type selectivity. The NZF of TAB2 (pink) also specifically binds to the I44 patches of two K63-linked diUbs (green). In this case the moieties have to bend in a way that is only possible with the K63-linkage (red). B) The NZF of HOIL (gray), in contrast specifically interacts with linear linked diUb. The NZF binds the distal moiety (blue) at its I44 patch while it contacts the proximal ubiquitin (pink and green) at a region close to the linker, which is only possible in the context of the linear linkage. Fig. 3A is modified from[24]. Structure RAP80-UIMs/diUb (PDB code 3A1Q[65]). Structure TAB2-NZF/diUb (PDB code 2WWZ[67]). Fig. 3B is modified from[69].

A different mechanism, how the ubiquitin chain linkage conveys UBD-binding specificity is exhibited by the interaction of the NZF-domain of the LUBAC-subunit HOIL with linear diUb (Figure 3B)[69]. Linear chains were recently discovered to be important for the activation of NF-κB-signalling and the E3-ligase LUBAC specifically produces linear ubiquitin chains[70]. The elongated structure of linear and K63-linked diUb is similar since the K63-residue is in close proximity to the M1-residue of the N-terminus, however, the linker region differs[20] (Figure 1). In contrast to previously described examples of UBD-ubiquitin interactions, the binding of the NZF of HOIL to diUb is facilitated by the

linker region. The distal moiety (the one where the C-terminus is attached to the methionine) interacts with the NZF via its hydrophobic I44 patch, while the proximal ubiquitin binds via its residues Q2, F4 and T12, which are close to the linker region. Only the linear linkage brings these residues close to their interacting residues in the HOIL-NZF, when at the same time the distal moiety binds the UBD with the I44 patch[69].

Taken together UBDs display decoding devises for ubiquitin signals. The ability of many ubiquitin receptors to interact with more than one site on ubiquitin increases their affinity. This is often also important for the specific interaction with a certain type of ubiquitin chain, which enables their specific function in a distinct cellular process[24].

1.1.4 Deubiquitylating enzymes (DUBs)

A set of deubiquitylating enzymes (DUBs), analogous to protein dephosphorylating phosphatases, is responsible for the removal of ubiquitin from a substrate[13, 71]. There are around 100 DUBs encoded in the human genome, which can be grouped in the five families USP, UCH, OTU, Josephin and JAMM.

Removal of ubiquitin by DUBs is important for various cellular events. DUBs are important for the production of monoubiquitin after the translation of ubiquitin genes[13, 14]. Since all ubiquitin genes get translated as a fusion protein, DUBs cut off monoubiquitin molecules from the translated pro-proteins, which brings ubiquitin into its activatable form. One of the main functions of the ubiquitylation of proteins is their proteasomal degradation (see chapter 1.1.5.1). Many DUBs are associated with the proteasome either for the coupled removal of ubiquitin from degraded proteins or to inhibit protein degradation directly at the proteasome[4]. Another important task of DUBs is to regulate signalling events that are mediated by ubiquitylation. By deubiquitylating proteins involved in cellular signalling, DUBs can inhibit or shut down the signal. Most signalling events only get initiated by ubiquitin chains with a certain linkage[2]. Many DUBs have a selective activity towards distinct linkage types and therefore specifically regulate cellular events. The importance of deubiquitylation is also reflected by

many diseases such as cancer or neurological disorders, that are linked to dysfunctions of DUBs[72]. The DUB USP7/HAUSP e.g. can deubiquitylate MDM2, which is an E3-ligase for p53. A high expression of USP7 can thus lead to higher MDM2-stability, which leads to degradation of the important tumour suppressor p53 due to its ubiquitylation by K48-linked ubiquitin chains.
An important function of DUBs is displayed in the NF-κB signalling pathway. This pathway is highly regulated by various ubiquitylation events, where certain types of ubiquitin chains have specific functions (see chapter 1.2.3). In order to shut down NF-κB signalling the DUBs CYLD and A20 perform important functions by deubiquitylating proteins in this pathway[73].

1.1.5 Physiological functions of ubiquitylation

The functions of ubiquitylation are extremely diverse covering almost every aspect of cellular physiology. Protein ubiquitylation is involved in various cellular processes, including cell-cycle regulation, signal transmission, DNA-repair, endocytosis and endosome sorting[4, 46, 74-77]. Many of these processes depend on the most prominent outcome of ubiquitylation, which is the degradation of proteins[4]. However, in the late 1990's it was discovered that ubiquitylation can have non-proteolytic functions and since than a myriad of non-degradative roles of protein ubiquitylation has been discovered[78, 79].

1.1.5.1 Proteasomal degradation

Responsible for the degradation of ubiquitylated proteins is a large protein complex called the proteasome[4]. The 26S proteasome consists of a 20S catalytic core and two 19S regulatory lids. The 20S core consists of four heptamer rings where the two rings in the middle possess protease activity. In the hollow cavity of the proteolytic 20S core proteins get degraded into short peptides consisting of about seven or eight amino acids. The 19S proteasome regulates substrate recognition and entry into the 20S proteasome. The 19S proteasome contains six ATPases, which are important for several processes leading to specific substrate degradation[4].

Proteins that have to be targeted for proteasomal degradation are usually modified with K48-linked polyubiquitin chains, however also other chain types as well as monoubiquitylation can function as degradation signals. These ubiquitin modifications constitute a specific signal, which is recognized by ubiquitin receptors. Some ubiquitin receptors, such as RPN10 and RPN13, are part of the 19S proteasome and directly recruit ubiquitylated substrates. Additionally these ubiquitin binding proteins might also bind to a ULD in, so called, shuttling receptors[9]. These ULD-containing shuttling receptors also contain a UBD and thus can bind ubiquitin chains, to recruit substrates to the proteasome via interaction of their ULD with the UBD of RPN10 or RPN13, giving the system a higher order of selectivity and specificity.

Protein degradation by the proteasome can serve two major purposes. The first is to remove misfolded and non-functional proteins that might damage the cell since these proteins might aggregate[4]. The second function is the regulation of the presence of a protein either for homeostasis or in cellular signalling. In cellular signalling, proteasomal removal of endogenous inhibitors can lead to a regulated activation of a signalling pathway. In the NF-κB signalling pathway (see chapter 1.2) e.g. the inhibitor of this transcription factor is ubiquitylated upon pathway activation leading to its destruction und thus activation of the transcription factor[80]. Another example how proteasomal protein removal regulates cellular processes is mitosis. The chromosomes in anaphase are attached to each other by a tethering complex during mitosis. Upon activation of the E3-ligase complex APC/C the proteins holding the chromosomes together get ubiquitylated and degraded which enables progression of mitosis[81].

1.1.5.2 Non-degradative roles of ubiquitylation

1.1.5.2.1 Ubiquitylation and DNA damage

Ubiquitylation plays a pivotal role in various DNA-repair or tolerance processes[75]. When a DNA-polymerase encounters a DNA-damage site during replication it stops. This is a signal for the monoubiquitylation of PCNA, the

polymerase leading DNA-clamp, which then induces the loading of the y-family polymerase pol η and pol ι via their UBDs[82]. These two polymerases can replicate DNA across lesions, preventing larger genomic defects, however, without repairing the DNA lesions. In contrast, the ubiquitylation of PCNA with K63-linked ubiquitin chains leads to an error-proof replication over DNA-damage sites[83].

A well analysed example for the scaffolding function of K63-linked ubiquitin chains is its role in the signalling cascade at the sites of DNA double strand breaks[84]. The unprotected open ends of DNA are recognised by the MRN-complex, which subsequently activates the kinase ATM. ATM phosphorylates a plethora of proteins, which, among other effects, leads to the recruitment of the E3-ligases RNF8 and RNF168. These ligases specifically synthesise non-degradative K63-linked ubiquitin chains on histones and possibly other proteins at the DNA-damage site. The K63-linked ubiquitin chains are a signal for the recruitment of the UIM containing protein RAP80 that specifically recognizes this chain type. This specificity gives the process a regulated directionality important for the efficient and ordered repair of the damage[85]. Recruitment of RAP80 is essential, since it is part of a complex, which among others, contains the tumour suppressor BRCA1 and this complex is crucial for the repair of the damage. BRCA1 itself is also an E3-ligase giving the ubiquitin signalling events at DNA double strand breaks another layer of complexity[86].

1.1.5.2.2 Ubiquitylation in endocytosis and endosome sorting

Endocytosis is another process where ubiquitin plays an important role. Many transmembrane receptors that get internalised get modified with (multi-) monoubiquitin or K63-linked chains[76, 87]. This constitutes a signal for the UBD-containing receptors Eps15 and Epsin, which assist clathrin driven endocytosis. The later sorting of vesicles in multivesicular bodies also depends on modification by (multi-) monoubiquitylation or K63-linked chains. This signal is received by some members of the ESCRT (Endosomal Sorting Complex Required for Transport)-complexes including STAM (Signal transducing adapter

molecule), Vps27 (vacuolar protein sorting) and Hrs (Hepatocyte growth factor receptor substrate), which all contain ubiquitin binding domains.

1.1.5.2.3 Ubiquitylation in protein expression

Ubiquitylation also has non-degradative roles in the splicing and translation process. Some spliceosomal proteins get modified with K63-linked chains, which stabilises the snRNP-complex and is important for efficient mRNA maturation[88, 89]. Polysomes on translated mRNA molecules get stabilised during translation by the modification of ribosomal proteins with K63-linked chains[90].

1.2 NF-κB signalling

An important cellular system, that exemplifies the different regulatory functions ubiquitin can have, is the NF-κB signalling pathway[91]. In this pathway ubiquitylation is important for degradative as well as non-degradative events and many forms of ubiquitin chains are present and perform distinct tasks. Most of the non-degradative functions of ubiquitin are transmitted by K63-linked chains or monoubiquitin[2]. However, recent findings suggest that also linear ubiquitin chains play a crucial role in NF-κB signalling[54, 56, 70].

NF-κB (Nuclear Factor of kappa light polypeptide gene enhancer in B cells) is a family of transcription factors that was discovered about 25 years ago as a binder to the kappa light chain gene enhancer in B cells[92]. Since then NF-κB has become one of the most thoroughly investigated transcription factors[80]. Although best known for its role in inflammation and the innate and adaptive immune system, NF-κB also has crucial effects on a plethora of other cellular processes, including the regulation of cell death, differentiation and proliferation. The NF-κB family consists of five members, p65/RelA, RelB, c-Rel, p50 (p105 as its precursor) and p52 (p100 as its precursor). All members bind to DNA in form of hetero- or homodimers, resulting in 15 potential dimer combinations, of which some activate and others inhibit gene transcription. All NF-κB family members contain a Rel-homology domain (RHD) that is necessary for

dimerisation and DNA binding. Only RelA, RelB and c-Rel have a transactivation domain (TAD), which mediates transcription, but the other two members can heterodimerise with a TAD-domain containing member and thus still assist gene activation[80].

1.2.1 NF-κB activation

NF-κB transcription factors are usually sequestered in the cytoplasm and get only activated upon a stimulus[80]. A family of ankyrin repeats containing inhibitors, the Inhibitor of NF-κBs (IκBs), bind to the RHD of the NF-κB dimer, keeping it inactive. IκBα, IκBβ and IκBε are the three classical members of the IκB-family, where IκBα is the prototype and the best studied one. Interestingly, the precursors of p50 (p105) and p52 (p100) also contain ankyrin repeats, which enables them to function as NF-κB inhibitors similar to the IκBs. p105 and p100 have to get processed by the proteasome, which removes the inhibitory ankyrin repeat, to transform them into their active forms p50 and p52 respectively. In order to remove the ankyrin repeats containing inhibitors, they first have to get phosphorylated, which is executed by the IκB Kinase (IKK)-complex. Phosphorylation triggers their K48-linked ubiquitylation by the cullin E3-ligase $SCF^{\beta TrCP}$, which leads to their proteasomal degradation. The removal of the inhibitor exposes a nuclear localisation signal of the NF-κB dimer, which enables it to translocate to the nucleus and bind to DNA to initiate gene transcription.

A huge variety of intra- and extracellular signals can activate NF-κB. For example many extracellular ligands bind to different kinds of transmembrane receptors that can activate NF-κB, including members of the TNF-receptor (TNFR) family (e.g. TNFR I and II, CD40, BAFF-receptor), IL-1β-receptor (IL-1βR), Toll-like-receptors (TLRs), T- and B-cell receptors (TCR, BCR) and growth factor receptors. Intracellular NF-κB activation signals are e.g. DNA-damage, ROS-production, and pathogen receptors, including RIG-I and NOD[80].

1.2.1.1 Canonical NF-κB activation

There are two major pathways that can activate NF-κB transcription factors: the canonical and the non-canonical pathway[80]. The canonical pathway is activated by intracellular signals as well as certain transmembrane receptors (Figure 4). The activation of any of these receptors leads to recruitment of adapter proteins, such as members of the TRAF-family. This protein recruitment cascade eventually activates the IKK-complex, which then phosphorylates IκBs. The IKK complex in the canonical pathway consists of the kinases IKKα, IKKβ and the regulatory subunit NEMO (IKKγ). Important and characteristic for the canonical pathway are NEMO and IKKβ, while IKKα is dispensable[80]. The main NF-κB members involved in the canonical pathway are RelA, c-Rel and p50. A well-investigated concept of canonical NF-κB signalling is its activation by extracellular signals via transmembrane receptors, including the TNFR, the IL-1βR, TLRs and B- and TCR (Figure 4). All these receptors have in common, that they don't have enzymatic activity and upon activation adaptor proteins that mediate the signal get recruited. All receptors have a different set of adaptor molecules but their activation always leads to the activation of the IKK-complex (see 1.2.2).

An important theme in the induction of NF-κB is protein ubiquitylation[91, 93]. The recruitment of E3-ligases has been observed for most of the NF-κB inducing transmembrane receptors. These E3-ligases produce different kinds of ubiquitin chains, which are important for the activation of NF-κB and will be discussed in more detail in chapter 1.2.3.

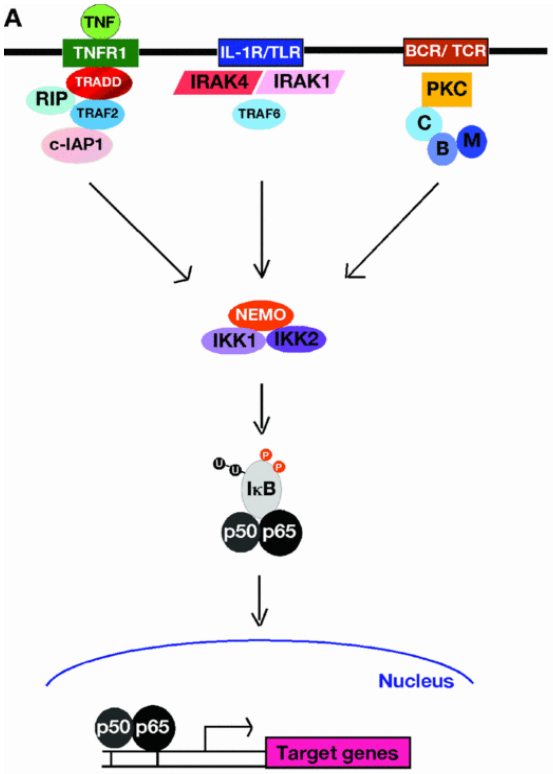


Figure 4: Canonical NF-κB activation. The ligand induced activation of transmembrane receptors leads to the recruitment of adapter proteins and enzymes. The enzymes catalyse PTMs such as phosphorylation and ubiquitylation. This recruits and activates the IKK-complex, mediated by its regulatory subunit NEMO. The IKK-complex phosphorylates IκB at a certain motif and this signal is recognized by the cullin E3-ligase $SCF^{\beta TrCP}$. The E3 facilitates K48-linked ubiquitylation of IκB, inducing its degradation, which exposes a nuclear localisation signal of the NF-κB-dimer. NF-κB can translocate to the nucleus and initiate gene transcription. (Modified from Wong and Tergaonkar Clin. Sci. (2009) 116, 451-465).

1.2.1.2 Non-canonical NF-κB activation

The non-canonical pathway is activated by certain receptors that belong to the TNFR family (e.g. BAFF-receptor, CD40, RANK, LTβR and FN14)[94]. A fundamental difference to the canonical pathway is that the regulatory IKK member NEMO, which is crucial for canonical NF-κB, is not involved in the activation of the non-canonical pathway. Instead, the non-canonical pathway is regulated by the MAP3-kinase NIK, which is indispensable for this NF-κB branch, but is not involved in the canonical. Under steady state conditions NIK gets modified with K48-linked ubiquitin chains by an E3-ligase complex consisting of TRAF2, TRAF3 and cIAP1/2, which leads to a constitutive NIK degradation by the proteasome. Upon receptor activation TRAFs and cIAPs get ubiquitylated and degraded, which stabilises NIK. NIK can then activate IKKα, the distinctive kinase for the non-canonical pathway, to activate NF-κB. The characteristic NF-κB members for the non-canonical pathway are RelB and p52 (p100). In unstimulated state RelB and p100 form an inactive heterodimer, since activation

is blocked by the ankyrin repeats of p100. When the non-canonical pathway gets activated, IKKα phosphorylates p100 at two serines in a recognition site for the E3-ligase $SCF^{\beta TrCP}$. This leads to the ubiquitylation and processing of p100 to p52 by the proteasome und thus to the activation of the RelB/P52 NF-κB dimer[94].

1.2.2 The IKK-complex

As described above, NF-κB signalling is a very complex system, where a huge amount of activators can lead to the activation of different kinds of NF-κB dimers that can be blocked by various endogenous inhibitors[80]. This activation can result in all kinds of cellular outcomes, mediated by transcription activation or inhibition. However, all these diverse signals, that induce canonical NF-κB activation, lead to activation of one single kinase complex which was already mentioned before: the IKK-complex[95] (Figure 4). The IKK-complex usually consists of the two kinases IKKα and IKKβ and a regulatory subunit NEMO (Figure 5). The composition of the IKK-complex, together with the utilisation of the IκBs, is the actual factor that distinguishes the canonical from the non-canonical pathway, reflecting its central role in NF-κB signalling[96]. In contrast to the canonical IKK-complex, where the IKK-holoenzyme consists of IKKα, IKKβ and NEMO, the non-canonical pathway relies on IKKα alone.

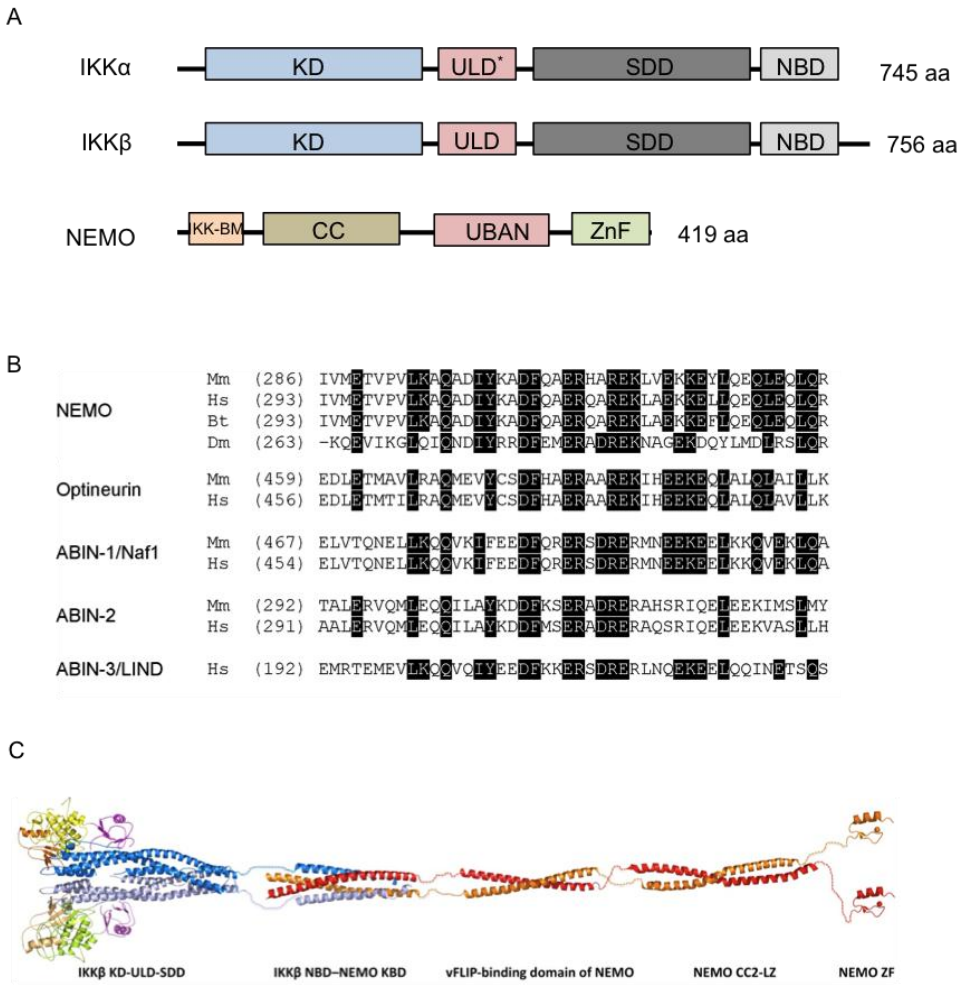


Figure 5: The IKK-complex. A) The canonical IKK-complex consists of the kinases IKKα and IKKβ and the regulatory subunit NEMO. B) Alignment of the UBAN-domain which is known to exist in five proteins C) Model of the IKK-complex assembled from different X-ray crystal structures. (Modified from[97]). *ULD from IKKα has been assumed from sequence comparison after solving the structure of IKKβ. KD (kinase domain), SDD (scaffold/dimerisation domain), NBD (NEMO binding domain), IKK-BM (IKK- binding motif), CC (coiled coil), ZnF (zinc finger).

1.2.2.1 The kinase subunits IKKα and IKKβ

The two kinases in the canonical IKK-complex, IKKα and IKKβ, have a similar structure and around 50% sequence identity[95]. The kinase domain is located at the N-terminus (Figure 5A). IKKβ has a ULD after the kinase domain and sequence comparison in combination with structural analysis of IKKβ suggests that also IKKα has a ULD[97]. The α-helical scaffold/dimerisation domain (SDD) is at the C-terminus and it contains 6 α-helices that are important for

kinase homo- or heterodimerisation, as well as for the binding to the regulatory subunit NEMO[95, 97]. At the very C-terminus of the SDD domain is the NEMO binding domain (NBD), which is highly conserved among different species and indispensable for the interaction with NEMO.

