ΧΑΡΑΚΤΗΡΙΣΜΟΣ ΤΩΝ ΜΟΡΙΑΚΩΝ ΜΟΝΟΠΑΤΙΩΝ ΠΟΥ ΣΥΝΔΕΟΥΝ ΤΑ ΜΕΛΗ ΤΗΣ ΟΙΚΟΓΕΝΕΙΑΣ ΤΩΝ TNF ΥΠΟΔΟΧΕΩΝ ΜΕ ΒΑΣΙΚΕΣ ΚΥΤΤΑΡΙΚΕΣ ΛΕΙΤΟΥΡΓΙΕΣ

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Characterization of novel molecular pathways that involve TNF receptor family members

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Only as a warrior can one withstand the path of knowledge. A warrior cannot complain or regret anything. His life is an endless challenge, and challenges cannot possibly be good or bad. Challenges are simply challenges.

To my parents

Περίληψη

Λόγω του εκτεταμένου επιστημονικού ενδιαφέροντος για την οικογένεια των TNF υποδοχέων, έχει δοθεί αρκετή σημασία στην περιγραφή των σηματοδοτικών μονοπατιών που συμμετέχουν τα μέλη αυτής της οικογένειας. Αυτό οδήγησε στην ανακάλυψη μιας νέας οικογένειας σηματοδοτικών μορίων που ονομάστηκαν TNF-Receptor-Associated Factors (TRAFs). Οι πρωτεΐνες TRAF λειτουργούν ως προσαρμογείς για μια ποικιλία μεμβρανικών υποδοχέων και μπορούν να ρυθμίσουν τόσο θετικά όσο και αρνητικά μονοπάτια που σχετίζονται με την απόπτωση αλλά και να επάγουν γονίδια που προωθούν την κυτταρική επιβίωση. Η TRAF3, μέλος αυτής της οικογένειας, δεν είχε αρχικά μελετηθεί με διεξοδικό τρόπο αλλά τα τελευταία χρόνια έχει δοθεί τεράστια σημασία στην ανάλυση των λειτουργιών της στο κύτταρο. Ως αποτέλεσμα, τώρα θεωρείται ως μια πρωτεΐνη με πολλαπλούς ρυθμιστικούς ρόλους σε μια ποικιλία σηματοδοτικών μονοπατιών. Παρόλα αυτά, υπάρχουν αρκετά ερωτήματα σχετικά με την ρύθμιση της ίδιας της TRAF3 από άλλους παράγοντες και την λειτουργία της. Η αναγνώριση νέων μορίων που αλληλεπιδρούν με την TRAF3 μπορεί να ρίξει φως στον τρόπο με τον οποίο η TRAF3 ¨στρατολογείται¨ στα διάφορα σηματοδοτικά συμπλέγματα καθώς και πιθανούς τρόπους με τους οποίους ρυθμίζεται η λειτουργία της.

Με την τεχνική ‘yeast-2-hybrid’ αναγνωρίστηκε η πρωτεΐνη Ubc9 ως νέος παράγοντας αλληλεπίδρασης με την TRAF3. Η Ubc9 είναι το μοναδικό E2 ένζυμο που έχει αναγνωριστεί μέχρι στιγμής να συμμετέχει στο μονοπάτι της Σουμουλίωσης (SUMOylation), μιας σημαντικής μετα-μετάφραστικής τροποποίησης. Ο σκοπός αυτού του διδακτορικού ήταν ο χαρακτηρισμός αυτής της αλληλεπίδρασης με λεπτομέρεια. Δείχνουμε ότι η TRAF3 σουμουλιώνεται *in vitro* και ότι η τροποποίηση αυτή οφείλεται στην Ubc9. Απουσία της τροποποίησης αυτής από την Ubc9 οδηγεί στην σταθεροποίηση του TRAF3 μετά από διέγερση των κυττάρων με CD40 και καθυστέρηση της έναρξης του εναλλακτικού μονοπατιού του NF-κB. Τέλος, ¨κλείσιμο¨ της Ubc9 με την μέθοδο του siRNA οδηγεί σε μειωμένη αλληλεπίδραση της TRAF3 με τον υποδοχέα του CD40, κάτι που οδηγεί στο συμπέρασμα ότι η συγκεκριμένη μετά-μεταφραστική τροποποίηση είναι σημαντική για το σχηματισμό των σηματοδοτικών συμπλεγμάτων που συμμετέχει η TRAF3.

Abstract

The tumor necrosis factor (TNF) and TNF receptor (TNFR) superfamily comprises a group of secreted or membrane-bound ligands and their receptors, respectively with a wide range of functions in apoptosis, bone regeneration and immune system regulation (Locksley *et al*, 2001). Given the immense interest in the functions of TNF receptor family members significant effort was put towards the characterization of the signal transduction pathways which mediate their pleiotropic effects. This led to the discovery of a family of signal transduction molecules called TNF receptor- associated factors (TRAFs). TRAFs serve as adapter proteins for a variety of cell surface receptors and are able to both negatively regulate programmed cell death pathways and induce genes that promote cell survival. TRAF3, a member of this family, has gone from a poorly understood protein to an important multifunctional regulatory protein within a few years. Nonetheless, there are still several open questions regarding the function and regulation of TRAF3. The possible identification of novel TRAF3 interacting proteins may shed light into how TRAF3 is recruited to different signaling complexes; yet un-identified molecules may be revealed that control TRAF3 function.

Given the essential functions of TRAF3 in different signaling pathways that control inflammation, antiviral immunity and cell survival and its already established role in human health, further mechanistic insights into TRAF3 signaling will go a long way towards understanding this mysterious TRAF. Unpublished results from Dr Eliopoulos' laboratory have utilized the yeast-two hybrid technique to identify novel TRAF3-interacting proteins. Using TRAF3 as bait and a HeLa cDNA library as prey, Ubc9, the sole E2 enzyme in the sumoylation process has been identified. The aim of this thesis is to validate and characterize in detail the nature of this protein's interaction with TRAF3. We demonstrate that TRAF3 is SUMOylated *in vivo* and its sumoylation is dependent on Ubc9. Absence of sumoylation leads to stabilization of TRAF3 following CD40 signaling and a delayed induction of the alternative NFκB pathway. Knockdown of Ubc9 leads to reduced affinity of TRAF3 with the CD40 receptor which suggests that TRAF3 SUMOylation is important for the formation of TRAF3 complexes.

Chapter 1A: Introduction to TNF-Receptor-Associated-Factor 3

1.1.1 TRAF family of proteins

The Tumor necrosis factor receptor (TNFR) superfamily comprises a group of secreted or membrane-bound ligands and their receptors, respectively with a wide range of functions (Locksley et al., 2001). All TNFRs are characterized as type I transmembrane proteins, with an extracellular N-terminus and intracellular C-terminus necessary for signaling initiation (Bodmer et al, 2002). The TNFR family includes TNFR1 and 2, the lymphotoxin β-receptor, CD27, CD30, CD40, Fas/CD95/Apo-1, OX-40, 4-1bb, RANK/TRANCE-R, TRAMP/DR3, TRAIL receptors and the low affinity NGF receptor. Receptor engagement leads to various biological outcomes ranging from cell survival to cell death and also include effects on cell metabolism, gene expression, differentiation, adhesion and motility (Shaulian and Karin, 2002). In the immune system, TNFR family members and their ligands are important for innate immunity, lymphoid organ development, co-stimulatory lymphocyte activation, cytokine production and immunoglobulin (Ig) class switching (Chang et al., 2006; Sakurai et al., 2006; Gallagher et al., 2007). Central in the intricate series of events following receptor engagement that culminates in a well-choreographed ballet of protein-protein interactions, are a small family of signaling proteins, comprising seven members: the TNF-receptor-associated factors (TRAFs).

Given the immense interest in the functions of TNF receptor family members significant effort was put towards the characterization of the signal transduction pathways which mediate their pleiotropic effects. This led to the discovery of a family of signal transduction molecules called TNF receptor- associated factors (TRAFs). The first TRAFs to be identified, TRAF1 and TRAF2, were cloned based on their interaction with the intracellular domain of TNFR2 (Rothe et al., 1994). During that time TRAF3 was also identified through its association with the cytoplasmic tails of CD40 and the Epstein-Barr Virus Latent Memebrane Protein (LMP1) (Hu et al., 1994; Sato et al., 1995; Cheng et al., 1995). TRAFs were proposed to be adaptor proteins in TNFR signaling owing to their activation-dependent receptor binding and concomitant recruitment of the

constitutively associated TRAF-binding proteins cellular inhibitor of apoptosis 1 (cIAP1) and cIAP2 (Rothe et al., 1995; Wang et al., 1998).

Subsequent studies have since led to the discovery of four more TRAF family members. The recognition that TRAFs interact with all TNFR family members has made clear that one important aspect of their function is the assembly of signaling complexes at the intracellular domains of transmembrane receptors, ultimately translating receptor ligation into the activation of downstream signaling pathways. TRAF4 was identified by its over-expression in breast carcinoma cells (Régnier et al., 1995); TRAF5 by its interaction with CD40 and LTβR (Ishida et al., 1996b; Nakano et al., 1996; Mizushima et al., 1998); TRAF6 was identified by yeast two hybrid system, utilizing CD40 as bait (Cao et al., 1996; Ishida et al., 1996a). TRAF7 was found to potentiate MEKK3-mediated AP-1 and CHOP activation and induce apoptosis through distinct domains as well as having a role in the negative regulation of c-Myb by sequestering it to the cytosol via SUMOylation (Xu et al., 2004; Morita et al., 2005). In the case of some TNFR family members, such as TNFR2 and CD40, the TRAFs bind directly to the intracellular domains of the receptors. In the case of other receptors, such as TNFR1 and members of the TLR/IL-1R family, additional adaptor proteins are used to recruit TRAFs (Häcker et al., 2011).

TRAFs serve as adapter proteins for a variety of cell surface receptors and are able to both negatively regulate programmed cell death pathways and induce genes that promote cell survival. They are genetically conserved and have been characterized in a variety of multicellular organisms including mammals, *Drosophila melanogaster*, *C. elegans* and *Dictyostelium discoideum* (Chung et al., 2002). Apart from their role as adapter proteins, TRAF proteins also act as E3 ubiquitin ligases, a function that is crucial for the activation of downstream signaling events. Such events include the activation of nuclear factor κB (NFκB) and activator protein 1 (AP-1), which are key transcription factors that control many immune response genes, and the activation of certain interferon factors (IRFs), which transcriptionally control the production of antiviral type I IFNs (Häcker et al., 2011).

1.1.2 Structure of TRAF proteins

TRAF proteins have been characterized on the basis of a conserved domain (TRAF domain) at the C-terminus with the exception of TRAF7. The TRAF domain has been divided into two sub-regions (Rothe et al., 1994): the carboxyl-terminal TRAF-C region is of high amino acid homology whereas the amino-terminal half of the TRAF domain, TRAF-N, is more divergent and adopts a coiled-coil configuration (Fig). The TRAF-N domain governs TRAF homotrimerization whereas the TRAF-C domain contributes to TRAF oligomerization and also promotes interactions with upstream regulators. These upstream regulators can either be the intracellular domains of receptors (such as TNFR2, CD40 and the BAFF receptor (BAFFR)) or intermediate adaptor proteins, such as TNFR1-associated death domain protein (TRADD; which functions downstream of TNFR1) and IL-1R-associated kinase (IRAK) family members which are involved in TLR and IL-1R signaling (Hsu et al., 1995; Wesche et al., 1997b).

In addition, both TRAF-N and TRAF-C domains interact with downstream effectors (Ha et al., 2009). These include cIAP and NFκB-Inducing-Kinase (NIK) which bind to the TRAF-N domain of TRAF2 and the TRAF-C domain of TRAF3 respectively (Rothe et al., 1995; Vince et al., 2009; Yamamoto et al., 2003; Sanjo et al., 2010; Zheng et al., 2010; Mace et al., 2010). The interplay between these proteins that occurs both constitutively and during receptor activation is essential for the regulation of the alternative NFκB pathway that will be thoroughly discussed later.

The structural differences among the TRAFs influence the range of receptors, heterodimerization partners, adapter molecules and signal transducers that each TRAF interacts with (Kaufman and Choi, 1999). All TRAFs, except TRAF1, contain N-terminal RING finger and several zinc finger motifs which are important for downstream signaling events. The RING domain of TRAF proteins is critical for downstream effector functions (Hsu et al., 1996).

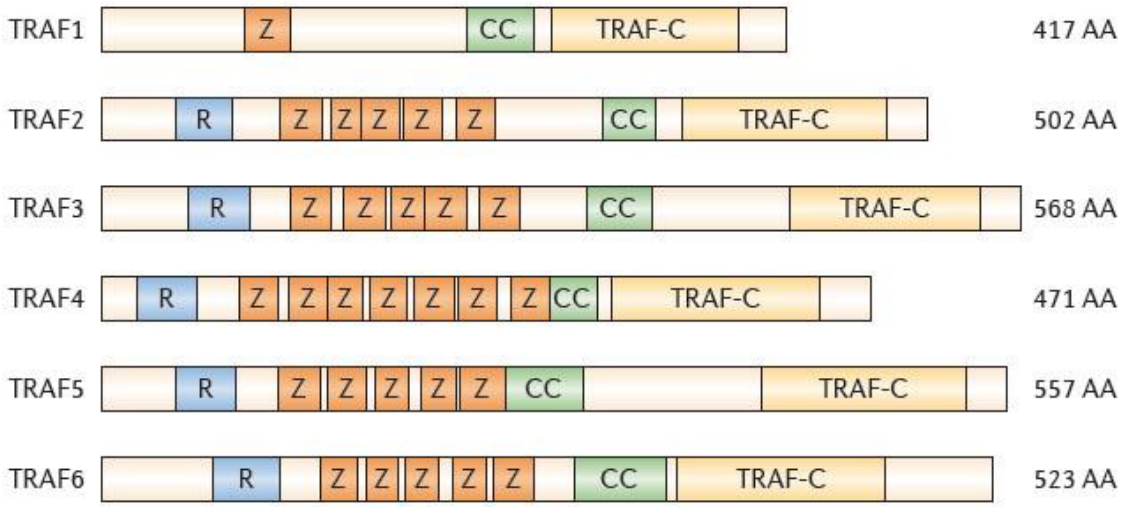


Figure 1.1: Domain organization of mammalian TRAF proteins. All TRAFs except for TRAF1 contain an N-terminal RING finger domain (R) which is a signature motif for E3 RING finger Ubiquitin ligases and several zinc finger motifs (Z). The TRAF domain contains a coiled-coil region (CC) and a C-terminal TRAF-C domain. (Figure reproduced from Häcker et al., 2011 All rights reserved).

1.1.3 TRAFS AND CD40

As this study focuses primarily on signaling on the CD40 axis, a brief introduction is essential. CD40, a member of the TNFR superfamily, was first identified in B cells and primary carcinomas and was found to share structural homology with TNFR and nerve growth factor receptor (NGFR) (Stamenkovic et al., 1989). This was followed by the discovery of CD40 ligand (CD40L) which was identified as a component of the plasma membrane of activated T cells that was able to stimulate B cells in an antigen non-specific and major histocompatibility complex (MHC) unrestricted manner (Noelle et al., 1992; Armitage et al., 1992). The structure of CD40L shares some homology with that of TNFα, with the biological unit being a homotrimer. CD40L is a type II transmembrane protein that is upregulated on activated T cells as well as in activated B cells (Higuchi et al., 2002) and platelets (Henn et al., 1998; Danese et al., 2003). CD40 is a transmembrane glycoprotein expressed in B cells, dendritic cells, monocytes, platelets and macrophages (van Kooten and Banchereau, 1997) and has been reported to be expressed in activated T cells as well (Bourgeois et al., 2002). It is also expressed by a number of mesenchymal

cells types including fibroblasts, epithelial and endothelial cells (Banchereau et al., 1995; van Kooten and Banchereau, 1997).

