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***New pieces in the NEMO-dependent NF- $\kappa$ B activation pathway.***

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## **1. INTRODUCTION**

## 1.1 Overview of the NF- $\kappa$ B pathway

NF- $\kappa$ B is a family of transcription factors that regulates a broad number of genes involved in immune response, cell survival, differentiation and proliferation (Hayden and Ghosh, 2008). This family consists of five members, p65, RelB, c-Rel, p100/p52 and p105/p50, which share a common Rel Homology Domain (RHD) in the N-terminal portion that is responsible for DNA-binding and homo- or hetero-dimerization (Figure 1A). Each member of the family, in fact, can potentially dimerize with all the other members, even if the most common dimers found in the cells are the p65/p50 or the p100/p100 (Hayden and Ghosh, 2008). In their inactive state, NF- $\kappa$ B dimers are associated to one of the Inhibitor of NF- $\kappa$ B (I $\kappa$ B) proteins, which are part of a gene family that contains six members, I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\epsilon$ , I $\kappa$ B $\gamma$ , Blc-3 and I $\kappa$ B $\zeta$ , all characterized by the presence of multiple Ankirin repeats, which are responsible for the interaction with NF- $\kappa$ B via its RHD (Malek et al., 1998). The classical cascade of NF- $\kappa$ B activation takes place through the activation of the I $\kappa$ B Kinase (IKK) complex, which is composed by two catalytic subunits, IKK $\alpha$  and IKK $\beta$ , and the regulatory subunit NEMO/IKK $\gamma$  (Ghosh and Karin, 2002; Hayden and Ghosh, 2008). IKK $\alpha$  and IKK $\beta$  share a common domain structure consisting in a N-terminal Kinase domain, a Leucine Zipper domain necessary for their dimerization, an Helix-Loop-Helix and a NEMO-Binding Domain in the C-terminal region (Figure 1A). These two proteins seem to have redundant functions, even if it has been shown that IKK $\beta$  is both necessary and sufficient for the phosphorylation of I $\kappa$ B $\alpha$  on Ser32 and Ser36, and I $\kappa$ B $\beta$  on Ser19 and Ser23. The role of IKK $\alpha$  in these events is unclear, although recent studies suggest that it may regulate gene expression in the nucleus by modifying the phosphorylation status of histones (Yamamoto et al., 2003). IKK $\alpha$ , however, is necessary for the activation of NF- $\kappa$ B in the so called “alternative” pathway (discussed below). NEMO/IKK $\gamma$  represents the key regulatory subunit of the IKK complex (Yamaoka et al., 1998). It is composed by two coiled-coil motifs



necessary for the interaction with IKK $\alpha$ , IKK $\beta$  and with many upstream intermediates, a Leucine Zipper and a Zinc Finger domain in the C-terminal region and by a recently characterized Ubiquitin Binding Domain (UBD) that confers to NEMO the ability to bind to polyubiquitinated proteins (Figure 1A), (Sebban et al., 2006).

## **1.2 Signaling to NF-kB**

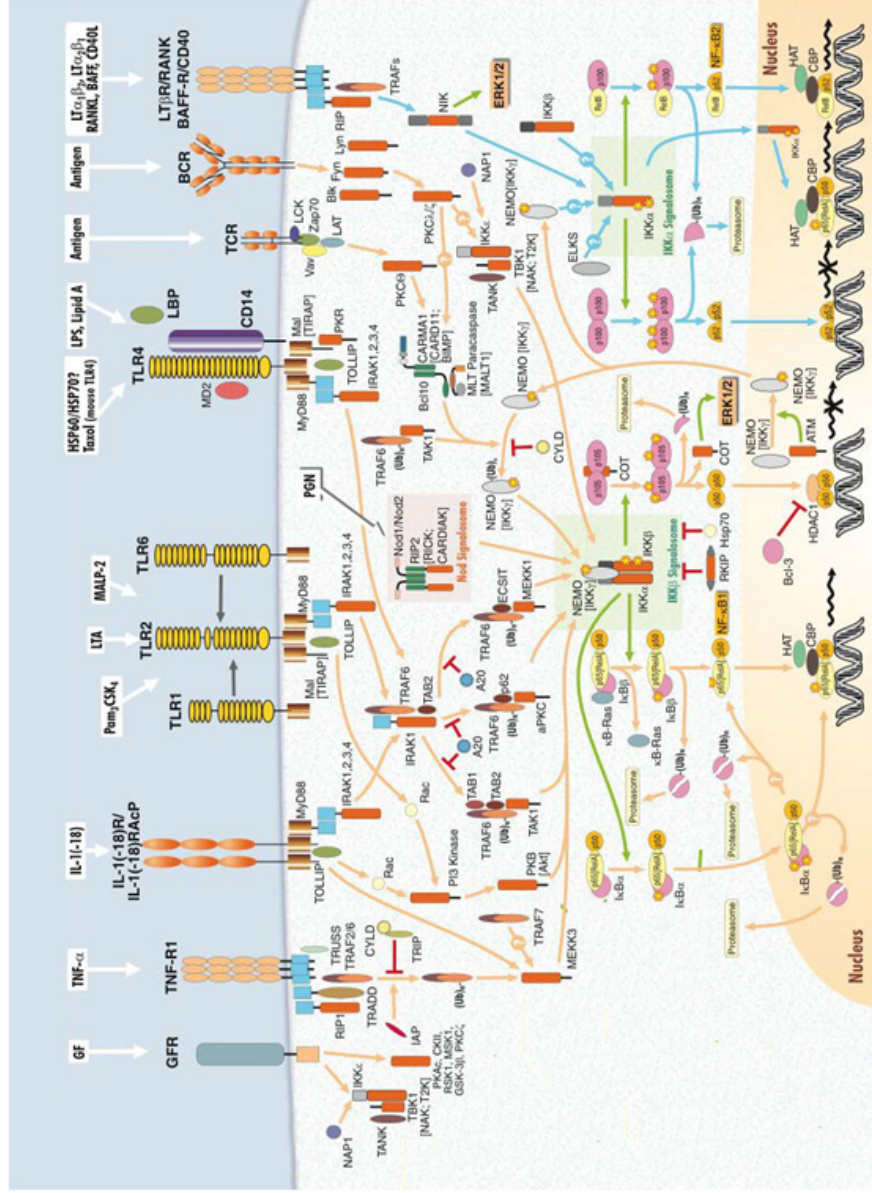
The basic scheme of NF-kB signaling counts several positive and negative regulators. NF-kB signaling is generally considered to occur through either the “canonical” or the “alternative” pathway (Figure 1B) (Bonizzi and Karin, 2004). In the canonical pathway, inducing stimuli trigger the activation of the IKK complex, which results in the phosphorylation and subsequent ubiquitination and degradation of I $\kappa$ B proteins (classically p65/p50-bound I $\kappa$ B $\alpha$ ). Free NF-kB is able to translocate into the nucleus and activate the transcription of its targets.

The non-canonical or “alternative” pathway operates mainly in B-cells in response to stimulation of a subset of the TNF-receptor superfamily, including receptors for BAFF, lymphotoxin- $\beta$  (LT $\beta$ ) and CD40 ligand. Stimulation of these receptors activates only the IKK $\alpha$  subunit, that then phosphorylates the p100/p100 dimers, thus causing its inducible processing to p52, entry into the nucleus and transcription of its targets.

While this NEMO- and IKK $\beta$ -independent pathway is restricted only to a subset of activating stimuli, the predominant NF-kB signaling pathway is the “canonical”, that comes into play upon the ligation of many receptors such as the Toll-like Receptors (TLRs) in the innate immune response, T cell Receptor (TCR) in the adaptive immunity, TNF Receptor (TNFR) or Interleukin-1 Receptors (IL-1R) in inflammation or upon stimulation of a variety of endogenous ligands (such as viral DNAs or RNAs) or chemical and physical stresses (such as oxidative or genotoxic stress) (Hayden and Ghosh, 2008). The binding of ligands to each receptor is able to activate a complex cascade of

events in which are involved many downstream molecules, although many of the signaling intermediates among the different pathways are shared. Beside the diversity of stimuli and molecules involved in the activation of NF- $\kappa$ B, the common feature of all the “canonical” pathways is the activation of the IKK complex (Figure 2). Consequently, this is the most important regulatory step in determining the NF- $\kappa$ B response to a given stimulus. Current evidence show that the activation of the IKK complex is mediated by its recruitment to receptor-containing complex signalosomes, where the final event for IKK activation seems to be its phosphorylation at specific Serines in the Kinase domain of IKK $\alpha$  or IKK $\beta$  (Figure 1A) (Hacker and Karin, 2006). However, the mechanism through which this phosphorylation occurs remains unclear. In particular, the fundamental question remains if the IKK phosphorylation occurs by transautophosphorylation or through phosphorylation by upstream kinases. In any case the regulatory role that NEMO plays in the activation of the IKK complex is essential. NEMO, in fact, represents the point of convergence of most stimuli activating NF- $\kappa$ B and in particular the physical interaction of NEMO with upstream signaling intermediates is essential for the IKK complex recruitment and activation. Indeed, numerous proteins producing either NF- $\kappa$ B activation or inhibition have been shown to interact with NEMO (Table 1) (Sebban et al., 2006). The interaction of NEMO with upstream molecules allows the formation of signaling complexes that could serve to position the IKK complex near to an IKK-kinase; alternatively, the oligomerization of NEMO-interacting proteins present in the signaling complexes, such as RIP, may provide a scaffold for oligomerization (Inohara et al., 2000), thus determining IKK transautophosphorylation and activation; this hypothesis is supported by the evidence that enforced NEMO oligomerization is sufficient to mediate IKK activation (Poyet et al., 2000) and that NEMO mutations in sequences required for the oligomerization results in a loss of IKK activity (Vinolo et al., 2006).

**Figure 2**



**Figure 2: The NF- $\kappa$ B pathway.** Schematic representation of the complex cascades producing IKK and NF- $\kappa$ B activation. Most activating pathways converge on IKK complex.



**Table 1. The NEMO interactome\***

Protein name <sup>a</sup>	Pathway or function	Part of the NEMO sequence bound
ABIN-1	TNF signaling pathway; negative regulator of IKK	51-100
A20	Negative regulator of RIP; K63 deubiquitinase; K48 E3 ligase	n.d.
CARDINAL	Inhibitor of IKK	n.d.
CARMA-1/3	TCR and BCR signaling pathways; scaffold	51-100
c-IAP-1	TNF signaling pathway; E3 ligase	n.d.
CIKS/Act-1	CD40 pathway; adaptor	50-100
v-CLAP	EHV-2-derived; Bcl10-like	200-300
CYLD	Negative regulator of IKK; K63 deubiquitinase	388-393
ELKS	Subunit of IKK; IκBs recruitment	n.d.
E3-14.7K	Adenovirus-derived; activator of IKK	397-419
p22-FLIP	Inhibitor of death receptor-mediated apoptosis	n.d.
v-FLIP	KSHV-derived; activator of IKK	150-272
HIF-2a <sup>e</sup>	Hypoxia-inducible factor	n.d.
Hsp70	Heat shock response; inhibitor of IKK	250-320
Htt	Mutant form of Huntingtin; neurotoxicity	n.d.
IKK-1	Subunit of IKK; phosphorylation of IκBs and p100	60-119
IKK-2	Subunit of IKK; phosphorylation of IκBs	60-119
Myo1c <sup>e</sup>	Unconventional myosin I; insulin signaling	n.d.
PP2A	TNF signaling pathway; phosphatase	121-179
RICK	TLR4, TCR, IL-12, IL-18 and Nod2 signaling pathways; adaptor	n.d.
RIP	TNF and TLR signaling pathways; adaptor	134-218
TANK	TNF signaling pathway; adaptor	200-250
Tax	HTLV-1-derived; activator of IKK	262-315
TRUSS	TNF signaling pathway; scaffold	n.d.
ZNF216	RIP <sup>b</sup> , TRAF-6- and A20-binding protein	n.d.