1.2.2.2 The regulatory subunit NEMO is a ubiquitin receptor

In order to be able to activate canonical NF-κB, the presence of IKKs regulatory subunit NEMO is absolutely essential, as is the interaction of the kinases with NEMO via their NBD[95]. NEMO itself forms a dimer that binds IKKα/β with an N-terminal IKK binding motif (IKK-BM), which is followed by a coiled coil domain (Figure 5). A UBD called UBAN (Ubiquitin Binding in ABIN proteins and NEMO) follows towards the C-terminus, and at the very C-terminus is a ZnF-domain.

The UBAN is composed of a coiled-coil and a leucine zipper that resides roughly between residues 280 and 340 in the human NEMO variant (Figure 5B). A highly homologous domain exists in at least four other proteins, optineurin and ABIN 1/2/3, of which at least some are involved in NF-κB signalling, and all UBANs have been shown to bind to ubiquitin chains[98-100]. First it was hypothesised that the UBAN is a UBD specific for K63-linked ubiquitin chains, but with the discovery of physiologically relevant linear ubiquitin chains, this concept has been challenged[70]. Several laboratories could show that the UBAN of NEMO binds with approximately 100 times higher affinity to linear than to K63-or other lysine-linked ubiquitin chains[99, 101, 102].

The importance of NEMO and especially its ability to bind ubiquitin is emphasised by human genetic diseases that are linked to mutations in NEMO and its UBAN domain[95]. Two examples are the X chromosome-linked human diseases, incontinentia pigmenti (IP) and anhydrotic ectodermal dysplasia with immuno deficiency (EDA-ID).

The predicted structure of the intact canonical IKK-complex was modelled based on data obtained from different structural studies[97, 99, 103-105]. Thus obtained structural model reveals a highly elongated protein complex that consists of a NEMO dimer and a kinase homo- or heterodimer (Figure 5C). This structure

underlines the important function of NEMO as a scaffolding protein, containing a large surface, which enables interactions with various proteins, including ubiquitin chains.

1.2.3 The role of ubiquitin in TNF-receptor induced signalling and NF-κB regulation

1.2.3.1 Activation of the TNFα-Receptor signalling complex

The binding of the cytokine TNFα activates the TNFα-receptor (TNFR) by inducing its trimerisation[106]. This leads to the recruitment of various adaptor proteins and enzymes in order to promote the transduction of the signal. The activation of the TNFR initiates four different signalling pathways: the NF-κB pathway, the MAP-kinase pathway, the Caspase-8-dependent apoptotic and the RIP1/RIP3-dependent necrotic pathway. The TNFR has a death domain (DD), which is important for the recruitment of the DD-containing proteins RIP1 and TRADD. The DD of TRADD can bind to FADD, which activates Caspase-8 and apoptosis[106].

The further composition of the TNFR signalling complex (TNFRSC) depends on ubiquitin and on members of the ubiquitylation machinery and different types of ubiquitin chains are essential for the proper regulation of the signalling events[91, 106, 107]. First, the putative E3-ligases TRAF2 and potentially TRAF5 bind to TRADD, which has a TRAF-binding domain. This TRADD bound TRAF-trimer binds the E3-ligases c-IAP1 and 2. cIAPs and possibly also TRAF2/5 then synthesise K63-linked ubiquitin chains on RIP1, TRADD, TRAFs and other members of the TNFRSC. Additionally, cIAPs modify RIP1 with K11-linked polyubiquitin[108]. All these ubiquitin chains help to recruit further members of the TNFRSC. The MAP3-kinase TAK1 e. g. localises to the complex via two of its adapter molecules, TAB2 and TAB3. TAB2/3 both contain a C-terminal NZF-domain that selectively binds K63-linked ubiquitin chains[68]. This interaction is important for the initiation of the MAP-kinase pathway by TAK1[109]. The non-degradative ubiquitin chains in the complex are also responsible for recruiting

the E3-ligase complex LUBAC, consisting of the proteins HOIP, HOIL and SHARPIN[54, 55, 110]. All LUBAC members have ubiquitin binding domains that are important for LUBAC recruitment and function in the complex. LUBAC is an E3-ligase that makes linear ubiquitin chains on RIP1, NEMO and itself and most likely also on other proteins[19, 54, 55, 70]. Linear ubiquitin chains have been shown to be crucial for an efficient activation of NF-κB. This novel ubiquitin chain type gives the ubiquitylation network in the complex another layer of complexity. NEMO as the regulatory subunit of the IKK-complex gets also recruited to the complex via its UBAN-domain and the binding of NEMO to ubiquitin chains is absolutely necessary for the activation of NF-κB[99, 111]. These different types of ubiquitin chains mentioned above form a platform that is important for the stability of the whole complex, since many participants are ubiquitylated and many also contain UBDs[106, 110].

1.2.3.2 Silencing of the TNFα-receptor signalling complex

The importance of the ubiquitin system also becomes evident in the cellular processes, which are implemented to deactivate NF-κB signalling. E3-ligases as well as DUBs participate in the attenuation of NF-κB. A20, CYLD and Cezanne are three DUBs that are involved in removing ubiquitin chains that are important for TNFRSC activity[73]. CYLD belongs to the USP-family of DUBs and also functions as a tumour-suppressor. It inhibits NF-κB signalling by removing K63-linked ubiquitin chains from various proteins in the TNFRSC. However, CYLD also has strong DUB-activity towards linear ubiquitin chains, making it possible that it also removes this chain type from the TNFRSC[20]. Cezanne is an OTU-domain containing DUB involved in silencing of NF-κB. It is upregulated by NF-κB and shows specific deubiquitylation activity towards K11-linked chains, which have been found on RIP1[108, 112]. However, the role of the presence and the removal of K11-linked chains has not been elaborated.

A20 is another DUB from the OTU-family that is usually expressed at low levels and gets induced by NF-κB[73]. A20 is a special member of the ubiquitylation machinery, since it was shown that it also has E3-ligase activity. *In vivo* it removes K63-linked ubiquitin chains from RIP1 (and possibly other substrates)

and attaches K48-linked ubiquitin chains instead, which removes RIP1 by the proteasome, thus acting as a fast and persistent silencer of NF-κB[113]. However, *in vitro* A20 preferably hydrolyses K48-linked ubiquitin chains, making it likely that additional factors are responsible for the A20 dependent removal of K63-linked ubiquitin chains from RIP1[20]. Indeed, several other proteins involved in the ubiquitin system interact with A20 and are important for the downregulation of NF-κB signalling and together they have been described as A20-ubiquitin editing complex[73]. Examples are the E3-ligases RNF11 and Itch, which have been shown to inhibit NF-κB signalling. Two other proteins that interact with A20 and inhibit NF-κB signalling are Tax1BP1 and ABIN1. Tax1BP1 contains two UBZ-domains at its C-terminus, of which only one binds ubiquitin and this ubiquitin interaction is important for A20 mediated NF-κB inhibition[114]. ABIN1, as already discussed, contains a UBAN that binds linear ubiquitin chains but also K63-linked ubiquitin chains and this ubiquitin interaction is also required for the downregulation of NF-κB[98].

2. Aims of the Study

Ubiquitin can form a diverse set of chains, which is based on the linkage type that connects two ubiquitin molecules[2]. The different ubiquitin chain types have distinct conformations and topologies. These diverse surfaces can be distinguished through binding of UBDs, which results in a selective interaction leading to a regulated and specific function[24]. NEMO is an important regulator of the NF-κB signalling pathway and has a UBD, called UBAN, which displays ubiquitin chain binding selectivity[98, 99, 111]. It was assumed that the UBAN selectively binds K63-linked ubiquitin chains. However, it was found that the newly discovered linear ubiquitin chains have an important role in the activation of NF-κB and an interesting question to address was if NEMO might also bind to linear ubiquitin chains and if this binding is involved in NF-κB activation[70].

To get a better understanding of the ubiquitin binding properties of NEMO, this study set out to thoroughly investigate in detail the direct interaction of purified NEMO domains and full-length NEMO with different types of ubiquitin chains in regards to the linkage as well as the length. This also included the determination of residues important for the NEMO-ubiquitin interaction, based on structural and homology comparison analysis.

On the functional side the aim of the study was to elucidate the role of NEMO binding to ubiquitin chains during the activation of NF-κB, with a focus on TNFR-dependent NF-κB signalling. The role of NEMO in other pathways regulated by the TNFR, such as apoptosis and MAP-kinase signalling, was analysed as well. Finally, it is crucial to dissect the ubiquitin binding details of NEMO in order to properly understand the signalling activation mechanisms at the TNFR. Therefore, the aim of this study was to gain insights into the functional outcomes of the binding selectivity of NEMO towards different types of ubiquitin chains, especially the different binding preference towards linear- and K63-linked ubiquitin chains.

3. Material and Methods

3.1 Material

3.1.1 Chemical compounds

Reagent	Source
Agarose	Lonza
Ampicillin	Roth
Amylose Resin	Biolabs
Aprotinin	Sigma-Aldrich
APS	Roth
Bovine serum albumin	Roth
Bromphenol Blue	Roth
Chemiluminescence Luminol Reagent	Santa Cruz
Coomassie Brilliant Blue R250	Roth
Cyclohexamide	Sigma-Aldrich
1,4-diazabicyclo (2,2,2) octan (DABCO)	Fluka
4',6-Diamidin-2-2phenylindol (DAPI)	Roth
Distilled water	GIBCO
1,4-dithio-DL-threitol (DTT)	Thermo scientific
dNTPs	Roche
Fetal calf serum	PAA Laboratories
FugeneHD	Roche
GeneJuice	Merck-Chemicals
Glutathione Sepharose 4B	GE Healthcare
IPTG	Invitrogen
Kanamycin	Roth
Leupeptin	BioMol
Lipofectamine	Invitrogen
β-Mercaptoethanol	Roth
Mounting medium	Biometra

Na-orthovanadate	Sigma-Aldrich
N-Ethylmaleimide (NEM)	Sigma-Aldrich
Ni-NTA	Qiagen
Penicillin/Streptomycin	Gibco, Invitrogen
Phenylmethylsulfonyl fluoride (PMSF)	Roth
Ponceau S	Roth
Puromycin	Roth
Random hexamer primer	Fermentas
RiboLock Ribonuclease inhibitor	Fermentas
Sodium vanadate	Sigma-Aldrich
TEMED	Roth
Triton X-100	Roth
Trypsin	Gibco, Invitrogen
Tween20	Roth

3.1.2. Kits

Kit	**Source**
QIAfilter plasmid Mini kit	Qiagen
QIAfilter plasmid Midi kit	Qiagen
QIAquick PCR purification kit	Qiagen
RNeasy mini kit	Qiagen
SensiMix real-time PCR kit	Bioline

3.1.3 Antibodies and recombinant or extracted proteins

Primary antibodies	
Antibody (source species; species reactivity)	**Source**
Ubiquitin-P4D1 (m; h)	Santa Cruz
Ubiquitin- (m; h)	Invitrogen
Phospho-IκBα (m; m and h)	Cell signaling
IκBα (r; h and m)	Cell signaling

Phospho-p38 (m; h and m)	Cell signaling
p38 (r; h and m)	Cell signaling
Phospho-JNK (m; h and m)	Cell signaling
JNK (r; h and m)	Cell signaling
IKKα (r; h and m)	Cell signaling
NEMO (IKKγ) (r; h and m)	Santa Cruz
Tubulin (m; h and m)	Sigma-Aldrich
Actin (m; h)	Sigma-Aldrich
Activated Caspase-3 (r; h and m)	Cell signaling
PARP (r; h)	Cell signaling
p65 (r; m and h)	Santa Cruz
MBP (m; Salmonella)	Sigma-Aldrich
GAPDH (r; h)	Cell signaling

h, human; m, mouse; r, rabbit

Secondary antibodies	
Antibody	**Source**
Rabbit-HRP	Goat (dako) p0448
Rabbit-HRP	Mouse anti light chain (Cell signaling) 3677
Mouse-HRP	Goat (Bio-Rad) (170-6516)

Recombinant or extracted proteins	
Reagent	**Source**
Murine TNFa	Peprotech
IL-1b	Peprotech
Ubiquitin from bovine erythrocytes	Sigma-Aldrich
T4-DNA ligase	NEB
PFU-Ultra DNA-polymerase	Aplicam
Reverse Transcriptase	Promega
PWO-DNA-Polymerase	Peqlab

DNaseI	Fermentas
Protein Marker I	Bio-Rad
Protein Marker II	Aplicam
PreScission™ Protease	GE Healthcare
Protein A/G	Roche
Thrombin	Novogen

3.1.4 Buffers, media and solutions

Solutions	**Composition**
Pull-down buffer	50 mM Tris pH 7.5 150 mM NaCl 5 mM DTT 0.1% NP-40
Ubiquitin chain reaction buffer	40 mM Tris, pH 7.5 10 mM $MgCl_2$ 0.6 mM DTT
Ubiquitin chain purification buffer A	50 mM NH_4Ac, pH 4.5
Ubiquitin chain purification buffer B	50 mM NH_4Ac, pH 4.5 1 M NaCl
Cationic exchange buffer	Buffer A: 50 mM NH_4Ac, pH 4.5 Buffer B: 50 mM NH_4Ac, pH 4.5 1000 mM NaCl
SDS-PAGE sample buffer	50 mM Tris HCl, pH 6.8 2% SDS 10% glycerol 0,1% bromphenol blue 25 mM DTT 5% b-Mercaptoethanol
SDS-PAGE running buffer	Tris 30 g glycine 144 g SDS 5 g

	dH_2O up to 1 L
Western blot transfer buffer	Tris 22.3 g glycine 105 g 20% Methanol dH_2O up to 1 L
Western blot blocking solution (TBS-BSA)	100 mM Tris, pH 7.5 150 mM NaCl 0.1% NaN_3 5% BSA
TBS-Tween	100 mM Tris, pH 7.5 150 mM NaCl 0.05% Tween20
TBS	100 mM Tris, pH 7.5 150 mM NaCl
Stacking gel buffer	0.5 M Tris, pH 6.8 0. 4% SDS
Resolving gel buffer	1.5 M Tris, pH 8.8 0.4% SDS
PBS	GIBCO 10x D-PBS (+$CaCl_2$, +$MgCl_2$) PAA Dulbecco's 1x PBS (-$CaCl_2$, -$MgCl_2$
Hypotonic buffer	20 mM HEPES, pH 7.9 10mM KCl 1mM EDTA 0.2% NP-40 10% Glycerol 1.5 mM $MgCl_2$ 1 mM Na-Vanadate 1mM PMSF 10 µg/ml Aprotinin 10 µg/ml Leupeptin

	1 mM DTT
Hypertonic buffer	20 mM HEPES, pH7.9 10 mM KCl 0.2 mM EDTA 20% Glycerol 400 mM NaCl 1.5 mM $MgCl_2$ 1 mM Na-Vanadate 1 mM PMSF 10 µg/ml Aprotinin 10 µg/ml Leupeptin 1 mM DTT
Lysis buffer	HEPES 50 mM, pH 7.5 NaCl 150 mM 10% glycerol 1mM EGTA 1 mM EDTA 25 mM NaF 10 µl $ZnCl_2$ 1 mM Na-Vanadate 1 mM PMSF 10 µg/ml Aprotinin 10 µg/ml Leupeptin
GST-buffer 1	20 mM Tris-HCl, pH 7.5 10 mM EDTA 5 mM EGTA 150 mM NaCl 1mM PMSF 10 µg/ml Aprotinin 10 µg/ml Leupeptin 0.1% b-Mercaptoethanol
GST-buffer 2	20 mM Tris-HCl, pH 7.5

	10 mM EDTA 150 mM NaCl 0.5% Triton X-100 1mM PMSF 10 µg/ml Aprotinin 10 µg/ml Leupeptin 0.1% b-Mercaptoethanol
GST-buffer 3	20 mM Tris-HCl, pH 7.5 0.1% b-Mercaptoethanol 10% NaN_3
PreScission™ protease cleavage buffer	50 mM Tris HCl, pH 8.0 100 mM NaCl 1 mM EDTA 1 mM DTT
Ponceau S	0.5% Ponceau S 10% acetic acid in H_2O
Coomassie brilliant blue staining	45% Methanol 10% Acidic acid 0,1% Coomassie brilliant blue in dH_2O
Coomassie brilliant blue destaining	45% Methanol 10% Acidic acid in dH_2O
50x TAE-buffer	240 g Tris 57.1 ml glacial acetic acid 100 ml 0.5 M EDTA ad to 1 l H_2O
Luria-Bertoni (LB) media	10g/L tryptone 5g/L yeast extract 10g/L NaCl
Dulbecco's modified Eagle' medium (DMEM)	Gibco, Invitrogen

3.1.5 Plasmids

For reconstitution of NEMO knock-out MEFs the retroviral derived vector pBABE puro (purchased from Addgene) was used. Isoform 1 of the NEMO-gene sequence from *Mus musculus* was cloned into pBABE using the restriction sites *Eco*RI/*Bam*HI. The human TAB2-NZF domain was exchanged with the NEMO UBAN domain (aa 250-339) or the ZnF (aa 389-412), in the full-length NEMO gene integrated into pBABE.

For expression of recombinant GST-tagged proteins the full-length sequence (aa 1-412), the UBAN (aa 250-338), the C-terminus (aa 250-412) or the ZnF (aa 389-412) of isoform 1 of the *Mus musculus* NEMO gene and the full-length gene of human RAP80 was cloned into pGEX-4T1 (GE Healthcare). The human TAB2-NZF domain was exchanged with the NEMO UBAN (aa 250-339) or the ZnF (aa 388-412), in the full-length NEMO gene cloned into pBABE.

For expression of recombinant MBP-tagged proteins the sequence of the full-length isoform 1 of the *Mus musculus* NEMO-gene (aa 1-412) was cloned into pMAL-c2X (New England Biolabs).

The plasmids pGEX-6P-1-Ubc13, pGEX-6P-1Uev1a and pET16His10-cdc34 were kindly provided by David Komander (MRC, Cambridge, UK).

3.1.6 Oligonucleotides

MBP-NEMO for	gcgaattcaacaagcacccctggaag
MBP-NEMO rev	gcggattcctactctatgcactccatgac
BamHI NEMOmM for	aaaggatccgccaccatgaacaagcaccc
EcoRI-NEMOmM rev	aaagaattcctactctatgcactccatgac
Nemo R309A a	gctgacttccaagctgaggcgcatgcccgggagaagc
Nemo R309A b	gcttctcccgggcatgcgcctcagcttggaagtcagc
Nemo R312A a	gctgagaggcatgccgcggagaagctggtgg
Nemo R312A b	ccaccagcttctccgcggcatgcctctcagc
Nemo R313A a	gctgagaggcatgcccgggcgaagctggtggagaagaagg
Nemo R313A b	ccttcttctccaccagcttcgcccgggcatgcctctcagc
Swap-NEMO-CoZi-	cgacagccacattaagagcagcaaggatgagggagctcagtggaattgtacc

TAB2-NZF megaprimer for	
Swap-NEMO-CoZi-TAB2-NZF megaprimer rev	cctcaatcctggctgactcatggcagaaatgccttggcatctcacactgttc
SWAP NEMO-UBZ-TAB2-NZF GST for	cctgaagaacctcctgacttcgatgagggagctcagtggaattgtacc
SWAP NEMO-UBZ-TAB2-NZF GST rev	gcggccgctcgagtcagaaatgccttggcatctcacactgttc
SWAP NEMO-UBZ-TAB2-NZF pB rev	ccactgtgctggcgaattcctagaaatgccttggcatctcacactgttc
SWAP NEMO-UBZ-TAB2-NZF AU1 rev	gttaacgcggccgctcctagaaatgccttggcatctcacactgttc

3.1.7 Additional materials and machines

Nitrocellulose membranes (GE healthcare)

PVDF membranes (Hybond)

Mono S 5/50 GL column (GE Healthcare)

Äkta Purifier (GE Healthcare)

Concentrators (GE Healthcare)

Empty Disposable PD-10 Columns (GE Healthcare)

3.1.8 Bacterial strains and cell lines

DH5a (Invitrogen)

BL21 (Amersham)

BL21 pRARE (Novagen)

MEFs (Marc Schmitt-Supprian)

HEK293-T (ATCC)

HEK293 Phoenix Ecotrophic (ATCC)

3.2 Methods

3.2.1 Mammalian cell culture methods

3.2.1.1 Cultivation of mammalian cells

Mammalian cells were grown in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum and 5 µg/l penicillin and 5 mg/l streptomycin (DMEM-FCS-P/S). The cells were incubated with 5% CO_2 in 95% humidity at 37° C. Cells were passaged by trypsinisation (0.05% (w/v) Trypsin, 0.5 mM EDTA in PBS) for 2-3 minutes at 37° C.

3.2.1.2 Transfection of mammalian cells

Mammalian cells were transfected using Genejuice™ or FugeneHD™. $3x10^5$ Cells were seeded into a dish with a diameter of 3.5 cm one day before transfection. Usually 1 µg of DNA was mixed with the recommended amount of transfection reagent and 100 µl of DMEM and the mix was added to the cells.

3.2.1.3 Viral transduction of cells and generation of MEFs stably expressing transduced genes

In order to produce viral particles containing the gene of interest Ecotrophic Phoenix retroviral packaging cells (ATCC) were transfected with pBABE-plasmids in which the gene was integrated. After 36-48 h the supernatant (2 ml) containing the virions was removed and filtered through a sterile filter with a pore size of 0.4 µm, which allows the virions to pass through. 500 µl of virion-containing medium was added to the MEFs that were seeded the day before in a dish with 3.5 cm diameter. The cells were incubated with the virions over night and the medium was exchanged the next day with DMEM-FCS-P/S. When the cells reached 90% confluency they were trypsinised and seeded in 10 ml DMEM-FCS-P/S and 3.5 mg/ml puromycin to select cells that have the plasmid stably

integrated into their genome. Positive selected cells were constantly kept under selection pressure with puromycin containing DMEM-FCS-P/S.

3.2.1.4 Cell lysis

Mammalian cells from cell cultures were lysed by adding chilled lysis buffer. Lysed cells were collected in a 1.5 ml tube and centrifuged for 15 min at 16,000 rcf to remove aggregates and membrane particles. The supernatant was transferred to another tube and used either for Western blot analysis, pull-down assays or immunoprecipitation.