CD40 can recruit the various TRAFs to its cytoplasmic tail, either directly through consensus sequences as is the case with TRAF1, -2, -3 and -6, (Pullen et al., 1998, 1999; Bishop et al., 2007) or indirectly, as with TRAF5 (Nakano et al., 1999; Hauer et al., 2005; Bishop et al., 2007). CD40 ligation can induce many signaling cascades including p38, Akt, c-Jun N-terminal kinase (JNK), and signal transducer and activator of transcription 5 as well as canonical and non-canonical NFκB cascades. These will be discussed, at least briefly, in later sections. A summarized overview of CD40 signaling through TRAFs is briefly depicted in Figure 1.2.

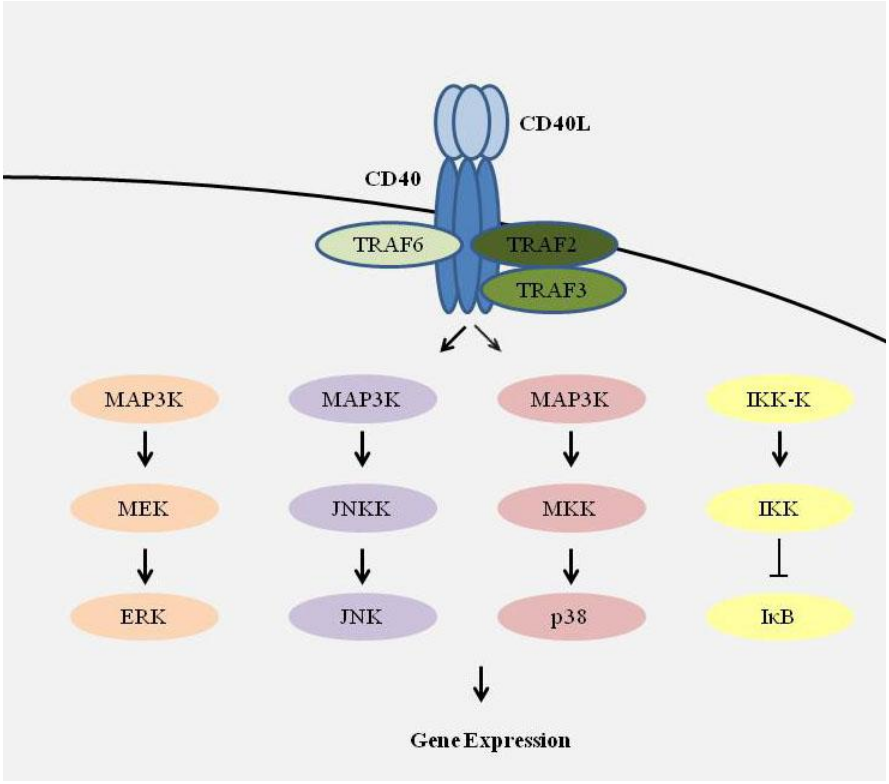


Figure 1.2: Following engagement of CD40 (or other TNFR family members) TNFR-associated factors (TRAFs) -2, -3, -6 are recruited to the receptor and this facilitates activation of MAP3Ks and the subsequent phosphorylation cascades that culminate in MAPK and IKK activation.

1.1.4 Biological functions of the mammalian TRAF proteins

As it has already been mentioned, the structural features of TRAF proteins suggest that these proteins function as cytoplasmic adapters, which may promote intracellular signal transduction through their ability to bind to receptors and potentiate the recruitment of proteins, including each other, to a signaling complex (Arch and Thompson, 1998). Extensive studies have shown that the specific biological function of each TRAF protein is not necessarily related to its origin of identification; most TRAF proteins can be considered as the molecular link between a number of different pathways and members of the TNFR family as well as the branching points between several diverse pathways (Hauer et al., 2005) (Table 1.1.).

TRAF1 and TRAF2 were first identified as TNFR2-interacting proteins. TRAF2, TRAF3 and TRAF6 were constitutively expressed in most cell types, whereas TRAF5 expression is restricted to immune cells. TRAF1, which is constitutively associated with TRAF2, is apparently involved in the fine-tuning of TRAF2 signaling, as in the case of CD40 activation (Xie et al., 2006). TRAF4 interacts with the transforming growth factor-β (TGFβ) receptor family and controls organ development (Shiels et al., 2000; Régnier et al., 2002; Kalkan et al., 2009). The role of TRAF members in the activation of NFκB and mitogen-activated protein kinsase (MAPK) has been thoroughly described (Liu et al., 1996; Rothe et al., 1995; Baud et al., 1999). Subsequently, TRAF6 and more recently, TRAF3, were found to be involved in signaling receptors that do not belong to the TNFR family, including TLRs and IL-1R family members (Cao et al., 1996; Häcker et al., 2000; Yamashita et al., 2008). Importantly, the outcome of TNFR or TLR engagement appears to be determined by the combination of TRAF proteins that are recruited by each receptor.

LIGAND	RECEPTOR	TRAF	RECEPTOR-EXPRESSING CELL TYPE
TNF, LTα	TNFR1 TNFR2	TRAF1, TRAF2, TRAF5	Ubiquitous expression
LTα-LTβ LIGHT	LTβR	TRAF2, TRAF3, TRAF4, TRAF5	Stromal cells
CD40L	CD40	TRAF1, TRAF2, TRAF3,TRAF5, TRAF6	Innate immune cells, B cells, T cells
BAFF	BAFFR	TRAF2, TRAF3	B cells
LPS	TLR4	TRAF3, TRAF6	Innate immune cells, B cells, activated T cells

Table 1.1. Various receptors are preferentially associated with different members of the TRAF family.

1.1.5 TRAF knock-out studies

TRAF2 is found in most tissues and therefore is considered to be the most widely expressed TRAF family member with a distinct cytoplasmic role (Rothe et al., 1994). Studies in mice have demonstrated that TRAF2-deficient mice die prematurely from severe runting and are more sensitive to TNF-induced death (Yeh et al., 1997) (Yeh *et al*, 1997). The generation of $TRAF2^{flox/flox}$ mice by ablation of *Traf2* in hematopoietic derived cells resulted in constitutive NFκ-B2/p100 processing and elevated c-Rel (Grech et al., 2004). CD40-induced B-cell proliferation is also significantly reduced in the absence of TRAF2 and mature $TRAF2^{-/-}$ cells exhibit reduced TRAF3 degradation following CD40 or BAFF-R engagement (Grech et al., 2004).

$TRAF3^{-/-}$ mice also exhibit post-natal lethality with a runted and hyper-inflammatory phenotype prior to death at day 10 (Xu et al., 1996). As in the case with TRAF2, $TRAF3^{flox/flox}$ mice showed elevated NF-κB2/p100 processing (Xie et al., 2007).

The biological importance of TRAF4 was revealed by the gross tracheal malformation in TRAF4 deficient mice (Shiels et al., 2000). Analysis of TRAF4 expression has also implicated TRAF4 in the function of neural multipotent cells and epithelial stem cells in adult mammals (Krajewska et al., 1998; Masson et al., 1998).

TRAF5 is considered to be a close functional and structural homologue of TRAF2 and overexpression of TRAF5 can also activate NFκB and AP-1 transcription factors (Ishida et al., 1996b; Nakano et al., 1996). However, deletion of TRAF5 did not cause prenatal lethality as in the case of TRAF2 deletion, maybe due to the more restricted expression pattern of TRAF5.

The analysis of TRAF6 function in mice by germline deletion also results in poor postnatal survival, with few mice surviving the 2-week mark due to osteoporosis and defective tooth eruption (Lomaga et al., 1999). The proliferation of B cells from these mice was reduced following stimulation with CD40 and lipopolysaccharide (LPS) (King et al., 2006).

1.1.6 TRAF-dependent signaling pathways

The best characterized TRAF-dependent effector pathways are those that lead to the activation of the IKK complex and NFκB, TANK-binding kinase 1 (TBK1) and IRF3, and the MAPK cascades. Even though this study focuses primarily on TRAF3, the roles of the rest of the TRAF proteins are also discussed, given their extensive overlapping in the various pathways.

1.1.6A Canonical and non-canonical NF-κB signaling

TRAF2, TRAF3, TRAF5 and TRAF6 have all been involved in the NFκB pathway activation. The signaling pathway that mediate NFκB activation can be classified into canonical and non-canonical (or alternative) pathways.

1.1.6A.i. Canonical NF-κB pathway

The canonical pathway responds to numerous stimuli including most TRAF-dependent receptors, antigen receptors, cytokine receptors, pattern recognition receptors etc (Hayden and Ghosh, 2008). The different pathways converge to an IκB kinase (IKK) complex, composed of catalytic (IKKα and IKKβ) and regulatory (IKKγ or NEMO) subunits. Upon

activation, IKK phoshorylates IκBα at two N-terminal serines, triggering its ubiquitination and proteasomal degradation; this leads to the nuclear translocation of commonly seen NF-κB complexes, predominantly p50/RelA and p50/c-Rel dimers (Figure 1.3. left panel). The classical NF-κB pathway, together with other signaling pathways, regulates the expression of a large number of target genes that are important for innate and adaptive immune responses, inflammation and cell survival (Vallabhapurapu and Karin, 2009).

RIP proteins have been shown to be crucial for NF-κB signaling as well as in the regulation of cell death (Meylan and Tschopp, 2005). RIP proteins generally act as scaffolds in the IKK activation pathway; RIPs function to recruit the IKK complex and also serve as a scaffold onto which ubiquitination is anchored, leading to IKK activation. In general, the linkage of ubiquitination onto RIPs is carried out in a TRAF-dependent manner. In TNFR1 signaling, TRAF2 in conjugation with cIAPs, mediates K63-linked ubiquitination of RIP1 (Karin, 2009). Ligation of TNFR1 by TNF results in the formation of a pro-inflammatory, multi-subunit signaling structure (Micheau et al., 2001), in which TRAF2 is recruited via an induced interaction with TRADD (Hsu et al., 1996). High-affinity interaction between TRAF2 and TRADD mediates robust activation of NF-κB and AP-1 pathways (Ayabe et al., 2001). However, despite deficiencies in JNK and AP-1 activation, TRAF2-deficient cells have relatively intact TNF-induced activation of NF-κB (Yeh et al., 1997).

TRAF5 is also part of the TNFR1 signaling complex and, although TRAF5 knockouts activate NF-κB normally, TRAF2/5 double knockouts are defective in IKK activation (Yeh et al., 1997; Nakano et al., 1999; Tada et al., 2001). While deletion of the RING finger domain of TRAF2 inhibits IKK activation, it may also prevent TRAF2-mediated recruitment of IKK to the receptor complex (Devin et al., 2000). Finally, knockdown of the E2 UBC13 or deletion of UBC13 in macrophages prevents TRAF2 ubiquitination with minimal effects on NF-κB activation (Habelhah et al., 2004; Yamamoto et al., 2006).

TRAF6 is necessary for MyD88-dependent activation of NF-κB (Cao et al., 1996; Wesche et al., 1997a; Yeh et al., 1997) and TRAF6-deficient cells fail to activate NF-κB in response to IL-1 and LPS (Lomaga et al., 1999; Naito et al., 1999). However, like TRAF2, the importance of E3 ligase activity of TRAF6 remains controversial. Reconstitution of TRAF6-deficient cells with a TRAF6 mutant, lacking the RING finger motif, completely restored IL-1-induced activation of NF-κB and JNK *in vitro* (Kobayashi et al., 2001). In addition UBC13 knockouts failed to show significant defects in TRAF6-mediated activation of NF-κB downstream of LPS, IL-1, CD40 or BAFF, despite impaired MAPK activation (Yamamoto et al., 2006). Ubiquitin replacement with K63R abrogates MyD88-mediated NF-κB activation by LPS and IL-1, suggesting that another E2/E3 pair may have important functions in this process (Xu et al., 2009).

1.1.6A.ii. Non-canonical NFκB pathway

In contrast to the activation of canonical NFκB by diverse receptor signals, only a specific subset of TNFR superfamily members mediates the induction of non-canonical NFκB signaling; these include LTβR (Dejardin et al., 2002), CD40 (Coope et al., 2002), BAFFR (Claudio et al., 2002; Kayagaki et al., 2002), RANK (Novack et al., 2003), TNFR2 (Munroe and Bishop, 2004; Rauert et al., 2010), CD27 (Ramakrishnan et al., 2004) etc. Hallmarks of non-canonical NFκB activation are the slow kinetics and dependence on *de novo* protein synthesis. Furthermore, a common feature among the non-canonical NFκB-stimulating receptors is the possession of a TRAF-binding motif, which recruits different TRAF members, particularly TRAF2 and TRAF3, to the receptor complex during ligand ligation (Bishop et al., 2007).

The induction of the non-canonical NF-κB pathway involves different signaling molecules and leads to the activation of the p52/RelB NF-κB complex using a mechanism that relies on the inducible processing of p100 instead of the degradation of IκBα (Figure 1.3, right panel) (Sun, 2011). In contrast to the constitutive and co-translational processing of p105 (Lin et al., 1998), the processing of p100 is a signal-induced and post-translational event (Xiao et al., 2001). As p100 preferentially interacts with RelB (Solan et al., 2002), the processing of p100 not only generates p52 but also

causes p52/RelB nuclear translocation (Sun, 2011). Genetic evidence suggests that the alternative NF-κB pathway regulates important biological functions such as lymphoid organogenesis, B-cell survival and maturation, dendritic cell activation and bone metabolism (Dejardin, 2006).