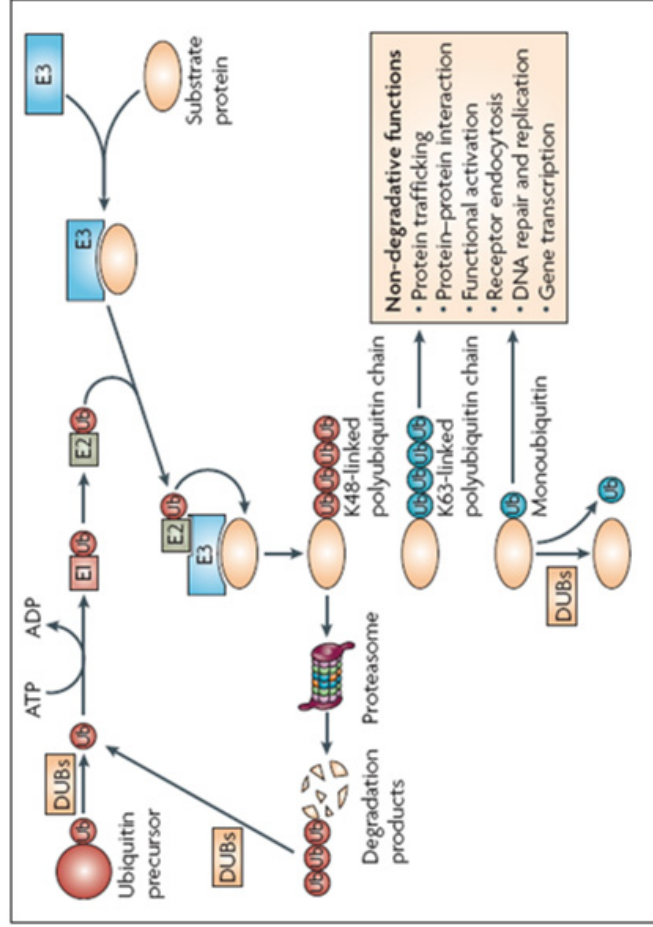
\*Courtois 2006, Trends in Cell Biology

In addition to the physical interaction of NEMO with upstream molecules, a novel mechanism of IKK recruitment and activation is linked to the ability of NEMO to recognize polyubiquitinated signaling intermediates.

### **1.3 Ubiquitin in the NF- $\kappa$ B signaling pathway**

Ubiquitin (Ub) is a highly conserved protein of 8 Kd that is covalently attached to lysine (Lys) residues of target proteins (Haglund and Dikic, 2005). Protein-linked Ub is a substrate for the attachment of further Ub residues, which leads to the formation of a polyubiquitin chain. Classically, polyubiquitination is a signal that directs proteins to the proteasome, where the Ub is recycled and the protein is degraded (Hershko and Ciechanover, 1992). Diverse forms of Ub modifications exist: monoubiquitination is the attachment of a single Ub to a protein; multiubiquitination occurs when several Lys residues of the target protein are tagged with single Ub molecules; and polyubiquitination, that consists in the addition of a Ub chain made of several ubiquitins in which an Ub molecule is linked to the next through a specific internal Lys residue (Haglund and Dikic, 2005). In particular, there can be at least seven different linkages between ubiquitins, because there are seven internal lysines in Ub. The role of different linkages in polyubiquitin chains has begun to be elucidated in recent years. Linkage through Lys48 is mainly used for targeting to the proteasome, and Lys63 linkages seem to play important roles in signal transduction. Ubiquitination is a reversible covalent modification that is catalyzed by three enzymatic steps (Figure 3). In the first step, ubiquitin is activated by a ubiquitin-conjugating enzyme (E1) in the presence of ATP. In the second step, the activated ubiquitin is transferred to another Ub-conjugating enzyme (E2). In the third step, a Ub-protein ligase (E3) mediates the attachment of the C-terminus of the ubiquitin to a Lys of the target protein. In the case of the polyubiquitination, Ub is attached to a Lys of the Ub molecule already linked to the target protein. Ubiquitination is a dynamic and reversible modification. The

**Figure 3**



Sun, 2008, Nat Imm Rev

**Figure 3: Ubiquitin conjugation**

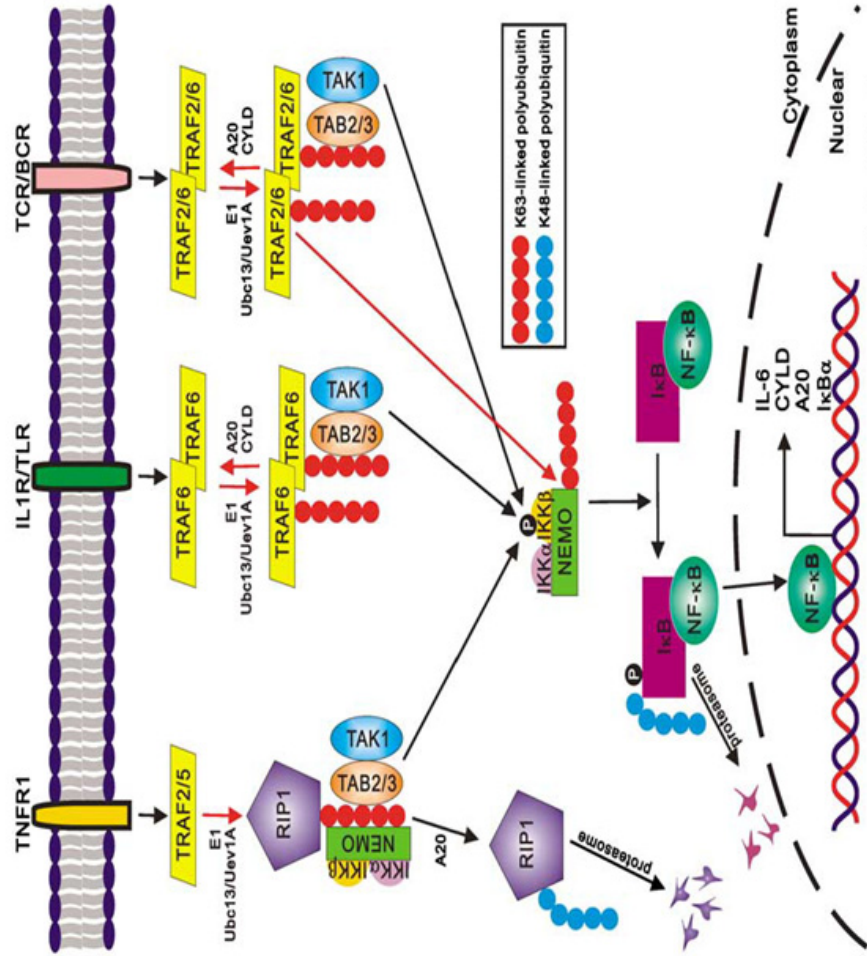
The ubiquitination is a reaction catalyzed by three enzymes: the Ub-activating enzyme (E1), the Ub-conjugating enzyme (E2), and the Ub ligase (E3). K48 polyubiquitin chains target substrates to proteasome while monoubiquitination or K63 polyubiquitination are important modifications for protein trafficking, protein-protein interactions, endocytosis, DNA repair and replication. Deubiquitinating enzymes (DUBs) deconjugate ubiquitin chains from ubiquitinated proteins.

rapid removal of Ub from substrates is catalyzed by De-Ubiquitinating enzymes (DUBs) (Sun, 2008).

Ubiquitination plays a fundamental role in the NF- $\kappa$ B pathway (Chen, 2005). In addition to the Lys-48 mediated polyubiquitination of phosphorylated I $\kappa$ Bs that targets them to the proteasomal degradation with the subsequent translocation of NF- $\kappa$ B into the nucleus, Lys-63 mediated polyubiquitination of signaling intermediates upstream the IKK complex is an essential event to activate NF- $\kappa$ B in many signaling pathways, such as TNF-R, TCR or IL1R/TLR (Figure 4) (Chen, 2005). Upon stimulation, the activation of downstream E3 ubiquitin ligases, mainly belonging to the TNF-R Associated Factor (TRAF) family, mediates the polyubiquitination of several signaling intermediates, determining the recruitment and the activation of the IKK complex. This can occur because of the ability of NEMO to recognize polyubiquitinated intermediates. Upon stimulation of the above mentioned receptors, K63-linked polyubiquitination of downstream molecules such as RIP (Li et al., 2006; Wu et al., 2006), IRAK1 (Conze et al., 2008), MALT1 (Oeckinghaus et al., 2007) and Bcl10 (Wu and Ashwell, 2008), promotes the binding of NEMO to these molecules, thus determining the recruitment and activation of the IKK complex. Although NEMO can directly bind to some of these molecules, their polyubiquitination is the essential event for their binding *in vivo*. This is the case of RIP, whose mutations in the Ub-acceptor lysines abrogates NEMO binding and IKK activation (Ea et al., 2006; Li et al., 2006). Although NEMO and RIP can directly interact *in vitro* (Zhang et al., 2000a), the polyubiquitination of RIP upon TNF stimulation seems to be the event determining their interaction *in vivo*. The example of RIP may serve as a paradigm for the role of Lys-63 ubiquitin in the NF- $\kappa$ B pathway and can be expanded to other molecules that operate in similar ways.

The TRAF family of E3 ubiquitin ligases plays a fundamental role in all these events. Seven members of this family have been identified (TRAF1-7)

**Figure 4**



Adhikari et al., 2007, Oncogene

**Figure 4:** The role of ubiquitin in the NF-κB pathway

(Bradley and Pober, 2001). Among them, TRAF2 and TRAF6 have been better characterized.

TRAF2 (probably together with TRAF5) is involved in the TNF pathway and represents the E3 ligase catalyzing the polyubiquitination of RIP upon TNF stimulation (Au and Yeh, 2007).

TRAF6 is the E3 ligase working in most NF- $\kappa$ B pathways such as those emanating from IL-1R (Cao et al., 1996), TLRs (Takeda and Akira, 2004), Nerve Growth Factor Receptor (NGFR) (Khursigara et al., 1999) or TCR (Sun et al., 2004), thus catalyzing the polyubiquitination of many molecules such as TRAF6 itself (necessary for its activation) (Yang et al., 2004), IRAK1 (Windheim et al., 2008), MALT1 (Oeckinghaus et al., 2007) or the NGF receptor TrkA (Geetha et al., 2005), all essential modifications for the activation of NF- $\kappa$ B. NEMO itself can undergo TRAF6-mediated Lys-63 polyubiquitination (Sebban-Benin et al., 2007), but the role of this event remains controversial.

The importance of Ub in the NF- $\kappa$ B pathway is also strengthened by the evidence that the overexpression of E3 ubiquitin ligases such as TRAF2 or TRAF6 is sufficient to activate NF- $\kappa$ B (Pineda et al., 2007), while overexpression of DUBs, such as CYLD or A20, that de-ubiquitinate several molecules belonging to the NF- $\kappa$ B pathway, inhibits it (Mauro et al., 2006; Regamey et al., 2003). However the targets on which both DUBs act to inhibit the NF- $\kappa$ B activity remain unclear.

#### **1.4 Physiological roles of the NF- $\kappa$ B components**

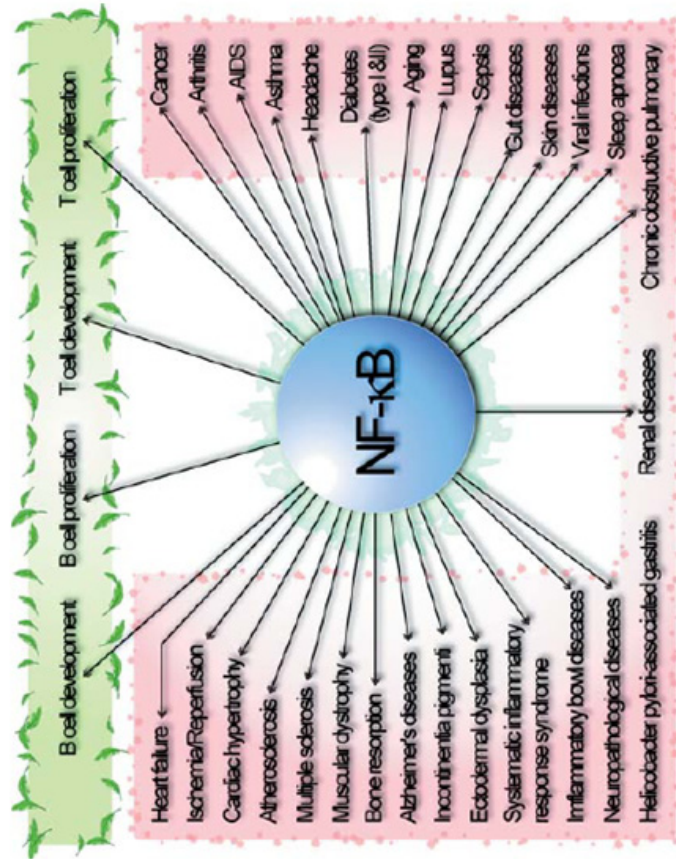
The diversity of inducers of NF- $\kappa$ B and the variety of processes in which it is involved highlight the intriguing and complex regulation of NF- $\kappa$ B activation, in which many signal transduction pathways from a wide variety of inducing mechanisms converge on a single target. Given the pivotal role that NF- $\kappa$ B plays in processes such as cell survival, inflammation, immunity, stress response and development it appears evident that dysfunctions in such a regulation,

determining an aberrant NF- $\kappa$ B activation, result in the pathogenesis of a variety of diseases including those related to enhanced cellular proliferation, viral or bacterial infection, inflammatory or genetic diseases (Figure 5).