3.2.2 Molecular biology techniques

3.2.2.1 Agarose gel electrophoresis

DNA fragments were separated with 0.5% to 2% agarose gels according to their size. For preparation of the gels agarose was solubilised in 1x TAE buffer and heated in the microwave. Before loading the sample on the gel 6x concentrated loading buffer was added. For running the gel electrophoresis the gels were embedded in 1x TAE and run at 100 Volt for 30 min. In order to visualize the DNA the gel was put in an ethidium bromide containing solution for 15 min and afterwards illuminated with UV-light.

3.2.2.2 DNA fragment amplification with PCR

DNA fragments of interest were amplified using polymerase chain reaction (PCR). PCR was performed in a Biometra thermocycler using Pfu or PWO DNA polymerases. A typical reaction contained 10 ng template cDNA, 10 pmol of primer-oligonucleotides and 0,5 µl of enzyme in a total volume of 50 µl. 35 reaction cycles were applied using annealing temperatures suitable for the primer and elongation time suitable for the DNA fragment length. 1 µl of 6x DNA-sample loading buffer was added to 5 µl of the PCR-product and the DNA was separated with an agarose gel and visualised with ethidium bromide staining.

3.2.2.3 Restriction digestion of plasmids and PCR-fragments

20 µl of purified PCR-product or 3 µg of vector or 1 µg of extracted plasmid from a ligation was digested in a volume of 50 µl using buffer and restriction conditions as suggested by the manufacturer. The reaction was stopped by the addition of 6X DNA-sample buffer. Restricted PCR products or vectors were purified with the QIAquick PCR purification kit™ and eluted in 40 µl distilled water (GIBCO).

3.2.2.4 Ligation of DNA fragments

In order to ligate two DNA fragments usually 4 µl of restriction enzyme digested and purified vector and PCR-product were incubated with T4-DNA ligase in 10 µl reaction volume for 1 h at RT or at 16° C o/n. For plasmid amplification *E. coli* bacteria of the DH5a strain were transformed with 10 µl of the mutagenesis reaction.

3.2.2.5 Site directed mutagenesis of plasmid DNA

DNA primer with the corresponding mutations were used to create point mutations in plasmids in a PCR reaction with usually 18 min elongation time in a 50 µl reaction volume. In order to remove methylated template DNA 1 µl of *Dpn*I-restriction enzyme was added to the finished PCR-reaction and incubated for 1 h at 37° C. For plasmid amplification *E. coli* bacteria of the DH5α strain were transformed with 10 µl of the mutagenesis reaction.

3.2.2.6 Exchange or insertion of larger DNA fragments with mega-mutagenesis primers

The insertion or exchange of the NZF-domain in NEMO was done by site directed mutagenesis using mega-mutagenesis primers. Mega-mutagenesis primers were produced by PCR using the human NZF-domain as template (aa 663-693) with oligonucleotides that contain the complementary NZF-sequence followed by the sequence of the site of insertion of the NEMO plasmid.

3.2.2.7 *E. coli* transformation with plasmid DNA

Competent *E. coli* strains DH5a or BL21 were transformed with plasmid DNA by heat shock. 0.5 µg, 10 µl of a mutagenesis PCR or 10 µl of a ligation were incubated on ice with 50-100 µl of competent bacteria for 10 min. Heat shock was performed for 90 seconds at 42° C. Immediately after the heat shock one ml of LB medium was added to the bacteria and the cells were mixed at 37° C for 1 h. The cells afterwards plated on LB-agar plates with the appropriate selection medium (100 µg/ml Ampicillin or 25 µg/ml Kanamycin).

3.2.2.8 Plasmid extraction from bacterial cells

Plasmids from bacterial cells were isolated using Miniprep or Midiprep kits from Qiagen. DNA from a miniprep was eluted with 40 µl distilled H_2O. DNA from a midiprep was washed in 70% ethanol and resuspended in 100 µl distilled H_2O.

3.2.2.9 RNA isolation and cDNA production

Total RNA was isolated with the RNeasy Mini Kit (Qiagen) from MEFs grown in a dish with a diameter of 3.5 mm having a confluency of ~90%. The RNA was eluted in distilled H_2O and the concentration was measured photometrically. To assess the quality of the RNA 2 µg were separated on a 1% agarose gel and the RNA was used for further experiments only if the 28S and the 18S rRNA were not degraded.

To remove DNA contaminations 2 µg of RNA were treated with 1 µl of DNaseI (Fermentas) with the appropriate buffer in a 20 µl reaction volume at 37° C for 30 min. In order to inactivate the DNaseI 1 µl of 25 mM EDTA was added to remove $MgCl_2$ and the reaction was heated for 10 min with 65° C.

To produce cDNA, 2 µg DNaseI treated RNA was incubated with 2 µg of random hexamer primer in a reaction volume of 20 µl, denatured for 5 min with 70° C and briefly chilled on ice. Subsequently 2 µl of RiboLock Ribonuclease inhibitor (Fermentas) and 4 µl of 10 mM dNTPs were added to a total volume of 39 µl and the reaction was incubated at RT for 5 min. 1 µl reverse transcriptase was added and the reaction was incubated for 10 min at 25° C and then for 60 min at 42° C.

Afterwards the reaction was stopped by increasing the temperature to 70° C for 10 min.

3.2.2.10 Quantitative Real-time PCR

qPCR was done using the SensiMix™ real-time PCR-kit (Bioline). 40 ng of cDNA were mixed with the appropriate amount of SensiMix™ real-time PCR-kit SYBR® green mix and 200 mM primer-oligonucleotides in a total volume of 10 µl.
The following program was used for quantitative PCR:

Initial denaturation 95° C, 10 min
Denaturation 95° C, 15 sec
Annealing 55° C, 20 sec
Elongation 72° C, 30 sec

3.2.3 Biochemical methods

3.2.3.1 SDS-PAGE

To separate proteins electrophoretically according to their molecular weight the denaturating SDS polyacrylamid gel electrophoresis (SDS-PAGE) was used. Resolving gels that were used had concentrations of 8-15% of acrylamid. Before loading samples were incubated with 2x SDS-PAGE sample buffer for 5 min at 95° C (ubiquitin chains were not boiled and ubiquitin pull-downs were boiled for 2 min). The electrophoresis was done using SDS-PAGE running buffer and voltage ranging from 80 (stacking gel) to 120 volt (resolving gel).

3.2.3.2 Coomassie staining

To stain proteins with Coomassie a SDS-PAGE gel was incubated in Coomassie brilliant blue staining solution for 4 h to o/n and afterwards incubated in Coomassie brilliant blue destaining solution for ~1 h.

3.2.3.3 Western blot

To detect specific proteins with antibodies they were separated by SDS-PAGE and transferred onto either a nitrocellulose membrane with a pore size of 0.45 µM (cell lysates) or onto a PVDF membrane (*in vitro* ubiquitin pull-down assays). Transfer was done in a wet electroblot system (Mino Trans-blot cell, Bio-rad), for 90 min with 90 volt in WB transfer buffer. The membranes were stained with Ponceau S to assess amount of GST- or MPB- fusion proteins used for pull-down assays. To block unspecific antibody binding sites the membranes were incubated for 1 h at RT or o/n at 4° C in WB blocking solution. The membranes were incubated with primary antibodies diluted in WB blocking solution o/n at 4° C or for 2-4 h at RT. Afterwards the membranes were washed 4x for 15 min in TBS-Tween and incubated with secondary antibody (coupled with horseradish peroxidase [HRP]) diluted in 5% milk in TBS for 45 min. The membranes were washed 4x for 15 min in TBS-Tween and incubated in chemiluminescence luminol reagent (Santa Cruz) for 1 min. A photosensitive film was laid on the membrane to visualise proteins bound to antibodies.

3.2.3.4 Concentration measurement of purified proteins

The concentration of purified recombinant proteins was measured photometrically at a wavelength of 280 nm, which corresponds to the absorption spectrum of the aromatic amino acids tryptophan and tyrosin. In order to get the exact concentration of the protein its extinction coefficient and molecular weight had to be correlated to the measured value. The extinction coefficient was determined with the ProtParam software (http://web.expasy.org/protparam/). The measured value was divided by the quotient of the extinction coefficient and the molecular weight of the measured protein.

Conc. (mg/ml) = conc. Measured (mg/ml)/[extinction coefficient/ molecular weight]

3.2.3.5 Recombinant protein purification

In order to purify GST-, MBP- or 6xHis-tagged recombinant proteins a 5 ml LB-culture (+ ampicillin or kanamycin) of BL21 *E. coli* bacteria containing the plasmid that encodes the recombinant protein was grown at 37° C o/n. The next day the culture was transferred to a 0.2–2 l LB medium (+ ampicillin or kanamycin) and grown at 37° C to an OD of ~0.6- 0.8 photometrically measured at a wavelength of 600 nm. Protein expression was induced with 0.1 mM IPTG and the culture was grown at 16° C o/n. The cells were harvested by centrifugation for 20 min at 5,500 rcf and 4° C. The cells were resuspended in 20-40 ml GST-buffer1 and lysed by sonication with 100% amplitude for 2 min with impulses of 1 sec on and 1 sec off. Triton X-100 with a final concentration of 0.5% was added and the lysates were centrifuged for 20 min at 10,000 rcf and 4° C. The supernatant containing the proteins was filtered through a filter with a pore size of 0.4 µm and glutathione sepharose beads (GST-tag), amylose beads (MBP-tag) or Ni-NTA beads (His-tag) were washed with 1x PBS and added to the lysates. The beads were incubated for 1-2 h and washed 3x in 30 ml GST-buffer2. Finally the beads were resuspended in 500 µl GST-buffer3 and stored at 4° C. In order to assess the quality of the purified proteins 5 µl of the beads suspension was separated by SDS-PAGE and visualised by Coomassie staining.

3.2.3.6 Cleavage of recombinant proteins with PreScissionTM protease

The E2 Ubc13 and Uev1a were inserted in the vector pGEX-6P-1 (GE healthcare) that contains a PreScission™ protease (GE healthcare) cleavage site between the GST gene and the multiple cloning site. GST-Ubc13 and GST-Uev1a were purified as described in 3.2.3.5. The purified GST-proteins were incubated with the recommended amount of PreScission™ protease in PreScission™ protease cleavage buffer at 4° C for 16 h under rotation in 1 ml reaction volume. Since the proteins were cleaved while they were coupled to the glutathione beads and the protease also has a GST-tag, which immobilises it to the beads, the supernatant of the cleavage reaction only contained the cleaved protein. The beads were loaded on a PD-10 column to obtain a complete separation of the supernatant and the beads. 3x 1 ml cleavage buffer was added to the beads on the column and

the flow through was collected combined with the first flow through and concentrated with a protein concentrator with a cut off of 10 kDa to a final concentration of ~2 mg/ml. The proteins were shock frozen in liquid nitrogen and stored at -80° C.

3.2.3.7 Production of linear ubiquitin chains

Linear ubiquitin chains were first purified as GST-di, tri- and tetraUb as described in 3.2.3.5. The ubiquitin gene sequences were inserted in the vector pGEX-4T-1, which has a thrombin protease restriction site between the GST-gene and the multiple cloning site. In order to cleave the ubiquitin chains from the GST-tag, the beads containing the fusion protein were incubated with thrombin protease according to the instructions of the provider in 1x PBS for 16 h at RT. The reaction was stopped by addition of the serine protease inhibitor PMSF to a final concentration of 1 µM. The separation of the cleaved ubiquitin chains in the supernatant from the beads was done as described for the separation of PreScission™ protease cleaved proteins in 3.2.3.6.

3.2.3.8 Lysine linked ubiquitin chain production

3.2.3.8.1 polyubiquitin chain reaction *in vitro*

Lysine linked polyubiquitin chains were produced *in vitro* using the following reaction compositions.

K63-linked ubiquitin chains:	K48-linked ubiquitin chains:
1.4 mM ubiquitin (Sigma-Aldrich)	2.8 mM ubiquitin (Sigma-Aldrich)
10 mM ATP	10 mM ATP
1 µM E1 enzyme	1 µM E1 enzyme
8 µM E2 Ubc13	25 µM E2 cdc34
8µM E2 Uev1a	1x Ubiquitin chain reaction buffer
1x Ubiquitin chain reaction buffer	ad 1 ml H_2O
ad 1 ml H_2O	

E1 enzyme was a kind gift from David Komander and was produced in and purified from insect cells. GST-Uev1a and GST-Ubc13 were bacterially purified and cleaved with PreScission™ protease.
Reactions were incubated for 4 h at 37° C and controlled by SDS-PAGE and Coomassie staining of 0.1% of the reaction.

3.2.3.8.2 Purification of polyubiquitin chains by cationic exchange

In order to purify and separate the polyubiquitin chains according to their length the finished polyubiquitin chain reaction was first diluted with 50 mM NH_4Ac, pH 4.5 to 25 ml and left on ice for 10 min to precipitate the enzymes. The ubiquitin chain dilution was loaded on a Mono S 5/50 GL (GE healthcare) column using the Äkta purifier system (GE healthcare). In order to separate the ubiquitin chains according to their size the bound ubiquitin was eluted with a linear gradient from 0 to 50% high salt buffer (50 mM NH_4Ac, pH 4.5, 1 M NaCl) over 180 column volumes. The elution of ubiquitin was monitored with UV-light at a wavelength of 230 nm. Fractions corresponding to a peak containing ubiquitin chains of a certain length were analysed by SDS-PAGE and Coomassie staining and pooled. Pooled fractions were concentrated with a sample concentrator with 3 kDa cut off (Vivaspin GE healthcare). To exchange the buffer the samples were diluted and concentrated 3x with 15 ml of 50 mM Tris, pH 7.6. Ubiquitin chains were stored at -80° C.

3.2.3.9 (Co-)Immunoprecipitation

The interaction of endogenous proteins was studied using immunoprecipitation combined with SDS-PAGE and Western blot analysis. Cell lysates were incubated with an antibody raised against the protein of interest with a ratio of 1 :100 o/n at 4° C. The next day the cell lysates were incubated with Protein A/G-sepharose beads for 1 h. The beads were washed 3x in lysis buffer and bound proteins were eluted with 25-50 µl of SDS-PAGE sample buffer. The immunoprecipitated proteins were analysed by SDS-PAGE and Western blot with antibodies against the directly immunoprecipitated or against co-immunoprecipitated proteins.

3.2.3.10 MBP- and GST- pull-down assays

In order to investigate the interaction of proteins with ubiquitin 10-20 µg of immobilised MBP- or GST-tagged bacterially expressed and purified recombinant proteins were incubated with 1 µg of ubiquitin in pull-down buffer on a rotator o/n at 4° C. In order to ensure incubation with equal amounts of ubiquitin a pull-down buffer/ubiquitin mix was prepared and the same amount of mix was added to each immobilised protein used for a certain experiment. After incubation the beads were 3x centrifuged at 300 rcf and washed with 500 µl pull-down buffer without BSA. The bound proteins were eluted with 50 µl SDS-PAGE sample buffer and analysed by SDS-PAGE and either Coomassie staining or Western blot. The usage of equal amounts of immobilised proteins was confirmed by staining the PVDF membranes with Ponceau S or the proteins were detected by probing with a specific antibody in a Western blot.

3.2.3.11 Nuclear extraction

Nuclei of MEFs were isolated by first lysing a 90% confluent 3.5 cm dish with 600 µl hypotonic buffer. Cells were transferred to a 1.5 ml tube and in order to burst the cell membrane cells were incubated with hypotonic buffer on ice for 15 min. Samples were centrifuged for 1 min at 16,000 rcf to harvest intact nuclei and the supernatant containing the cytoplasm was transferred to another 1.5 ml tube. To remove traces of cytoplasm the nuclei were washed 2x with 500 µl of hypotonic buffer and centrifuged at 16,000 rcf. After the last wash the pelleted nuclei were lysed in 30 µl hypertonic buffer and incubated for 30 min. The lysates were centrifuged for 5 min at 16,000 rcf and the supernatant containing the nucleoplasma was collected.

3.2.4 Confocal laser scanning microscopy

Cells were grown on cover slips under conditions described in 3.2.1.1. After treatment cells were fixed in 2% PFA and mounted on mounting media containing DAPI (1:1000). Images were taken by Carl-Zeiss 510 UV confocal microscope with 400 times magnification.

4. Results

4.1 *In vitro* production and purification of K48- and K63- linked ubiquitin chains

An important aim of this study was to thoroughly analyse the interaction of NEMO with different types of ubiquitin chains and to investigate how these interactions influence the activation of NF-κB. For the biochemical interaction analysis it was necessary to first produce sufficient amounts of polyubiquitin chains. Linear linked ubiquitin chains were expressed in and purified from bacteria as described in (3.2.3.7). However, it is not possible to produce lysine-linked ubiquitin chains this way, since they cannot be genetically encoded. Instead K48- and K63- linked ubiquitin chains were synthesised in an enzymatic reaction *in vitro* (protocol modified from[115]). In order to obtain polyubiquitin chains with a certain linkage type, bacterially purified ubiquitin conjugating E2 enzymes that specifically produce only one ubiquitin chain type, were used.

4.1.1 Production and purification of K48-linked ubiquitin chains

K48-linked polyubiquitin chains were produced by incubating 25 mg of monoubiquitin with the ubiquitin activating enzyme E1 and the E2 cdc34, which specifically catalyses the conjugation of the C-terminus of one ubiquitin to the lysine 48 of another ubiquitin[116]. In order to assess the quality of the chains produced by this reaction 1% of the reaction volume was separated by SDS-PAGE and the gel was stained with Coomassie, which visualised a ladder of K48-linked ubiquitin chains with defined length, ranging from diUb to tetraUb, as well as higher molecular weight polyubiquitin chains of undefined length (Figure 6A). Since for the interaction experiments it was necessary to have isolated ubiquitin chains of defined length, K48-linked ubiquitin chains of mixed length were separated by cationic exchange. Eluted ubiquitin chains were measured with UV-light at a wavelength of 230 nm and fractions corresponding to a single peak on the chromatogram contained chains with the same length (Figure 6B). The fractions containing K48-linked ubiquitin chains with two or four ubiquitin

molecules or monoubiquitin were pooled, concentrated and the purity was checked by SDS-PAGE and Coomassie-staining (Figure 6C). This confirmed that K48-linked di- and tetraUb were separated sufficiently to perform binding studies with these ubiquitin chains.

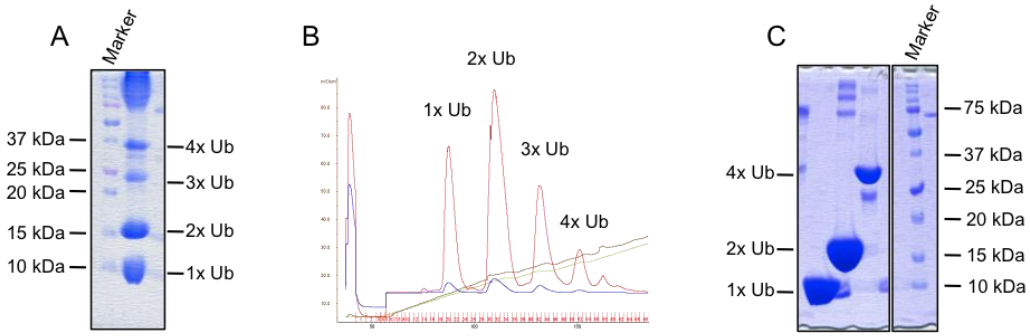


Figure 6: Synthesis and purification of K48-linked ubiquitin chains. A) 1% of an *in vitro* reaction to produce K48-linked ubiquitin chains was separated by SDS-PAGE and the gel was stained with Coomassie. B) Chromatogram of eluted ubiquitin chains by cationic exchange from a MonoS 50/5 column. Peaks represent fractions with a K48-linked chain of defined length. C) Coomassie stained SDS-PAGE gel with purified and concentrated K48-linked ubiquitin chains.

4.1.2 Production and purification of K63-linked ubiquitin chains

The production of K63-linked ubiquitin chains followed principally the same procedure as with the K48-linked chains. The E2s used to produce K63-linked chains were Ubc13 and Uev1a, which form a heterodimer that specifically synthesises this type of chains[79]. The fractions containing K63-linked chains of the same length, judged by the peaks of the elution chromatogram, were pooled, concentrated and buffer exchanged (Figure 7A). Afterwards the samples were separated by SDS-PAGE and visualised by Coomassie staining (Figure 7B). This revealed a purity of the K63-linked chains from di- to heptaUb sufficient for *in vitro* interaction studies. The fractions that contained chains of different lengths were also pooled and concentrated to obtain K63-linked ubiquitin chains of mixed lengths (Figure 7B, lane 8).

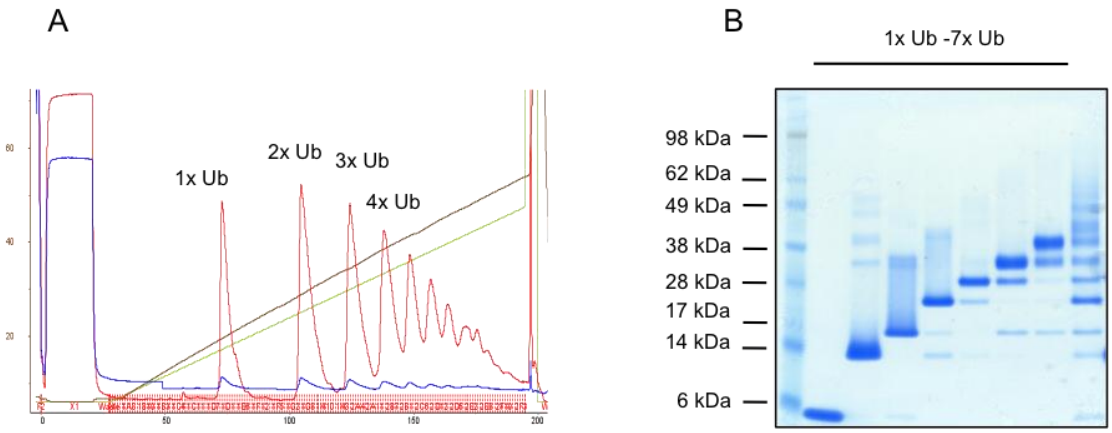


Figure 7: Synthesis and purification of K63-linked ubiquitin chains. A) Chromatogram of eluted ubiquitin chains by cationic exchange from a MonoS 50/5 column. Peaks represent fractions with K63-linked chains of defined length. B) Coomassie stained SDS-PAGE gel with purified and concentrated K48-linked ubiquitin chains.