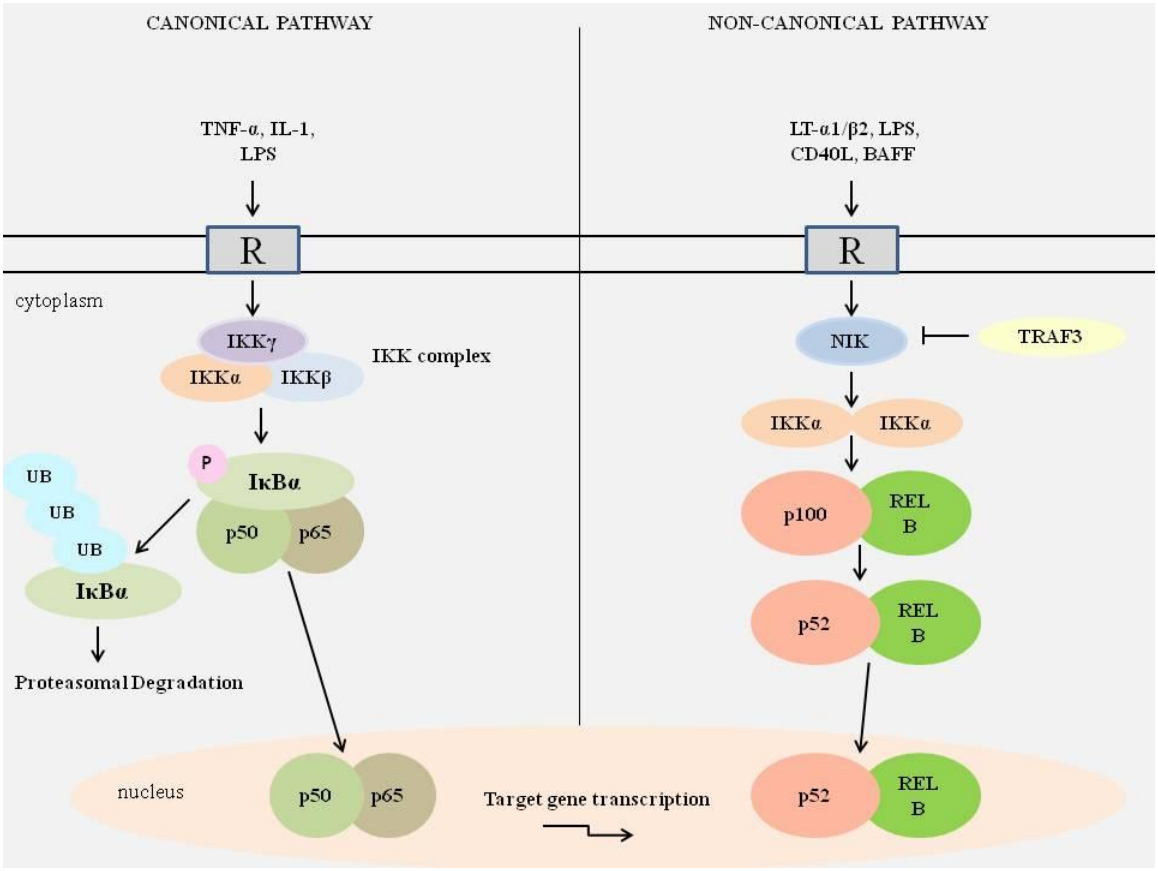


Figure 1.3 The canonical and the non-canonical NF-κB pathways. In the case of the canonical pathway, TRAF molecules activate the IKK complex which induces the phosphorylation of IκBa, triggering its ubiquitination and subsequent proteasomal degradation. NFκB complexes translocate to the nucleus. The non-canonical NF-κB pathway involves the processing of the NF-κB2 precursor protein p100 to p52. The processing of p100 generates p52 but also causes p52/RelB nuclear translocation.

A central signaling component of the non-canonical NF-κB pathway is NIK (NFκB-Inducing-Kinase) (Xiao et al., 2001), a mitogen-associated protein 3 kinase (MAP3K) originally thought to mediate NF-κB activation by cytokines, including TNFα and IL-1 (Malinin et al., 1997). Although over-expressed NIK activates canonical NFκB, it is dispensable for NFκB activation under physiological conditions (Shinkura et al., 1999; Yin et al., 2001). On the contrary, NIK is essential for the induction of the p100

processing, suggesting that, when utilizing mice carrying *NIK* gene mutation in the alymphoplasia (aly) or NIK knockouts, the pathway is blocked completely (Shinkura et al., 1999; Yin et al., 2001; Xiao et al., 2001). To date, all of the known cellular stimuli and inducers of the non-canonical NFκB pathway are known to signal through NIK (Coope et al., 2002; Claudio et al., 2002; Dejardin et al., 2002; Kayagaki et al., 2002; Novack et al., 2003), although some viral oncoproteins seem to induce p100 processing in a NIK-independent manner (Sun and Cesarman, 2011).

A unique feature of the non-canonical NFκB pathway is its dependence on the steady levels of NIK expression. Under normal conditions, the steady state level of NIK protein is extremely low, which is apparently due to its constant degradation targeted by a ubiquitination-dependent mechanism (Liao et al., 2004). A major player of this negative regulatory mechanism is TRAF3, which was identified as a NIK binding protein in a yeast-two-hybrid screening (Liao *et al,* 2004). Originally, NIK was identified as a TRAF2-binding protein (Malinin et al., 1997) although the interaction between TRAF2 and NIK is substantially weaker than TRAF3 and NIK (Liao *et al*, 2004). TRAF3 binds to an N-terminal domain of NIK upon *de novo* synthesis of NIK and targets NIK for continuous degradation through the proteasome. Thus, this negative regulatory mechanism ensures steady and quite low levels of NIK and prevents signal-independent processing of p100 in unstimulated cells (Figure 1.4.a). TRAF3 knockdown with RNAi or TRAF3 knockout by gene targeting is sufficient for triggering NIK accumulation and constitutive p100 processing (Liao et al., 2004; He et al., 2006).

Although TRAF3 induces NIK ubiquitination and degradation *in vivo*, TRAF3 has no intrinsic function to catalyze the formation of K48-linked Ubiquitin chains, as purified TRAF3 does not display E3 Ubiquitin ligase activity. This led to the hypothesis that TRAF3 may not directly ubiquitinate NIK but rather function as a component of a Ubiquitin ligase mediating NIK ubiquitination (Liao et al., 2004). In fact, several studies have since identified a multi-subunit Ubiquitin ligase complex, composed of cIAP1/2,

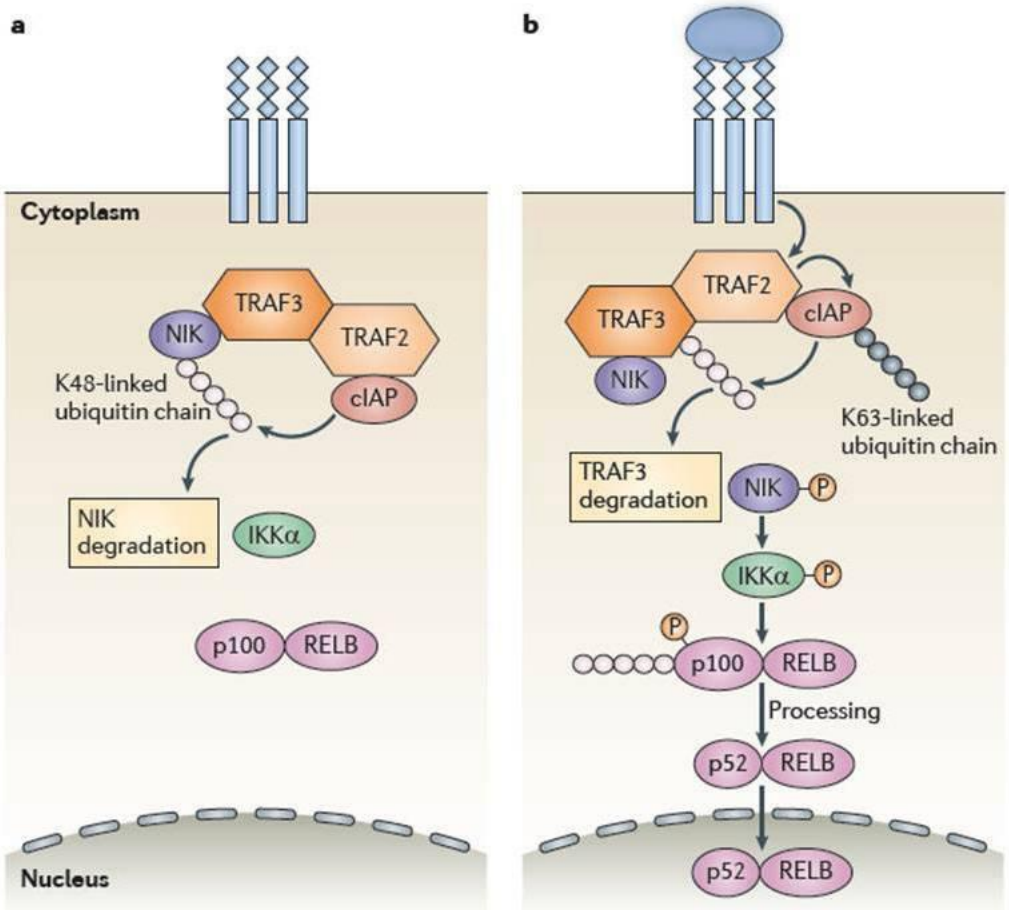


Figure 1.4. (a) Role of TRAF3 in the regulation of NIK turnover and activity. In unstimulated cells NIK is associated with TRAF3 . TRAF3 interacts with a TRAF2/cIAPs complex to direct K48, cIAP-mediated poly-ubiquitination of NIK. This results in continuous proteasomal degradation of NIK thus, stabilizing its levels. (b) Engagement of certain TNF receptor family members result in the recruitment of the NIK-TRAF3-TRAF2-cIAPs complex to the receptor and the activation of TRAF3 K63-specific Ubiquitin ligase activity. TRAF2 then ubiquitinates cIAP to direct the K48-specific Ubiquitin ligase activity of cIAPs towards TRAF3. Degradation of TRAF3 prevents the association of NIK with the cIAPs-TRAF2 complex which leads to its accumulation. NIK accumulation leads to the phosphorylation and processing of p100 and the eventual nuclear translocation of the p52/RELB dimers. (Image reproduced from Häcker et al, 2011, all rights reserved).

TRAF2 and TRAF3 (Varfolomeev et al., 2007; Vince et al., 2007; Vallabhapurapu et al., 2008; Zarnegar et al., 2008). TRAF2 directly interacts with cIAP1/2 whereas TRAF3 binds to cIAPs through dimerization with TRAF2 and serves as an adapter to recruit this multi-subunit E3 complex to NIK (Vallabhapurapu et al., 2008; Zarnegar et al., 2008).

Under basal conditions TRAF3-bound NIK is constitutively targeted for degradation through cIAP1/2-mediated ubiquitination, which depends on TRAF2. Genetic deficiencies in TRAF2 or TRAF3, or degradation of cIAP1/2 by specific agonists, lead to accumulation of NIK and aberrant p100 processing (He et al., 2004; Xie et al., 2007; Gardam et al., 2008).

1.1.6B TRAFs and type I IFN production- the important role of TRAF3

The induction of type I IFNs (IFNα and IFNβ) results from the activation of various receptor systems, including TLRs and cytoplasmic RLRs after pathogen recognition. Both receptor families utilize TRAF3 for type I IFN induction. TLRs initiate signal transduction via the recruitment of TLR/IL-1R (TIR) domain-containing adaptor proteins, which bind to the intracellular part of the receptor via homotypic TIR-TIR interactions. Four TIR domain-containing adaptor proteins are known and whereas MyD88 is used by all TLR and IL-1 family members with the exception of TLR3, TIR domain-containing adaptor protein inducing IFNβ (TRIF) is only used by TLR3 and TLR4. MyD88 also contains a death domain through which it interacts with IRAK family members, which participate in TRAF6 activation (Wesche et al., 1997a).

MyD88 and TRIF interact with TRAF3 to activate signaling pathways leading to type I IFN production, although how TRAF3 can bind to either has not been fully elucidated (Häcker et al., 2006). The expression of type I IFNs and related molecules is controlled by IRF proteins, in particular IRF3 and IRF7, together with other transcription factors (Kawai and Akira, 2008). Depending on the particular cell type and TLR agonist being examined, a specific pattern of IRF activation and type I IFN gene induction is observed. Activation of the plasmocytoid dendritic cells via TLR9 leads to robust IRF7-driven type I IFN response, which is dominated by the production of IFNβ (Kawai and Akira, 2008). The differences in IRF usage, and hence the IFN gene expression pattern, are determined to some extent by the TIR domain-containing adaptor proteins, as MyD88 activates IRF7, whereas TRIF activates IRF3 (Yamamoto et al., 2003; Kawai et al., 2004). However, it should be noted that IRF7 itself is encoded by an IFN-inducible gene, the expression of

which is upregulated in an autocrine manner via type I IFN receptor I (IFNR1) during the initial phases of TLR engagement (Marié et al., 1998; Sato et al., 2000).

TRAFs have a crucial role in TLR-mediated IFN response. TRAF6 is involved in the MyD88-dependent, but not TRIF-dependent type I IFN response, whereas TRAF3 is involved in both MyD88- and TRIF-dependent IFN induction (Häcker et al., 2006; Oganesyan et al., 2006).

More specifically, TRAF3 is crucial for TLR-induced type-I IFN and IL-10 production by macrophages and DCs. TRAF3 deficient cells show lower levels of type I IFNs and IL-10 but higher levels of pro-inflammatory cytokines (TNF, IL-6, IL-12) compared to wild type cells following TLR4, TLR3 or TLR9 activation (Oganesyan et al., 2006; Karin and Gallagher, 2009). Both MYD88 and TRIF were found to recruit TRAF3 following TLR9 and TLR4 activation respectively (Häcker and Karin, 2006) but given that the TLR4 activation through LPS makes for a simpler model, the TRIF-dependent TRAF3 engagement and subsequent IFN is better understood. In short, activation of TLR4 in LPS-stimulated macrophages leads to TRIF-dependent auto-ubiquitination of TRAF3 which leads to the phosphorylation and activation of IRF3 (Häcker and Karin, 2006; Tseng et al., 2010). Mutations on the RING-finger of TRAF3 blocks the K63-linked ubiquitination and subsequent IFN production (Häcker and Karin, 2006) although type I IFN production is also inhibited by the TRAF3-specific de-ubiquitilating enzyme A (DUBA) (Kayagaki et al., 2007).

Similarly, in RLR-induced IFN production (Saha et al., 2006; Tang and Wang, 2009; Paz et al., 2011), TRAF3 recruits MAVS (Mitochondrial Antiviral Signaling Protein) which leads to the activation of ITF3, IRF7 and NFκB pathways (Takeuchi and Akira, 2010). TRAF3 binds directly to MAVS which results in K63-polyubiquitination and the recruitment of IRF-3 activating kinase TBK1 (Saha et al., 2006; Paz et al., 2011).

1.1.6C MAPK pathways and TRAFs

The MAPK signaling pathways are hierarchically organized phosphorylation cascades, consisting of a MAPK, a MAPK kinase (MAPKK) and a MAPKK kinase (MAP3K) (Chang and Karin, 2001). TRAF proteins have been shown to activate several MAP3Ks including MEKK1, MEKK3, TGFβ-activated kinase 1 (TAK1) and Tumor Progression Locus 2 (TPL2) (Karin and Gallagher, 2009). TPL2 has a role in CD40- and TLR4-induced ERK1 and ERK2 activation, but how TPL2 is activated is not clear (Eliopoulos et al., 2003). MEKK3-deficient fibroblasts exhibit defects in IL-1 and TRAF6-dependent NFκB activation, which depends in part on TAK1 (Yamazaki et al., 2009). However, due to early embryonic lethality, no data are available on the role of MEKK3 in primary immune cells (Yang et al., 2000).

By contrast a large set of experimental data is available for TRAF-dependent TAK1 and MEKK1-mediated signaling. TAK1 was initially described as a MAP3K that is activated upon TGFβ or bone morphogenic protein (BMP) treatment of cultured mammalian cells that may also be involved in the early stages of *Xenopus* development (Yamaguchi et al., 1995). TAK1 has been implicated in TNFR signaling along with its binding partners TAB1 and TAB2 (Wang et al., 2001). TAK1 is activated via TRAF-dependent and independent pathways and controls NFκB and MAPK in a cell type and stimulus-specific manner. TAK1 activation relies on TRAF6 mainly, and is activated downstream of both CD40 (which signals via TRAF2, TRAF3 and TRAF6) and TLR4 (which signals via TRAF3 and TRAF6) (Gohda et al., 2004; Sato et al., 2005; Häcker et al., 2006). MEKK1 activation depends on TRAF2 (Matsuzawa et al., 2008) and along with TRAF2, TRAF6 and TAK1 is involved in JNK and p38 activation but this is receptor- and cell-type specific (Sato et al., 1995; Gallagher et al., 2007). TRAF3 was found to be a negative regulator of TNFR- and TLR- mediated MAPK activation and has to be degraded in order for MAPK activation to take place (Matsuzawa et al., 2008; Cheng et al., 2010) .