Gene-knockout studies in mice have been useful to understand the physiological roles of each component of the NF- $\kappa$ B pathway and have revealed both specific and redundant functions of each member of NF- $\kappa$ B family. For example, the deletion of the *RelA* (p65) gene in mice causes embryonic lethality due to extensive apoptosis in the liver (Beg et al., 1995), which indicates that the function of p65 cannot be compensated for by other NF- $\kappa$ B family proteins and is indispensable for the survival of the mouse embryo. On the other hand, mice lacking p50 or RelB are immunodeficient but develop normally to adulthood (Burkly et al., 1995; Sha et al., 1995; Weih et al., 1995). Mice lacking other NF- $\kappa$ B proteins, including c-Rel and p52, also have immune defects (Kontgen et al., 1995). The knockouts of multiple members of NF- $\kappa$ B family results in even more severe phenotypes, which suggests that there is some functional redundancy between the NF- $\kappa$ B family members (Li and Verma, 2002).

The gene-targeting experiments have also revealed the importance of other key components of NF- $\kappa$ B signaling pathways in mouse development. Although both IKK- $\alpha$  and IKK- $\beta$  are necessary for survival of mouse embryos, their respective roles in embryonic development and survival are quite different (Hayden and Ghosh, 2008). IKK- $\alpha$  has a unique function in skin and skeletal development, as well as in B cell maturation, and its absence cannot be compensated for by IKK- $\beta$  (Hu et al., 1999). In contrast, IKK- $\beta$  appears to play an indispensable role in inducible NF- $\kappa$ B activation in response to pro-inflammatory and pro-apoptotic stimuli. Lack of IKK- $\beta$  leads to embryonic lethality and liver degeneration in knockout mice similar to p65 knockout mice (Li et al., 1999). Severe liver degeneration and early lethality were also observed in embryos that were deficient in the IKK $\gamma$ /NEMO subunit of IKK complex (Rudolph et al., 2000). The *NEMO* gene is located on the q28 region of the X

**Figure 5**



**Figure 5:** Major physiological roles of NF-κB and its repercussion in Human diseases



chromosome. While the *nemo* knock-out is lethal in males, *nemo*<sup>+/-</sup> females can survive developing a phenotype that is very similar to the Incontinentia Pigmenti disease in Humans (Makris et al., 2000; Schmidt-Supprian et al., 2000), that is caused by mutations in the *NEMO* gene of affected females (Fusco et al., 2004). The clinical presentation of the disease is characterized by skin defects, that can be quite dramatic, although the most significant medical problems in IP are blindness, due to retinal detachment, and Central Nervous System (CNS) defects, which cause mental retardation or seizures (Nelson, 2006). A few minor signs include hair loss, conical or absent teeth and nail dystrophy .

Incontinentia Pigmenti is not the only disease in which an impaired NF- $\kappa$ B activity correlates with CNS defects. An abnormal regulation of NF- $\kappa$ B has also been correlated to neurodegenerative diseases such as the Alzheimer or Parkinson diseases, multiple sclerosis, atherosclerosis, demonstrating that alterations in NF- $\kappa$ B activity could play important roles in both developmental or neurodegenerative diseases in the CNS (Memet, 2006).

### **1.5 NF- $\kappa$ B and the Central Nervous System**

NF- $\kappa$ B activity in the Nervous system is involved in neuron survival, neurodegeneration, injury response, cognitive functions and behavior (Meffert and Baltimore, 2005). NF- $\kappa$ B activation during development reflects diverse functions according to stages and/or cell types. The neuroprotective function of NF- $\kappa$ B during neural development is now well established and has been shown to be cell- and/or time-restricted. Complete abrogation of NF- $\kappa$ B activity in *IKK $\alpha$ <sup>-/-</sup>IKK $\beta$ <sup>-/-</sup>* mice leads to demise at E12.5 of embryos, which present defects in neural tube closure due to enhanced apoptosis in the neuroepithelium, as well as increased apoptosis of sensory neurons is also described in *p65<sup>-/-</sup>* mouse embryos (Hayden and Ghosh, 2008). If NF- $\kappa$ B activity results to be essential for its neuroprotective roles, on the other hand it can participate to the pathogenesis of neurodegenerative disorders. NF- $\kappa$ B-dependent production of

proinflammatory mediators is crucial for autoimmune demyelinating disease such as multiple sclerosis. CNS specific KO of NEMO or IKK $\beta$  has been shown to ameliorate the phenotype in mice with Experimental Autoimmune Encephalomyelitis (EAE) (van Loo et al., 2006), the best known model of multiple sclerosis in mice. Moreover the NF-kB signaling pathway results to be altered in neurodegenerative diseases such as Alzheimer, Parkinson or Huntington diseases (Memet, 2006).

In addition to its opposite role in contributing both to neuroprotection, by controlling the transcription of neuronal anti-apoptotic genes, and neurodegeneration, by regulating proinflammatory genes, NF-kB plays important roles also in CNS specific processes. Indeed many stimuli specific of neuronal cells, such as NGF (Carter et al., 1996; Wood, 1995), glutamate (Guerrini et al., 1995; Kaltschmidt et al., 1995), amyloid  $\beta$  peptide (Behl et al., 1994), membrane polarization and sleep deprivation (Brandt et al., 2004; Chen et al., 1999), can activate NF-kB. NF-kB plays also essential roles in regulating growth of neural processing in developing nervous system. Indeed, inhibition of NF-kB activity with super-repressor I $\kappa$ B $\alpha$  resulted to substantially reduce the complexity of neurite arbors of sensory neurons (Gutierrez et al., 2005). Moreover, neurite outgrowth during NGF-induced differentiation of PC12 cells requires several components of the NGF-induced NF-kB activating pathway, such as TRAF6 (Geetha et al., 2005), p62 (Wooten et al., 2005) or IKK $\beta$  (Azoitei et al., 2005).

In the nervous system, the features of the canonical NF-kB activation cascade are conserved. Among the specific stimuli activating NF-kB in the nervous system, those emanating from the TrkA and p75<sup>NTR</sup> receptors in the NGF pathway are well characterized. Binding of NGF to TrkA induces dimerization (Khursigara et al., 1999), autophosphorylation (Friedman and Greene, 1999) and internalization to signaling vesicles (Riccio et al., 1997), which mediates NGF-induced differentiation (Zhang et al., 2000b). An important role in this process is

played by the E3 ubiquitin ligase TRAF6. The interaction of TRAF6 with the adapter molecule p62 (Sanz et al., 2000) allows both the dimerization of the TrkA and p75<sup>NTR</sup> receptors (Wooten et al., 2001) and the TRAF6-mediated polyubiquitination of TrkA (Geetha et al., 2005). This last modification seems to be necessary for the receptor internalization and signaling to NF- $\kappa$ B (Geetha et al., 2005). All these events occur at specific time points upon NGF stimulation (Figure 6).

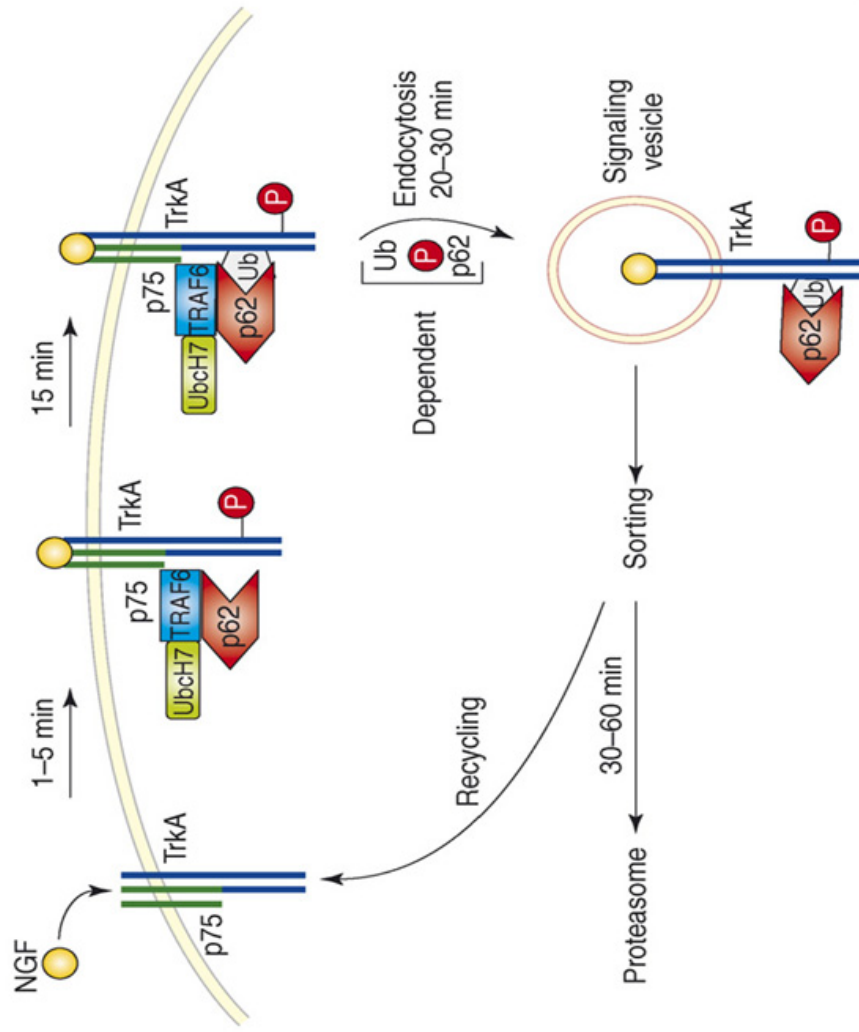
Also atypical PKCs have been shown to be involved in NGF pathway by binding to p62 (Samuels et al., 2001); it has been demonstrated that their overexpression can induce NF- $\kappa$ B activation in neurons (Wooten et al. 1999) probably by determining the phosphorylation of IKK $\beta$ , as it has been shown to happen *in vitro* (Lallena et al., 1999), although it is not clearly understood how the IKK complex can be recruited and activated.

### **NESCA/RUSC1 and RUSC2**

NESCA (new molecule containing a SH3 carboxy-terminal, vedi) and RUSC2 (RUN and SH3 containing protein 2) are two paralagous proteins that share a common domain structure consisting in a RUN, a LZ and a SH3 domain. NESCA is an ubiquitous protein 433 aminoacids long, whose function has been described by MacDonald et al (2004). By a Yeast Two-Hybrid screen using the TrkA receptor of the NGF as a bait, the authors found NESCA as a novel molecule involved in the NGF pathway. Moreover they showed that this molecule is important in the NGF-mediated neurite growth of neurites in PC12 cells because this process can be enhanced or repressed by overexpression or downregulation by RNAi of NESCA.

RUSC2 is a 1516 aa long protein that has been found as a Rab1b interacting protein (Bayer et al., 2005). Rab1b is a molecule involved in the trafficking of vesicles from Endoplasmic Reticulum to Golgi, but what is the function of RUSC2 in this process is to date unknown.

**Figure 6**



**Figure 6: NGF signaling kinetics**  
 Upon 1-5 NGF stimulation a molecular complex formed by TRAF6, p62, TrkA and p75 is formed. The TRAF6-mediated polyubiquitination of the TrkA receptor mediates its internalization within 15-30 minutes. The receptor can be then sorted to the proteasome or recycled to the cell membrane in 30-60 minutes.