4.2 Analysis of the interaction mode of NEMO with polyubiquitin chains

4.2.1 Analysis of the interactions of full-length NEMO and NEMO domains with different types of tetraUb

It is known that NEMO can interact with ubiquitin and that this interaction is important for its function as a regulator of the NF-κB pathway[102, 111, 117]. It has been proposed, that NEMO selectively interacts with K63-linked ubiquitin chains, however a detailed analysis of the ubiquitin chain binding preference of full-length NEMO hasn't been done. In order to better understand the interaction of NEMO with ubiquitin, it is essential to analyse in detail how full-length NEMO and certain NEMO domains interact directly with different types of ubiquitin chains. To this end *in vitro* binding studies with bacterially purified recombinant NEMO proteins and K48-, K63- and linearly linked tetraUb chains were performed (Figure 8). The binding of GST-full-length NEMO as well as the UBAN, the ZnF and the C-terminus (comprising the UBAN and the zinc finger (ZnF)) of NEMO to different types of ubiquitin chains was compared (Figure 8A). The ZnF of NEMO has been shown to bind monoUb,

though with a rather low Kd of ~250 μM[118]. However, when the interaction of tetraUb with the ZnF of NEMO was assessed in pull-down experiments, no interaction of the GST-ZnF with all types of tetraUb chains tested was observed (Figure 8B). The UBAN domain has recently been identified as a UBD in ABIN-proteins and, with high sequence homology, also in the proteins optineurin and NEMO[98, 99] (Figure 5B). In agreement, a region comprising the UBAN has been shown to be important for the interaction of NEMO with ubiquitin[111]. In order to analyse the interaction of the NEMO-UBAN with ubiquitin in more detail, purified GST-UBAN was incubated with different types of tetraUb. In accordance with previous interaction studies[111, 117], the UBAN of NEMO strongly interacted with ubiquitin chains (Figure 8B). However, in contrast to aforementioned studies, reporting a selective binding to K63-linked ubiquitin chains, this interaction showed strong chain type selectivity towards linear tetraUb while interaction with K63-linked tetraUb was extremely weak and only visible after very long exposure of the film (Figure 11B). The C-terminus of NEMO interacted with linear tetraUb with a similar strength as the UBAN. Interestingly, the C-terminus also interacted with K63- and K48-linked tetraUb, although the interaction was weaker than with linear tetraUb. The increase in affinity of the C-terminus of NEMO towards lysine-linked ubiquitin chains in comparison to the UBAN alone was not surprising, since Laplatine *et al.* could show that the whole C-terminus of NEMO has a higher affinity towards K48- and K63-linked tetraUb than the isolated UBAN domain, when analysed in a fluorescence spectroscopy assay[119]. In line with this, similar binding strengths of the C-terminus to K48- and K63-linked tetraUb were observed (Figure 8B; binding in comparison to the input), suggesting that the C-terminus has only weak binding selectivity towards K63- and K48-linked ubiquitin chains. When the binding of full-length GST-NEMO was analysed, it revealed that the binding of K63- and K48-linked tetraUb was rather weak. On the other hand, full-length NEMO showed a strong interaction with linear tetraUb, indicating a selective binding mechanism towards linear ubiquitin chains of full-length protein.

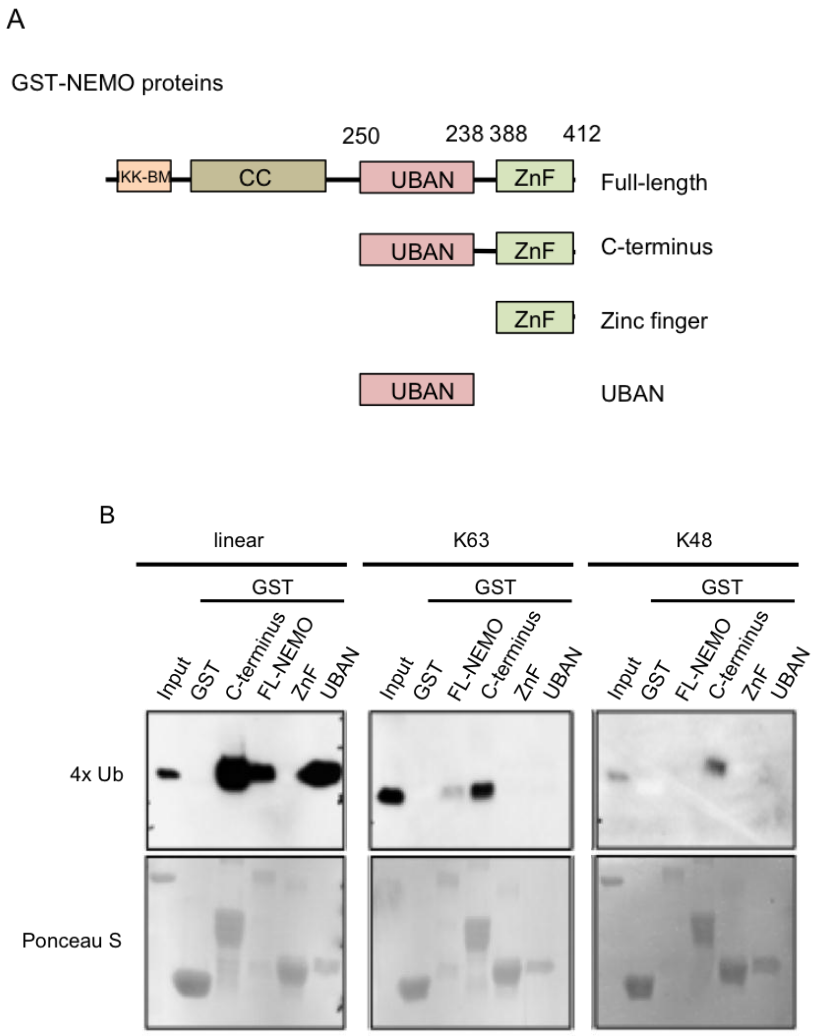


Figure 8: Analysis of the interaction of NEMO with ubiquitin. A) Schematic model of recombinant NEMO peptides fused with GST. Full-length NEMO or indicated domains were fused with GST and expressed in and purified from bacteria. B) Interaction of NEMO and NEMO-domains with different types of tetraUb. 1 µg of linear, K63- or K48-linked tetraUb were incubated with the indicated recombinant GST-tagged NEMO peptides, that were bound to glutathion-sepharose beads and a pull-down assay was performed. Bound proteins were eluted with SDS-sample buffer and separated by SDS-PAGE. Ubiquitin was visualised by Western blot analysis. IKK-BM, IKK-binding motif; CC, coiled coil; FL, full-length; ZnF, zinc finger.

Since the previous interaction studies revealed a rather weak interaction of full-length NEMO with K63-linked tetraUb (Figure 8B), the interaction of NEMO with longer K63-linked ubiquitin chains was assessed. To this end, full-length NEMO was incubated with K63-linked chains of mixed length and pull-down assays were performed (Figure 9). Interestingly, only longer K63-linked ubiquitin chains

of unclear length bound efficiently to GST-full-length NEMO, while shorter chains only weakly interacted with full-length NEMO. This is consistent with previous studies, where also only longer K63-lined chains bound efficiently to NEMO, but shorter chains, as tetraUb, did not[111, 117]. Results in Figure 8 and Figure 9 together show that full-length NEMO is not a strong and efficient binder of K63-linked chains, but only very long chains seem to bind NEMO more potently. This indicates that the interaction of full-length NEMO with K63-linked chains is rather unspecific, while already linear tetraUb strongly interacts with full-length NEMO. Moreover, the interaction between all NEMO domains and linear tetraUb is much stronger than with K63- and K48-linked tetraUb (Figure 8B).

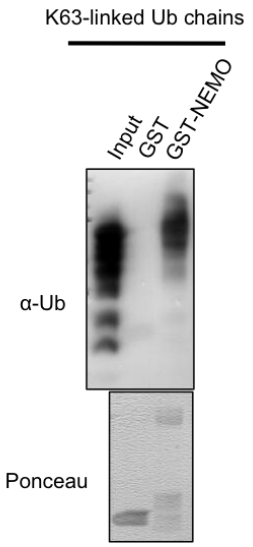


Figure 9: Full-length NEMO interacts preferentially with longer K63-linked ubiquitin chains than with K63-linked tetraUb. Mixed K63-linked ubiquitin chains were incubated with immobilized GST-NEMO and GST-pull-down assays were performed. Bound proteins were eluted with SDS-sample buffer and separated by SDS-PAGE. Ubiquitin was visualised by Western blot analysis.

4.2.2 Binding selectivity of NEMO to linear ubiquitin is determined by an unconventional ubiquitin interaction surface

An important method to determine the interaction mode of proteins is the structural analysis of a crystal of these proteins in complex. Since NEMO seems to interact preferentially with linear ubiquitin chains and this interaction seems to depend on the UBAN, a co-crystal structure of the NEMO-UBAN and linear diUb should shed light on the nature of this strong interaction. In a collaborative effort Prof. Soichi Wakatzuki and his co-workers were able to solve the co-crystal structure of the mouse NEMO-UBAN and linear diUb, which revealed a novel

modus of how ubiquitin interacts with a UBD[99] (Figure 10A). The UBAN of NEMO forms a coiled-coil dimer and one diUb molecule binds to each side of the dimer in an almost symmetrical fashion. The distal ubiquitin with the free N-terminus in the linear diUb molecule binds the UBAN at its N-terminal part, thus called the distal patch (the distal ubiquitin makes a peptide bond with the N-terminus of the proximal ubiquitin, which has a free C-terminus that could be ligated to a substrate). The proximal ubiquitin moiety interacts with the, so-called proximal patch of the UBAN, which is at the C-terminal end of the UBAN-domain.

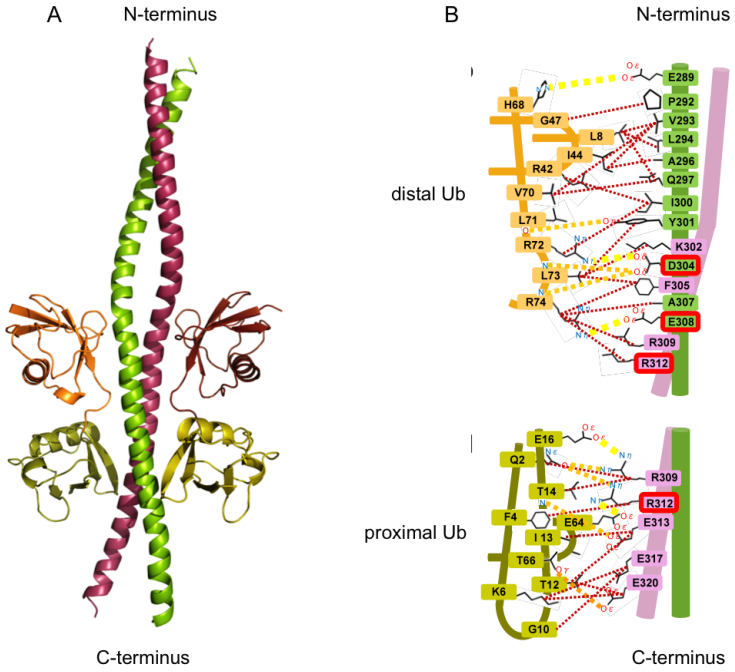


Figure 10: Co-crystal structure of the NEMO-UBAN and linear diUb. A) Structure of the UBAN-homodimer in complex with two linear diUb molecules. B) Schematic diagram of the interaction of the distal and the proximal ubiquitin with the UBAN-domain. Residues circled in red are found mutated in patients with ectodermal dysplasia and interact with residues of either the distal or the proximal ubiquitin. The crystal structure was obtained in the lab of Prof. Wakatsuki, Tokyo, Japan[99].

The distal ubiquitin binds mainly via hydrophobic residues of the classical UBD interacting I44 patch to hydrophobic regions of the N-terminal part of the UBAN (Figure 10B). Interestingly, the proximal ubiquitin binds to the more C-terminal part of the UBAN in a novel fashion, involving residues around the linker region. This includes residues R72 and R74 of the distal moiety and Q2, E16, T14, E64,

T12 and G10 of the proximal moiety, which engage mainly in polar interactions with residues R309, R312, E313, E117 and E120 of the UBAN. These residues around the linker of linear diUb are crucial for the interaction with NEMO, since mutating R72, R74 and Q2 could inhibit the binding[99]. This explains the high binding selectivity of the UBAN towards linear ubiquitin chains, since the proximal patch of the UBAN interacts with certain residues of the proximal and the distal moiety and these residues are only in the right orientation for UBAN interaction with a linear linkage, leading to a higher affinity. Moreover, the co-crystal structure of the UBAN in complex with K63-linked diUb explains the weak binding to this type of chains[120] (Figure 20B). The distal moiety of K63-linked diUb also interacts with its hydrophobic patch with the distal patch of the UBAN. However, the UBAN cannot bind the linker region in K63-linked diUb, which means there is only one interaction site between the UBAN and K63-linked diUb. In conclusion, the high affinity of linear ubiquitin towards the UBAN is enabled by a second binding site at its linker region and this binding is only possible with this type of chain and gives this binding a high selectivity.

To confirm the interaction modes discovered in the crystal structure, certain amino acid residues of the UBAN and ubiquitin were analysed biochemically for their importance for the interaction. Derived from the co-crystal structure of NEMO with linear diUb several residues were chosen and mutated to alanine, using site directed mutagenesis. Work done by colleagues in the lab revealed that bacterially purified GST-UBAN interacts with linear diUb but not with K63-linked diUb[99]. This interaction with linear diUb was lost when the amino acids V293, Y301 and K302 were mutated. These residues are part of the distal patch of the UBAN, which binds to the distal ubiquitin in diUb molecules and they are also important for the interaction with K63 and linear tetraUb[99] (Figure 10B). When the residues R309, R312 and E313 were mutated the interaction to linear di- and tetraUb was lost while the interaction with K63-linked tetraUb was still intact, indicating a qualitatively different binding mode. In order to further investigate the ubiquitin binding relevance of UBAN residues in the proximal patch, which is important for the selective high affinity interaction with linear ubiquitin, a UBAN mutant where only residue E313 was mutated to alanine was

used for interaction studies (Figure 11). This revealed, that this single point mutation was sufficient to abrogate the interaction between GST-UBAN and linear diUb, emphasising the importance of the proximal UBAN-patch for the selective interaction with linear ubiquitin chains (Figure 11A). The next question was if the mutation of residue E313 inhibited the interaction of the UBAN with ubiquitin in general or if residue E313 is of specific importance for the interaction with linear ubiquitin chains. Since the UBAN doesn't interact on a detectable level with K63-linked diUb using GST-pull-downs, but a weak interaction is observed for K63-linked tetraUb, these longer K63-linked chains were used for this analysis. K63-linked tetraUb chains were incubated with GST-wild-type UBAN and various UBAN mutants and pull-down experiments were performed (Figure 11B). This showed, that the triple mutant V293A, Y301A and K302A, as well as the mutant F305A blocked the binding of the UBAN to K63-linked tetraUb. These are all residues in the UBAN that bind to the distal ubiquitin. In contrast a mutation in residue E313 didn't inhibit the interaction of the GST-UBAN to this ubiquitin chain type. This shows, that a single point mutation in the part of the UBAN that binds to the proximal ubiquitin of linear diUb is sufficient to selectively block this interaction, while this mutant is still able to bind with low affinity to K63-linked tetraUb.

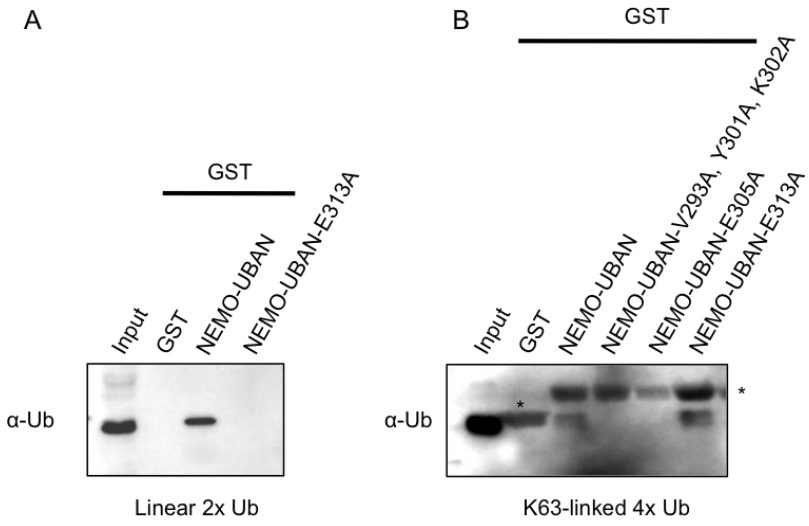


Figure 11: UBAN residue E313 is important for the interaction with linear diUb but not with K63-linked tetraUb. A) 1 µg linear diUb was incubated with the indicated recombinant GST-tagged NEMO peptides, that were immobilised on glutathion-sepharose beads and a GST-pull-down assays were performed. Bound proteins were

eluted with SDS-sample buffer and separated by SDS-PAGE. Ubiquitin was visualised by Western blot analysis. B**)** 1 µg K63-linked tetraUb was incubated with the indicated recombinant GST-tagged NEMO peptides and GST-pull-down and Western blot analysis were performed as in A. *Background from GST-proteins.

4.3 A competitive interaction study reveals preferential interaction of full-length NEMO with linear ubiquitin chains

The results shown above, together with structural data, indicate that the UBAN needs at least two interaction sites to efficiently bind ubiquitin[99, 120] (Figure 10 and Figure 11). This is suggested since both, the binding of K63-linked diUb with the wild-type UBAN or linear linked tetraUb with the UBAN that is mutated at one patch, and thus can only interact with the other patch, doesn't seem to be sufficient for a high affinity interaction. On the other hand, K63-linked tetraUb does interact with the wild-type UBAN, indicating that this longer K63-linked chain has an additional binding site with this UBD. Linear diUb has a up to 100 times higher affinity towards the UBAN than diUbs of other chain types and also linear tetraUb shows stronger interaction with NEMO than other chain types[99, 101, 102] (Figure 8). However, the UBAN combined with the ZnF has a higher affinity towards K63 and K48-linked tetraubiquitin than the UBAN alone, possibly by providing an additional binding site (Figure 8). In line with this, it was also shown, in a fluorescence spectroscopy based interaction study, that the C-terminus of NEMO, containing the UBAN and the ZnF, has a higher affinity towards longer K63- and K48-linked ubiquitin chains compared to the diUb variant of each chain type[119]. If longer lysine linked chains have an additional binding site with NEMO, increasing their affinity, the question is, if NEMO preferably binds to lysine linked chains or to linearly linked ubiquitin chains that have also two binding sites with the UBAN in a longitudinal orientated interaction (Figure 10A). Since it seems that most, if not all types of ubiquitin chains have to bind to the distal patch of the UBAN to be able to interact with NEMO[99, 120] (Figure 11), it is not possible that different ubiquitin chain types bind to NEMO at the same time. This means, that binding of NEMO to different chain types, present at a given time, occurs in a competitive manner. To address the

question of ubiquitin binding preference of NEMO, here an approach was used, where equal concentrations of different ubiquitin chain types were incubated with purified MPB- or GST-tagged NEMO in the same tube and binding to NEMO was monitored by washing off unbound chains and subsequent Western blot analysis (Figure 12 and Figure 13). First the binding preference of GST-tagged NEMO-UBAN as well as the full C-terminus towards K63 and linear tetraUb was compared. To this end, 1 µg of each tetraUb chain was incubated with the indicated recombinant GST-proteins and a competitive pull-down assay was performed (Figure 12). Since ubiquitin chains of the same length but with different linkage display distinctive mobility on SDS-gels[20], it is possible to distinguish the different types of chains, using the same anti-ubiquitin antibody. In this experiment we observed a clear binding preference of the UBAN and the C-terminus of NEMO towards linear tetraUb, while an interaction with K63-linked tetraUb could not be detected. This shows that although the affinity towards K63-linked tetraUb increases when the ZnF is added to the UBAN of NEMO[119] (Figure 8), the binding to linear ubiquitin is still clearly preferred in a situation where both types of chains are present (Figure 12).

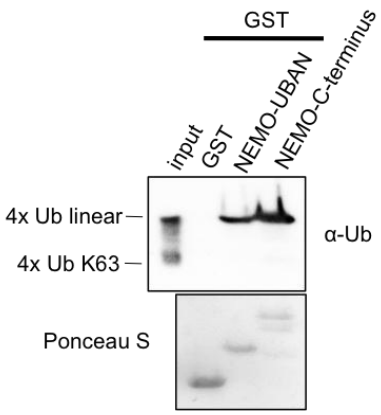


Figure 12: The UBAN and the C-terminus of NEMO preferentially interact with linear tetraUb in competition with K63-linked tetraUb. 1 µg of K63- and linear tetraUb were incubated with the indicated recombinant GST-Proteins, that were bound to glutathion-sepharose beads and a GST-pull-down assay was performed. Bound proteins were eluted with SDS-sample buffer and separated by SDS-PAGE. Ubiquitin was visualised by Western blot analysis.

After discovering that the UBAN as well as the C-terminus of NEMO preferentially interact with linear tetraUb the questions arose, if full-length NEMO shows a binding preference towards a certain type of ubiquitin chain and if a possible binding preference might depend on the length of the chains.