1.1.6C.i. The role of TRAF3 in TNFR-induced MAPK activation

TRAF3 was found to be unique among TRAFs in its role of inhibiting CD40-mediated NFκB activation (Cheng et al., 1995; Nakano et al., 1996) following over-expression

experiments. Studies have subsequently revealed that TRAF3 exerts control over spatial organization and composition of receptor-associated signaling complexes (Vallabhapurapu et al., 2008; Matsuzawa et al., 2008).

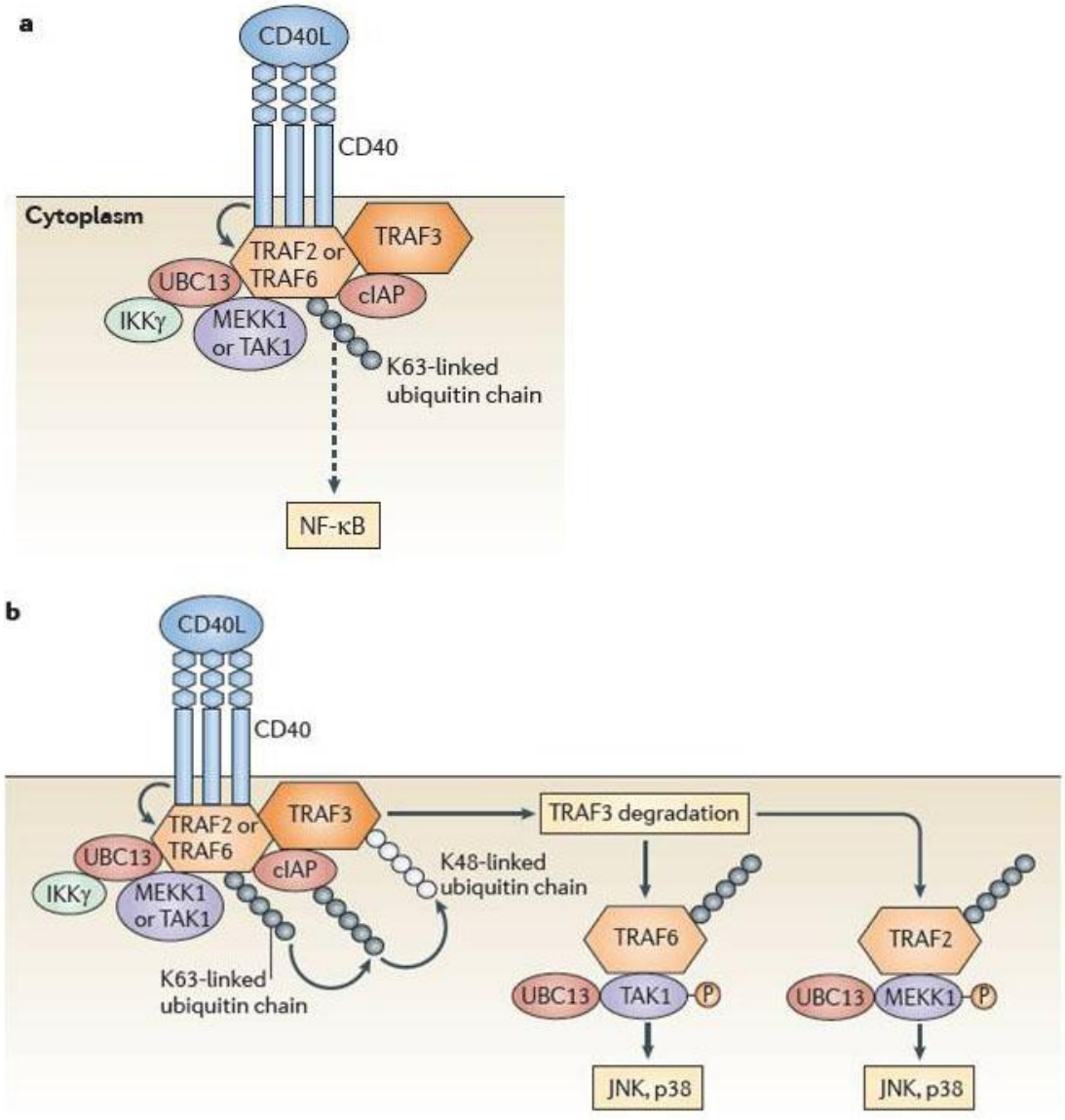


Figure 1.5. Following stimulation with CD40 ligand, two large complexes are assembled to the receptor. TRAF3, cIAPS, Ubc13 and IKKγ are components of both complexes whereas TRAF2 and MEKK1 are part of one complex and TRAF6 and TAK1 are parts of the other. Activation of TRAF2 or TRAF6, through auto-ubiquitination results in the activation of the canonical NF-κB pathway (a) or to the eventual K48-specific Ubiquitin ligase activity of cIAPs towards TRAF3 which leads to its degradation by the proteasome (b). This leads to the activation of MEKK1 and TAK1 and the eventual activation of p38 and JNK (Image adapted from Häcker et al, 2011, all rights reserved).

Following CD40 activation of the receptor, TRAF2, TRAF3, TRAF6, cIAPs, Ubc13, MEKK1, TAK1 and IKKγ are recruited to the cytoplasmic domain of the receptor. Although TRAF2 and TRAF6 are recruited through separate sites, they are both recruited to the activated receptor along with TRAF3, cIAP1/2 and Ubc13. The TRAF2 signaling complex also includes IKKγ and MEKK1 whereas the TRAF6 signaling complex includes TAK1. The signaling complexes that first form on CD40 within the plasma membrane translocate into the cytoplasm within 10 minutes or so after receptor engagement (Matsuzawa et al., 2008). This step is controlled by at least two proteins with opposing actions. The first is TRAF3 which acts as an inhibitor of the MEKK1 and TAK1- associated complexes into the cytoplasm and the others are the cIAP protein which target TRAF3 for K48-linked poly-ubiquitination, thereby counteracting its inhibitory function (Matsuzawa et al., 2008; Vallabhapurapu et al., 2008).

The cytoplasmic translocation and activation of both the TRAF2-MEKK1 and the TRAF6-TAK1 complexes is dependent on cIAP1/2 E3 activity. Treatment of B cells with IAP agonists or a proteasome inhibitor prevented CD40-induced TRAF3 ubiquitination and degradation, cytoplasmic translocation of the signaling complexes and MAPK activation. Neither of these treatments affected receptor recruitment, activation and cytosolic release of the IKK complex, further confirming the spatial and temporal separation between IKK and MAPK signaling (Matsuzawa et al., 2008).

1.1.6C.ii. The role of TRAF3 in the MYD88-mediated MAPK activation

As mentioned before, TRAF3 has a critical role in type I IFN production through the TLR receptors. Following engagement of the TLR4 receptor it has also been shown that TRAF3 is part of a complex that is recruited to the receptor and includes TRAF6, Ubc13, IKKγ and TAK1 (Tseng et al., 2010). As with CD40 engagement, TLR4 activation is accompanied by TRAF3 degradation which depends on the cIAP-mediated K48-linked poly-ubiquitination of TRAF3. The downstream signaling events rely heavily on TRAF3 degradation and subsequent TAK1 release into the cytoplasm (Tseng et al., 2010). Although TAK1-mediated JNK activation and the production of pro-inflammatory cytokines are reduced following interference with TRAF3 degradation, this has no effect

on the NFκB activation which is consistent with the observation that although TAK1 is required for TLR-dependent JNK and p38 activation, this is not the case in TLR-mediated IKK activation (Sato et al., 2005; Yamamoto et al., 2006).

1.1.7 TRAF3 in disease

Loss of function mutations that prevent the interaction between TRAF3 and NIK or complete *traf3* gene deletions were identified in malignant cells from patients with multiple myeloma. Moreover, a point mutation in *traf3* resulting in an amino acid substitution (R118W) that decreases TRAF3 stability thereby resulting in loss of function, was also identified in multiple myelomas (Annunziata et al., 2007; Keats et al., 2007). This mutation was originally described as a germline mutation in a patient with paediatric herpes simplex encephalitis (Pérez de Diego et al., 2010). These *TRAF3* mutations in multiple myelomas result in the accumulation of NIK and aberrant NFκB signaling which promotes cancer cell survival (Annunziata et al., 2007; Keats et al., 2007). Interestingly, the same result can be achieved through some mutation in the *NIK* gene, namely small deletions, which can be found in patients with multiple myeloma, and cause a truncated form of NIK to be synthesized that cannot bind TRAF3.

The R118W mutation of TRAF3 described as a heterozygous, germline mutation in a patient with pediatric HSE, led to decreased TRAF3 stability and overall, exhibited a dominant-negative effect through TRAF3 homotrimerization. The patient, in contrast with TRAF3 deficient mice, did not display evident disease symptoms apart from the HSE, which leads to the conclusion that the cell type that promotes disease in the TRAF3-deficient mice might still contained functional levels of TRAF3. Cells from this patient also displayed, as expected, constitutive NFκB2 processing, although it is not clear how this may had contributed to the disease phenotype (Pérez de Diego et al., 2010).

1.1.8 Brief outline of this PhD's objectives

It appears that TRAF3 has gone from a poorly understood member of the TRAF family to an important multifunctional regulatory protein within a few years. Nonetheless, there are still several open questions regarding the function and regulation of TRAF3. The possible identification of novel TRAF3 interacting proteins may shed light into how TRAF3 is recruited to different signaling complexes; yet unidentified molecules may be revealed that control TRAF3 function.

Given the essential functions of TRAF3 in different signaling pathways that control inflammation, antiviral immunity and cell survival and its already established role in human health, further mechanistic insights into TRAF3 signaling will go a long way towards understanding this mysterious TRAF. Unpublished results from Dr Eliopoulos' laboratory have utilized the yeast-two hybrid technique to identify novel TRAF3-interacting proteins. Using TRAF3 as bait and a HeLa cDNA library as prey the human Ubc9, the sole E2 enzyme in the sumoylation process has been identified. Background to help the reader understand the nature of this molecule1 is given in the second part of the Introduction. The aim of this thesis is to characterize in detail the nature of these proteins interactions with TRAF3.

Objectives

1. To characterize in detail the nature of the novel TRAF3:Ubc9 interaction.
2. To determine the role of this interaction in TRAF3-mediated signal transduction.

Chapter 1B: Introduction to Ubc9

1.2.1 General function of Ubc9

Ubc9 was initially described in humans by virtue of its interaction with HsRad51 Recombinase, a human homolog of RecA protein (Kovalenko et al., 1996). Its role as an important protein for normal mitosis and cell cycle progression in lower eukaryotes had already been described previously as its absence was associated with cell cycle arrest and abortive mitosis. Repression of Ubc9 synthesis prevents cell cycle progression at the G2 or early M phase, causing the accumulation of large budded cells with a single nucleus, a short spindle and replicated DNA (Seufert et al., 1995).

In yeast and higher eukaryotes gene disruption is lethal (Seufert et al., 1995; Hayashi et al., 2002). Mice deficient in Ubc9 die at early post-implantation stage due to chromosome defects such as polyploidy and abnormal metaphase plates and anaphase bridges (Nacerddine et al., 2005). Ubc9 loss-of-function studies similarly showed mitotic defects in hemopoietic tissues whereas Ubc9 yeast mutants are more sensitive to DNA damaging agents (Mao et al., 2000; Chiu et al., 2005; Jacquiau et al., 2005). Similar results to yeast have been described during zebrafish development (Nowak and Hammerschmidt, 2006).

RNAi-mediated knockdown of Ubc9 in the nematode *Caenorhabditis elegans* results in several specific developmental defects that resemble phenotypes produced by mutations in known developmental regulators such as Hox genes (Jones et al., 2002). Similarly, in the fruit fly *Drosophila melanogaster*, loss of Ubc9 in the *semushi* mutant causes patterning defects associated with the mis-regulation of the anterior-posterior morphogen bicoid (Epps and Tanda, 1998). Inducible studies of Ubc9 function in a chick cell line leads to poly-nucleated cells and cell-cycle independent apoptosis (Hayashi et al., 2002). Together these studies indicate that in addition to the regulation of particular cellular processes that will be analyzed later, Ubc9 can influence developmental programs of higher multicellular organisms, accounting for spatial pattern formation and differential cellular specification.

This only further underlines the notion that Ubc9 is involved in many critical pathways something reinforced by the fact that it is conserved from yeast to humans and is expressed ubiquitously (Kovalenko et al., 1996). Ubc9 has been found to exert a central function for the sumoylation pathway, interacting with almost all the partners required for sumoylation. It is perhaps its most well-defined role and its crucial involvement in the various cellular pathways is exerted through the SUMO pathway.

1.2.2 SUMOylation and Ubc9

The coordination of signaling pathways within the cell is vital for human embryonic development and post-natal tissue homeostasis (Vaillancourt and Lafond, 2009). This requires the regulation of gene expression at multiple levels (Chen and Rajewsky, 2007), including protein post-translational modification (PTM). Post-translational modifications of proteins and the domains that recognize these modifications have central roles in creating a highly dynamic relay system that reads and responds to alterations in the cellular microenvironment (Deribe et al., 2010).

Protein PTMs involve the addition of a chemical group, following protein translation, providing organisms with added control of protein activity, localization and stability and are largely responsible for the plasticity of protein interaction networks. PTMs are usually reversible (Hannoun et al., 2010) and a wide variety has been described to date. Apart from small molecule modifications, such as phosphorylation, acetylation or methylation, larger molecules may be attached to target proteins and therefore provide larger and more chemically diverse surfaces for interaction. Ubiquitin is the most extensively studied large molecule modifier which via a three step process is covalently attached to a lysine residue of a target protein (Moschos and Mo, 2006).

1.2.3 The SUMO proteins

SUMOylation, after ubiquitination, represents the best studied example of post-translational modification that stably joins one protein to another and elicits a wide range

of effects within the cell (Johnson, 2004). SUMO proteins are highly conserved in a large number of species and have been shown to be important in many eukaryotic cell processes (Hannoun et al., 2010). Yeast have only one form of SUMO, encoded by *SMT3* (suppressor of the mitotic fidelity gene 3). Four homologues exist in mammals, SUMO-1, -2,-3 and -4, each encoded by a distinct gene. SUMO-2 and -3 share 95% homology with each other, but only share 50% with SUMO-1 (Johnson, 2004) resulting in different biological activities. SUMO-2 and -3 (as well as SUMO proteins from *S.cerevisiae* and *S.pombe*) possess the ability to form poly-SUMO chains covalently binding to themselves via the lysine residue at the N terminus consensus motif (Müller et al., 2001). SUMO-1 lacks this consensus site and is therefore unable to form poly SUMO chains (Kroetz, 2005) and often acts as a poly SUMO chain terminator (Ulrich, 2009). SUMO-4 has a predicted 86% amino acid homology with SUMO-2 but its mRNA is expressed in a limited number of tissues, mainly kidney, lymph and spleen (Bohren et al., 2004; Guo et al., 2004). It has been found that over-expressed mature SUMO-4 can be conjugated to targets under conditions of extreme cellular stress (Wei et al., 2008) but, in contrast to the other SUMO genes, the *SUMO-4* gene lacks introns, which presents the possibility that it could be a pseudogene (Bohren et al., 2004).