## **2. AIM**

NF- $\kappa$ B is an ubiquitous transcription factor involved in a variety of biological processes, such as inflammation, immunity, cell survival and development. In most pathways, NF- $\kappa$ B activation occurs upon the activation of the IKK complex, which mediates the phosphorylation and subsequent degradation of the I $\kappa$ B inhibitory proteins, so that NF- $\kappa$ B can translocate into the nucleus and promote the transcription of its target genes. The IKK complex is composed by two catalytic subunits, IKK $\alpha$  and IKK $\beta$ , and by NEMO, which is the key regulatory subunit of this complex and represents the point of convergence of most stimuli activating NF- $\kappa$ B. NEMO contains two coiled-coil motifs, a leucine zipper, a C-terminal Zinc Finger domain and a recently characterized Ubiquitin-binding domain. These domains are required for the correct assembly of the IKK complex and for the recruitment of upstream signaling molecules, whose interaction with NEMO is essential for the IKK-mediated NF- $\kappa$ B activation. Indeed, numerous proteins producing either NF- $\kappa$ B activation or inhibition (Kovalenko et al., 2003; Mauro et al., 2006; Zhang et al., 2000a) have been shown to interact with NEMO/IKK $\gamma$ ; the ability of NEMO to interact with upstream components as well as its ability to bind to signal-induced polyubiquitinated-intermediates of the NF- $\kappa$ B pathway is essential for the correct recruitment and activation of the IKK complex, and for this reason NEMO represents a key fundamental element for the activation of NF- $\kappa$ B in most pathways.

In the nervous system, the features of the canonical NF- $\kappa$ B activation cascade are conserved. NF- $\kappa$ B in this tissue can be activated by a wide array of stimuli, among which those specific of neuronal cells, such as NGF (Carter et al., 1996; Wood, 1995), glutamate (Guerrini et al., 1995; Kaltschmidt et al., 1995), amyloid  $\beta$  peptide (Behl et al., 1994), membrane polarization and sleep deprivation (Brandt et al., 2004; Chen et al., 1999). NF- $\kappa$ B activation in CNS is involved in many processes such as neuron survival, cognitive functions and behavior (Meffert and Baltimore, 2005) and also in regulating growth of neural

processing in developing nervous system. Moreover NF- $\kappa$ B can contribute to neurodegeneration and aberrant NF- $\kappa$ B activation has been correlated to CNS disorders. To date, the molecular link(s) between NEMO activity and central nervous system function is still unclear. Mutations in the *NEMO* gene are the most common cause of Incontinentia Pigmenti, an X-linked pathology often associated with severe defects such as mental retardation, microcephaly or seizures (Fusco et al., 2004; Fusco et al., 2008; Smahi et al., 2000) suggesting that NEMO has an important role in the nervous system development.

Despite its well understood mechanism of activation in the immune system, the molecular interactions determining the activation of NF- $\kappa$ B in the CNS are poorly characterized. Most studies concerning NF- $\kappa$ B in this tissue focused on the type of inducing stimuli and on the effect of NF- $\kappa$ B inhibition/hyperactivation in neuronal cells, but there is a little knowledge on the molecules converging on the IKK complex to mediate its activation. Given the important role that NF- $\kappa$ B plays in CNS development and pathogenesis, clarifying the modality of NF- $\kappa$ B activation in this tissue results to be essential.

The aim of my thesis is to get insight in the molecular mechanisms activating NF- $\kappa$ B in CNS. Given the importance of NEMO in the NF- $\kappa$ B pathway and in particular of the molecular interactions with upstream signaling intermediates, I searched for new NEMO-interacting proteins in the CNS, by the Yeast Two-Hybrid method. The finding of new players in this tissue could be important to better understand the complex regulation of IKK/NF- $\kappa$ B activation in this tissue, that is necessary in developing new drugs for the treatment of pathological conditions.

### **3. METHODS**



### *Cell Culture and Biological Reagents*

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 1% glutamine. PC12 cells were grown in RPMI (Invitrogen) containing 10% horse serum, 5% fetal bovine serum, 1% glutamine and antibiotics (100 units/ml penicillin, 100 mg/ml streptomycin).

Monoclonal and polyclonal antibodies against HA epitope and polyclonal antibodies against NEMO/IKK $\gamma$  were purchased from Santa Cruz Biotechnologies. Monoclonal and polyclonal anti-FLAG antibodies coupled to agarose or not were purchased from Sigma.

### *Plasmids*

NESCA, RUSC2 and TRAF6 were amplified by PCR from a Human Fetal Brain cDNA library (Clontech) and cloned into pcDNA3.1-HA and -FLAG (Invitrogen) for expression in mammalian cells. NEMO/IKK $\gamma$ , IKK $\beta$ , and ubiquitin expression vectors were already present in the lab. NESCA, RUSC2 and NEMO deletion mutants were prepared by conventional PCR and cloned into pcDNA3.1-HA or -FLAG vectors.

### *Yeast Two-hybrid Screening*

The cDNA encoding the N-terminal part of Human NEMO/IKK $\gamma$  (amino acids 1–399) was cloned in-frame into the GAL-4 DNA-binding domain vector pGBKT7 (Clontech). The resulting plasmid pGBKT7-NEMO/IKK $\gamma$  was used as a bait in a yeast two-hybrid screening of a Human Fetal Brain cDNA library (Clontech) in *Saccharomyces cerevisiae* strain AH109.

This strain was transformed with the bait vector and grown for a week in selective medium (SD-Leu), then was transformed with the cDNA library and grown on selective media to select colonies in which the bait interacted with a clone of the library. About fifty colonies were selected and their plasmidic

DNAs extracted and transformed in the DH5 $\alpha$  *E. coli* strain to specifically select the vectors of the cDNA library, that putatively interacted with the bait, that were subjected to direct sequencing.

#### *Transfection and Immunoprecipitation Assay*

Transfections were performed by using Lipofectamine or Lipofectamine 2000 (Invitrogen). DNA complexes were prepared and mixed to Lipofectamine or Lipofectamine 2000 in a 1:2 ( $\mu\text{g DNA}:\mu\text{L Lipofectamine}$ ) ratio in Optimem medium (Invitrogen) without antibiotics. Each mix was added to 90% confluent cells (plated 24 hours before) and was replaced with fresh medium 5 hours later. Cells were lysed 24 hours after transfection.

All transfections included supplemental empty vector to ensure that the total amount of transfected DNA was kept constant in each dish culture.

#### *Coimmunoprecipitation assays*

For immunoprecipitation of transfected proteins, HEK293 cells ( $4 \times 10^6$ ) were transiently transfected with Lipofectamine and 24 hours after transfection cells were lysed in Triton X-100 lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and Complete Protease Inhibitor mixture). After an additional 10 min on ice, cell extracts were centrifuged for 10 min at 14000 x g at 4 °C and supernatants were incubated for 4 hours at 4 °C with anti-FLAG antibodies bound to agarose beads (M2, Sigma). The immunoprecipitates were washed five times with Triton X-100 lysis buffer, subjected to SDS-PAGE and Western Blot analysis using anti-HA antibodies. Total extracts were previously analyzed by anti-HA or anti-FLAG Western Blotting to verify the correct expression of transfected proteins.

#### *In Vivo Ubiquitination Assays*

HEK293 cells ( $1 \times 10^5$ ) were transfected with expression vectors containing Epitope-tagged Ubiquitin, FLAG-NEMO/IKK $\gamma$ , HA-NESCA and HA-TRAF6, in different combinations. 24 hours after transfection, cell lysates were prepared as above, proteins were dissociated by heating for 10 min at 95 °C in 1% SDS, samples were diluted 1:10 in Lysis Buffer and immunoprecipitated with anti FLAG antibodies as described above. Immunoprecipitated extracts were analyzed for polyubiquitination of NEMO/IKK $\gamma$  or NESCA by Western blot with anti-HA antibodies.

#### *In Vitro Translation*

*In vitro* transcription and translation were carried out according to the TNT Quick Coupled Transcription/Translation System protocol (Promega). This kit contains all the transcriptional and translational machinery. T7 promoter-containing vectors can be transcribed by the T7 RNA polymerase and then translated by the rabbit reticulocyte translational machinery. 1  $\mu$ g of each plasmid was added to 49  $\mu$ L of the mix and incubated 90 minutes at 30°C. Proteins were immunoprecipitated as described above.

#### *Confocal analysis*

PC12 cells were plated on poly-L-lysine-coated glass slides, transfected with Lipofectamine 2000; 24 hours later were fixed in 4% paraformaldehyde and treated with 1% Triton X-100 in phosphate-buffered saline. Non-specific protein binding was prevented by blocking cells with 3% bovine serum albumin in phosphate-buffered saline. Cells were stained with the appropriate primary antibodies (1:250 dilution) for 1 hour at room temperature. After three washes in phosphate-buffered saline the slides were stained with the appropriate secondary antibodies (labeled with Alexa Fluor 488 or Texas Red, 1:250 dilution) for 1 hour and washed with phosphate-buffered saline. Images were acquired with the TCS AOBS SP2 scan head mounted on the DM IRE2 microscope (Leica

Microsystems Heidelberg GmbH, Wetzlar, Germany). Data acquisition and analysis were done using Leica Confocal Software v.2.45.

## **4. RESULTS**

#### **4.1 RUSC1/NESCA and RUSC2 are two novel NEMO-interacting proteins**

The molecular mechanisms driving NF- $\kappa$ B activation through NEMO in the CNS are to date unclear. To get insight into how NEMO modulates the activation of NF- $\kappa$ B in this tissue, I decided to search for new NEMO interacting proteins, via the yeast two-hybrid system. For this purpose, I cloned the cDNA encoding for the amino acids 1-399 of NEMO, lacking of the C-terminal Zinc Finger domain to avoid non-specific interactions, into the pGBKT7 vector in frame with the GAL4 DNA binding domain. This vector was used as a bait to screen a Human Foetal Brain cDNA library, in which each cDNA is fused to the trans-activation domain of GAL4. About fifty clones encoding for putative NEMO-interacting proteins were isolated and sub-cloned in frame with the HA- or FLAG- epitopes in mammalian expression vectors in order to test their effective interaction with NEMO in coimmunoprecipitation experiments. In particular, three of them encoded for overlapping fragments of a 1516 aa long protein of unknown function, RUSC2, previously reported to bind to Rab1b, that is involved in the transport of vesicles from the Endoplasmic Reticulum to Golgi. Furthermore I found another clone encoding for the paralagous of RUSC2, RUSC1/NESCA, a 433 aa long protein that has been recently shown to be involved in the NGF pathway emanating from the TrkA receptor and to be required for the NGF-mediated growth of neurites in PC12 cells.

RUSC1/NESCA and RUSC2 share a common domain structure consisting of a RUN domain, a Leucine Zipper and an SH3 domain; furthermore they present a high sequence similarity (49% in the C-terminal region, 73% in the conserved domains such as RUN, LZ and SH3) (Figure 7).