Recently it was shown that the UBAN also binds to K11-linked chains with similar affinity than K48- and K63-linked chains[108]. In order to analyse in a competition assay which chain type full-length NEMO preferentially binds to, MPB-NEMO was incubated with K11-, K63- and linear-triUb (Figure 13A). This showed that full-length NEMO selectively bound linear triUb, while no binding to K11- and K63-linked chains was observed (Figure 13A). So far it was shown that longer lysine linked ubiquitin chains with at least four moieties bind to full length NEMO (Figure 8) and structural as well as biochemical analysis indicates that they bind only to the distal patch of the NEMO UBAN domain as compared to a longitudinal arrangement of linear chains that recognizes both, the proximal and the distal patch[99] (Figure 10 and Figure 11). In accordance with results shown in Figure 12, when GST-NEMO or MBP-NEMO was incubated with linear and K63-linked tetraUb, linear tetraUb was able to block binding of K63-linked tetraUb (Figure 13B). In contrast to NEMO, receptor-associated protein 80 (RAP80) selectively bound to K63-linked tetraUb, while no binding to linear tetraUb was detected (Figure 13C). This is in agreement with a previous study, showing that the two UIM-domains of RAP80 as a unit selectively bind K63-linked, but not K48- or linearly linked diUb[65, 121] (Figure 3). Since the length of ubiquitin chains influences the affinity towards NEMO and especially longer lysine-linked ubiquitin chains show an increase in binding, possibly by providing additional binding sites[99] (Figure 9 and Figure 11), the binding of K63-linked hexa- and heptaUb to NEMO was investigated in competition with linear tetraUb (Figure 13D). In both cases NEMO bound only to linear tetraUb, while binding to hexa- or to hepta- K63-linked ubiquitin chains could not be observed. Comparing the ubiquitin binding preference of NEMO with a mixture of long K63-linked ubiquitin chains and linear tetraUb revealed that the selective interaction preference for linear chains is high enough to compete out even very long K63-linked chains, since NEMO didn't pull down K63-linked chains up to at least decaUb in the presence of linear tetraUb (Figure 13E). Chains that were longer than decaUb showed interaction with NEMO despite the presence of linear tetraUb, but since ubiquitin chains longer than decaUb were not separated, it's not clear what size of K63-linked chains was necessary for binding and also if

this precipitation of high molecular weight ubiquitin chains is a specific interaction. In order to get more detailed information about the ubiquitin binding preference of NEMO towards linear chains, 1 µg of K63-linked tetraUb were incubated together with different amounts of linearly linked tetraUb (Figure 13F). Interaction of NEMO with K63-linked tetraUb alone is rather weak (Figure 13F). In contrast, already the addition of 20 ng of linear tetraUb leads to a strong linear chain binding, emphasising the high binding preference of NEMO to this chains type. The increase of binding of NEMO to linear chains correlates strongly with an increasing input where already 100 ng of linear ubiquitin chains shows a much stronger binding than 1 µg of K63-linked ubiquitin chains. Moreover, the linear chains compete out the binding toward K63-linked ubiquitin chains, clearly visible by a decreased K63-linked chain binding, already in the presence of only 200 ng of linear ubiquitin chains. Taken together, these results demonstrate the quantitative distinction in ubiquitin chain binding selectivity of full-length NEMO towards linear ubiquitin chains compared with long K63-linked ubiquitin chains.

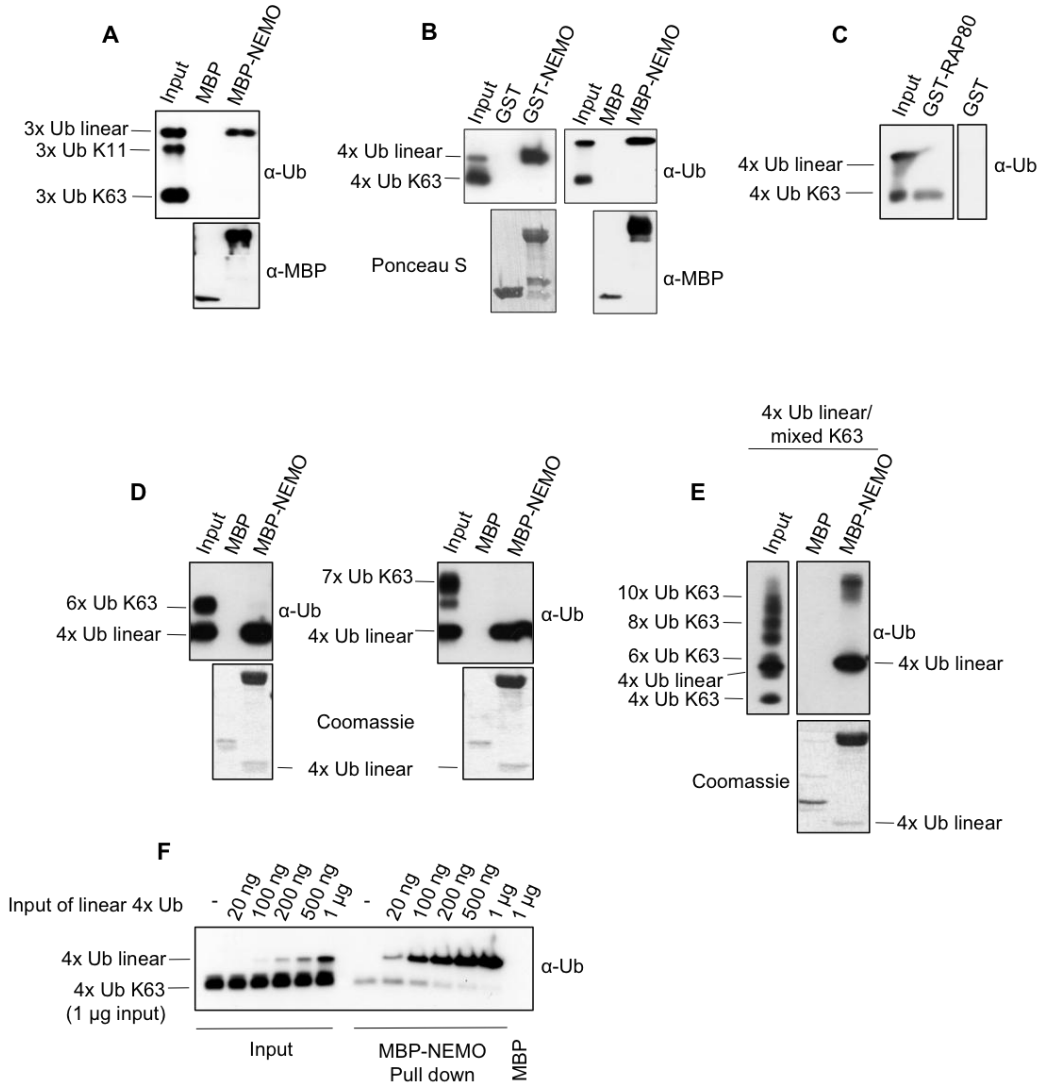


Figure 13: Full-length NEMO selectively interacts with linear ubiquitin chains. A-E) 1 µg of the indicated ubiquitin chains (in F the indicated amounts of ubiquitin chains were used) were incubated with MBP or GST-full-length NEMO, or GST-full-length RAP80, which were immobilised by sepharose beads and pull-down assays were performed. Bound proteins were eluted with SDS-sample buffer and separated by SDS-PAGE. Ubiquitin was visualised by Western blot analysis.

4.4 Analysis of the interaction of the A20-ZnF7 with different types of ubiquitin chains

Ubiquitin chain binding of NEMO is important for the activation of NF-κB and as shown, NEMO preferentially interacts with linear ubiquitin chains[99, 111] (Figure 9, Figure 12 and Figure 13). The DUB A20 is a ubiquitin editing enzyme,

that can also act as an E3-ligase[113]. A20 is crucial for silencing NF-κB activity in various ways and for many of its NF-κB inhibiting functions the seven zinc finger domains at its C-terminus are important[113, 122, 123]. Recently, the ZnF4 and ZnF7 have been shown to be involved in ubiquitin chain binding with a possible preference of the ZnF4 for K63-linked ubiquitin chains[123, 124]. In collaboration with the group of Rudi Beyaert, the biochemical evaluation of the binding of the A20 ZnF7 to ubiquitin chains and its functional relevance in the TNFα-induced activation of NF-κB was assessed[125].

Since linear ubiquitin chains play an important role in the activation of TNFα-induced activation of NF-κB[54, 70, 99], and A20 is important for silencing NF-κB, the A20-ZnF7 was probed for its ability to bind linear ubiquitin chains in competition with other chain types (Figure 14). Incubation of GST-A20-ZnF7 with linear- and K48-linked tetraUb together exhibited a clear binding preference for linear tetraUb, while a ZnF7 mutant was not able to bind ubiquitin at all (Figure 14A). Interestingly, when linear tetraUb was incubated with the structural similar K63-linked ubiquitin chains (Figure 1B), there was a weaker binding preference of the ZnF7 for linear ubiquitin chains (Figure 14B compare binding to input). Since the length of ubiquitin chains might influence the interaction with a UBD (Figure 11), the competitive binding of A20-ZnF7 to linear- and K63-linked triUb was assessed (Figure 14C). Interestingly, in contrast to tetraUb, the ZnF7 showed a high binding preference towards linear ubiquitin with these shorter chains. To further investigate the role of the length of the ubiquitin chains in binding to the A20-ZnF7, a pull-down analysis with GST-A20-ZnF7 and different types of diUb was performed (Figure 14D). Surprisingly, with diUb no interaction between the ZnF7 and K63-linked chains was observed, which was in harsh contrast to longer K63-linked chains that even bound in competition with linear chains (Figure 14B). Even more surprising was the clear and strong binding between the ZnF7 and linear diUb, revealing a strong contrast in the interaction quality of the ZnF7 with these two chain types depending on their length (Figure 14 compare B and D). K11- and K48- linked diUb, the two other chain types examined for their binding with the ZnF7, hardly showed any interaction and also longer K48-linked ubiquitin chains don't

interact with the ZnF7 of A20[125] (Figure 14D). These results indicate that the A20-ZnF7 is a highly selective binder of short linear ubiquitin chains, but it can also interact with longer ubiquitin chains of other linkages.

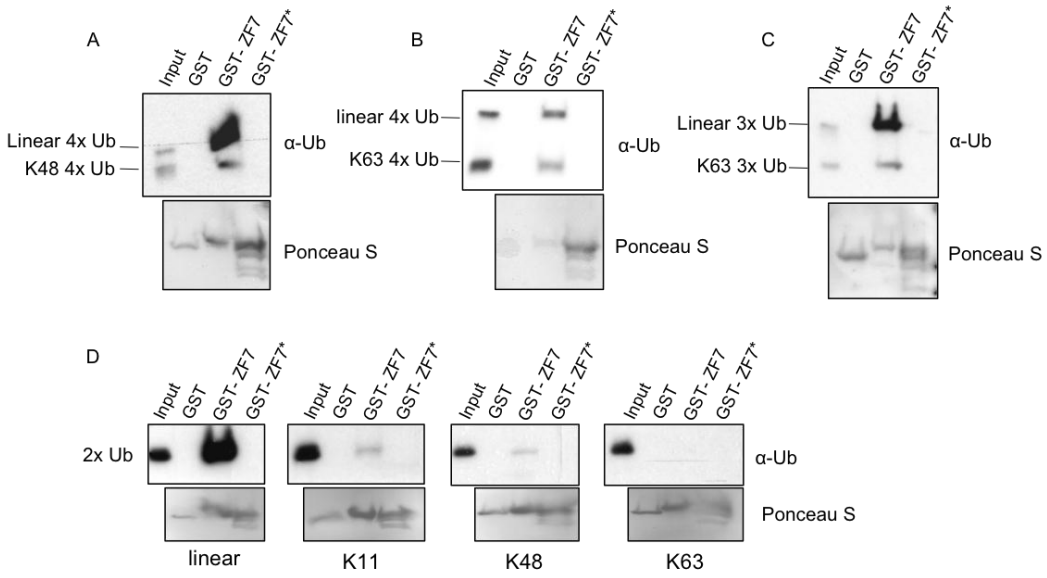


Figure 14: The ZnF7 of A20 preferentially interacts with linear ubiquitin chains and this selectivity depends on the length of the chains. A-D) 0.5 µg of the indicated ubiquitin chains were incubated with GST-A20-ZnF7 (wild-type and ubiquitin non-binding mutant), which were immobilised by sepharose beads and pull-down assays were performed. Bound proteins were eluted with SDS-sample buffer and separated by SDS-PAGE. Ubiquitin was visualised by Western blot analysis. GST-ZnF7* represents a mutant that is not able to bind ubiquitin.

4.5 Functional relevance of the interactions of NEMO with linear polyubiquitin

4.5.1 Interaction of NEMO with linear ubiquitin chains is necessary for the activation of NF-κB

Several studies investigated the role of the interaction of NEMO with ubiquitin chains in the activation of NF-κB[111, 117, 126, 127]. It was proposed that especially the interaction with K63-linked ubiquitin chains is the key mechanism how NEMO and IKK get activated by various NF-κB inducers. However, it was also shown that K63 of ubiquitin is not important for the TNFα-induced

activation of NF-κB and the knock-out of the K63-specific E2 Ubc13 in mice didn't affect NF-κB-signalling[128, 129]. Considering aforementioned and the discovery that NEMO preferentially interacts with linear ubiquitin chains, the question arose what relevance that interaction has for the activation of NF-κB (Figure 8, Figure 12, Figure 13).

After identifying NEMO mutants that selectively lose the interaction with linear but not with K63-linked ubiquitin chains[99] (Figure 11), the impact of the interaction of NEMO with linear ubiquitin chains on NF-κB signalling could be tested *in vivo* (Figure 15). To this end NEMO knock-out MEFs were reconstituted with wild-type and mutant NEMO and their function in the TNFα induced activation of NF-κB was examined. When NEMO knock-out MEFs, reconstituted with wild-type NEMO were treated with TNFα, similar activation of NF-κB as with wild-type MEFs was observed, as judged by the phosphorylation status and the subsequent degradation of IκBα 5 and 15 minutes after treatment respectively. In contrast, cells reconstituted with the triple NEMO mutant V293A, Y301A, K302A were not able to activate NF-κB. These residues are in the distal UBAN patch and thus are important for the binding to linear and lysine linked ubiquitin chains[99]. However, also the mutant R309A, R312A, E313A was not able to efficiently propagate the signal to activate NF-κB. This mutant selectively inhibits the interaction of NEMO with linear ubiquitin chains, but not with K63-linked tetraUb[99], indicating that the specific interaction of NEMO with linear ubiquitin chains is important for the full activation of NF-κB.

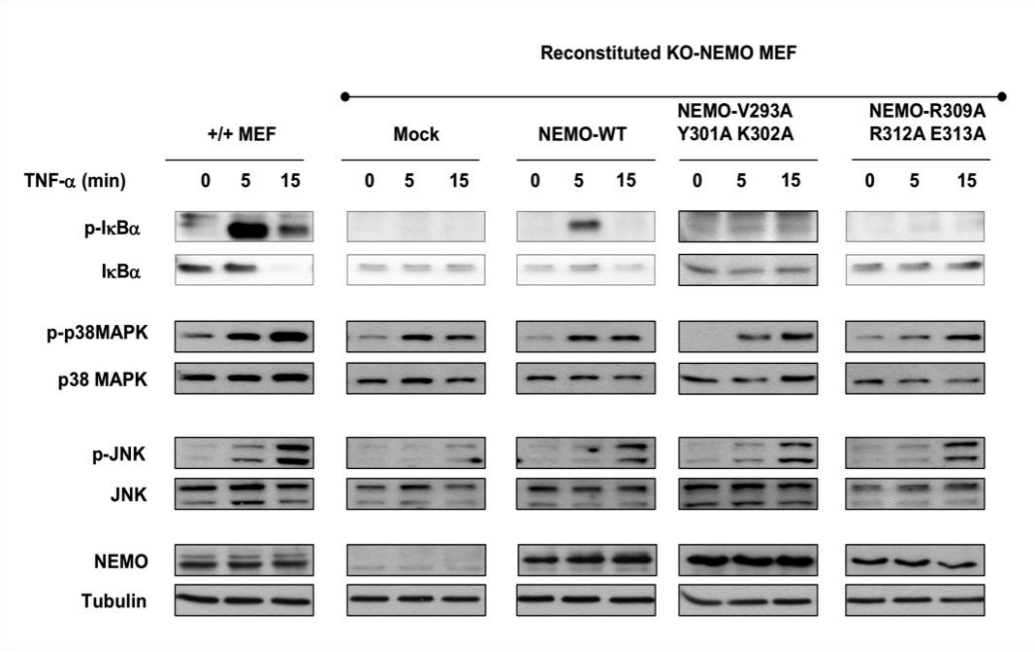


Figure 15: The interaction of NEMO with linear ubiquitin chains is necessary for the activation of NF-κB. Wild-type or NEMO deficient MEFs, stably reconstituted with NEMO or different NEMO-mutants, were untreated or treated with TNFα (20 ng/ml) for the indicated times. The cell lysates were separated by SDS-PAGE and protein content was analysed by Western blot.

TNFα is also an inducer of MAP-kinase signalling pathways that lead to the activation of p38- and JNK- MAP-kinases[129]. Both MAP-kinases, p38 and JNK were activated similarly in wild-type MEFs or NEMO knock-out MEFs, reconstituted with wild-type or mutant NEMO constructs, indicating that the inhibition of the NF-κB pathway in cells expressing ubiquitin binding selective NEMO-mutants is not affecting the MAP-kinase pathway and that TNF-receptor signalling in these cells was intact (Figure 15).

4.5.2 Interaction of NEMO with the proximal UBAN patch is necessary to rescue cells from apoptosis

Apart from the NF-κB and the MAP-kinase-signalling pathway, is the activated TNFR also responsible for the induction of TNFα activated cell death via Caspase-8 and -3 dependent apoptosis[130]. NEMO and the NF-κB pathway are crucial negative regulators of TNFα induced apoptosis[131]. In order to investigate

the role of NEMO binding to ubiquitin in the pro-survival regulation of TNFα treated cells, we used the reconstituted NEMO knock-out MEFs and treated them for 6 hours with TNFα and cyclohexamide (to inhibit cellular TNFα-production) (Figure 16). Cells that didn't express NEMO (mock) massively underwent apoptotic death upon TNFα treatment, evaluated by the degree of Caspase-3 cleavage and the condensation of DAPI-stained nuclei (Figure 16). In contrast, wild-type MEFs or NEMO knock-out MEFs, reconstituted with wild-type NEMO, did not show signs of apoptotic death. However, cells reconstituted with NEMO protein that is not able to bind linear ubiquitin chains showed strong activation of Caspase-3 as well as nuclear condensation (Figure 16). This indicates that the interaction of NEMO with linear ubiquitin chains is important for rescuing cells from TNFα induced apoptosis.

Overall these results strongly support the hypothesis that the interaction of NEMO with ubiquitin chains and especially with linear ubiquitin chains is crucial for its function as a regulator of signalling by TNFα.

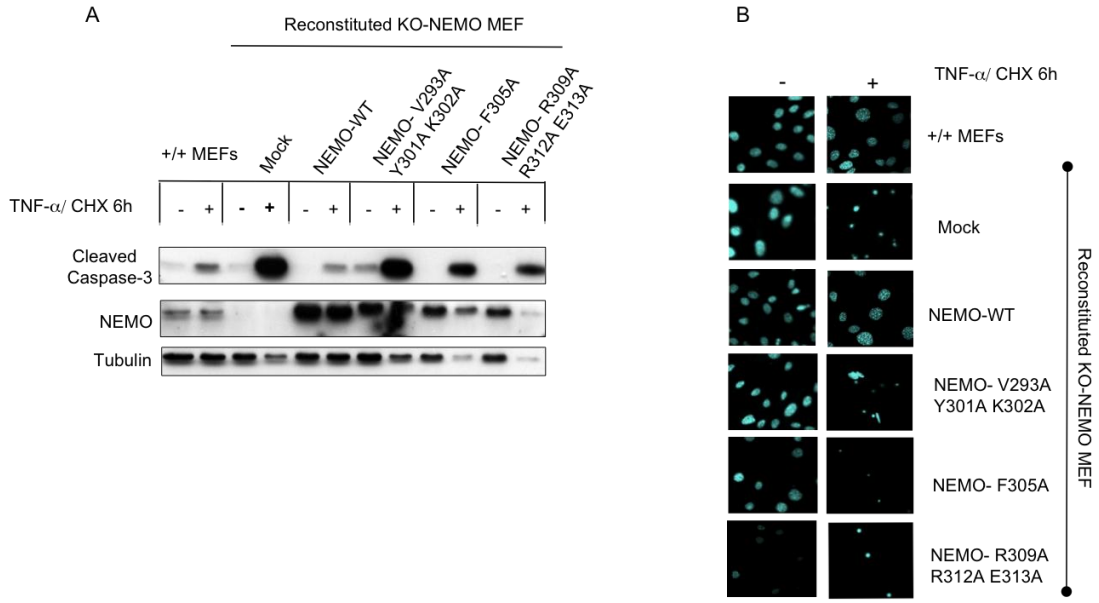


Figure 16: NEMO mutants that are not able to bind linear ubiquitin chains are impaired in rescuing cells from TNFα induced apoptosis. A) Wild-type and reconstituted NEMO knock-out MEFs were treated with TNFα/CHX for 6 h. Total cell lysate was separated by SDS-PAGE and Western blot analysis was performed. B) Cover slips with cells grown in the same dishes as in A were fixed in 2% PFA and mounted on mounting media (Biometra) containing DAPI (molecular probes 1:1000).

Apoptosis was assessed by epifluorescense microscopy showing condensed chromatin in apoptosis positive cells.

4.6 The interaction of NEMO with K63-linked ubiquitin chains is not sufficient for full NF-κB activation

4.6.1 TAB2-NZF containing NEMO has a high affinity towards K63-linked ubiquitin chains

Previous studies proposed that complex formation between NEMO and K63-linked ubiquitin chains is important for the TNFα-induced activation of NF-κB signalling[111, 117]. In order to test, whether a high affinity binding of NEMO to K63-linked ubiquitin chains can be sufficient to activate the NF-κB pathway, NEMO chimeras that contain the NZF-domain of TAB2 were engineered (Figure 17). This NZF-domain is an exclusive binder of K63-linked polyubiquitin[67, 68]. To this end, the NEMO-ZnF was swapped with the TAB2-NZF domain in combination with or without UBAN mutation at the residue F305 that blocks interaction with ubiquitin (F305A-NZF-ΔZnF-K63 or NZF-ΔZnF-K63/M1) (Figure 17A). In addition, a NEMO chimera was constructed, where the UBAN of NEMO was replaced with the NZF (NZF-ΔUBAN-K63) (Figure 17A). With these selective K63 binding NEMO chimeras the relevance of NEMO binding to K63-linked ubiquitin chains in the activation of NF-κB was analysed.

To first confirm the ubiquitin-binding properties of these NEMO variants, the respective GST-fusion proteins were purified and incubated with linearly or K63-linked tetraUb (Figure 17B). As expected, an exclusive binding of the two NZF-containing chimeras F305A-NZF-ΔZnF-K63 and NZF-ΔUBAN-K63 to K63-linked ubiquitin chains was observed. On the other hand, NEMO mutant NZF-ΔZnF-K63/M1 bound to both linearly and K63-linked ubiquitin chains. In contrast, GST-wild-type-NEMO is a selective linear ubiquitin chain binder. The F305A mutation abolished the binding to ubiquitin, as also previously shown (Figure 11B).

To further test how these chimeras bind to different ubiquitin chain types, binding competition assays with the different GST-NEMO-chimeras and linearly- and K63-linked tetraUb was performed (Figure 17C). While wild-type NEMO and the NZF-containing but UBAN-deficient chimeras selectively bound linearly and K63-linked tetraUb chains respectively, the NEMO mutant NZF-ΔZnF-K63/M1 bound to linearly and K63-linked tetraUb chains with similar strength. This is an interesting fact, since thus it is possible to compare the physiological relevance of the interactions of NEMO-proteins with similar affinity towards K63-linked or linear ubiquitin chains.