1.2.4 The SUMO pathway

SUMO proteins, as many other Ubiquitin and Ubiquitin-like proteins (UbLs), are translated as inactive precursors that must undergo a C-terminal cleavage modified by a family of SENP (Sentrin/SUMO-specific protease) enzymes. This cleavage exposes a di-glycine motif that allows SUMO to be conjugated to lysine residues in target proteins (Figure 1.6.). During each conjugation cycle, SUMO proteins are first activated in an ATP-dependent manner by the E1 activating enzyme, a heterodimer of SAE1 (SUMO-activating enzyme E1) and SAE2 in mammals (Gong et al., 1999). This step involves the formation of a thioester bond between the active-site cysteine residue of SAE2 and the C-terminal glycine residue of SUMO. SUMO is then passed to the active site cysteine of the conjugating enzyme Ubc9 (Ubiquitin-conjugating 9), again via a thioester linkage

(Johnson and Blobel, 1997; Gong et al., 1997; Desterro et al., 1997; Schwarz et al., 1998; Saitoh et al., 1998; Lee et al., 1998).

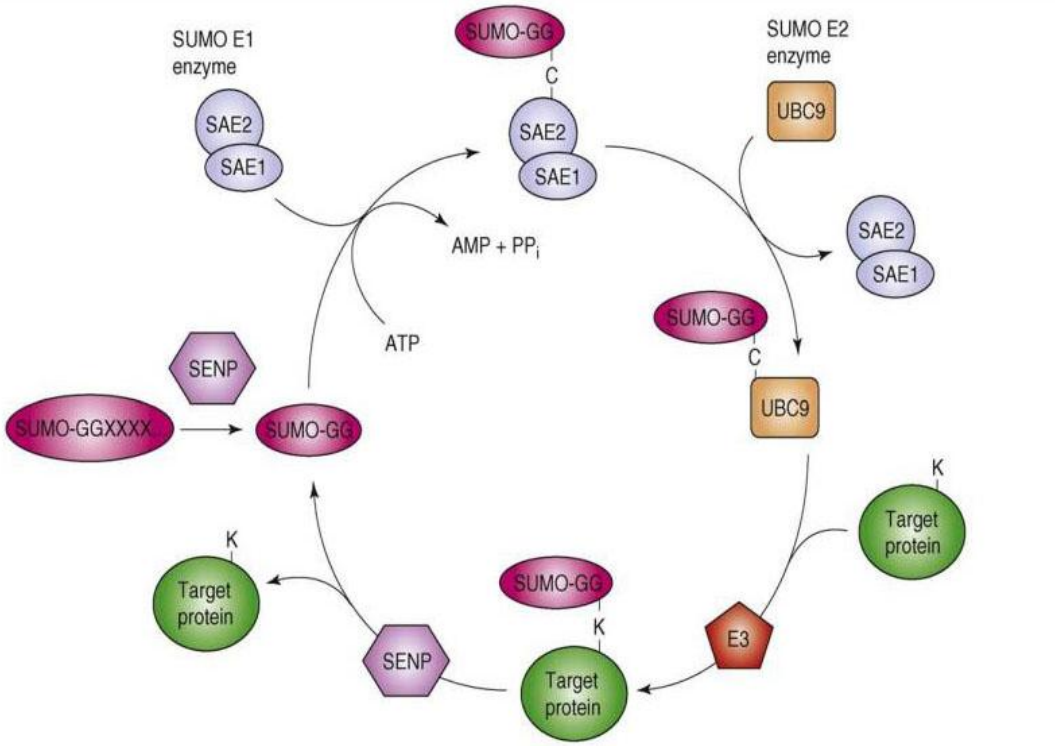


Figure 1.6. Overview of the SUMOylation pathway. SUMO is conjugated to its substrates through three distinct enzymatic steps: activation involving the E1 enzyme, conjugation, involving the E2 enzyme Ubc9 and substrate modification through the cooperation of the E2 and E3 protein ligases.

It has to be noted that Ubc9 is the only known SUMO-conjugating enzyme and Ubc9 itself binds directly to the consensus SUMOylation motif on substrate proteins (Rodriguez et al., 2001; Sampson et al., 2001). The presence of only one SUMO E2 contrasts with the Ubiquitin pathway where multiple E2s have been identified that participate in ubiquitilating distinct sets of substrates. Ubc9 shares considerable sequence similarity with the ubiquitilating E2s and also assumes essentially the same folded structure, although Ubc9 has a strong overall positive charge (Tong et al., 1997).

In the Ubiquitin system, E3 ligases are generally a requirement for ubiquitination. They can be broadly classified into two distinct types: the HECT (homologous with E6-associated protein C-terminus) domain-containing E3s and the RING domain containing

E3s. Both, however, facilitate the conjugation of Ubiquitin to the target proteins via the cognate E2 (Hu, 2012). Ubc9, on the other hand, is capable of directly recognizing and SUMOylating many substrates *in vitro*, making the presence of an E3 ligase in the SUMO pathway dispensable and has, thereby, sparked a huge debate in the field about the importance of E3 ligases in the SUMOylation process. To date, numerous proteins have been reported that possess E3 ligase activity in the SUMO pathway *in vivo*. Each of the reported SUMO E3 ligases appear to function in a similar manner to the RING-domain E3s of the Ubiquitin pathway although, instead of directly receiving SUMO through a thioester linkage, they facilitate the process of transferring the SUMO to substrate acceptor Lys residues, through two mechanisms. They can either recruit the E2-SUMO thioester and substrate into a complex to promote specificity or, in the cases of substrates that interact directly with Ubc9, they can enhance conjugation by stimulating the ability of Ubc9 to discharge SUMO to substrates. (Wilkinson and Henley, 2010; Gareau and Lima, 2010).

The three main categories of SUMO E3 ligases, that are also highly conserved, are: the protein inhibitor of activated STAT-signal transducer and activator of transcription (PIAS) family (Hochstrasser, 2001), the nuclear pore proteins Ran binding protein 2 and nucleoporin 358 (RanBP2/Nu358) (Pichler et al., 2002) and the Polycomb group protein Pc2 (Kagey et al., 2003). Although they have slightly different mode of function in the cell and different cellular localization, they appear to be important for the sumoylation process as nearly all of yeast sumoylation is E3-dependent (Johnson and Gupta, 2001; Takahashi et al., 2001), and E3s enhance SUMO attachment *in vitro* to all substrates that have been tested (Johnson and Gupta, 2001; Kahyo et al., 2001; Sachdev et al., 2001; Takahashi et al., 2001; Pichler et al., 2002; Kotaja et al., 2002; Kirsh et al., 2002; Schmidt and Müller, 2002).

1.2.5 Consensus Motifs for the conjugation of SUMO

Many SUMO-modified proteins identified contain an acceptor Lysine with a consensus motif ψKxD/E (where ψ is a large hydrophobic residue) (Rodriguez et al., 2001). These residues directly interact with Ubc9 and consequently have a crucial role in regulating the stability of interactions between the E2 enzyme and the substrate (Sampson et al., 2001). However, it is important to emphasize that whereas ~75% of known SUMO substrates are modified within a consensus motif (Xu et al., 2008), SUMOylation can also occur at lysine residues outside this motif and not all ψKxD/E motifs are SUMOylated. Thus, although useful as an initial predictor, the presence of ψKxD/E motif on potential SUMO substrates is certainly not a definitive indicator that a protein is SUMOylated and neither the absence of such a motif excludes the possibility that the protein is modified. Other factors, such as sub-cellular localization or appropriate presentation of the sequence may be required for modification. The ψKxD/E motif is bound directly by Ubc9 (Girdwood et al., 2003), and this direct interaction explain why so many SUMOylation substrates have been identified via their interaction with Ubc9 in the yeast two-hybrid system.

1.2.6 De-SUMOylation

SUMOylation is a highly dynamic process that can be readily reversed by the action of the same SENP enzymes that are required for the maturation of pro-SUMO. These enzymes cleave precisely between the terminal glycine of SUMO and the substrate Lys (Hickey et al., 2012). In mammals there are six SENPs, designated SENP1-3 and SENP5-7, which vary in their cellular distribution, SUMO paralog specificity and selectivity for SUMO maturation compared with deconjugation activities (Yeh, 2009; Mukhopadhyay and Dasso, 2007). Moreover, recent studies have identified three new SUMO proteases in humans, deSUMOylating isopeptidase 1 (DESI1), DESI2 and Ubiquitin-specific protease-like 1 (USPL1) which share little sequence similarity with the SENP protease class (Suh et al., 2012; Schulz et al., 2012). (Table 1.2.)

SENP1 and SENP2 have a broad specificity for SUMO-1 and SUMO-2/3 and function both in their processing and deconjugation (Gong et al., 2000; Hang and Dasso, 2002; Zhang et al., 2002). SENP3 and SENP5 favor SUMO-2/3 (Nishida et al., 2001; Di Bacco and Gill, 2006; Gong and Yeh, 2006) as do SENP6 and SENP7 (Mukhopadhyay et al., 2006; Shen et al., 2009). Neither SENP6 or SENP7 seem to be involved in the maturation of pro-SUMO proteins and they show minimal activity in the deconjugation of monomeric SUMO2/3 from substrate proteins. Rather, SENP6 and SENP7 efficiently edit and/or deconjugate poly-SUMO-2/3 chains (Mukhopadhyay et al., 2006; Shen et al., 2009). There are clear distinctions between the functions of different SENP enzymes. SENP1/2 are primarily responsible for cellular maturation of the SUMO proteins and perform roles in the deconjugation of both SUMO-1 and SUMO-2/3 from substrates. SENP3/5 function in the removal of monomeric SUMO-2/3 from substrates and SENP6/7 act as editors of the SUMO-2/3 chains. In addition, paralogue-specific SUMOylation through preferential removal of particular SUMO paralogues from substrate proteins (Wilkinson and Henley, 2010).

DESI1, expressed diffusely throughout the cytoplasm and nucleus, has been found to deconjugate both SUMO-1 and SUMO-2/3 and weakly cleaves poly SUMO-2/3 chains from B2EL (BTB-2F protein expressed in effector lymphocytes) which is currently its only known substrate (Shin et al., 2012; Suh et al., 2012). Importantly, the substrate specificity of DESI1 is distinct from SENPs as DESI1 cannot de-SUMOylate known substrates of SENPs and as it does not alter bulk SUMO profiles, it appears to have a limited number of substrates. DESI2, also located in the cytoplasm, appears to compensate for DESI1 (Shin et al., 2012). USPL1 was identified as protein in HeLa extracts that crosslinked to either SUMO-1 or SUMO-3 and shows broad sUMO protease specificity *in vitro* (Schulz et al., 2012).

Name	Subcellular localization*	Isoform preference	Precursor processing	Deconjugation	Chain editing‡
Saccharomyces cerevisiae					
Ulp1	Nuclear pore	Smt3	Yes	Yes	No
Ulp2	Nucleoplasm	Smt3	No	Yes	Yes
Mammals					
SENP1	Nuclear pore and nuclear foci	SUMO1 and SUMO2/3	Yes	Yes	No
SENP2§	Nuclear pore and nuclear foci; cytoplasm	SUMO2/3 > SUMO1	Yes	Yes	No
SENP3	Nucleolus	SUMO2/3	Unknown	Yes	No
SENP5	Nucleolus and mitochondria	SUMO2/3	Yes	Yes	No
SENP6	Nucleoplasm	SUMO2/3	No	Yes	Yes
SENP7	Nucleoplasm	SUMO2/3	No	Yes	Yes
DESI1	Cytoplasm and nucleus	SUMO1 and SUMO2/3	Weak	Yes	Weak
DESI2	Cytoplasm	Unknown	No	Unknown	Unknown
USPL1	Cajal bodies	SUMO2/3 > SUMO1	Weak	Yes	Yes

DESI, desumoylating isopeptidase; SENP, sentrin-specific protease; Smt3, suppressor of the mitotic fidelity gene 3; SUMO, small ubiquitin-related modifier; Ulp, UBL-specific protease; USPL1, ubiquitin-specific protease-like 1. *The primary subcellular localizations are listed, and minor fractions have been observed elsewhere. ‡Chain editing refers to the ability of a protease to remove SUMO moieties from a SUMO chain without removing the entire SUMO chain from SUMO-modified proteins. §SENP2 has at least three splice variants that localize differentially in cells. The 589 amino acid version localizes primarily to the nuclear pore.

Table 1.2. Properties of SUMO proteases.

1.2.7 Knockout studies on SUMOylation

The critical importance of SUMOylation in mammals has been confirmed through knockout and knockdown studies of Ubc9. As Ubc9 is required for the conjugation of every SUMO paralogue, deletion of Ubc9 prevents all SUMO conjugation. As already mentioned, removal of Ubc9 in the chicken DT40 lymphocyte cell line resulted in detrimental effects: abnormalities in chromosome segregation, nuclear organization and cell death through apoptosis (Hayashi et al., 2002). Similarly, Ubc9-knockout mice die at an early embryonic stage due to defects in chromosomal segregation at mitosis and aberrant nucleolar organization (Nacerddine et al., 2005).

In contrast, *SUMO* gene knock-outs are more complicated. Alkuraya and colleagues (Alkuraya et al., 2006) through the generation of mice hetero- or homo- zygous for a β-galactosidase insertion in the SUMO-1 gene, showed that most animals died in late

embryonic or early postnatal periods, indicating that *SUMO-1* gene is probably involved in important developmental functions. However, studies carried out since then, display animals that are viable and have an apparently normal phenotype (Zhang et al., 2008; Evdokimov et al., 2008). This leads to the conclusion that SUMO2/3 may be able to compensate for the loss of SUMO-1. In the study by Kuehn's lab, RanGAP1, which is preferentially modified by SUMO-1, displayed enhanced modification by SUMO-2/3 in SUMO-1-knockout embryonic extracts (Evdokimov et al., 2008) thus, supporting the hypothesis that under extreme circumstances SUMO2/3 can effectively compensate for the lack of SUMO-1.

1.2.8 Functional heterogeneity within the SUMO family: SUMO-1 versus SUMO-2/3

Despite the evidence that SUMO-1 and SUMO-2/3 are conjugated to only partially overlapping subsets of proteins and have unique properties and functions it remains unclear how different proteins are selectively modified by one paralog relative to another. Examples of proteins that are preferentially modified by one of the SUMO isoforms exist in literature, such as RanGAP1 which is modified almost exclusively by SUMO-1, or PML-nuclear body component Sp100 which is modified by SUMO-2/3 (Saitoh and Hinchey, 2000; Vertegaal et al., 2006). However, there are many substrates that have been identified to be modified by both SUMO-1 and SUMO-2/3 (Vertegaal et al., 2006) although the exact mechanism for the distinction between the different paralogues by the SUMO machinery for the conjugation to the target proteins is not understood and in many cases it is difficult to elucidate the functional differences of SUMO-1 versus SUMO-2/3 conjugation. Nonetheless, observations that SUMO-1 and SUMO-2/3 differ in their conjugation dynamics and show distinct patterns of localization in the cell, indicate specific regulation of the SUMO paralogues (Saitoh and Hinchey, 2000; Ayaydin and Dasso, 2004). The levels of unconjugated SUMO-1 and SUMO-2/3 differ dramatically; in resting cells, there is very little free SUMO-1 and a large pool of free SUMO-2/3 although various cellular stresses invoke a massive increase in SUMO-2/3

conjugation (Bossis and Melchior, 2006; Agbor and Taylor, 2008; Tempé et al., 2008). It is thus suggested that SUMO-2/3 may act as a cellular reserve of SUMO (Saitoh and Hinchey, 2000).