In order to confirm the interaction between NEMO and RUSC1/NESCA or RUSC2 I cloned the full-length cDNA encoding for NESCA and RUSC2 in expression vectors in fusion with the HA- epitope (HA-NESCA and HA-RUSC2). Each of these constructs was transfected in HEK293 cells together with the construct containing the full-length cDNA of NEMO fused to the

Figure 7

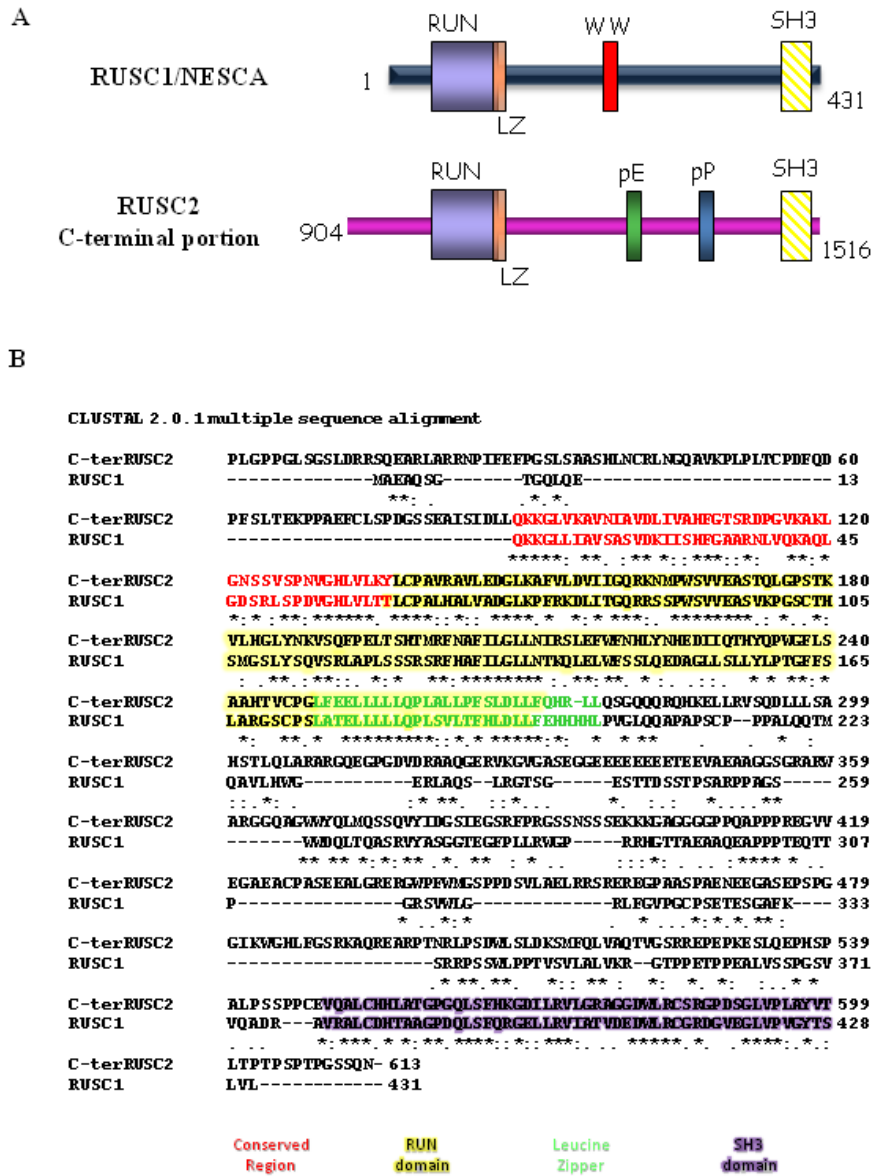


Figure 7: NESCA and RUSC2 proteins. A. Schematic representation of NESCA and carboxi-terminal portion of RUSC2. RUN, RPIP8 UNC14 and NESCA; LZ, Leucine Zipper; SH3, Src Homology domain 3. B. Clustal W sequence alignment between NESCA and the C-terminal portion of RUSC2.

FLAG- epitope (FLAG-NEMO) and cell lysates were immunoprecipitated with anti-FLAG antibodies. Anti-HA western blotting on immunoprecipitated extracts revealed that both HA-NESCA and HA-RUSC2 interact with NEMO (Figure 8).

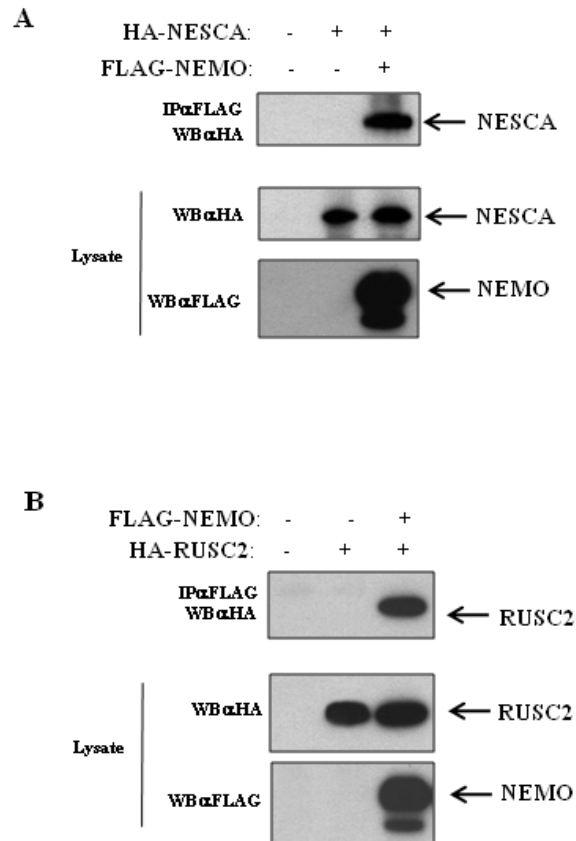
Given the absence of data about the function of RUSC2 and since NESCA has been demonstrated to be involved in the NGF pathway, that is specific of the CNS and that culminates in the activation of various transcription factors among which NF- $\kappa$ B, I decided to focus my attention mainly on the study of the NEMO-NESCA interaction, although I will also present preliminary results on RUSC2.

#### **4.2 Mapping the NEMO interaction site**

In order to biochemically characterize the interaction between NEMO and NESCA I decided to map the site by which NESCA can interact with NEMO. For this reason I cloned several N- or C-terminal deletion mutants of NESCA (Figure 9A) in frame with the HA- tag: HA-NESCA 304-433, lacking of the N-terminal portion and containing only the SH3 domain; HA-NESCA 1-306, lacking only of the SH3 domain and containing the RUN, LZ and WW domains; HA-NESCA 1-203 contains the RUN and the LZ domains; HA-NESCA 1-136 covers the first 136 amino acids of NESCA, in which is present only a part of the RUN domain and lacking of the LZ, WW and SH3 domains. HEK293 cells were co-transfected with FLAG-NEMO together with HA-NESCA or each of the HA-NESCA mutants. Upon immunoprecipitation of lysates with anti-FLAG antibodies, anti-HA western blotting on immunoprecipitates revealed that only HA-NESCA, HA-NESCA 1-306 and 1-203 were able to co-precipitate with NEMO (Figure 9b), although all mutants are expressed in the total fractions, revealing that the region necessary for the interaction is located between amino acids 137-305; this region contains part of the RUN and the LZ domain.

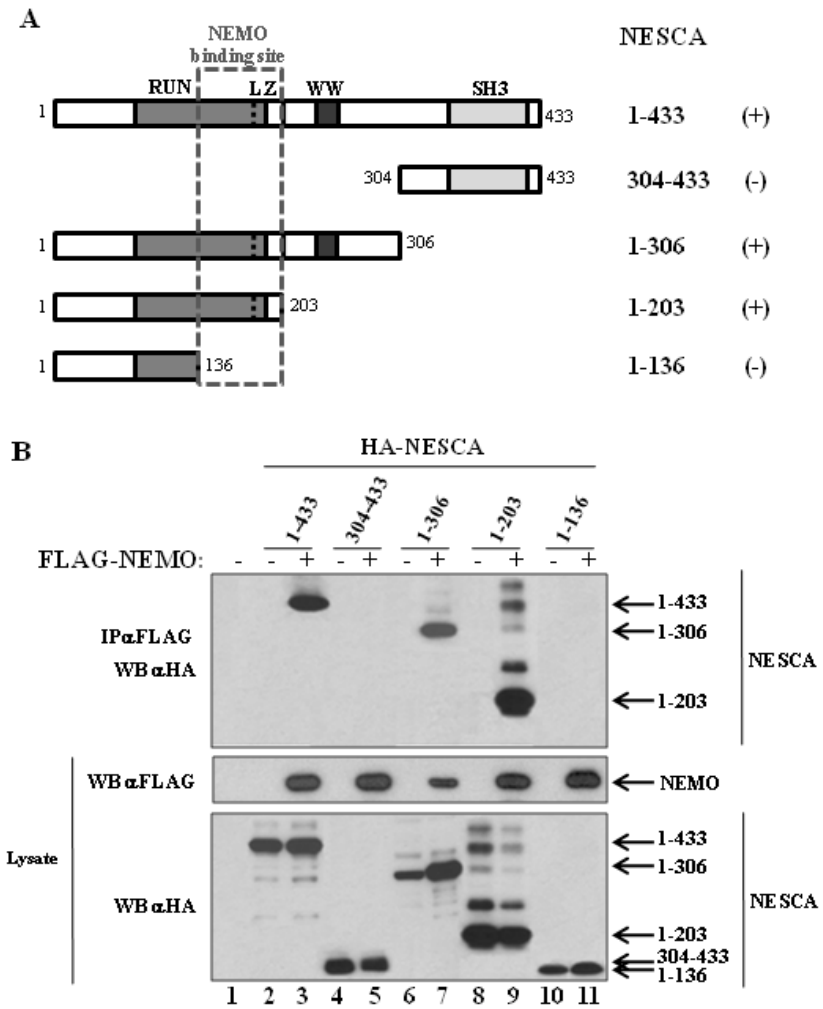


**Figure 8**



**Figure 8: NESCA and RUSC2 interact with NEMO.** *A and B.* HA-NESCA or HA-RUSC2 and FLAG-NEMO were co-transfected in HEK293 cells; cell lysates were immunoprecipitated with anti-FLAG antibodies. Immunoprecipitates were subjected to SDS-PAGE and subsequently to Western immunoblotting

**Figure 9**



**Figure 9: Mapping the NEMO interaction site.** *A.* Schematic representation of the NESCA deletion mutants used to map the NEMO-binding site (indicated in the dashed panel). The interaction of each construct with NEMO is indicated with a *plus*. RUN, Leucine Zipper (LZ), WW and Src Homology 3 (SH3) domains are indicated. *B.* Mapping of the NEMO-interaction site; HEK293 cells were transfected with the indicated combinations of expression constructs encoding for FLAG-NEMO and HA-NESCA deletion mutants. Cells extracts were analyzed by immunoblotting either directly or after immunoprecipitation with anti-FLAG antibodies.

### **4.3 Mapping the NESCA binding site of NEMO**

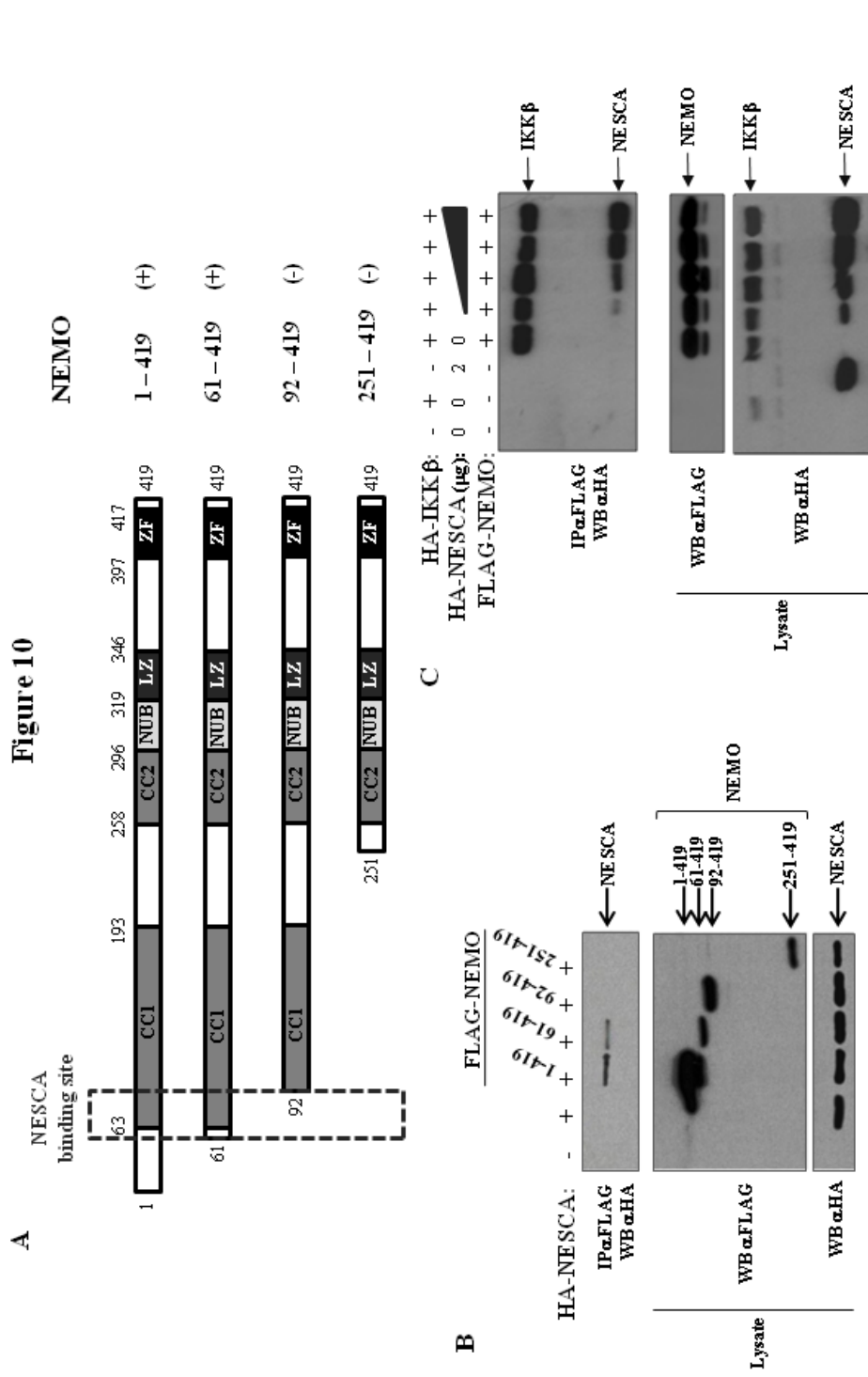
In order to define the region of NEMO that is critical for the interaction with NESCA, I generated three N-terminal deletion mutants of NEMO (Figure 10A): NEMO 61-419, lacking the first 60 amino acids; NEMO 92-419 lacks of a small part of the first Coiled-Coli domain (CC1); NEMO 251-419 lacks of the entire CC1 but still contains the CC2, NUB, LZ and ZF domains. These mutants were cloned in frame with the FLAG- epitope. FLAG-tagged mutants and HA-NESCA were co-transfected in HEK293 cells and their interaction was examined by coimmunoprecipitation experiments. From this screening I found that HA-NESCA was able to co-precipitate only with FLAG-NEMO and FLAG-NEMO 61-419, demonstrating that the region from amino acid 61 to 92 is essential for the association between the two proteins (Figure 10B).