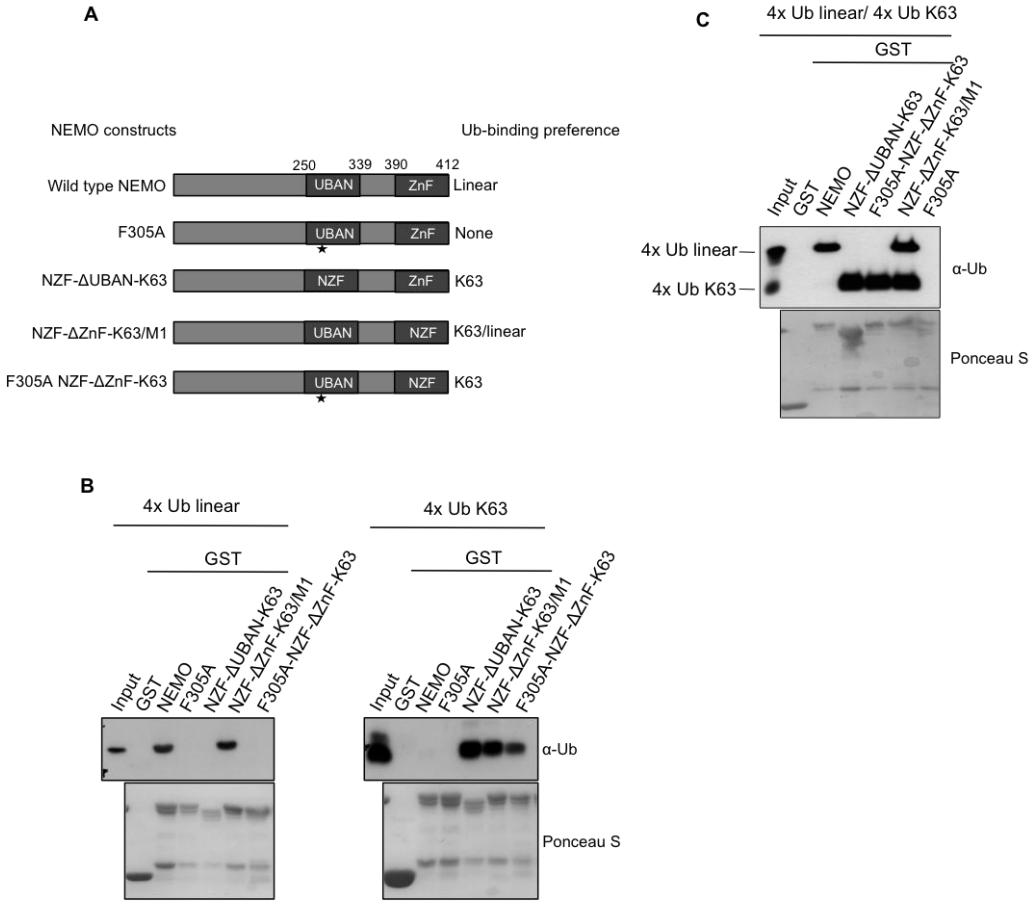


Figure 17: TAB2-NZF-containing NEMO chimeras with UBAN-mutation selectively bind K63-linked ubiquitin chains. A) Domain structures of wild-type and different NEMO-mutant constructs. NEMO constructs with indicated ubiquitin binding selectivity were used to analyse ubiquitin binding and reconstitute NEMO deficient MEFs. B) NEMO with intact UBAN has high affinity to linear ubiquitin chains and NEMO-chimeras with NZF-domain have high affinity to K63-linked ubiquitin chains. Purified GST-tagged NEMO-mutants were incubated with 1 µg of either K63- or linearly linked ubiquitin chains and pull-downs were performed. Bound proteins were eluted with SDS-sample buffer and separated by SDS-PAGE. Ubiquitin was

visualised by Western blot analysis. C) NZF- and UBAN-containing chimeras bind K63- or linearly linked ubiquitin chains with similar strength, while UBAN-only containing wild-type-NEMO selectively binds linear ubiquitin chains and NZF- but UBAN-mutant NEMO-proteins selectively bind K63 ubiquitin chains when exposed to both chain types. Purified GST-wild-type and mutant NEMO proteins were incubated with 1 µg of indicated ubiquitin chains and pull-downs were performed. Bound proteins were eluted with SDS-sample buffer and separated by SDS-PAGE. Ubiquitin was visualised by Western blot analysis.

4.6.2 TAB2-NZF containing NEMO is impaired in the activation of NF-κB

In order to address, if these K63-selective NEMO chimeras are able to mediate TNFα-induced NF-κB signalling, NEMO-deficient MEF cell lines were reconstituted with the NEMO mutants or wild-type NEMO. In these cell lines wild-type NEMO and the mutants were expressed at similar levels and could form an IKK complex when immunoprecipitated with an anti-NEMO antibody (Figure 18).

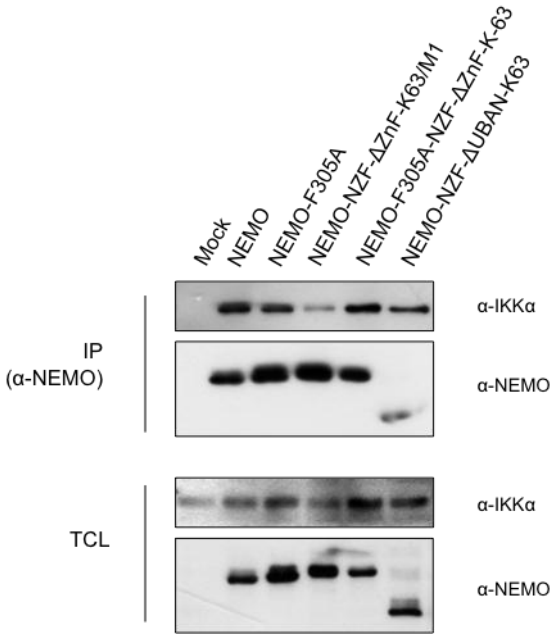
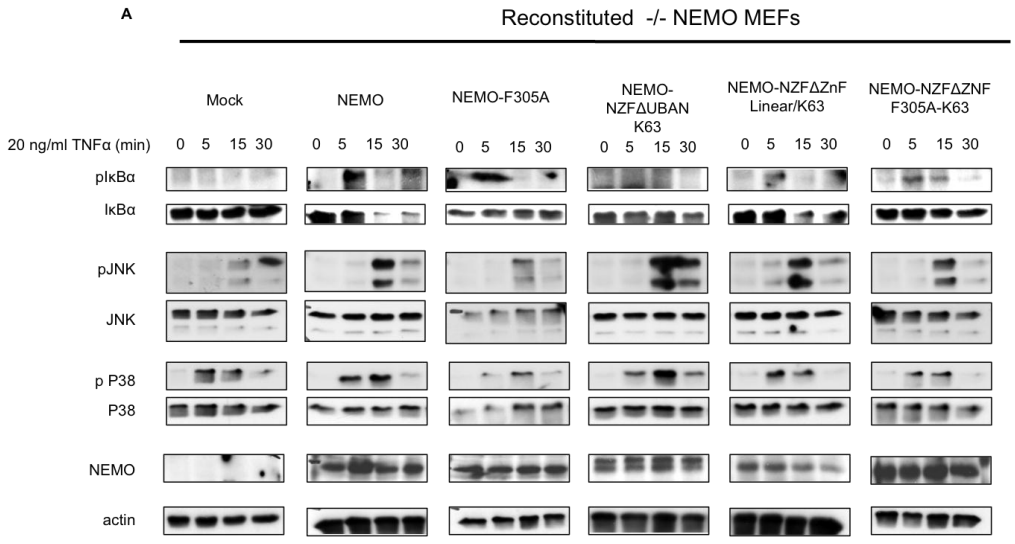


Figure 18: NZF-containing NEMO-chimeras can form an IKK complex. NEMO deficient MEFs, stably reconstituted with wild-type or different NEMO-mutants were lysed and immunoprecipitated using a NEMO-specific antibody. Immunoprecipitated proteins were eluted with SDS-sample buffer and immunoblotted. Co-immunoprecipitation of IKKα was verified with anti-IKKα antibody and expression level of NEMO variants was analysed with anti-NEMO specific antibody.

With these tools in hand, the effect of strong K63-linked ubiquitin chain binding NEMO-chimeras on TNFα-induced IKK-dependent NF-κB activation was examined (Figure 19). In NEMO deficient MEFs reconstituted with wild-type NEMO or the linear and K63-linked chain-binding mutant NEMO-NZF-ΔZnF-K63/M1, IκBα got phosphorylated and degraded after treatment with TNFα for 5 and 15 minutes respectively. In contrast, NEMO deficient MEFs or cells expressing the mutant F305A, that cannot bind ubiquitin, were not able to induce IκBα phosphorylation and degradation (Figure 19A). Interestingly, MEFs which express NEMO-NZF-ΔUBAN-K63 or NEMO-F305A-NZF-ΔZnF-K63 showed only partial degradation of IκBα with both mutants. Additionally, NEMO-K63 only binding chimeras expressing MEFs were impaired in phosphorylation of IκBα, indicating a defect in IKK-activity. These observations suggest that binding of NEMO to K63-linked ubiquitin chains is not sufficient to efficiently regulate TNFα-induced activation of NF-κB signalling. In order to further examine if the signalling defects in NEMO mutant-expressing cells were specific for NF-κB activation, TNFR-dependent activation of MAP kinase signalling pathways was tested (Figure 19A). As indicated by their phosphorylation, both MAP-kinases, p38 and JNK, are activated in cells expressing wild-type NEMO and NEMO-chimeras, suggesting that strong K63-linked ubiquitin chain binding NEMO-chimeras do not non-specifically affect MAP kinase signalling functions at the TNFR.



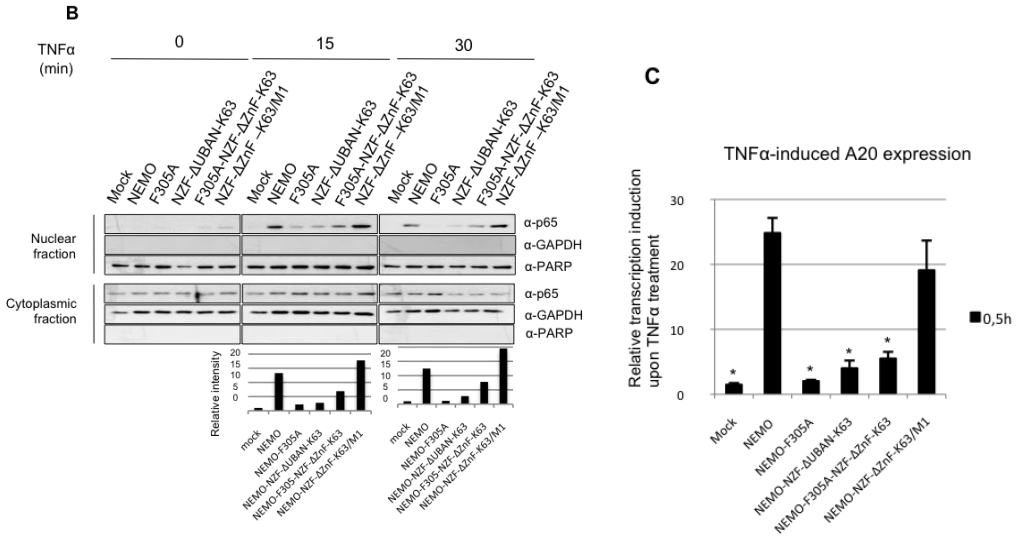


Figure 19: K63-linked binding specific NEMO-chimeras are impaired in the activation of NF-κB. A) NEMO deficient MEFs, reconstituted with linear chain binding deficient and K63-binding selective NEMO mutants are impaired in IκBα degradation upon TNFα stimulation. NEMO-deficient MEFs stably reconstituted with wild-type NEMO or different NEMO mutants were untreated or treated with TNFα (20 ng/ml) for the indicated times. The lysates were separated by SDS-PAGE, and protein content was analysed by Western blotting. B) NEMO deficient MEFs reconstituted with linear chain binding-deficient and K63 binding selective NEMO mutants are hampered in nuclear translocation of p65 upon TNFα stimulation. NEMO-deficient MEFs stably reconstituted with wild-type NEMO or different NEMO mutants were untreated or treated with TNFα (20 ng/ml) for the indicated times. Nuclear and the cytoplasmic fractions were separated by SDS-PAGE, and the presence of p65 in the nuclear fraction was analysed by Western blotting. Densitometrical quantification of p65 in the nuclear fraction in the shown Western blot is indicated in the graphs, where the intensity for vector (Mock) containing cells was set to 1, and the intensity of the other cell lines was related to mock. The p65 signal was measured using the software ImageJ and normalized by PARP level. C) NEMO-deficient MEFs, reconstituted with linear ubiquitin chain binding-deficient but K63 binding selective NEMO mutants are impaired in the induction of NF-κB target gene expression. NEMO-deficient MEFs stably reconstituted with wild-type NEMO or different NEMO mutants were untreated or treated with TNFα (20 ng/ml) for 30 minutes and mRNA was extracted. Quantitative PCR was performed as described under "Methods". Ct values of target gene A20 were normalized to Ct values of α-actin. Induction of gene expression in a cell line was determined by comparison with the expression level of the untreated sample of the same cell line. Values shown are means ±SEM (error bars) of at least three individual experiments. *$p < 0.05$: significant difference between wild-type NEMO expressing and NEMO deficient MEFs or NEMO mutants expressing MEFs.

To analyse NF-κB activation downstream of IκBα-degradation in these MEFs the nuclear translocation of the NF-κB subunit p65 was examined in TNFα-stimulated cells (Figure 19B). MEFs reconstituted with wild-type NEMO or NEMO-NZF-ΔZnF-K63/M1 showed strong activation of NF-κB, as determined by the amount of nuclear p65 after 15 and 30 minutes of TNFα-treatment. On the other hand, MEFs reconstituted with the ubiquitin binding deficient F305A-mutant did not mediate efficient p65 translocation. In contrast to MEFs with linear ubiquitin chain binding NEMO and in agreement with the results of the degradation of IκBα (Figure 19A), was the nuclear translocation of p65 markedly impaired in MEFs expressing NEMO-NZF-ΔUBAN-K63 or NEMO-F305A-NZF-ΔZnF-K63 (Figure 19B).

To further assess the NF-κB activation potential of K63-selective NEMO chimeras, the induction of the NF-κB target gene A20 was analysed (Figure 19C). After 30 min of TNFα treatment, A20 gene expression was induced 24.8 ± 2.33 SEM fold higher in wild-type NEMO expressing cells and 19.1 ± 4.6 SEM fold higher in NEMO-NZF-ΔZnF-K63/M1 cells compared to NEMO deficient cells. In contrast, induction of A20 gene expression was significantly impaired in cells with K63- only binding NEMO or NEMO-F305 mutant. Although only wild-type NEMO or the chimeras that bind linear ubiquitin chains activated NF-κB properly, the K63-binding selective NEMO chimera NEMO-F305A-NZF-ΔZnF-K63 was also able to induce A20-expression at a certain level, which is significantly higher than with the non-ubiquitin binding NEMO-F305A-mutant (Figure 19C). This is in agreement with a recent study showing that linear chain binding deficient NEMO-mutants could still partially activate NF-κB through their lower affinity binding towards K63-linked ubiquitin chains intrinsic in the wild-type protein[132]. However, even a higher affinity binding of NEMO to K63-linked chains doesn't activate NF-κB as efficiently as NEMO that is able to bind to linear chains, emphasising the importance of the binding of NEMO to linear ubiquitin chains for an efficient activation of TNFα-induced NF-κB-signalling.

5. Discussion

The modification of proteins with ubiquitin is a versatile process, to regulate many aspects of cellular physiology[2]. The many different ways how ubiquitin can modify proteins, provides the cell with a myriad of potential and specific signals that can participate in cellular functions. Most functions controlled by ubiquitylation are mediated by ubiquitin receptors, which read the signal with their UBDs[24]. Since ubiquitin can form so many different signals, receptors have evolved that specifically recognize only a small variety of the palette of potential ubiquitin forms, such as different types of ubiquitin chains or monoubiquitin. In order to understand the signalling networks that are regulated by ubiquitin it is eminent to unravel the detailed recognition processes of ubiquitin signals. In this light this study mainly aimed at elucidating the detection specificity of the ubiquitin receptor NEMO, the central regulator of the NF-κB transcription system, as well as the role these interactions play in the activation of this transcription factor.

5.1 NEMO chain binding selectivity

Previous studies reported, that NEMO selectively binds K63-linked ubiquitin chains[111, 117]. However, it was also shown that the UBAN has an up to 100 fold higher affinity towards linear diUb than towards K63-linked diUb[99, 101]. In line with that, a detailed analysis of the ubiquitin binding modes of NEMO in the present study showed, that the UBAN alone, as well as full-length NEMO, have a high binding selectivity towards linear ubiquitin chains (Figure 8B, Figure 10 and Figure 13). Here it was revealed, that the binding of K63- and K48-linked ubiquitin chains to the UBAN is rather weak (Figure 8B and Figure 11). On the other hand, the C-terminus of NEMO, which includes the UBAN and the ZnF, exhibited an increased binding to tetraUb of these chain types compared to the UBAN alone. This is in agreement with previous studies that discovered that the addition of the ZnF increases the affinity to longer lysine linked chains compared to the UBAN alone[119, 132]. However, this binding is still weaker than the binding of the C-terminus to linear chains and additionally does full-length NEMO bind

weaker to the tested lysine-linked chains than to linear polyubiquitin (Figure 8B). K63-linked tetraUb has a higher affinity to the UBAN compared to K63-linked diUb[99] and the UBAN plus the ZnF have an even higher binding strength towards K63-linked tetraUb in comparison to the UBAN alone[119]. On the other hand, there is no ZnF dependent increase in the binding to the shorter K63-linked diUb[132]. It is possible that the longer K63-linked ubiquitin chains have a second binding site with the UBAN to increase the binding strength, which doesn't exist for K63-linked diUb as seen by the structure[120]. Moreover, the ZnF might provide an even better additional binding site for longer K63-linked chains, since the UBAN plus ZnF has an increased affinity towards K63-linked tetraUb compared to the UBAN alone[119] (Figure 8B).

Indeed, current data point to the fact that NEMO needs to bind at least at two sites to ubiquitin to get an efficient interaction. The structure of the UBAN in complex with linear diUb reveals an interaction site of one moiety via its I44 patch and a second interaction site at the linker region of the diUb molecule[99] (Figure 10). This binding mode with the unconventional linker-UBD interaction is responsible for the selective high affinity between the UBAN and linear diUb, since a structure of a complex between the UBAN and K63-linked diUb shows that in this case only one moiety interacts with the UBAN[120]. The K63 diUb-UBAN interaction also happens via the hydrophobic patch of the distal moiety, as it is the case with the distal moiety of linear diUb[120] (Figure 20B). A second interaction site in this crystal was observed between the hydrophobic patch of the proximal moiety and the distal patch of the UBAN of another UBAN dimer. This cross-UBAN interaction however might also be due to the tight packaging of the molecules in the crystal and thus be a forced second interaction site that is necessary to get sufficient binding for crystal formation, yet may not reflect a physiological situation[133]. In contrast to K63-linked diUb, it could be that longer chains with this linkage have an additional binding site with the UBAN, explaining the higher binding strength. This binding site could depend on the distal patch, since mutating it also abrogates the interaction with K63-linked tetraUb[99] (Figure 11). A modelled structure of the UBAN in complex with K63-linked tetraUb displays a possible interaction mode how longer K63-linked

chains bind to two sites of the UBAN-dimer (Figure 20A). In this scenario a ubiquitin moiety at one end of the chain interacts with the distal patch on one side of the UBAN, while another ubiquitin binds to the distal patch on the other side of the UBAN.

As outlined above, it seems that NEMO needs at least two interaction sites to efficiently interact with ubiquitin. However, there are probably differences in the binding strength, depending on the sites utilised for the interaction. For example K63-linked tetraUb interacts much stronger with the C-terminus of NEMO than with the UBAN alone, though in both cases additional binding sites might be the reason for binding compared to the non-binding K63-linked diUb[99, 119] (Figure 8). It was shown that the ZnF has a weak affinity towards ubiquitin with a Kd of ~250 μM[118], thus it might be that this is a second binding site for K63-linked tetraUb which is favoured to a possible second interaction site with the second distal patch of the UBAN. It might also be that the longitudinal binding arrangement for ubiquitin chains on UBAN is favoured since this orientation is also the way the high affinity binding linear diUb interacts with the UBAN[99] (Figure 10). Thus, a stronger interaction between longer K63 linked chains with the C-terminus compared to the UBAN alone might be favoured due to a preferred additional binding site or a favoured orientation or a combination of these models. A previous study using a fluorescence spectroscopy assay showed that the C-terminus of NEMO has an affinity towards longer K63-linked chains with a Kd of 0.22 μM, which in this case was even stronger than the interaction of the C-terminus with linearly linked ubiquitin which had an affinity of 1.7 μM[119]. Another study on the other hand revealed, that the C-terminus of NEMO can exhibit a different binding behaviour with K63-linked tetraUb, depending on if the C-terminus was fixed or in solution[132]. Using a method where the C-terminus was in solution didn't lead to a binding of K63-linked tetraUb, while it still bound strongly to linear di- and tetraUb. Surprisingly, when the C-terminus of NEMO was fixed to a surface it bound to longer K63-linked chains[132]. However, results from experiments in the present study revealed that when the C-terminus of NEMO was incubated with linear- and K63-linked tetraUb together there was a clear binding preference towards linear ubiquitin chains (Figure 12). This shows

that linearly linked polyUb can outcompete the binding of longer K63-linked chains even though the C-terminus of NEMO is fixed to sepharose beads, reflecting a more solid state, that seems to be more favourable for binding to longer K63-linked chains than being in solution[132]. Possibly the special binding mode of the UBAN has a higher interaction efficiency compared to a putative binding mode of longer K63-linked chains at the ZnF. An explanation for this clear preference might be that an interaction between the UBAN and linearly linked ubiquitin chains is more efficient, maybe due to a cooperative binding of its binding sites, which might cause a faster association rate, thus this different kinetics outcompete the interaction with longer K63-linked chains. Additionally, it should be mentioned that the physiological relevant full-length NEMO has a lower affinity towards K63-linked chains than the C-terminus alone, while full-length NEMO binds linear ubiquitin chains with only a bit less efficiency, indicating some kind of regulatory role for the N-terminus for the ubiquitin chain binding of NEMO (Figure 8). Until structural data is available the reason for the binding difference of full-length NEMO and the C-terminus as well as the actual binding mode of longer K63-linked ubiquitin with the C-terminus remains enigmatic. In accordance with the preference of the C-terminus for linear chains in competition with K63-linked tetraUb (Figure 12) as well as the less decrease in affinity of the full-length NEMO for linear tetraUb (Figure 8), full-length NEMO preferentially interacts with linear chains, when incubated together with K63 linked chains (Figure 13). Moreover, even longer K63-linked chains, up to at least decaUb don't bind efficiently to full-length NEMO in the presence of the shorter linear tetraUb, although these longer chains might offer even further interaction sites (Figure 13B, D and E). Recently it has been shown, that RIP1 gets modified with K11-linked ubiquitin chains at the activated TNFR and that the UBAN of NEMO also binds to K11, with a similar affinity than to K63- and K48- linked chains[55, 108]. However, when K63-, K11- and linearly linked triUb were incubated together with full-length NEMO, there was a clear binding preference of NEMO for linear chains (Figure 13A). This, and the analysis of the binding to K48-and K63-linked tetraUb (Figure 8), indicates that full-length NEMO is a low affinity receptor for lysine linked ubiquitin chains, while being a selective and highly

efficient receptor for linear linked ubiquitin chains, due to its special interaction mode that is only possible with this linkage type. This is also emphasised by semi-quantitative interaction experiments, that estimated that the binding preference of full-length NEMO towards linear tetraUb is at least 50 fold higher than for K63-linked tetraUb (Figure 13F), although single measurements of the C-terminus suggested that the difference in affinity for these two chain types is smaller[119] (Figure 8B). Taken together these results revealed, that there is a binding preference for linear chains of full-length NEMO and the efficient binding of NEMO to linear chains through its UBAN outcompetes the binding of any other chain type that might otherwise bind with lower affinity when linear chains are not there (Figure 8, Figure 10 and Figure 13).