Both SUMO-1 and SUMO-2/3 use identical core conjugation machinery which presents a major challenge to determine how paralogue specificity is achieved. The three major points are discussed briefly below.

1.2.8A Preferential de-SUMOylation as a means of paralogue specificity

To address this, it is best to use the example of RanGAP1 protein which is the very first SUMO substrate to be identified (Matunis et al., 1996; Mahajan et al., 1997). RanGAP1 protein can be modified equally well by both SUMO-1 and SUMO-2/3 *in vitro*. However, *in vivo*, SUMO-2/3 RanGAP1 is readily deconjugated by SUMO proteases, leading to low steady state levels of RanGAP1-SUMO-2/3. Protection of RanGAP1-SUMO-1 is mediated by the formation of a stable complex between RanGAP1, Ubc9 and RanBP2 and it is thought that this complex is less stable with RaGAP1-SUMO-2 although there is no way to determine any quantifiable level of specificity from the SUMO machinery with respect to the specificity of conjugation of SUMO-1 or SUMO-2/3 to RanGAP1 (Zhu et al., 2009).

1.2.8B SIM-mediated paralogue specificity

Often, the SUMOylation of many substrates is enhanced via non-covalent interactions between the SUMO substrate and SUMO via SIMS (SUMO-interacting motifs) (Kerscher, 2007). Similarly to the Ubiquitin system, protein sumoylation provides an interaction platform for the recruitment of SIM-containing proteins that bind SUMO non-covalently. In addition to SIMs mediating effects of SUMOylation, a growing number of proteins have been identified for which SUMOylation is dependent on the presence of the SIM in the substrate. This suggests that recruitment of SUMO-loaded Ubc9 may

represent a general mechanism of substrate recognition and, possibly, paralogue specificity, through paralogue-specific binding to the substrate protein (Wilkinson and Henley, 2010).

1.2.8 E3-mediated paralogue specificity

E3-mediated SUMO-1 or SUMO-2/3 specificity has also been suggested as a mechanism of paralogue specificity (Tatham et al., 2005). The SUMO E3s, do not form covalent intermediates with SUMO but instead bring together Ubc9 and the substrate. To date, there have been cases of E3 SUMO ligases that appear to preferentially promote attachment of either SUMO-1 or SUMO-2/3. More specifically, in the case of E3 RanBP2, mutational disruption of the RanBP2-Ubc9 binding, affected the SUMO-2 but not SUMO-1 conjugation to Sp-100 and PML (Tatham et al., 2005). Furthermore, PIASy, another type of SUMO E3, preferentially conjugates SUMO-2 rather than SUMO-1 to the transcription factors LEF1 and GATA-2, and it strongly enhances overall SUMO-2 conjugation (Sachdev et al., 2001; Chun et al., 2003).

1.2.9 Regulation of SUMOylation

Although the list of potential SUMOylation substrates grows rapidly, the precise mechanism of regulation of SUMOylation for these proteins remains largely unclear. In theory, SUMOylation can be regulated at the level of either attachment or removal of SUMO; a change in either rate would alter the steady-state amount of protein modified. It seems that SUMOylation of many substrates is regulated through a complex interplay between SUMOylation and other post-translational modifications of the substrate protein. Furthermore, in addition to co-regulating substrate proteins, there is also direct reciprocal interplay between SUMOylation and other post-translational modifications through modification of the proteins involved in their enzymatic pathways.

1.2.9A Cellular stress

Numerous stimuli have been reported that lead to global changes in cellular SUMOylation (Bossis and Melchior, 2006) such as various cellular stresses cause global increases in SUMOylation in mammalian cells. For example, in COS-7 cells, following heat shock a large increase of SUMO-2/3 conjugation occurs although SUMO-1 remains unaffected. Oxidative, ethanol and osmotic stresses have similar results in that there is rapid increase of SUMO-2/3 conjugation (Saitoh and Hinchey, 2000). Furthermore, hibernation torpor, as a model of natural tolerance to ischemia, has been shown to be responsible for a massive increase of global SUMOylation (Lee et al., 2007) in ground squirrels (*Ictidomys tridecemilineatus*). Stress-dependent accumulations in SUMO-conjugation have also been reported in various cellular systems including oxygen/glucose deprivation and hypothermia in neurons (Yang et al., 2008; Loftus et al., 2009; Cimarosti et al., 2008).

Although increases in SUMOylation resulting from cellular stress appear to be a widespread phenomenon, it is important to emphasize that specific substrates are differentially modified. For example, although there is a net increase in SUMOylation following heat shock, the SUMOylation level of some substrates is unchanged and for others decreases, indicating that substrate SUMOylation under these conditions is a regulated stress-specific response, rather than a generalized non-specific increase (Golebiowski et al., 2009).

1.2.9B Cross-talk between SUMO and other post-translational modifications

SUMOylation takes place on lysine residues, which can also be modified by other post-translational modifications, including acetylation, methylation and ubiquitination. This provides ample opportunity for regulatory cross-talk between different pathways that culminate in different modification events. (Guo et al., 2007).

1.2.9B.i. SUMOylation and acetylation

Studies have indicated a specific motif in many potential SUMO substrate proteins that has been reported to target both SUMOylation and acetylation to a target lysine residue. This motif, the 'SUMO-acetyl switch' consists of a SUMOylation consensus motif flanked by a C-terminal proline residue that can also direct acetylation to the SUMOylated lysine residue (Stankovic-Valentin et al., 2007). An interesting example of complex interplay between the SUMOylation and acetylation machinery exists in the case of HDACs, which function in the removal of acetyl groups from substrate proteins and have been reported to function as SUMO E3s for some substrates. In addition, some HDACs can also be SUMOylated, as well as binding SUMO proteins non-covalently, highlighting intricate inter-relationship between the pathways (Yang and Sharrocks, 2004). HDACs also control acetylation of SUMO-1 (Lys^{37}) and SUMO-2 (Lys^{33}) which in turn selectively modulate the binding of SUMO to SIMs. More specifically, Lys^{33}/Lys^{37} acetylation abolishes binding to PML, Daxx and PIAS family members, thereby revealing a new level of control of this modification on the SUMO pathway (Ullmann et al., 2012). Furthermore, acetylation of the SUMO E2 Ubc9 at Lys^{65} has recently been shown to selectively cause downregulation of the SUMOylation of substrates with negatively charged amino-acid dependent SUMOylation motifs (NDSM) but not substrates with a typical SUMO motif or SIM such as CBP or Elk-1. Ubc9 acetylation results in attenuated binding of Ubc9 to NDSM substrates causing a reduction in NDSM SUMOylation (Hsieh et al., 2013).

1.2.9B.ii. SUMOylation and ubiquitination

Many proteins are substrates for Ubiquitin and SUMO, often at the same lysine residue which led to the proposal that they act antagonistically. It has been made clear however, that the interplay between the two systems is much more complex and in many cases they may act either sequentially or in concert to regulate a substrate (Geoffroy and Hay, 2009; Ulrich, 2009). In addition to co-regulating substrate proteins, ubiquitination and SUMOylation can directly cross-regulate each other by modification of components of their respective enzymatic machinery. The recent discovery of SUMO-targeted Ubiquitin ligases has revealed a previously unsuspected co-operation between the ubiquitination

and SUMOylation pathways in regulating the proteasomal degradation of the SUMO substrate proteins (Geoffroy and Hay, 2009).

1.2.9B.iii. SUMOylation and phosphorylation

Another post-translational modification that is critical in many cellular pathways and is also an important regulator in SUMOylation is protein phosphorylation. Subject to the substrate, it can either inhibit or enhance SUMOylation, depending on the existence of a specific motif (Phosphorylation-Dependent SUMOylation motif or negative charge-dependent SUMOylation motif) (Shalizi et al., 2006; Grégoire et al., 2006; Hietakangas et al., 2006). Furthermore, phosphorylation can modify components of the SUMO pathway to directly regulate SUMO conjugation to proteins or influence the consequences of protein SUMOylation. On the other hand, SUMOylation can regulate phosphorylation dynamics through modification of the phosphorylation machinery as in the case of phosphatase PTP1B (Dadke et al., 2007). Interestingly, SUMO-1 protein itself can be phosphorylated and this modification is highly conserved amongst eukaryotes, suggesting it may play an important role in SUMO-mediated processes (Matic et al., 2008).

1.2.9C SUMO regulation by Ubc9

Unlike the Ubiquitin pathway where multiple E2 ligases exist there is only a single E2 protein in the SUMO pathway. Thus, any regulation of Ubc9 is likely to produce a global effect and that is indeed the case. Many well-documented examples exist: low doses of H_2O_2 cause reversible oxidation of Ubc9 that causes a global decrease of SUMOylation levels (Bossis and Melchior, 2006). Similar reductions in global SUMOylation can be seen upon infection with the CELO (chicken embryo lethal orphan) adenovirus which apart from Ubc9, also target the E1 activating enzyme SAE1/SAE2, through the Gam1 protein, for destruction (Boggio et al., 2004). Global reduction of SUMO levels have also been shown after infection with *Listeria monocytogenes* which cause Ubc9 to be targeted for degradation which overall suggests that pathogens dampen the host response by decreasing the SUMOylation level of proteins critical for infection (Ribet et al., 2010).

1.2.9D Regulation by E3 ligases

Spatial and temporal regulation of the E3 enzymes controls the SUMO modification for some substrates. Several E3s have been identified to date and their number is set to increase over the next years. For example, the E3 ligase Pc2, which has been shown to be regulated in response to DNA-damaging agents, is phosphorylated by HIPK2, which is activated following DNA damage. This enhances its E3 ligase activity and that affects HIPK2 SUMOylation and thereby the ability to act as a transcriptional repressor. This provides a nice illustration of a feedback loop where mutual regulatory modifications are exchanged between Pc2 and HIPK2 (Roscic et al., 2006).

1.2.10 SUMO regulation of Ubc9

The sole SUMOylation E2 enzyme was identified as a SUMO substrate. Initially it was described during *in vitro* SUMOylation assays for yeast where Ubc9 was used at high concentrations and it was therefore tentative whereas the phenomenon was physiologically relevant (Bencsath et al., 2002). Subsequent proteomic studies have verified the SUMO modification in both yeast and mammalian cells (Wohlschlegel et al., 2004; Zhou et al., 2004; Zhao et al., 2004) and *in vitro* assays followed by MS have identified that the SUMOylation site on Ubc9 is Lys^{14} in mammals (Knipscheer et al., 2008). In yeast, sumoylation occurs at a different lysine residue (Lys^{153}) suggesting a different function of Ubc9 SUMOylation in yeast and in mammals (Knipscheer et al., 2008).

SUMOylation of Ubc9 does not appear to influence the Ubc9-SUMO-1 thioester formation but rather, it alters the capability of Ubc9 to modify particular SUMO substrates. For example, SUMOylation of RanGAP1 by SUMOylated Ubc9 is dramatically reduced, whereas SUMO modification of Sp100 is enhanced when Ubc9 is SUMOylated. This is consistent with the idea that SUMOylation of Ubc9 influences its target specificity towards a particular subset of substrates (Knipscheer et al., 2008).

1.2.11 Biological Functions of SUMO

Although the SUMO machinery has been relatively well described, investigation of how SUMOylation affects biological processes is at the early stages. Several factors contribute to this fact: usually the levels of modification are low, the presence of protease activity in native lysates and a number of complex interactions between enzymes and substrates. For some proteins reported to be SUMOylated *in vitro*, it is not clear if there is a function or even if the protein can be sumoylated in native conditions.

The most common way to study the function of SUMO conjugation to a particular protein is through mutational elimination of the SUMO attachment sites. This presents a different set of challenges as by mutating a possible attachment-site lysine, the possibility that it doesn't destroy a putative attachment site for a different modification cannot be excluded. Over-expression, dominant –negative or knockdown experiments complement results but the experiments need to be carried out with both the wild type substrate and the substrate that cannot be SUMOylated to confirm that the effects are direct. Often the same results are seen whether the SUMO attachment site is present or not thereby suggesting that the effect involves the SUMOylation of another protein in the same pathway.

In general, protein modification by SUMO may lead to one of three non-mutually exclusive effects (Geiss-Friedlander and Melchior, 2007). First, SUMOylation may mask the binding site of a protein that interacts with the substrate protein, essentially acting to occlude the interaction in a SUMOylation-dependent manner. Secondly, the covalently attached SUMO may act as an interaction 'hub' that recruits new interacting proteins to the substrate either by direct non-covalent interaction with the SUMO-moiety, or via a novel interaction domain created at the SUMO-substrate interface. Thirdly, SUMOylation can lead to a conformational change in the SUMOylated substrate, directly regulating its function (Wilkinson and Henley, 2010).

Various studies have shown that disruption of the SUMO pathway causes abnormal cellular differentiation. Moreover, disruption of the SUMO pathway as has already been

mentioned, can lead to embryo lethality (Nacerddine et al., 2005; Nowak and Hammerschmidt, 2006), demonstrating that SUMOylation is required during development. Perhaps more elaborate experimental strategies are called for to determine the precise role of SUMOylation in early development.

1.2.12 SUMO and diseases

Rapidly growing evidence has been linking SUMO pathways and SUMOylation to human diseases. These diseases include cancer, neurodegenerative diseases such as Alzeimer's, Parkinson's, familial amyotrophic sclerosis (FALS) and Huntington's disease, diabetes, and the developmental disease cleft lips with or without cleft palate (CLP). The evidence results from either deregulated expression or chromosomal locations (in most cases through chromosomal translocations) of SUMO pathway machineries or altered functions of sumoylation substrate proteins. Although the causative relationships between the deregulation and pathogeneses of the diseases and underlying molecular basis need extensive investigations, studies so far have provided strong suggestions that SUMO pathway molecules or SUMO target proteins could eventually be targeted for therapeutic intervention (Zhao, 2007).

1.2.13 CONCLUDING REMARKS

Work over the last years has shown SUMO to be a remarkably versatile regulator of protein function, both in the number of different biological pathways it affects and in the different sorts of mechanisms by which it controls the activities of other proteins. Conversely, as it is a relatively new field, much remain to be answered and the difficulties associated with detecting the SUMO-modified proteins as well as the poorly-described overlapping with other pathways such as Ubiquitin have significantly delayed progress. It is certain however, that as these difficulties are overcome, the SUMO (and in extension Ubc9) role in various diverse cellular processes will be further elucidated and expanded upon.

Chapter 2: Materials & Methods

2.1 Tissue culture techniques

2.1.1 Maintenance of cell lines

HEK293 and HEK293T cells (Human embryonic kidney) were grown in Dulbeco's Modified Eagle Medium (D-MEM), (Gibco, Paisley, UK) containing 1000mg/L Glucose, GlutaMAX^{TM}I and Puruvate supplemented with 10% Fetal Bovine Serum (vol/vol) (Gibco, Paisley, UK). HeLa (Human Cervical carcinoma immortalized line), HeLa SUMO1 and HeLa SUMO2 cells (a kind gift from Prof. Ronald Hay) were grown in Dulbeco's Modified Eagle Medium (D-MEM), (Gibco, Paisley, UK) containing 4500mg/L Glucose, GlutaMAX^{TM}I and Puruvate supplemented with 10% Fetal Bovine Serum (vol/vol) (Gibco, Paisley, UK) and puromycin (Sigma) at final concentration of 2μM. The antibiotic was omitted when cells were used for experimentation. EJ (Human Bladder carcinoma) cells were grown in RPMI media (Gibco, Paisley, UK) containing 1000mg/L Glucose and 25mM HEPEs supplemented with 10% Fetal Bovine Serum (vol/vol) (Gibco, Paisley, UK). BJAB (Human Lymphoma line) cells were grown in Dulbeco's Modified Eagle Medium (D-MEM), (Gibco, Paisley, UK) containing 4500mg/L Glucose, GlutaMAX^{TM}I and Puruvate supplemented with 10% Fetal Bovine Serum (vol/vol) (Gibco, Paisley, UK). HaCaT cells were grown in Dulbeco's Modified Eagle Medium (D-MEM), (Gibco, Paisley, UK) containing 1000mg/L Glucose, GlutaMAX^{TM}I and Puruvate supplemented with 10% Fetal Bovine Serum (vol/vol) (Gibco, Paisley, UK). All cells were kept in a Forma Series II Water Jacketed CO_2 (5%) Incubator (Thermo Electron Corporation, Ohio, USA). Cells were routinely passaged to new culture dishes when they reached 80-90% cofluency by detaching them with trypsin-EDTA (Gibco, Paisley, UK).