As the NESCA-binding site overlaps with the IKK-binding region of NEMO (Leonardi et al., 2000), I wondered if the interaction between NEMO and NESCA can interfere with the stability of the IKK complex. To test this hypothesis I co-transfected increasing amounts of HA-NESCA together with FLAG-NEMO and HA-IKK $\beta$  in HEK293 cells and I examined their interaction. Upon incubation with anti-FLAG antibodies, immunoprecipitates were subjected to SDS-PAGE and anti-HA western blotting. I found that NEMO is able to bind to both IKK $\beta$  and NESCA in all experimental points (Figure 10C), demonstrating that the interaction with NESCA does not disrupt the IKK complex.

### **4.4 NEMO and NESCA co-localize in PC12 cells in an NGF-dependent manner**

To establish the subcellular localization of NESCA and to know whether this protein is able to co-localize with NEMO, I co-transfected HA-NESCA and FLAG-NEMO in the neuronal cell line PC12. After transfection, cells were incubated with monoclonal anti-HA and polyclonal anti-FLAG antibodies,

**Figure 10**



**Figure 10: NESCA interacts with the IKK complex by the N-terminal region of NEMO.**  
**A** Schematic representation of the NEMO-deletion mutants tested for their ability to interact with NESCA. The interaction of each construct with NESCA is indicated with a plus. The NESCA-interaction site is indicated in the dashed square. Coiled-coil 1 (CC1), Coiled-coil 2 (CC2), NEMO-Ubiquitin Binding (NUB), Leucine Zipper (LZ) and Zinc Finger (ZF) domains are shown. **B** HEK293 cells were transiently transfected with the indicated FLAG-tagged NEMO deletion mutants and HA-NESCA; cell extracts were immunoprecipitated with anti-FLAG antibodies and immunoblotted to assess the ability of NESCA to co-immunoprecipitate with each deletion mutant. **C** Increasing amounts of HA-NESCA (0.1 to 2 μg) were transfected in HEK293 cells together with FLAG-NEMO and HA-IKKβ. Cell lysates were generated after 24 hours and subjected to direct Western blotting or immunoprecipitated with anti-FLAG antibody. Immunoprecipitates were immunoblotted with anti-HA antibodies.

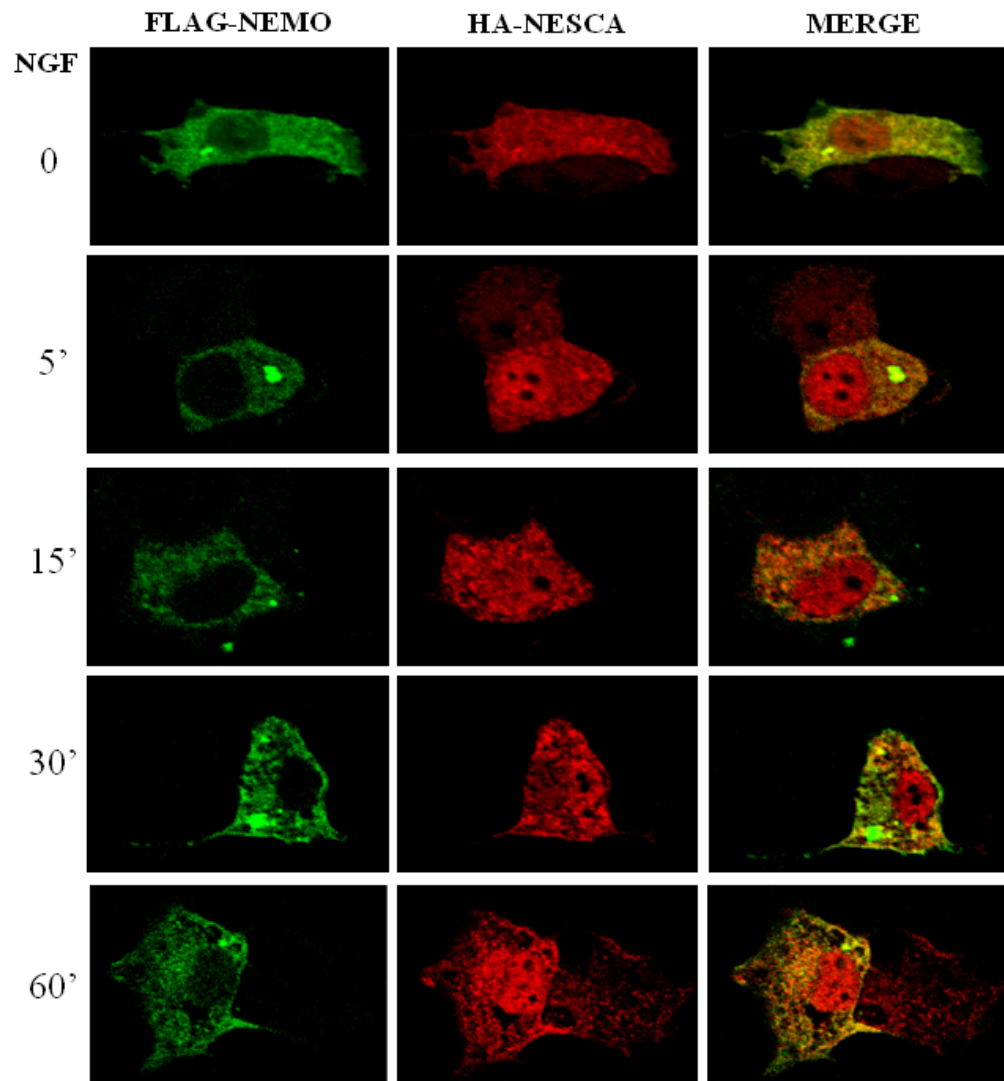
respectively recognized by Red anti-mouse and Green anti-rabbit antibodies. As shown in the Figure 11, HA-NESCA and FLAG-NEMO co-localize in the cytoplasm of PC12 cells, although NESCA shows also a nuclear localization. Of note, the distribution of the two proteins seems to change in the presence of NGF. In particular, 15 minutes upon NGF stimulation, the two proteins appear with a spotted pattern while they are also localized under the cell membrane upon 30 to 60 minutes of NGF stimulation. This localization seems to recapitulate the events that have been shown to occur upon NFG stimulation (Figure 6), in which the receptor is internalized in signaling vesicles upon 10-15 minutes of NGF stimulation and it re-localizes under the cell membrane upon 30-60 minutes.

#### **4.5 NESCA directly associates with TRAF6 by its N-terminal portion**

Adapter molecules are generally involved in regulating the dynamics of molecular interactions. Since NESCA represents a new adapter in the NGF-pathway (MacDonald et al., 2004), I sought to verify the presence of other NESCA-interacting proteins belonging to the NGF-mediated NF- $\kappa$ B pathway. In particular, when I transfected the mutant HA-NESCA 1-203, western blot analysis revealed the presence of upper bands (Figure 9B, lanes 8-9), that could be the result of covalent post-translational modifications, such as polyubiquitination. Since TRAF6 is the E3 ubiquitin ligase involved in the NGF pathway, I wondered if NESCA and TRAF6 could interact.

I cloned TRAF6 in frame with the FLAG- epitope by amplifying it from a Human Brain cDNA library. Coimmunoprecipitation experiments in HEK293 cells revealed that NESCA is able to interact with TRAF6 when both proteins were co-transfected in HEK293 cells (Figure 12A). To further confirm the direct interaction between the two proteins I produced *in vitro* translated proteins; also in this case I could detect the interaction between FLAG-TRAF6 and HA-NESCA, demonstrating the direct interaction between the two proteins (Figure 12B).

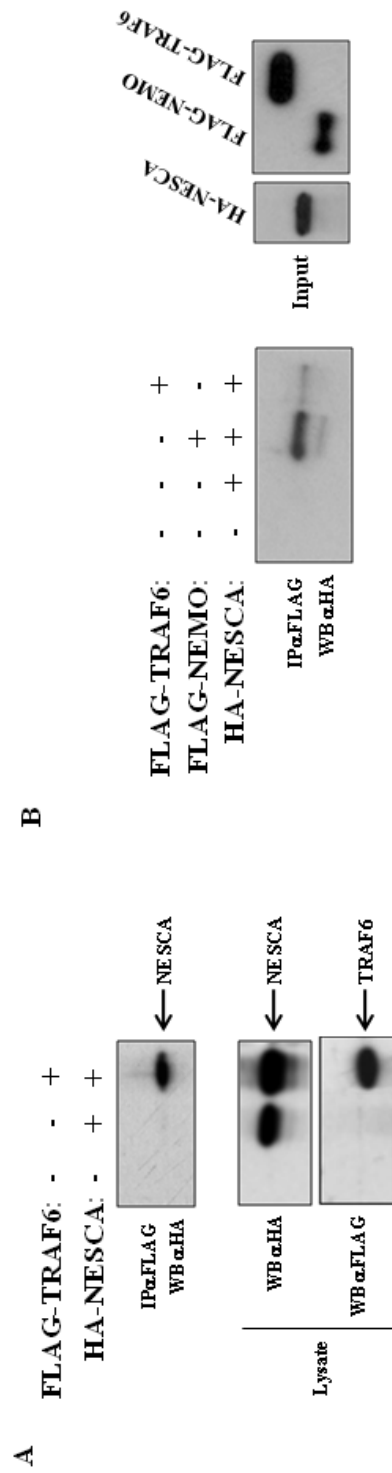
**Figure 11**



**Figure 11: NEMO and NESCA colocalize in PC12 cells.**

PC12 cells were plated on poly-L-lysine-coated glass slides and transfected with HA-NESCA and FLAG-NEMO. 24 hours after transfection cells were treated with 100ng/mL NGF for the indicated times and then treated with anti-FLAG or anti-HA antibodies and with the appropriate secondary antibodies labeled with Alexa Fluor 488 or Texas Red. Cells were examined by confocal microscopy.