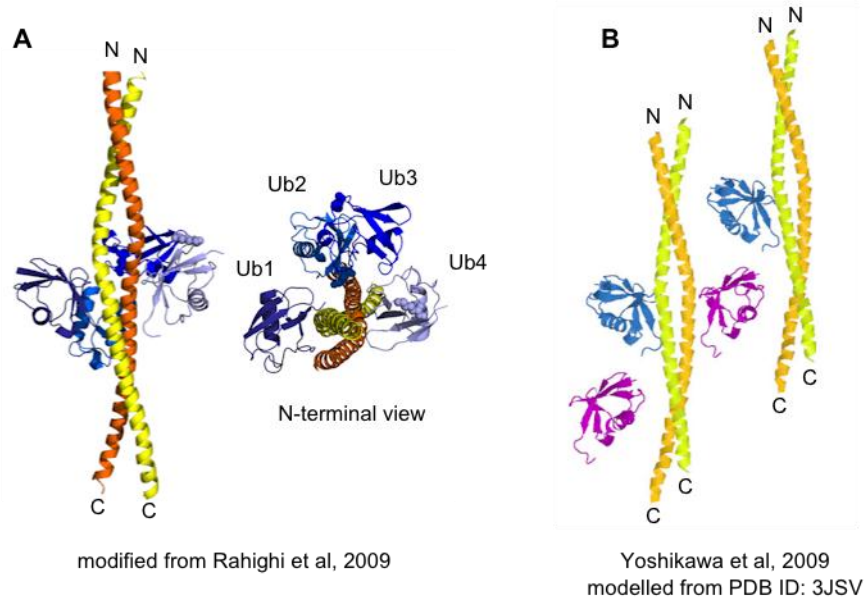


Figure 20: Structures of K63-linked ubiquitin chains in complex with the NEMO-UBAN. A) A model of a putative structure, showing how K63-linked tetraUb might bind the NEMO-UBAN in a perpendicular way, thus interacting with both distal patches of the UBAN-dimer. Modified from[99]. B) Structure of K63-linked diUb in complex with the NEMO-UBAN. The distal diUb-molecule binds with its I44 patch to the distal patch of one UBAN while the proximal diUb binds with its I44-patch to the distal patch of another UBAN homodimer. Modified from[120].

5.2 Ubiquitin chain length can influence UBD selectivity

The interaction with ubiquitin is in many cases influenced by the length of the chains[85]. For example K48-linked tetraUb seems to be the minimal chain length for optimal proteasome receptor recognition[22,23]. In this case a chain with four moieties is the optimal length for the interaction with two ubiquitin receptors at the proteasome[134]. In other instances the chain length can determine the specificity of interaction with one ubiquitin receptor or even only one UBD. The binding selectivity of NEMO and its UBAN e.g. partially depend on the length of the chains[99] (Figure 11). The UBAN has a ~100 fold higher affinity towards linear diUb than towards K63-linked diUb, which is explained by their different binding modes seen in the respective structures[99, 101, 120] (Figure 10 and Figure 20). However, this selectivity becomes less with longer lysine-linked chains, which weakly interact with the UBAN (Figure 11). These distinct binding features might play an important role in terms of signal regulation. Short chains might be a stringent signal for chain type selective UBDs, such as the UBAN, while the interaction of NEMO with longer chains could have different effects in terms of quality, meaning different outcome, or quantity, meaning more or less of the same outcome. In this light it is interesting that linear diUb fused to NEMO is already sufficient to fully activate NF-κB, when overexpressed in HEK293-T cells, since the fusion of NEMO with tri- to heptaUb didn't increase the activation[135]. This full activation of NF-κB depends on the specific binding of a linearly linked diUb by the UBAN, since the non-binding UBAN mutant F312A fused with polyubiquitin is not able to activate NF-κB and also monoubiquitin fused to wild type NEMO didn't activate NF-κB. On the other hand it might be that the selective binding of NEMO to long K63- or other lysine-linked chains is also able to activate NF-κB, however only weakly, since UBAN mutants defective only in linear chain binding have been reported to partially activate NF-κB[132]. Thus the presence of a certain chain type and the chain length could decide about the outcome by determining a strong or a weak activation of NF-κB.

Another indication for the relevance of diUb as a selective signal comes from a study where a mass spectrometrical analysis showed that RIP1 at the activated TNFR is modified with K48-, K11-, K63- and linear diUb[55]. These facts indicate

that diUb is a preferred chain length for stringent signalling at the TNFR, which might be recognized by selective ubiquitin receptors. Indeed, also the activation of the MAP3-kinase TAK1 depends on the interaction of its adapter proteins TAB2/3 with ubiquitin and the TAB2/3-NZF specifically interacts with K63-linked diUb, but not with linear diUb[67, 136]. Thus linear diUb and K63-linked diUb might provide distinct selective signals for the activation of different signalling pathways.

The importance of the chain length for UBD-selectivity is also displayed by the ubiquitin-binding of ZnF7 of A20 (Figure 14). ZnF7 has a clear binding preference for linear diUb compared to lysine-linked diUb (Figure 14D). A clear binding preference is also observed in a binding competition assay between K63- and linearly linked triUb (Figure 14C). However this preference is not so clear anymore between K63- and linearly-linked tetraUb (Figure 14B). On the other hand, the ZnF7 clearly distinguishes between the structurally more different K48, -and linear tetraUb (Figure 14A). This is a good example how the two features of a ubiquitin chain, the length and the linkage type, can individually or combined determine binding selectivity for a UBD. Short chains show a clear ZnF7 binding preference depending on the linkage type (Figure 14D), while this selectivity gets gradually lost with longer chains (Figure 14B and C), however, still keeping a selective distinction between structurally very different chains (Figure 14A), such as elongated linear and more compact K48-linked chains. As A20 is an important negative regulator of NF-κB-signalling[137] and many different ubiquitin chains are involved in the activation of this transcription factor[106], this characteristic ubiquitin binding of the A20 ZnF7 as well as other A20-domains, seems to be important for its function[124, 125].Taken together not only the ubiquitin topology, depending on the linkage, but also the chain length seems to be an important factor for UBD selectivity and thus cellular function.

5.3 NEMO binding to linear ubiquitin chains is important for NF-κB activation and TNFR-signalling

Many different types of ubiquitin chains are involved in TNFR induced NF-κB signalling[106]. For some of these chain types their role is clear, e. g. K48-

linked chains are responsible for the degradation of the NF-κB inhibitors[80]. It has been known for a while that the first discovered non-degradative ubiquitin chain type, K63-linked chains, functions in NF-κB signalling and it has also been found recently that NEMO can interact with this chain type[111, 113]. Upon these findings it has been proposed that NEMO selectively binds to K63-linked ubiquitin chains to activate NF-κB. This led to a model where ubiquitin receptors such as TAB2/3 and NEMO interact with long K63-linked ubiquitin chains, which are attached to adaptor proteins at the activated TNFR and these interactions activate MAP-kinases and NF-κB[77].

However, recently it has been discovered, that the structurally similar linear ubiquitin chains are also important for the activation of NF-κB[70, 99]. This finding was surprising, since before the discovery of the linear ubiquitin chain specific E3-ligase LUBAC it was believed that these chains don't have a physiological role in the cell, but are mere precursors for monoubiquitin[11, 19]. With the discovery of the role of linear chains, K63-linked chains as single non-degradative ubiquitin regulators of NF-κB activation got questioned. Moreover, the knock-out of Ubc13, the only K63 chain specific E2, in mice, as well as the replacement of endogenous ubiquitin with K63-mutant ubiquitin in human cells, had no effect on the activation of NF-κB via the TNFR[128, 129]. This suggested that K63-linked chains at the TNFR, and thus K63-linked chain binding of NEMO, are not necessary for the activation of NF-κB. Interestingly, the first linear ubiquitylation substrate discovered was NEMO itself and this modification was shown to be important for NF-κB signalling[70]. Supporting the role of linear chains in NF-κB-signalling were studies revealing that LUBAC is recruited to the TNFR and cells lacking LUBAC components were defective in NF-κB activation[54-56, 110]. In the light of the importance of linear ubiquitin chains for NF-κB-signalling at the activated TNFR it was interesting to find that the ubiquitin receptor NEMO is a high affinity binder of linear ubiquitin chains, while detailed analysis showed that the affinity to K63-linked chains is actually rather low[99, 101, 102] (Figure 8B, Figure 10 and Figure 13). The clear preference of NEMO binding to linearly- over lysine-linked ubiquitin chains indicated a functional relevance of this interaction (Figure 12 and Figure 13). And indeed, mutants that are still able to bind K63-

linked ubiquitin[99] (Figure 11), but selectively lost binding to linear chains, were impaired in the activation of NF-κB induced by TNFα[99] (Figure 15 and Figure 19). This strongly indicates that K63-linked chains at the TNFR are not essential for the activation of NF-κB while linear chains are, and at least one of their functions, if not the most important one, is the regulation of the activity of the IKK-complex by binding to NEMO[135].

The importance of NEMO binding to linear ubiquitin chains in the activation of NF-κB was confirmed in NEMO deficient B-cells that were reconstituted with the linear chain binding deficient triple-mutant R309A, R312A, E313A[99]. Stimulation of these cells expressing the R309A, R312A, E313A-mutant with different NF-κB inducing compounds lead to significantly less activation of NF-κB compared to those reconstituted with wild-type NEMO. Further support for the relevance of linear chains in NF-κB signalling brought the finding that LUBAC components are also present in the activated CD40-receptor, which is also a member of the TNFR superfamily, and that LUBAC is important for CD-40 signalling[55]. Additionally, the activation of NF-κB in different cell types, which lack any of the three LUBAC components, is defective[54-56, 70, 110]. Cells that lack SHARPIN seem especially defective in TNFR-family dependent activation of NF-κB while IL-1βR or TLR-signalling is not as much attenuated[54-56]. Interestingly, although the non-canonical NF-κB-pathway gets mainly activated by TNFR-family members it is not affected by the lack of expression of LUBAC-components, which is in sharp contrast to the canonical pathway[56]. For instance the lymphotoxin β-receptor (LTβR) induces the canonical and the non-canonical pathway[138], however upon stimulation of SHARPIN deficient cells with LTβ only the phosphorylation and degradation of IκB is inhibited, while the processing of p100 to the transcriptional active p52 is not effected[56]. Since one of the main differences between the canonical and the non-canonical NF-κB-pathway is that NEMO is not involved in the non-canonical pathway[94], and the lack of linear chains leads to a specific impairment of the canonical pathway, this indicates that one of the main function of linear ubiquitin chains in NF-κB activation is constituting a signal for the IKK-regulator NEMO.

The TNFR can also activate Caspase-8 dependent apoptosis, which is usually inhibited by the pro-survival activity of NEMO and NF-κB[130]. Without NEMO and the activation of NF-κB cells undergo rapid TNFα induced apoptosis[131] (Figure 16). Additionally to the activation of anti-apoptotic genes there is also an NF-κB independent role of NEMO in the inhibition of TNFα-induced apoptosis, since when NEMO knock-out MEFs, reconstituted with wild-type NEMO, were treated with TNFα they didn't die by apoptosis, despite the fact that gene expression was blocked by cyclohexamide (Figure 16). Interestingly, the ability of NEMO to rescue cells from TNFα -induced apoptosis depended on its ability to bind to ubiquitin, since when the cells were reconstituted with ubiquitin binding deficient NEMO-mutants they died from apoptosis, just as NEMO knock-out cells (Figure 16). Moreover, the specific binding of NEMO to linear chains was necessary to rescue the cells from apoptosis, since mutants in the proximal UBAN-patch also died upon treatment with TNFα (Figure 16). This indicates that the specific high affinity binding of NEMO to linear ubiquitin chains, additionally to the regulating of IKK-activity, is also crucial for the regulation of the pro-survival pathway. This is in line with studies showing that linear chains are important to rescue cells from TNFα-induced apoptosis[54, 139]. For example SHARPIN deficient mice have the immunodeficiency disease CPDM (chronic proliferative dermatitis) that also has hallmarks of apoptosis and this phenotype is rescued when crossed with mice that lack TNFα, indicating a linear chain dependent pro-survival signal at the TNFR[54, 55]. Additionally, there is another survival pathway that is regulated by the binding of NEMO to ubiquitylated RIP1[140]. The activated TNFR can also induce necrosis when the pro-survival pathway and apoptosis are inhibited[141]. In a recent study by O'Donnell et al. it was shown that NEMO deficient cells die from TNFα-induced necrosis, when Caspases are inhibited, and this can be rescued by reconstitution with wild-type NEMO but not with ubiquitin binding deficient NEMO[140]. This is in line with the necessity of NEMO to bind linear ubiquitin chains to rescue cells from TNFα-induced apoptosis. Taken together these studies reveal an overall importance of NEMO binding to linear ubiquitin chains in the regulation of TNFR-signalling.

The discovery of many different types of ubiquitin chains revealed a complex signalling system at the TNFR that can give signalling a regulated directionality through the binding of selective ubiquitin receptors but also provide certain redundancy in the signalling events[85, 91, 107]. For example the interaction of linear chain binding deficient NEMO with K63-linked ubiquitin can in some instances still partially activate NF-κB[132] (Figure 19). Also the NF-κB inhibiting protein A20 can interact with K63-linked and linearly linked chains via its C-terminal zinc fingers and the ZnF7 shows selective binding to short linear chains, but also a bit more plasticity towards longer ubiquitin chains[123] (Figure 14). However, many ubiquitin receptors at the TNFRSC have a selective affinity towards a certain chain type, at least up to a certain length or concentration[67, 68] (Figure 11, Figure 13 and Figure 14). Thus it might be that signalling events at the TNFR are tightly regulated by an orchestrated production of different types of ubiquitin chains with different lengths. Possibly short ubiquitin chains produced at the right time on the right place could give the TNFR a regulated directionality that is necessary for deciding which pathway will be activated. This is in accordance with the mass spectrometrical analysis of the activated TNFRSC that found that RIP1 was modified with K63-, K48-, K11- and linear diUb[55]. Thus, the activation of NF-κB at the TNFR seems to have a regulated sequence of events that was partially delineated by a study, which analysed the recruitment of components to the activated TNFR[110]. Upon activation the proteins TRADD, RIP1 and TRAF2/5 bind the TNFR. TRAF2 recruits cIAP1/2, which can synthesise different kinds of lysine linked ubiquitin chains[108], and this process is required for TNFR-function[110]. These E3-ligases build lysine-linked ubiquitin chains on many components at the receptor that might have different lengths[55], which then recruit HOIP and thus LUBAC by the interaction of its UBD[110]. Long lysine-linked chains could recruit and activate a small amount of IKK by binding to NEMO[132] (Figure 8B and Figure 13F). LUBAC then synthesises linear ubiquitin chains on NEMO-molecules already present, RIP1 and possibly other substrates[54, 55, 70]. Finally these linear chains constitute a strong signal to recruit and activate NEMO for a full and efficient activation of NF-κB. Here it is interesting to note that the steady state concentration of linear ubiquitin chains in the cell is rather

low[54]. However, experiments using the absolute protein quantification method AQUA could show that upon activation of LUBAC their concentration increases rapidly[54]. Considering the highly efficient binding of NEMO to linear ubiquitin this could be a strong specific signal for efficient recruitment and activation of IKK and thus NF-κB (Figure 13 and Figure 15). Indeed, already linear diUb is able to activate the purified IKK-complex while K63- or K48-linked diUb is not[135]. Moreover, linear diUb-fused to NEMO is sufficient to fully activate NF-κB because adding longer chains doesn't increase the activation. This stringent regulation of NF-κB activation by linear ubiquitin chains also became apparent by the experiments that showed that the TAB2-NZF containing NEMO-chimeras, with high binding selectivity for K63-linked chains, are also impaired in the activation of NF-κB, although the affinity is similar to that of the UBAN towards linear chains (Figure 17 and Figure 19). This confirms that binding of NEMO to K63-linked chains cannot serve as an efficient NF-κB activating signal at the TNFR. K63-linked chains on the other hand can activate the MAP-kinase pathway by selective interaction with the TAK1-adapters TAB2/3 via their NZF-domains[67, 136]. Thus, due to the chain type selective receptors TAB2/3 and NEMO the TNFR has the possibility to regulate the MAPK- and the NF-κB-pathway independently, which is supported by the fact that the impairment of NEMO to bind linear chains and activated NF-κB, doesn't affect the activation of JNK and p38 (Figure 15 and Figure 19). Taken together, the activation of the TNFR leads to the recruitment of proteins, of which many can get ubiquitylated by cIAPs (Figure 21). This can lead to a weak recruitment and activation of IKK through weak interactions of NEMO with lysine-linked ubiquitin chain. Only when LUBAC produces linear ubiquitin chains at the TNFR the signal for NEMO recruitment and IKK activation is strong enough to fully activate NF-κB (Figure 15, Figure 19 and Figure 21).

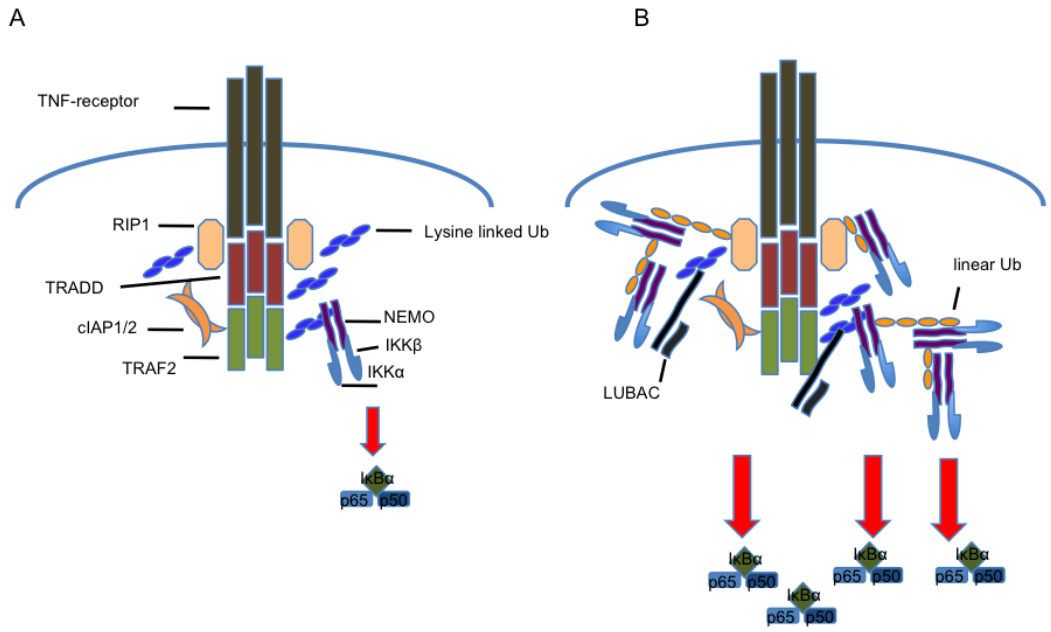


Figure 21: Schematic model how linear ubiquitin chains induce strong and efficient NF-κB activation. A) Activation of the TNFR triggers its trimerisation and the recruitment of TRADD, RIP1, TRAFs, cIAP1/2 and other proteins. TRAFs and cIAPs are E3-ligases that can produce lysine linked ubiquitin chains. NEMO can weakly bind to these chains and induce NF-κB, though inefficiently. B) The recruitment of LUBAC to the TNFR through lysine linked ubiquitin chains enables the production of linear ubiquitin chains, e.g. on RIP1 to which NEMO can bind strongly. NEMO itself gets linearly ubiquitylated and due to its high affinity to linear chains many IKK-complexes can cluster very stably, which creates a strong NF-κB activation signal.

The model where different ubiquitin chain types facilitated a tight regulation, but also some redundancy, of the signalling events at the TNFR might also hold true for the downregulation of TNFR induced NF-κB-signalling. Apart from NEMO, there are at least four other protein containing a UBAN-domain, which are called ABIN1/2/3 and optineurin[98]. It was shown that the other UBANs also bind linear ubiquitin chains and ABIN1 and optineurin are negative regulators of NF-κB[98, 100, 142]. ABIN1 seems to bind to ubiquitin chains on NEMO to recruit A20 which can deubiquitylate NEMO[98]. For optineurin it was shown that it competes with NEMO for the binding to ubiquitylated RIP1 to inhibit NF-κB-signalling[142]. In this study it was assumed that it is the binding of optineurin to K63-linked chains that inhibits NF-κB-signalling, however, knowing now about the role of linear ubiquitin chains and that both, NEMO and optineurin are linear chain

receptors[100, 143] (Figure 10 and Figure 13), the concept of competitive binding of NEMO and optineurin might also be true for linear chains. Thus, UBAN containing NF-κB-inhibitors might specifically downregulate linear ubiquitin chain mediated signalling but might also be involved in the inhibition of K63-linked ubiquitin chain signalling. Another study discovered that optineurin inhibits NF-κB-signalling by recruiting the DUB CYLD to the TNFRSC, which is an important DUB in the downregulation of NF-κB[73, 144]. CYLD has a high DUB activity against K63-linked ubiquitin chains, which has been shown to be important for silencing NF-κB[145]. However CYLD also deubiquitylates linear ubiquitin chains and since this chain type and CYLD play important roles in the TNFRSC it is possible that this DUB also removes linear chains from proteins that can activate NF-κB[20].