2.1.2 Stimulation of cells with CD40L

Cells were seeded in 12-well dishes at $3x10^5$ or 10cm dishes at 10^6, transfected with appropriate plasmids and/or siRNA and left to reach cofluency. CD40L was resuspended in PBS according to the manufacturer's instructions (Bender). The cells were stimulated

with CD40L (500-1000ng/ml) for the desired amounts of time and proceeded to be assayed according to the purpose of each experiment.

2.1.3 Transfection of cells

2.1.3aTransient transfection in 60mm dishes using Lipofectamine™

HEK 293T cells were seeded in 60mm culture dishes and transfected the following day with various expression plasmids using the Lipofectamine™ method as follows: For each transfection sample the DNA plasmids (2μg/reaction) were diluted in 160μl of Serum-Free D-MEM (Gibco, Paisley, UK). Lifofectamine™ (5μl/reaction) (Invitrogen, CA, USA) was mixed with 160μl of Serum Free D-MEM according to the manufacturers' instructions and were left to stand for 5 minutes at room temperature. The two solutions were combined, mixed and incubated at room temperature for 15 minutes. All the medium was removed from the dishes and the cells were washed once with Serum Free D-MEM. After the lapse of the incubation period, 300μl of the transfection cocktail and 1ml of Serum Free D-MEM were added to the cells. After 6-7 hours incubation in a humidified atmosphere containing 5% CO_2, 1ml of full growth D-MEM was added to each dish. 24-30 hours later the cells were washed twice with cold phosphate-buffered saline (PBS) and lysed in RIP lysis buffer [Tris-HCI pH 7.5 20mM, 150mM NaCI, 1% Triton X-100 (vol/vol), 1mM EDTA] supplemented with full protease inhibitors (Sigma, CA) and Sodium Orthovanadate (Sigma, CA).

2.1.3b Transient transfection in 12-well plates using Lipofectamine™

HEK 293T or EJ cells were seeded in 12-well plates at $6x10^5$ cells per well, 24 hours prior to transfection. The following day 100μl of a mastermix containing the appropriately diluted plasmids (concentrations from 0.3-1.5 μg) and 100Xn μl OptiMEM (Gibco, Paisley, UK) (where n is the no of samples) was aliquoted in eppendorfs (100μl/well) containing 100μl OptiMEM and 2.5μl Lipofectamine™ (for HEK293) or 4μl Lipofectamine™ (for EJ cells) (Invitrogen, CA, USA) and incubated for 15 minutes at room temperature. Following incubation, 200μl of transfection cocktail and 500μl OptiMEM were added to the cells and the plate was transferred to a 5% CO_2 humidified chamber. After 6-7 hours, 500μl of D-MEM supplemented with Fetal Bovine Serum

(10%) were added and the cells were left to grow for 12-18 hours before proceeding with cell lysis. Cell lysates were normalized for total protein content (maximum 50μg per sample).

2.1.3c Transient transfection using Calcium Phosphate

HEK293T cells were seeded in 10cm culture dishes 24 hours prior to transfection. The following day, the transfection cocktail was prepared as follows: in a bizoux and for 10 ml of F-12 medium (supplemented with 10% FBS), 450μl TE pH 8.0, 50μl 2.5M $CaCI_2$, 500μl 1xHBS and the DNA plasmids were mixed and incubated at room temperature for 30 minutes before added to the dishes. The cells were incubated for 8-12 hours in a 5% CO2 humidified chamber, then washed with complete medium and incubated for further 36 hours before proceeding with cell lysis with Lysis Buffer A (as above).

2.1.3d Transient transfection using Lipofectamine^{TM} siRNA max

12 well plates

Cells were seeded in 12 well plates at $2,5x10^4$ cells/well (no antibiotics were added). One set of eppendorfs was prepared as follows (mastermixes were prepared as necessary): Mix A contained 125 μl of OptiMEM GlutaMAX (Gibco, Paisley UK) and 0.25μl of the desired siRNA or control siRNA (from a stock of 20μM which corresponded to a final concentration of 5nmol per plate). Mix B contained 125 μl of OptiMEM GlutaMAX (Gibco) and 1.5μl of Lipofectamine siRNAmax (Invitrogen Life Technologies). The mixes were combined and left to incubate at RT for 20-30 minutes. Meanwhile, the wells were washed twice with OptiMEM+GlutaMAX (Gibco). 250μl of transfection mix were added per well and supplemented with 250μl OptiMEM+GlutaMAX (Gibco) and the plate was transferred to a 5% CO_2 humidified chamber. After 6-7 hours the transfection mix was replaced with 1 ml of OptiMEM+GlutaMAX (Gibco). Cells were assayed 48-72 hrs later.

10cm-dishes

Cells were seeded in 10cm dishes at $7,5x10^5$ cells/dish (no antibiotics were added). One set of bijoux was prepared as follows (mastermixes were prepared as necessary): Mix A contained 1000 μl of OptiMEM GlutaMAX and 2μl of the desired siRNA or control siRNA (from a stock of 20μM which corresponded to a final concentration of 5nmol per plate). Mix B contained 1000 μl of OptiMEM GlutaMAX and 12μl of Lipofectamine

siRNAmax (Invitrogen Life Technologies). The mixes were combined and left to incubate at RT for 20-30 minutes and subsequently 2ml of transfection mix were added per dish and supplemented with 2ml OptiMEM+GlutaMAX (Gibco). The plate was transferred to a 5% CO_2 humidified chamber. After 6-7 hours the transfection mix was replaced with 6 ml of fresh OptiMEM+GlutaMAX (Gibco) . Cells were assayed 48-72 hrs later.

2.2 Molecular Biology Techniques

2.2.1 PCR amplification

The Ubc9 fragment was amplified using polymerase chain reaction (PCR). The primers that were used were as follows: sense Bam-UBC-FORW 5'-CTTTGAACGGATCCGGGATCGCCCTC- 3' and antisense Eco-UBC-REV 5'-CACAAGGTGAATTCTTATGAGGGCGCAAAC- 3' (MWG, Germany). It was amplified using Expand High Fidelity PCR System (Roche, Germany) and dNTPs (10x) (Promega, WI, USA). PCR reaction was as follows: 94ºC for 3 minutes followed by 29 cycles of 94 ºC for 30 seconds, 64ºC for 40 seconds and 72ºC for 45 seconds and then extended by 72ºC for 5 minutes. In the case of cloning into the pCR®2.1 TOPO vector (Invitrogen, CA, USA) an extra PCR step was performed for the addition of A' overhangs. The purified PCR product was incubated for 30 minutes at 72ºC with Taq Polymerase (Minotech, Heraklion) and 2.5M of dATP (Promega, WI, USA). The reactions were performed in a MJ Research Peltier Thermal Cycler (PTC-200).

2.2.1a Generation of myc-Ubc9

Ubc9 was expressed as an N-terminally tagged myc fusion protein using the pRK5 vector. The Ubc9 fragment was first cloned into a TOPO vector (pCR®2.1) using TA Cloning® Kit (Invitrogen, CA, USA) according to the manufacturer's instructions. The fragment was excised from a 2% gel agarose after restriction digestion and purified with QIAquick® Gel Extraction Kit (Qiagen, UK) according to the manufacturer's instructions. It was cloned into the pRK5-myc tagged vector using the unique restriction

sites for BamHI (GˇGATCC) and EcoRI (GˇAATTC). The ligation reaction was performed at 16ºC using the T4 DNA ligase (Roche, Germany).

2.2.1b Generation of GST-Ubc9

Ubc9 was expressed as an N-terminally tagged GST fusion protein using the pGEX 2TK-P vector. The Ubc9 fragment was first cloned into a TOPO vector (pCR®2.1) using TA Cloning® Kit (Invitrogen, CA, USA) according to the manufacturer's instructions. The fragment was excised from a 2% gel agarose after restriction digestion and purified with QIAquick® Gel Extraction Kit (Qiagen, UK) according to the manufacturer's instructions. The fragment was cloned into the pGEX 2TK-P vector using the unique restriction sites for BamHI (GˇGATCC) and EcoRI (GˇAATTC). The ligation reaction was performed to a final volume of 10µl at 16ºC using the T4 DNA ligase (Roche, Germany).

2.2.1c Generation of TRAF3 mutants using PCR amplification

Deletion mutants of TRAF3 were generated by using the polymerase chain reaction. The primers used were as follows (Table 2.2.1C.):

	SENSE	**ANTISENSE**
1	5' GGAATTCAGTAAAAAGATGGACTCTCCTG 3'	5'CGACTCGAGTCACTCCTTCAGCAGG 3'
2	5' GGAATTCAGTAAAAAGATGGACTCTCCTG 3'	5'ACTCGAGCAGGCCTCAGTTCCGAGC3'
3	5' GGAATTCAGCAACTCGCTCGAAAAGAAG 3'	5' CCTCGAGTCAGGGATCGGGCAG 3'
4	5' GGAGGAATTCGACAGCATGAAGAGCA 3'	5' CCTCGAGTCAGGGATCGGGCAG 3'

Table 2.1. Primers used in the construction of TRAF3 mutants

PCR reactions were as follows: for mutant 1 and 4, 94ºC for 2 minutes followed by 30 cycles of 94 ºC for 30 seconds, 61ºC for 40 seconds and 72ºC for 50 seconds and then extended by 72ºC for 10 minutes; for mutants 2 and 3, 94ºC for 2 minutes followed by 30 cycles of 94 ºC for 30 seconds, 61ºC for 40 seconds and 72ºC for 67 seconds and then

extended by 72ºC for 10 minutes. The TRAF3 mutants were cloned as N-terminally flag-tagged fusion proteins into the pcDNA3 5.4Kb (Invitrogen, CA, USA) vector on the basis of the existence of two unique restriction sites: EcoRI (G˅AATTC) and XhoI (CTCGAG).

2.3 DNA manipulation techniques

2.3.1 Transformation of competent E.coli bacteria

Plasmid DNA or Ligation Products were transformed into chemically-competent E.Coli bacteria DH5α or DH5β. The bacteria were transferred from -80°C and thawed on ice. The DNA was added to the cells (approximately 0.5ng for plasmid DNA or 10μl of the ligation reaction) and after a 15-minute-incubation on ice, they were treated by heat shock (42°C for 1 minute) followed by a 5-minute quick chill on ice. Pre-warmed SOC media was added to the bacteria (1ml) and they were incubated for 1 hour at 37°C on a shaking rotor. They were subsequently plated on LB-agar containing the appropriate antibiotics (mainly 100μg/ml Ampicillin or 50μg/ml Kanamycin) and incubated overnight at 37°C.

2.3.2 DNA extraction from bacteria

2.3.2a Minipreps

Single colonies were selected from the LB agar plates, placed into starter cultures of 2ml of LB media (see appendix) containing the appropriate antibiotic (mainly 100μg/ml Ampicillin or 50μg/ml Kanamycin) and grown overnight onto a shaking rotor at 37°C. The liquid cultures were centrifuged at 3500rpm (Kubota 5800) for 15 minutes to pellet the bacteria and resuspended in 200μl GTE buffer [25mM Tris-HCI pH 7.5, 10mM EDTA pH 8.0, 1% glucose (vol/vol)] supplemented with RNAse A (250μg/ml). The bacterial cells were lyzed in 400μl of freshly-made Lysis Buffer [200mM NaOH, 1%

SDS (wt/vol)] and the mix was neutralized after a 5-minute incubation on ice with 300µl of Solution III [3M CH_3COOK in acetic acid]. The DNA was precipitated in ice-cold ethanol for 30 minutes on ice and pelleted at 13000rpm for 30 minutes (4°C). The pellets were washed in 200µl 70% ethanol, and left to air-dry. The DNA was resuspended in 25-50µl of TE buffer and left overnight at 4°C prior to determination of DNA concentration to maximize the yield. The concentration of DNA was measured using NanoDrop ND-1000 Spectrophotometer.

2.3.2b Midipreps and maxipreps

The plasmid DNA was extracted from the bacteria using the Plasmid Midi Prep Kit or the Plasmid Maxi Prep Kit (Qiagen) or the Macherey-Nagel according to the manufacturer's instructions. The resulting DNA pellet was resuspended in 50-150 µl of TE buffer (see appendix). The concentration of the DNA was determined using the NanoDrop ND1000 Spectrophotometer and/or gel agarose analysis.

2.3.3 Restriction digestion of DNA plasmids

Plasmids were routinely checked with restriction digestion to verify successful cloning. Briefly, approximately 1µg of plasmid DNA was cut with the desired restriction enzymes according to the individual restriction map. Roughly 1 units of each enzyme was used per 1µg of DNA in a 20µl reaction. BSA was added if necessary to a final concentration of 100µg/ml and the reactions were incubated at 37°C for 2 hours and analyzed by agarose gel electrophoresis.

2.3.4 Detection of nucleic acids

Nucleic acids were mixed with loading buffer [20 % Ficoll 400, 0.1 M Na_2EDTA, pH 8, 1.0% sodium dodecyl sulfate (vol/vol), 0.25 % bromphenol blue (wt/vol), 0.25 % xylene cyanol(vol/vol)] and run on a 1-2% agarose/ethidium bromide/1xTAE gel. 2-3µl of λ DNA digested with PstI was run as a standard on a Power 300 Electrophoresis Power Supply (Fisher Scientific).

2.4 Protein biochemistry techniques

2.4.1 Determination of protein concentration- Bradford Assay

The protein concentration of the samples was determined by the BioRad Bradford Assay (BioRad Lab Ltd, U.K.). In standard test tubes, 980μl of the BioRad Bradford Assay reagent (diluted 1:4 in sterile water) and 20μl of each BSA standards were mixed (see table below) or 998μl of the BioRad Bradford Assay reagent and 2μl of each protein sample to be measured.

Mg of protein/20μl	BSA stock (5mg/ml)	ddH_2O	Final Concentration
2μg	20μl	980μl	100μg/ml
5μg	50μl	950μl	250μg/ml
10μg	100μl	900μl	500μg/ml
15μg	150μl	850μl	750μg/ml
20μg	200μl	800μl	1mg/ml

Table 2.2.: Quantities required to prepare Protein Standards for Protein concentration determination.

The OD was measured at 595nm in a DigiScan Plate Reader (Asys-Hitech) and the measurements were analyzed with the DigiWin software or a BioRad Microplate Reader 680. The protein concentration was calculated according to a standard curve $y=\alpha x$ (y is the OD, x is μg/2μl).