**Figure 12**



**Figure 12: NESCA directly binds to TRAF6**

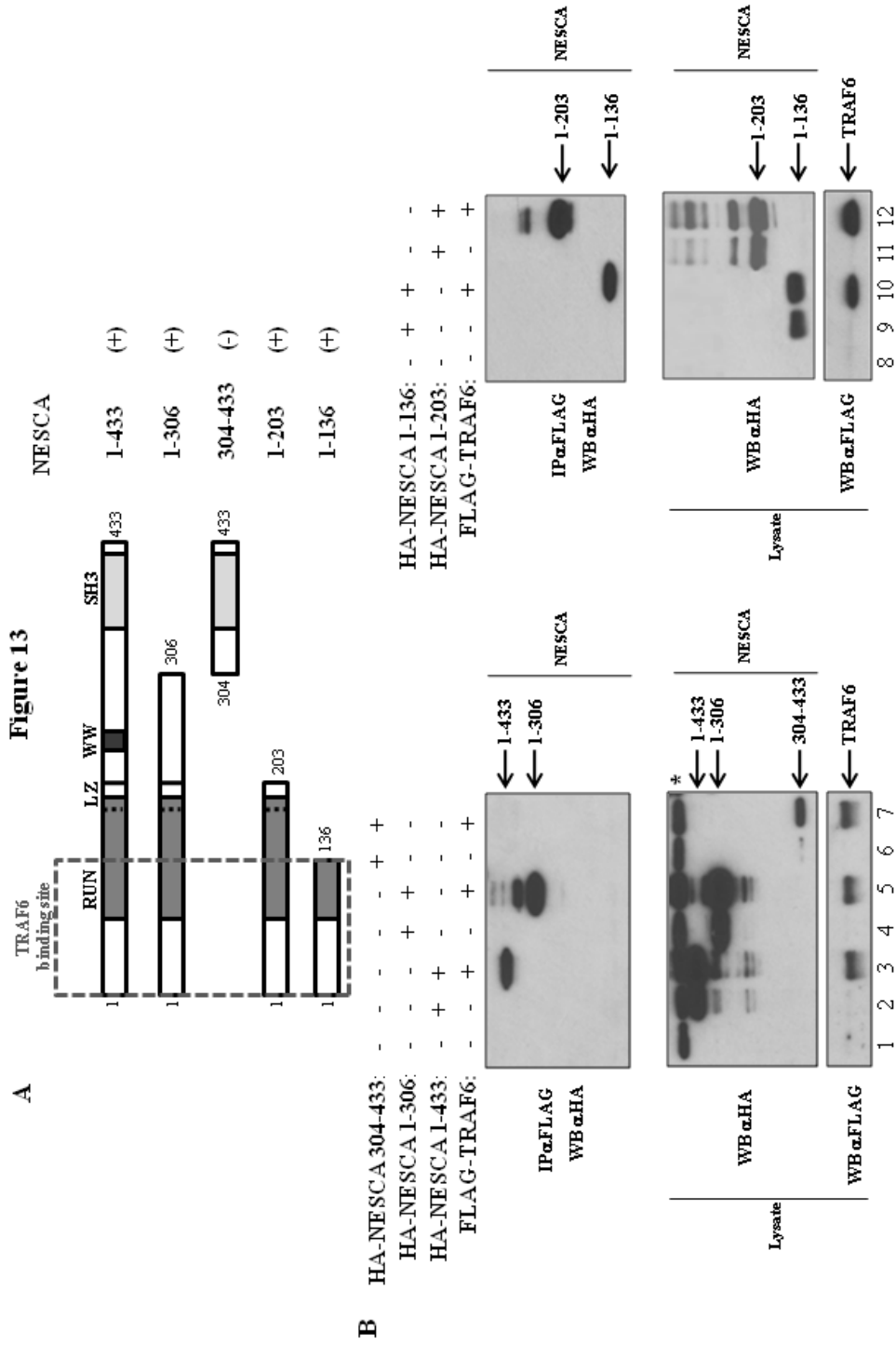
*A.* HEK293 were transfected with the indicated constructs encoding for HA-NESCA and FLAG-TRAF6. Cells were lysed and immunoprecipitated with anti-FLAG antibodies. Bound proteins were subjected to SDS-PAGE and immunoblotted with anti-HA antibodies. *B.* *in vitro* translated HA-NESCA and FLAG-NEMO or FLAG-TRAF6 were incubated 4 hours with anti-FLAG antibodies. Immunoprecipitates were subjected to anti-HA Western blotting.

To map the region of NESCA responsible for the interaction with TRAF6, I co-transfected the HA-tagged deletion mutants of NESCA already used to map the interaction with NEMO together with FLAG-TRAF6 in HEK293 cells (Figure 13A). Coimmunoprecipitation assays revealed that all the C-terminal deletion mutants are able to co-precipitate with TRAF6, while NESCA 304-433 is not able to do it, revealing that the N-terminal region of NESCA is required for the binding to TRAF6 and in particular the first 136 amino acids of NESCA, that contain part of the RUN domain, are sufficient to produce this interaction (Figure 13B).

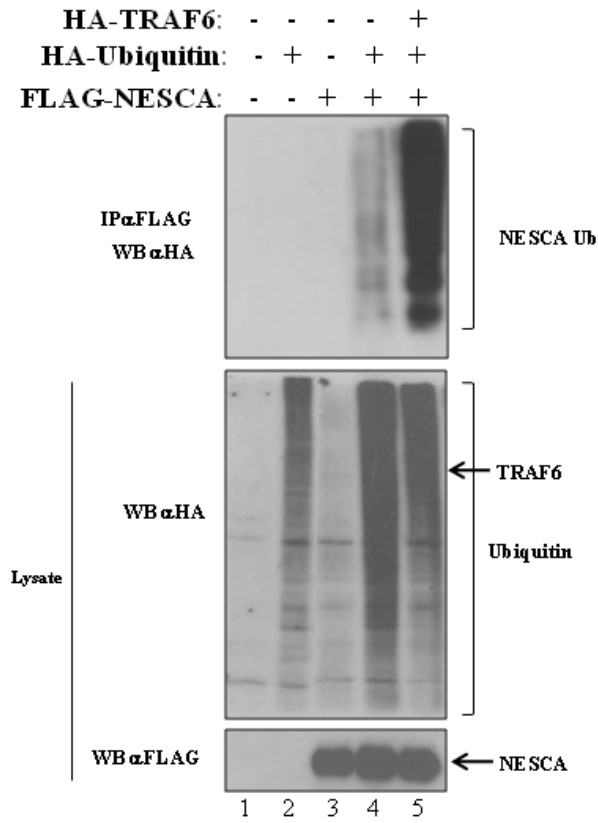
#### **4.6 NESCA is Lys-63 polyubiquitinated by TRAF6**

Polyubiquitination of signaling intermediates of the NF- $\kappa$ B pathway has been shown to be an essential modification occurring upon stimulation of many external triggers (Chen, 2005; Perkins, 2006), including the NGF. Since NESCA is able to interact with TRAF6, I sought to verify whether it may undergo polyubiquitination. To test this hypothesis, I performed a polyubiquitination assay using NESCA as a substrate. I transfected HEK293 cells with NESCA in the absence (Figure 14, lane 3) or in the presence of exogenous ubiquitin (Figure 14, lane 4). Cell extracts were boiled in 1% SDS and then subjected to anti-FLAG immunoprecipitation. Western blotting analysis on immunoprecipitates with anti-HA antibodies revealed that NESCA undergoes polyubiquitination (Figure 14, IP panel, lane 4). Of note, NESCA polyubiquitination was strongly enhanced in the presence of TRAF6, indicating that this molecule acts as an E3 ubiquitin ligase for NESCA (Figure 14, lane 5). Under stringent SDS-denaturing conditions, TRAF6 did not co-immunoprecipitate with NESCA, excluding the possibility that the polyubiquitination observed was the result of auto-ubiquitinated TRAF6 (not shown).





**Figure 14**



**Figure 14: NESCA undergoes TRAF6-mediated polyubiquitination.**

HEK293 were transfected with the indicated plasmids encoding FLAG-NESCA with or without HA-Ubiquitin and HA-TRAF6. Cells extracts were boiled in 1% SDS for 5 minutes, immunoprecipitated with anti-FLAG antibodies and subjected to SDS-PAGE. Ubiquitin-bound NESCA was revealed by anti-HA Western blotting.

#### **4.7 NEMO polyubiquitination is affected by the overexpression of NESCA**

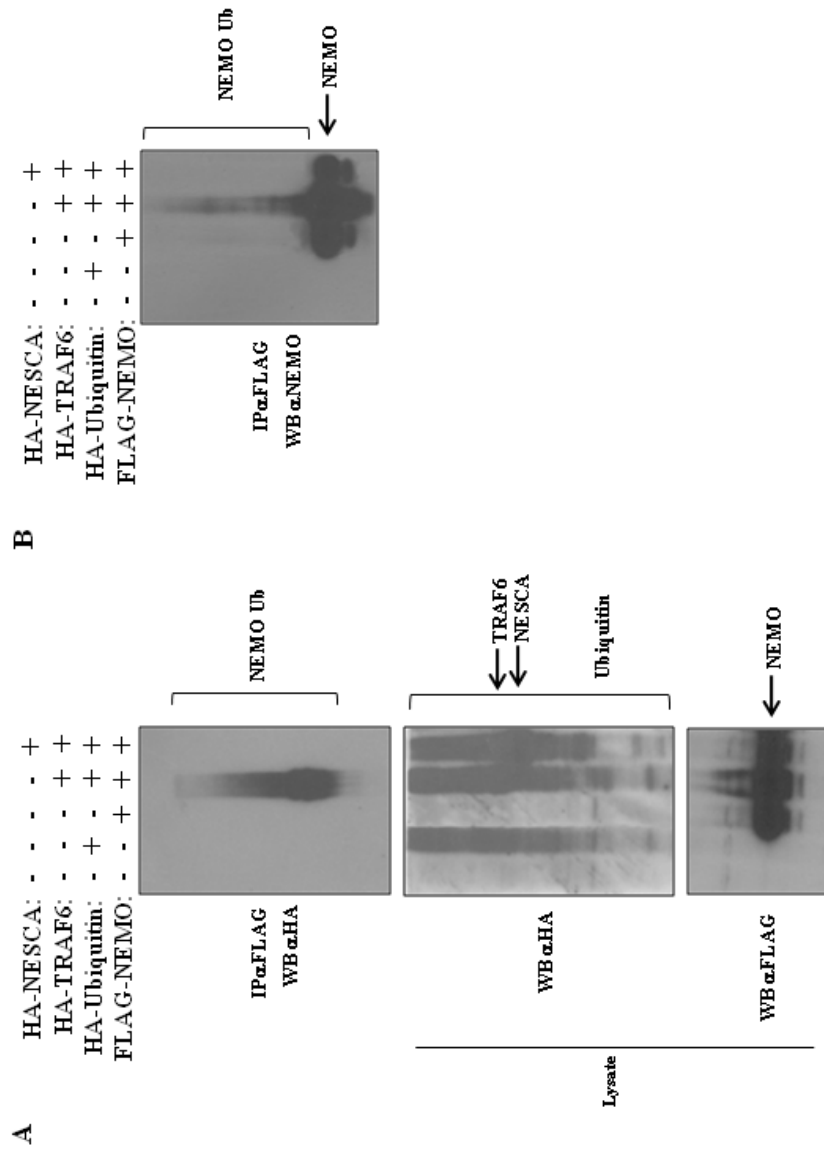
NEMO is a well characterized target for TRAF6-mediated polyubiquitination (Sebban-Benin et al., 2007). Because NESCA interacts with both NEMO and TRAF6, I wondered whether NESCA may have a role in the process of polyubiquitination of NEMO. To test this hypothesis I transfected FLAG-NEMO in the presence of HA-Ubiquitin and HA-TRAF6 without (Figure 15A, lane 4) or with HA-NESCA (Figure 15A, lane 5). This experiment revealed that the overexpression of NESCA completely abolished the TRAF6-mediated polyubiquitination of NEMO, as it appears by using either anti-HA (Figure 5A) or anti-NEMO (Figure 15B) antibodies on immunoprecipitated extracts.

TRAF6 is a well known activator of NF- $\kappa$ B and its overexpression results in both polyubiquitination of signaling proteins and NF- $\kappa$ B activation (Conze et al., 2008; Sebban-Benin et al., 2007; Sun et al., 2004). Although the overexpression of NESCA is able to abolish the polyubiquitination of NEMO, this event does not seem to correlate with an impaired NF- $\kappa$ B activation, measured by an NF- $\kappa$ B driven luciferase plasmid transfected in each experimental point (data not shown).

#### **4.8 Preliminary results on the protein RUSC2**

In order to characterize the interaction between NEMO and RUSC2 I began to verify if this binding can occur through the same regions necessary for the NEMO-NESCA interaction. For this reason I generated various HA-tagged deletion mutants of RUSC2 lacking several portions of the protein, that I tested for their ability to bind to NEMO in coimmunoprecipitation assays in HEK293 cells (Figure 16A). From this screening emerged that only the mutants containing the region between amino acids 904-1182 of RUSC2, containing the RUN and the LZ domain, are able to co-precipitate with FLAG-NEMO, demonstrating that this region is responsible for the interaction (Figure 16B).