Recently K11-linked ubiquitin chains have been implicated in TNFR-signalling and they also interact with the UBAN of NEMO[55, 108]. Interestingly, the DUB Cezanne, that is known for silencing NF-κB at the TNFR[146], preferentially hydrolyses this chain type[112]. Thus, K11-linked ubiquitin chains constitute another chain type in the ubiquitin network and my help to activated signalling. K11-linkd chains can weakly interact with the UBAN, but so far no UBD is known that specifically interacts with this chain typ. However, since Cezanne is important for silencing NF-κB and preferentially removes K11-linked chains, there might be a specific role for this chain type which is yet unknown.

A20, another important DUB for silencing TNFR-signalling, has been shown to have a special function at the TNFR by removing K63-linked chains from and adding K48-linked chains to RIP1[113]. However, the function of A20 is more complicated, because *in vitro* it preferentially hydrolyses K48-linked chains, which suggests that there are other proteins that assist A20 in the aforementioned process[20]. Recently it was discovered that A20 can interact with K63-linked ubiquitin chains via its ZnF4 and 7 and this interaction is important for the inhibition of NF-κB signalling[123, 124]. In line with this, the present study shows a certain affinity of the A20 ZnF7 for K63-linked ubiquitin chains (Figure 14B). However, these binding experiments revealed an overall preference of ZnF7 for linear ubiquitin chains, which becomes most evident with

diUb, a chain length where linear chains are the exclusive binder of the ZnF7 (Figure 14D). In functional studies it could be shown that this ubiquitin interaction of the ZnF7 is important for inhibiting TNFα-induced NF-κB-signalling[125]. Moreover, the ZnF7 was specifically responsible for the inhibition of LUBAC induced NF-κB activation, while the ZnF4 didn't have an inhibitory effect. This indicates a certain specificity but also some redundancy in the inhibitory function of A20 by binding to ubiquitin chains. For shorter chains the ZnF7 can specifically inhibit the NF-κB-inducing function of linear chains. This might be important because of the strong signal inducing effect of this chain type, since it was shown that already diUb is enough to activate NF-κB[135]. On the other hand can the ZnF4 and the ZnF7 together interact with longer K63- and possibly other lysine-linked chains and thus also influence their signalling capacity.

Taken together, it seems that short ubiquitin chains can regulate specific signalling events at the TNFR by selectively interacting with dedicated UBDs, while longer ubiquitin chains might confer a certain redundancy, which might have a weaker and more general function by binding with lower affinity, more promiscuously, to a broader range of ubiquitin receptors. Linear ubiquitin chains have an important signalling function at the TNFR and it seems that many of these functions are mediated by binding to the IKK-regulator NEMO.

6. Summery and Future Perspectives

Many types of ubiquitin chains can modify several proteins at the activated TNFR. Additionally many UBD-containing ubiquitin receptors localise there to mediate the signal constituted by these chains. Among these ubiquitin receptors is the NF-κB regulator NEMO and in the present study it was shown that NEMO preferentially binds linear ubiquitin chains and that this interaction is important for its function at the TNFR. The ZnF7 of A20 also has a selective affinity towards linear ubiquitin chains, however, it can interact with longer lysine linked ubiquitin chains as well and its ZnF4 was shown to preferentially interact with K63-linked chains.

A lot of progress has been made in the understanding of the role of ubiquitin at the TNFRSC. However, many details are still not very clear. Although various proteins have been identified to be ubiquitylated, the specific role of the respective modification is most of the time not clear or controversial. E. g. the ubiquitylation of RIP1 was shown to be important for the activation of NF-κB[113], however in another study RIP1 deficient MEFs can still activate TNFR-dependent NF-κB signalling[147]. Thus a future task is to exactly identify the substrates for ubiquitylation as well as their residues and then analyse the role of this modification *in vivo*. This could be accomplished with sophisticated protein purification and separation methods combined with mass spectrometrical analysis. For instance ubiquitylated substrates at the activated TNFR could be isolated with ubiquitin-linkage specific antibodies or UBDs and separated by 2D-gel electrophoresis. Such purified ubiquitylation substrates could then be identified by mass spectrometrical analysis. In order to confirm the importance of a given modification, knock-out cells could be utilised to reconstitute them with mutants of residues of proteins discovered to be modified in the mass spectrometrical analysis and the activation of the TNFR-signalling could then be monitored. This way it might also be possible to detect further substrates for linear ubiquitin chains in the TNFRSC that might be crucial for the interaction with NEMO and the induction of NF-κB signalling.

It is also important to dissect when ubiquitylation happens and how long the chains are. This could be accomplished by utilising ubiquitin chain selective sensors *in vivo*. The usage of such sensors has recently been shown in a study from van Wijk et al., where the K63-linked chain specific UIMs of RAP80 and the linear ubiquitin chain specific UBAN-domain of NEMO were applied to selectively detect these chain types in fixed as well as in living cells[148]. In order to detect the production of certain ubiquitin chains at the TNFR and to get a temporal resolution of this process, FRET (fluorescent resonance energy transfer)-sensors could be utilised. To this end e. g. a TNFR-RAP80-UIM-chimera could be tagged with a YFP-protein, while ubiquitin mutants that can only form K63-linked chains could be tagged with CFP, which together form a FRET-couple that enables detection of binding in real-time. A sudden production of K63-linked ubiquitin chains upon TNFR activation could thus be detected as a FRET-signal as soon as these chains would interact with the UIMs at the TNFR and this would indicate the time of the chain production.

So far the role of the interaction of NEMO with linear ubiquitins chain has mainly been studied at the TNFR. Some reports indicated a role for LUBAC in the CD40[56] the RIG-1[149] and the NOD2[150] signalling pathways and there is also evidence that different stimuli rely on the interaction of NEMO with linear ubiquitin chains to activate NF-κB, however detailed analysis of the role of linear ubiquitin chains hasn't been performed[99]. Thus it would be interesting to analyse in more detail if the interaction of NEMO with linear ubiquitin chains is also responsible for the regulation of NF-κB with other stimuli and in other cell types.

Additionally, it is mechanistically not clear how the interaction of NEMO with linear ubiquitin chains activates the IKK-complex. The structure of the UBAN with linear diUb shows that the binding of diUb induces a conformational change in the UBAN, which might play a role in the activation of IKK[99]. However, it is also possible that the interaction with linear ubiquitin chains provokes just the right concentration of the IKK-complex on the right site at the right time, which is necessary for its efficient activation. A more detailed structural analysis of the IKK-complex with and without linear ubiquitin chains could help to understand ubiquitin induced conformational changes that might lead to IKK activation.

An immune system that is not properly regulated can lead to autoimmune diseases or uncontrolled inflammation, which might be responsible for the development of cancer[151]. Since NEMO is the central regulator of NF-κB, which is an important transcription factor for genes promoting inflammation, the detailed understanding of the processes that lead to NEMO dependent activation of NF-κB by ubiquitin interaction, could help to develop drugs that specifically target these interactions.

7. References

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8. Appendix

8.1 Abbreviations

ABIN – A20 binding inhibitor of NF-κB

APC/C – anaphase-promoting complex/cyclosome

APS – ammonium persulfate

ATG – autophagy related

ATM – Ataxia telangiectasia mutated

ATP – adenosine-5'-triphosphate

BAFF – B-cell activating factor

BCR – B-cell receptor

BRCA1 – breast cancer 1, early onset

BSA – Bovine serum albumin

di-, tri,- tetra,- hexa-, hepta-, deca-, polyUb – x-ubiquitin

CD40 – cluster of differentiation 40

Cdc34 – cell-division cycle

CHX – cyclohexamide

cIAP – cellular inhibitor of apoptosis

CRL – cullin ring ligase

Ct – cycle threshold

CYLD – cylindromatosis

DAPI – 4'-6-Diamidin-2-phenylindol

DD – death domain

DMEM – Dulbecco's Modified Eagle's Medium

DTT – 1,4-dithio-DL-threitol

DUB – deubiquitylating enzyme

E1 – ubiquitin activating enzyme

E2 – ubiquitin conjugating enzyme

E3 – ubiquitin ligating enzyme

E6AP – E6-associated protein

EDTA – ethylenediaminetetraacetic acid

EGTA – ethylene glycol tetraacetic acid

ERAD – Endosplasmatic reticulum associated degradation

FADD – Fas-Associated protein with Death Domain

GABARAP – Gamma-aminobutyric acid receptor-associated protein

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GST – glutathione S-transferase

H – hour

HECT – Homologous to the E6-AP Carboxyl Terminus

HEK-cells – human embryonic kidney cells

HOIL-1L – haem-oxidized iron-regulatory protein 2 ubiquitin ligase-1

HOIP – HOIL-1L interacting protein

HRP – horse reddish peroxidase

IL-1β – interleukin-1 β

IκB – inhibitor of NF-κB

IKK – inhibitor of NF-κB kinase

IPTG – Isopropyl β-D-1-thiogalactopyranoside

ISG15 – interferon stimulated gene 15

IsoT – isopeptidase T

JAMM – Jab1/Mov34/Mpr1 Pad1 N-terminal+

JNK – c-Jun N-terminal kinase

Kd – dissociation constant

kDa – kilo Dalton

LC3 – microtubule-associated protein 1 light chain 3

LTβR – lymphotoxin β receptor

LUBAC – linear ubiquitin chain assembly complex

M – molar

MAP – Mitogen-activated protein kinase

MBP – Maltose binding protein

MEF – mouse embryonic fibroblast

MVB – multiple vesicular bodies

NBD – NEMO binding domain

Nedd8 – Neuronal precursor cell expressed, developmentally downregulated 8

NEM – N-Ethylmaleimide

NEMO – NF-κB essential modulator

NF-κB – Nuclear Factor of kappa light polypeptide gene enhancer in B cells

NIK – NF-κB inducing kinase

Ni-NTA – nickel-nitrilotriacetic acid

NOD – Nucleotide-binding oligomerization domain-containing protein

NZF – Npl4 zinc finger

OTU – ovarian tumour

PARP – Poly (ADP-ribose) polymerase

PBS – phosphate buffered saline

PCNA – Proliferating Cell Nuclear Antigen

PCR – polymerase chain reaction

PDB – protein database

PFA – paraform aldehyde

PH-domain – pleckstrin homology domain

PTM – posttranslational modification

PVDF – polyvinylidene fluoride

RAD23A – RADiation sensitivity abnormal

RANK – receptor activator of NF-κB

RAP80 – receptor associated protein 80

RBR – RING-between-RING

Rcf – relative centrifugal force

RHD – Rel homology domain

RIG-I – retinoic acid inducible gene I

RING – really interesting new gene

RIP1 – Receptor-Interacting Protein 1

RNF – ring finger

ROS – reactive oxygen species

RPN – regulatory particle, non-ATPase-like family member

RPS27A – 40S ribosomal protein S27a

RT – room temperature

SAE1 – SUMO-activating enzyme subunit 1

$SCF^{\beta TrCP}$ – Skp1-cullin-F-box/β-transducin repeat-containing protein

SDS – Sodium dodecyl sulfate

SDS-PAGE – SDS-polyacrylamide gel electrophoresis

SEM – standard error of the mean

SHARPIN – SHANK-associated RH domain interactor

SUMO – small ubiquitin like modifier

TAB2 – TAK1-binding protein 2

TAD – transactivation domain

TAE – Tris-acetate-EDTA

TAK1 – TGFβ-activated kinase 1

TBS – tris buffered saline

TCR – T-cell receptor

TEMED – Tetramethylethylenediamine

TLR – toll like receptor

TNF – Tumour necrosis factor

TNFR – TNF-receptor

TNFRSC – TNFR signalling complex

TRAF – TNF-receptor-associated factor

UBA – ubiquitin associated

Uba – ubiquitin-activating enzyme

UBA52 – ubiquitin A-52 residue ribosomal protein fusion product

UBAN – UBD in ABIN proteins and NEMO

UBB – ubiquitin gene b

UBC – ubiquitin gene c

Ubc – ubiquitin conjugating

UbcH –human ubiquitin conjugating

UBD – ubiquitin binding domain

UBL – ubiquitin-like protein

UBZ – ubiquitin binding zinc finger

UCH – ubiquitin c-terminal hydrolase

Uev1a – ubiquitin-conjugating enzyme variant

UFM1 – Ubiquitin-fold modifier 1

UIM – ubiquitin interacting motif

ULD – ubiquitin like domain

URM1 – ubiquitin-related modifier

USP – ubiquitin specific protease

UV – ultra violet

ZnF – zinc finger

8.2 List of original publications

1.) Analysis of Nuclear Factor-κB (NF-κB) Essential Modulator (NEMO) binding to Linear and Lysine-linked Ubiquitin Chains and Its Role in the Activation of NF-κB.
T. Kensche, F. Tokunaga, F. Ikeda, E. Goto, K. Iwai and I. Dikic.
J Biol Chem, Vol. 287, no. 28, Jul 6 2012, pp. 23626-34.

2.) Specific recognition of linear ubiquitin chains by NEMO is important for NF-κB activation.
S. Rahighi, F. Ikeda, M. Kawasaki, M. Akutsu, N. Suzuki, R. Kato, T. Kensche, T. Uejima, S. Bloor, D. Komander, F. Randow, S. Wakatsuki and I. Dikic.
Cell, Vol. 136, no. 6, Mar 20 2009, pp. 1098-109.

3.) A20 inhibits LUBAC-mediated NF-κB activation by binding linear polyubiquitin chains via its zinc finger 7.
K. Verhelst, I. Carpentier, M. Kreike, L. Meloni, L. Verstrepen, T. Kensche, I. Dikic and R. Beyaert.
EMBO J, online pub Aug 28, 2012

4.) Fluorescence-Based Sensors to Monitor Localization and Functions of Linear and K63-Linked Ubiquitin Chains in Cells.
S. J. van Wijk, E. Fiskin, M. Putyrski, F. Pampaloni, J. Hou, P. Wild, T. Kensche, H. E. Grecco, P. Bastiaens and I. Dikic.
Mol Cell, online pub Jul 19, 2012

5.) Human Wrnip1 is localized in replication factories in a ubiquitin-binding zinc finger-dependent manner.
N. Crosetto, M. Bienko, R. G. Hibbert, T. Perica, C. Ambrogio, T. Kensche, K. Hofmann, T. K. Sixma and I. Dikic.
J Biol Chem, Vol. 283, no. 50, Dec 12 2008, pp. 35173-85.

8.3 Acknowledgements

I would like to thank all former and present members of the Institute of Biochemistry II who helped to create a great atmosphere to work in.

My gratitude goes to my supervisor, Prof. Ivan Dikic, who always kindly supported my project with guidance, generosity and enthusiasm and whose door was always open for fruitful discussions. I want to thank Prof. Volker Dötsch for supervising my thesis and being always very helpful with any kind of concern.

Many thanks go to Fumiyo Ikeda, who was always of great help in any kind, beginning with introducing me to the work in the lab till later collaborative work on the project. The scientific discussions we had were always a great support.

Many thanks also to the former members of the lab, Magda Bienko, Nicola Crosetto and Yonathan Lissanu-Deribe, who became good friends and with whom I could discuss scientific and other issues for hours.

Many thanks to the members of the lab, Koraljka Husnjak, Philipp Wild, David McEwan, Michael Olma, Evgeni Fischkin, Sascha Khaminets, Sjoerd van Wijk, Doris Popovic, Jaime Lopez and Veronique Schaeffer as well as to Igor Kovacevic, who were always helpful in terms of discussions and work and with whom I also enjoyed spending my free time with. Masuda Sader and Birgit Stein were an indispensible help in the lab and it was great to work together with them. I am very thankful to Birgit Lipke and Rebecca Pfeiffer, who were a great support with all the administrative issues. Many thanks to Farshid Miri, who was able to solve any problem concerning computers.

I want to thank Ron Hay and David Komander, who kindly gave me the opportunity to work in their labs, enabling me to learn new methods.

Many thanks to my companion Nina Dümke, who was always patient with my long working hours and who supported me throughout the years wherever she could.

Finally I want to thank my family, Lore Bücke, Anja Kensche, Natalie Kensche and my mother Dorothee Kensche who were always there for me, whenever it was necessary. Without their help I would not be where I am today.

I dedicate this work to my grandmother Katharina Kensche, my grandfather Emil Bücke and to my father Matthias Kensche.

8.4 Lebenslauf

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8.5 Erklärung

Hiermit erkläre ich an Eides Statt, dass ich die vorliegende Dissertation selbständig und nur mit den angegebenen Hilfsmitteln angefertigt und dass ich noch keinen Promotionsversuch unternommen habe.

Frankfurt am Main, den

Tobias Kensche

Abstract

Posttranslationale Modifikationen regulieren wesentliche Eigenschaften von Proteinen, wie z. B. Lokalisation, Konformation, Aktivität, Stabilität und Interaktionsfähigkeit. Eine besondere Form der Proteinmodifikation ist die Ubiquitylierung, bei der das kleine Protein Ubiquitin mit seinem C-Terminus kovalent an ein Substratprotein gebunden wird.

Die am besten untersuchte Funktion der Ubiquitylierung ist die Markierung eines Substrates für den Abbau durch das Proteasom. In den letzten Jahren wurde jedoch entdeckt, dass Ubiquitylierung in vielen Bereichen der Zelle eine wichtige Rolle spielt. Dazu gehören der Transport von Vesikeln, die Reparatur von DNA-Schäden und zelluläre Signalübertragung. Ubiquitin kann verschieden-artige Ketten bilden, indem ein Ubiquitin an eines der sieben Lysine (K6, K11, K27, K29, K33, K48, K63) oder den N-Terminus eines anderen gebunden wird. Diese unterschiedlichen Kettentypen regulieren verschiedene Prozesse. Z. B. dienen K48-verknüpfte Ubiquitinketten als Signal für den proteasomalen Abbau, wohingegen über K63 verknüpfte Ketten hauptsächlich eine Rolle bei Signalübertragungen spielen.

Die meisten Funktionen die durch Ubiquitylierung reguliert werden, werden durch Ubiquitinrezeptoren vermittelt, die eine Ubiquitinbindedomäne (UBD) besitzen. Manche UBDs binden selektiv nur einen Ubiquitinkettentyp und sind somit in der Lage gezielt Prozesse regulieren zu können, indem sie nur durch diesen speziellen Kettentyp aktiviert werden.

Das Protein NEMO ist ein Ubiquitinrezeptor, dessen UBD UBAN selektiv bestimmte Ubiquitinketten bindet. NEMO spielt eine zentrale Rolle bei der Aktivierung der Transkriptionsfaktorfamilie NF-κB, indem es den IKK-Kinasekomplex reguliert. Dieser Kinasekomplex sorgt durch die Phosphorylierung des NF-κB-Inhibitors IκBα für dessen proteasomalen Abbau, wodurch schließlich NF-κB aktiviert wird. Die NF-κB-Aktivierung kann u. a. durch den TNF-Rezeptor (TNFR) induziert werden. Am aktivierten TNFR werden viele Proteine durch verschiedene Ubiquitinketten modifiziert. Bisher wurde angenommen, dass die spezifische Bindung von NEMO an K63-verknüpfte Ubiquitinketten ausschlaggebend für die Aktivierung von IKK ist. Jedoch spielen lineare Ubiquitinketten, die über den N-Terminus verknüpft sind, auch eine

wichtige Rolle bei der Aktivierung von NF-κB und die UBAN von NEMO hat eine sehr hohe Affinität zu linearen Ubiquitinketten.

Um die genauen Vorgänge zu verstehen, die zur Aktivierung von NF-κB am TNFR führen, ist es nötig, zu analysieren, welche Proteine mit welchen Ubiquitinketten modifiziert werden und welche Ubiquitinrezeptoren daran binden.

In dieser Studie sollte detailliert untersucht werden, mit welchen Ubiquitinketten NEMO bevorzugt interagiert. Dazu wurden *in vitro*-Bindungsstudien mit bakteriell aufgereinigtem NEMO und verschiedenen Ubiquitinketten durchgeführt. Des Weiteren sollte geprüft werden, wie die Bindung von NEMO an bestimmte Ubiquitinketten die Aktivierung von NF-κB reguliert.

Dabei ergab sich, dass sowohl NEMO in voller Länge, als auch die UBAN, bevorzugt mit linearen Ubiquitinketten interagieren, wohingegen die Interaktion von NEMO mit anderen Ubiquitinketten relativ schwach ist. Ausgehend von einer Kristallstruktur eines Komplexes aus der NEMO-UBAN und linearem di-Ubiquitin, wurden NEMO-Mutanten generiert, die seletkiv die Bindung von NEMO an lineare Ubiquitinketten verhindern, während die schwache Bindung von NEMO an längere K63-verknüpfte Ketten erhalten blieb. Um die Relevanz der Interaktion von NEMO mit linearen Ubiquitinketten für die Aktivierung von NF-κB zu überprüfen, wurden diese NEMO-Mutanten dann verwendet um Zellen die kein NEMO exprimieren zu rekonstituieren. Nach Stimulation dieser Zellen mit TNFα wurde NF-κB kaum aktiviert, womit gezeigt werden konnte, dass NEMO gezielt an lineare Ubiquitinketten binden muss, um NF-κB zu aktivieren.

Zusätzlich zu seiner Rolle bei der Aktivierung von NF-κB ist NEMO ein wichtiger Inhibitor der durch den TNFR induzierten Apoptose. In dieser Studie wurde gezeigt, dass diese Apoptoseinhibierung abhängig von der Bindung von NEMO an lineare Ubiquitinketten ist, da die Zellen die NEMO-Mutanten exprimierten, die keine linearen Ketten binden können, durch Apoptose starben, währen Wildtyp-Zellen überlebten.

Zusammenfassend konnte in dieser Studie gezeigt werden, dass NEMO bevorzugt und mit vergleichsweise hoher Affinität an lineare Ubiquitinketten bindet und dass diese spezifische Bindung wichtig für die Inhibierung von TNFR-induzierter Apoptose sowie für die Aktivierung von NF-κB ist.