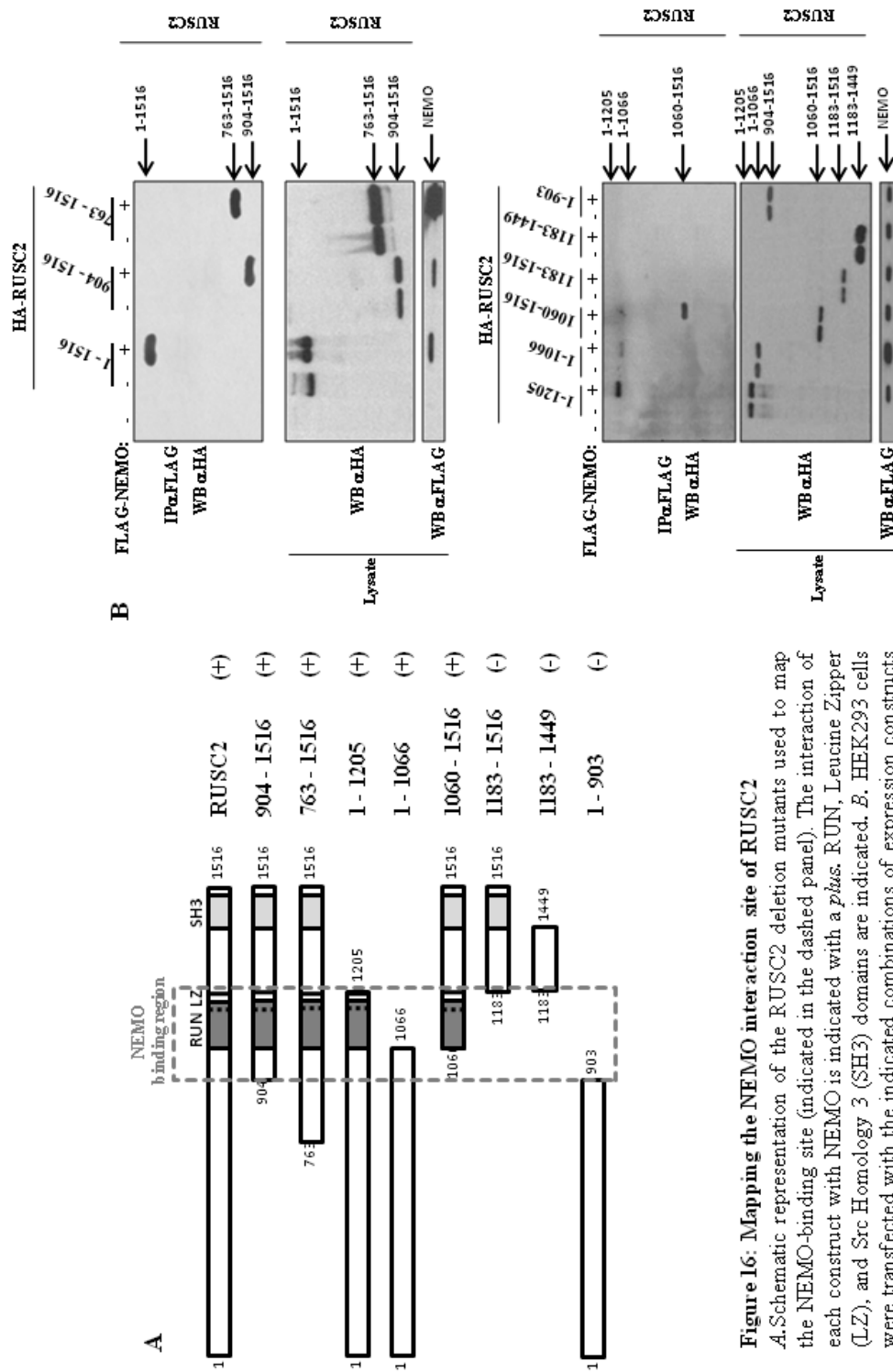
**Figure 15**



**Figure 15: NEMO polyubiquitination is abolished in the presence of NESCA.**

*A.* HEK293 cells were transfected with the indicated combination of constructs encoding FLAG-NEMO, HA-Ubiquitin and HA-TRAF6 with or without HA-NESCA. After boiling in 1% SDS, cell extracts were immunoprecipitated with anti-FLAG antibodies. Ubiquitin-bound NEMO was revealed by Western blotting using both anti-HA or *B.* anti-NEMO antibodies.

**Figure 16**

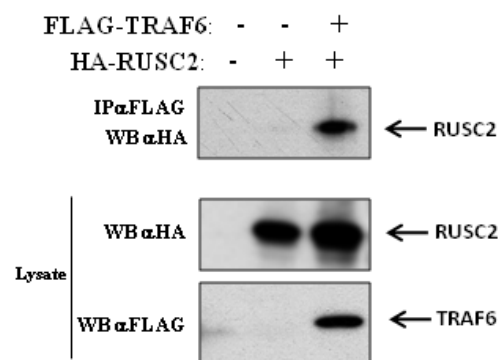


**Figure 16: Mapping the NEMO interaction site of RUSC2**

A. Schematic representation of the RUSC2 deletion mutants used to map the NEMO-binding site (indicated in the dashed panel). The interaction of each construct with NEMO is indicated with a *plus*. RUN, Leucine Zipper (LZ), and Src Homology 3 (SH3) domains are indicated. B. HEK293 cells were transfected with the indicated combinations of expression constructs encoding for FLAG-NEMO and HA-RUSC2 deletion mutants. Immunoprecipitates were analyzed by anti-HA immunoblotting.

In order to map the RUSC2-interaction site of NEMO, I used the same FLAG-NEMO-deletion mutants shown in Figure 10. Each of these mutants was co-transfected with HA-RUSC2 in HEK293; coimmunoprecipitation assays revealed that RUSC2 is able to co-precipitate only with FLAG-NEMO and FLAG-NEMO 61-419, demonstrating that the NESCA- and RUSC2-interaction sites are the same (not shown). Finally, HA-RUSC2 is also able to co-precipitate with FLAG-TRAF6 in HEK293 cells (Figure 17), suggesting that redundant functions between NESCA and RUSC2 may occur.

**Figure 17**



**Figure 17: RUSC2 interacts with TRAF6**

HEK293 were transfected with the indicated plasmids encoding FLAG-TRAF6 and HA-RUSC2. Cells extracts were immunoprecipitated with anti-FLAG antibodies and subjected to SDS-PAGE. Anti-HA western blotting revealed the interaction between RUSC2 and TRAF6.

## **5. DISCUSSION**



In the present study I report on the identification of two novel NEMO and TRAF6 interacting protein: NESCA and RUSC2. These are two paralogous protein sharing a common domain structure and a high sequence similarity. While RUSC2 is a protein of unknown function, NESCA has been previously shown to be part of the TrkA-mediated NGF pathway and to be important in the NGF-mediated neurite growth of PC12 cells (MacDonald et al., 2004). NF- $\kappa$ B is one of the transcription factors activated upon NGF stimulation, whose activity is necessary for neuronal differentiation (Carter et al., 1996),(Foehr et al., 2000a). Several molecules have been shown to belong to the NGF-induced NF- $\kappa$ B pathway, such as the E3 ubiquitin ligase TRAF6 (Khursigara et al., 1999), the adapter molecule p62 (Wooten et al., 2005) and the PKC atypical kinases (Wooten, 1999; Wooten et al., 2001). Albeit the effects of overexpression or downregulation of these molecules impact on neuronal survival or differentiation and on NF- $\kappa$ B activation (Foehr et al., 2000b; Joung et al., 2005; Yeiser et al., 2004), it is not known how these molecules can recruit and regulate the IKK complex. NESCA represents (one of) the molecular link(s) connecting the IKK complex to upstream molecules. Because it is able to bind to the TrkA receptor (MacDonald et al., 2004) and also to TRAF6 and to NEMO, NESCA can be considered a novel central adapter in the NGF-induced NF- $\kappa$ B pathway. This raises the question of which is the functional role of NESCA in such pathway. In general, NF- $\kappa$ B signaling requires adapter molecules, such as RIP, which binds to NEMO to recruit the IKK complex to the receptor and therefore to induce its activation (Poyet et al., 2000). A central event in this process is the polyubiquitination of RIP upon TNF $\alpha$  stimulation. Therefore, polyubiquitinated RIP provides a platform for the recruitment and modulation of the IKK complex through the Ubiquitin-binding domain of NEMO (Ea et al., 2006; Li et al., 2006; Wu et al., 2006). One can imagine that NESCA may operate in a way quite similar to RIP. This hypothesis is supported by some experimental findings: first, despite its binding to the region between amino acids 61-91 of NEMO, that

overlaps with the IKK-binding region, NESCA does not disrupt the IKK complex, considering that NEMO can interact with both NESCA and IKK $\beta$  simultaneously (Figure 10C) and that IKK $\beta$  can co-immunoprecipitate with NESCA in the presence of NEMO (not shown). Second, NESCA can be polyubiquitinated by TRAF6, because we observed that TRAF6 overexpression strongly enhances the polyubiquitination of NESCA (Figure 14). Third, polyubiquitinated forms of NESCA are able to co-immunoprecipitate with NEMO, as it appears both by transfecting HA-NESCA and exogenous ubiquitin (not shown) or by transfecting a deletion mutant of NESCA (HA-NESCA 1-203) that seems to be constitutively ubiquitinated (Figure 9B, lane 9). These data suggest that NESCA could be poly-ubiquitinated by TRAF6 and that this event could stimulate the IKK complex recruitment.

Which is the functional significance of the NESCA–IKK complex binding? Since NESCA can bind to both NEMO and TRAF6, we wondered whether polyubiquitination of NEMO could be altered by the overexpression of NESCA. Surprisingly, NESCA overexpression completely abolishes the TRAF6-dependent NEMO polyubiquitination, without producing any changing in NF- $\kappa$ B activity, measured by luciferase assay. The polyubiquitination of NEMO has been generally accepted to have a positive effect on the activation of the IKK complex (Tang et al., 2003; Yamamoto et al., 2003; Zhou et al., 2004), although some recent papers bring this thesis into question. Indeed, it has been shown that a point mutation in the C-terminal region of NEMO (K392R) results in a defective LPS-induced NEMO polyubiquitination, without affecting the activation of NF- $\kappa$ B (Ni et al., 2008). Moreover, another C-terminal NEMO mutant (K399R) shows a defective CARMA1-Bcl10-Malt1 induced polyubiquitination, even if this mutation has only a slight effect on inducible NF- $\kappa$ B activation in T cells (Oeckinghaus et al., 2007). These last data are in agreement with my findings suggesting that additional and not yet clearly

understood mechanisms of IKK complex regulation through the polyubiquitination of NEMO may occur.

In summary, I have identified NESCA as a novel adapter involved in the NGF-mediated NF- $\kappa$ B pathway. In addition to its ability to bind to the TrkA receptor (MacDonald et al., 2004), NESCA can also bind to NEMO and to TRAF6, which in turn catalyzes the polyubiquitination of NESCA. The functional consequence of these interactions is that NESCA could recruit the IKK complex and regulate the levels of NEMO polyubiquitination, even if the functional significance of this latter event in the NGF signaling remains to be established (Figure 18).

It still remains to establish which role RUSC2 can play in these events, in particular whether it has redundant functions, as it is suggested by the similarity between NESCA and RUSC2 interaction with NEMO and TRAF6. RNAi experiments downregulating both NESCA and RUSC2 expression could help to understand the redundant or individual role that each of the two proteins may play in the NGF-mediated NF- $\kappa$ B pathway.

Figure 18

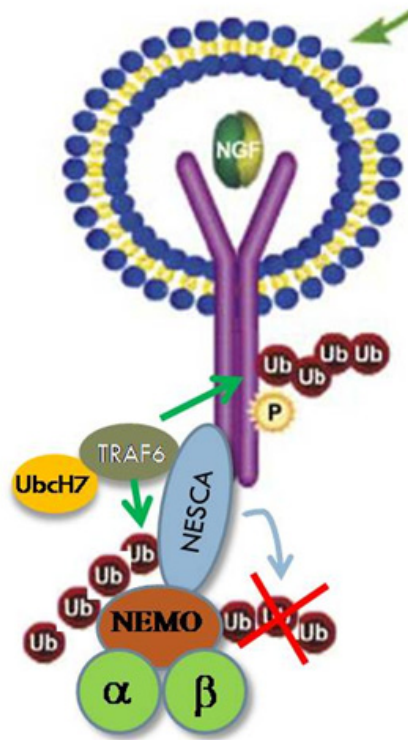


Figure 18: Model of the possible mechanism of action in the NGF pathway

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