2.4.2 Direct Lysis for SUMOylation detection

EJ cells were seeded in 60mm dishes and stimulated with CD40L. Cells were washed with ice-cold PBS supplemented with full protease inhibitors (Sigma) and 25mM NEM (Sigma) and lyzed in Buffer for Direct Lysis [10mM Tris-HCI pH 6.8, 20% glycerol

(v/v), 4% SDS (wt/v), 20mM NEM (freshly prepared)]. The extracts were incubated for 15 minutes with DNAse and subsequently boiled for 10 minutes. A 20-gauge syringe was used to reduce viscosity followed by a 20-sec sonication at medium power. The lysates were cleared at 13000rpm for 10 minutes (RT) and analyzed by SDS-PAGE.

2.4.3 Immunoprecipitation Studies

2.4.3a Immunoprecipitation of over-expressed proteins

Cells were washed twice with ice-cold Phosphate Buffer Saline (PBS) and subsequently lyzed in 500µl (60mm dishes) or 1ml of RIP Lysis Buffer [20mM Tris-HCI pH 7.5, 150mM NaCI, 1% Triton-X-100 (v/v), EDTA 1mM] (10cm dishes) supplemented with protease inhibitors (Sigma) and sodium orthovanadate (1mM). Lysates were incubated for 10 minutes on ice and centrifuged for 10 minutes at 4°C (13000rpm). Protein concentration was determined using the Bradford assay and approximately 500µg to 2mg of total protein was used for each immunoprecipitation reaction. The protein lysate was pre- cleared with 10µl of Protein G Sepharose for 30 minutes at 4°C. After the pre-clearing, each supernatant was transferred to a clean tube and incubated with the appropriate amount of antibody (Usually 1µg of antibody per 1mg of total protein lysate. Minimum of 1µg of antibody was used if total protein was less than 1mg) for 4-12 hours at 4ºC on a rotor. The following day 20µl of G-Sepharose Beads were added to each immunoprecipitation reaction and the samples were rotated for 1 hour at 4°C. The beads were then washed at least 5 times in Lysis Buffer A and boiled with 30µl Protein Loading Buffer [50mM Tris pH 6.8, 4% SDS (w/v), 10% glycerol, 5% mercaptoethanol, 0.01% bromophenol blue (w/v)] for 8 minutes.

2.4.3b Immunoprecipitation of endogenous proteins

Cells were washed twice with ice-cold Phosphate Buffer Saline (PBS) and subsequently lyzed in 500µl (60mm dishes) or 1ml of RIP Lysis Buffer [20mM Tris-HCI pH 7.5, 150mM NaCI, 1% Triton-X-100 (v/v), EDTA 1mM] (10cm dishes) supplemented with protease inhibitors (Sigma) and sodium orthovanadate (1mM). Lysates were incubated for 10 minutes on ice and centrifuged for 10 minutes at 4°C (13000rpm). Protein concentration was determined using the Bradford assay and approximately 2mg to 6mg

of total protein was used for each immunoprecipitation reaction. The samples were incubated with the appropriate amount of antibody (Usually 1µg of antibody per 1mg of total protein lysate) for 10-15 hours at 4ºC on a rotor. The following day, 20µl of G-Sepharose Beads were added to each immunoprecipitation reaction and the samples were rotated for minimum of 1 hour at 4°C. The beads were then washed at least 2 times in RIP Lysis Buffer and boiled with 30µl Protein Loading Dye [50mM Tris pH 6.8, 4% SDS (w/v), 10% glycerol, 5% mercaptoethanol, 0.01% bromophenol blue (w/v)] for 8 minutes.

2.4.3c Immunoprecipitation of endogenous proteins for SUMO experiments

The dishes were quickly washed twice with ice cold PBS supplemented with protease inhibitors and either 25mM NEM (Sigma) or 25mM Iodoacetamide (Sigma). The cells were lysed in modified cold RIPA buffer (500µl buffer/10cm dish) supplemented with protease inhibitors (Sigma), sodium orthovanadate (1mM) and either NEM or iodoacetamide (25mM), were briefly sonicated (20 sec at medium power) and incubated on ice for 10 minutes. The samples were centrifuged at 13000rpm for 15 minutes at 4°C (the protein concentration was determined using the Bradford Assay) and minimum of 4mg of total protein was used for each immunoprecipitation reaction. The samples were incubated with the appropriate amount of antibody (1µg of antibody per 1mg of total protein lysate) overnight at 4°C on a rotor. The following day 20µl of G-Sepharose Beads were added to each immunoprecipitation reaction and the samples were rotated for 1 hour at 4°C. The beads were washed at least 3 times with modified RIPA buffer supplemented with 25mM iodoacetamide or NEM (both Sigma) and subsequently boiled in the presence of 30µl SDS protein loading dye for 8 minutes.

2.4.4 Detection of SUMOylated proteins with Ni+ pulldown

The method was carried out according to the protocol described by Tatham and colleagues (Tatham et al., 2009). Briefly, HeLa-SUMO1, HeLa-SUMO2 and HeLa parental cells or EJ cells ectopically expressing His-SUMO1 or His-SUMO2 plamids

were grown in 10-dishes (minimum 3 dishes for each condition) to 80-90% cofluency. The cells were collected in 10ml ice-cold PBS, pelleted at 1200rpm for 5 minutes (4°C) and resuspended in 10ml ice-cold PBS. For each sample, 1ml was obtained for crude protein expression by pelleting the cells at 1200 rpm for 5 minutes (4°C) and resuspended in RIPA lysis Buffer (25mM Tris-HCI pH 7.2, 50mM NaCI, 0.5% NP40, 0.5% Sodium Deoxycholate, 0.1% SDS supplemented with full protease inhibitors) supplemented with 25mM iodoacetamide. The remaining samples were spun down (1200rpm, 5min, 4°C), resuspended in 5ml Buffer-1 (6M Guanidinium-HCI, 10mM Tris, 100mM Sodium Phosphate Buffer pH 8.0.) and sonicated for 30 seconds at medium power in the presence of 5mM imidazole and 5mM β-mercaptoethanol. The lysates were centrifuged for 15 minutes at 4000rpm to pellet out any solid material and the supernatants were incubated overnight (4°C) with 50µl packed volume of Ni^{2+} NTA-sepharose beads that had been pre-equilibrated with Buffer-1. The following day the beads were washed once with 4ml Buffer-1 supplemented with 5mM β-mercaptoethanol and 0.1% Triton X-100 (v/v), once with 4ml Buffer-2 [8M Urea, 10mM Tris, 100mM sodium phosphate buffer pH 8.0, 0.1% (vol/vol) Triton X-100, 5mM β-mercaptoethanol] and three times with 4ml of Buffer 3 [8M Urea, 10mM Tris, 100mM sodium phosphate buffer pH 6.3, 0.1% (vol/vol) Triton X-100, 5mM β-mercaptoethanol]. The samples were eluted in 30-50µl elution buffer [200mM Imidazole, 5% (wt/vol) SDS, 150mM Tris-HCI pH 6.7, 30% (vol/vol) glycerol, 720mM β-mercaptoethanol, 0.0025% (wt/vol) bromophenol blue] for 20 minutes at RT and boiled briefly (2 minutes).

2.4.5 GST-pulldown assays

BL21 cells were transformed with pGEX-5x-1/Ubc9 or pGEX-5x-1/CD40 fusions, grown to an optical density of 0.4-0.5 and induced with 0.01mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) for minimum of 3 hours at 37°C, on a shaking rotor. Bacterial lysates were lyzed by pulse sonication (UltraSonic Processor, Sonics VibraCell) in Phosphate Buffer Saline with Protease Inhibitors (Sigma), Sodium Orthovanadate (1mM) and 1% Triton-X-100. Soluble GST-tagged proteins or GST control were purified by incubating overnight at 4°C with GST-Sepharose beads and

washed three times with PBS/1% Triton. The resultant Sepharose beads/GST-tagged proteins or Sepharose beads/GST-tagged control were resuspended in a volume of cold PBS to obtain a 1:1 slurry. For the GST-Ubc9 pull-downs, unstimulated HEK293T cells transiently expressing flag-TRAF3 or any of the flag-tagged TRAF3 mutants were lysed in RIP Lysis Buffer [20mM Tris-HCI pH 7.5, 150mM NaCI, 1% Triton-X-100 (v/v), EDTA 1mM] and 500μg of protein incubated with 20μl of Sepharose/GST-Ubc9 or Sepharose/GST beads for 4 hours at 4°C. The beads were washed three times with RIP buffer and boiled in the presence of SDS gel loading buffer. The proteins were resolved by 9% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) prior to immunoblotting. For the GST-CD40 pull-downs, unstimulated HEK293T cells or EJ cells were lysed in modified RIPA Lysis Buffer (25mM Tris-HCI pH 7.2, 50mM NaCI, 0.5% NP40, 0.5% Sodium Deoxycholate, 0.1% SDS supplemented with full protease inhibitors) in the presence or absence of 25mM iodoacetamide (Sigma) and 1-2mg of protein were incubated with 10μl of Sepharose/GST-CD40 or Sepharose/GST-CD40 MT for 1.5 hour at 4°C. The beads were washed extensively with RIPA buffer containing 25mM iodoacetamide and boiled in the presence of SDS gel loading buffer. The proteins were resolved by 9% SDS-polyacrylamide gel electrophoresis prior to immunoblotting.

2.4.6 Western Analysis

Samples were analyzed by SDS-page on polyacrylamide gels (prepared according to Tables 18.3 and 18.4 in Molecular Cloning-A Laboratory Manual by Sambrook *et al*, modified by Harlow and Lane in 1988), run at 100V constant in a BioRad Mini-PROTEAN 4 cell Apparatus (BioRad Laboratories Inc, Hertfordshire, UK) and electrophoretically transferred to Whatman Protran® Nitrocellulose Transfer Membrane (0.45μm) for 90 minutes or 2 hours (SUMO experiments) at 400 mA constant. Blocking was performed for 1 hour in 5% non-fat milk (Regilait, France)–TBS/Tween 0.1%. The membranes were incubated with various polyclonal or monoclonal antibodies (Table 3.1.) overnight at 4ºC (with the exception of actin which was incubated for 1 hour at RT) and detected by the appropriate secondary HRP-conjugated antibodies for 1 hour at room temperature (Table 3.2.). The detection was performed using equal volumes of Solutions

A and B of the ECL Western blotting detection Plus reagents and analysis system (PerkinElmer), exposed in autoradography films (Fuji Medical X-Ray film 100NIF Super RX) and developed in a Kodak X-OMAT 1000 Processor (Kodak Int, Rochester USA) or in a Fujifilm Las-3000.

ANTIBODY	SPECIES	COMPANY	CATALOG-NUMBER	DILUTION
α-myc 9E10	Mouse monoclonal	Cancer Research UK Antibody Production Fcl	N/A	1:500
α-flag M2	Mouse monoclonal	Sigma	F1804	1:2000
α-TRAF3 C20	Goat polyclonal	Santa Cruz	SC949	1:500
α-TRAF3 C20	Rabbit polyclonal	Santa Cruz	SC949	1:500
α-TRAF2 H20	Rabbit polyclonal	Santa Cruz	SC947	1:500
α-TRAF2 C20	Rabbit polyclonal	Santa Cruz	SC876	1:500
α-NFkappaB2	Rabbit polyclonal	Cell Signaling	4810	1:1000
α-actin	Mouse monoclonal	Millipore	MAB1501	1:30000
α-SUMO-1	Sheep polyclonal	Homemade Hay Lab	n/a	1:1000-1:500
α-SUMO-2	Sheep polyclonal	Homemade Hay Lab	n/a	1:1000-1:500
A-GMP1	Mouse Monoclonal	Invitrogen	21C7	1:500
α-p65	Mouse	Cell Signaling	6956	1:700

Table 2.3. Primary antibodies

ANTIBODY	COMPANY	CATALOG-NUMBER	DILUTION
Anti-Rabbit HRP-congugated	Sigma	A0545	1:25000
Anti-Sheep HRP conjugated	Santa Cruz Biotech	SC2473	1:5000
Anti-mouse HRP-congugated	Sigma	A9917	1:20000
Anti-goat HRP-congugated	Santa Cruz Biotech	A5420	1:8000

Table 2.4. Secondary antibodies.

2.4.7 Reprobing of membranes

2.4.7a Neutralization of HRP

The membranes were incubated on a shaking rotor (Stuart Scientific Orbital Shaker, SO1) at room temperature for 30 minutes in TBS/Tween-0.2% NaN_3. After extensive washing with TBS/Tween 0.1%, the membranes were incubated overnight at 4ºC with the desired primary antibody on a Stuart Roller Mixer SRT1.

2.4.7b Stripping of membranes

The membranes were incubated on a rotor at 50°C for 30 minutes in Stripping Buffer [60mM Tris-HCI pH 6.8, 0.7% β-mercaptoethanol (vol/vol), 2% SDS (wt/vol)] and washed extensively under tap water for 30 minutes. The stripped membrane was blocked in 5% non-fat milk (Regilait, France)–TBS/Tween 0.1% for 1 hour at RT and re-incubated overnight with the desired primary antibody.

2.4.8 Preparation of Nuclear and Cytoplasmic Extracts

Cells were firstly lysed in a hypotonic buffer (20 mM Hepes pH 7.6, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% NP-40, 20% glycerol, 1 mM DTT, 1 mM Na_3VO_4 and

protease inhibitor cocktail) and incubated on ice for 10 min. Following centrifugation (5 min, 1500 rpm) the supernatant was collected as the cytoplasmic fraction. The remaining pellet was washed twice with hypotonic buffer and then resuspended in hypertonic buffer (composed as the hypotonic with NaCl supplemented to final concentration of 0.5 M) and was incubated on ice for 30 min with agitation. Debris and nuclear remnants were removed with centrifugation (20 min, 13000 rpm) and the supernatant was collected as the nuclear fraction.

2.4.9 Expression of recombinant Proteins and in Vitro SUMOylation Assays

For *in vitro* SUMOylation assays, ^{35}S labeled proteins were generated using in TNT® Quick Coupled Transcription/ Translation System (Promega) according to the manufacturer's protocol. SUMO conjugation assays were carried out as follows: 1µl of translation product was incubated for 2-4 hours at 37°C (control unmodified tubes were kept at -20°C) in a 20µl reaction mix (50mM Tris pH 7.5, 5mM $MgCl_2$, 2mM ATP, 10mM Creatine Phosphate, 3.5U/ml Creatine Kinase, 0.6U/ml Inorganic pyrosphosphatase, 5mM DTT, full Protease Inhibitors) containing 120ng E1 (SAE1/2), 5-750ng E2 (Ubc9) and 500ng of SUMO-1 or SUMO-2. PIAS proteins were added in a concentration range from 10 to 100ng.

Chapter 3: RESULTS

3.1 Identification of Ubc9 as a novel TRAF3-interacting protein through yeast 2-hybrid

In order to identify novel proteins that interact with TRAF3 the yeast two-hybrid method was selected. The basis of this method is the activation of a downstream reporter gene by the binding of a transcription factor onto an upstream activation sequence. The transcription factor is essentially split into two fragments, the binding domain and the activating domain. The BD domain binds to the activating sequence whereas the AD domain is responsible for the activation of transcription (Young, 1998).

In the case of TRAF3, yeast two hybrid assay was utilized by fusing full-length TRAF3 to Gal4 DNA binding domain and using it as a bait to screen a HeLa cDNA library. Out of a total of 10 million transformants, 10 independent positive clones were obtained. One of them encoded the full-length Ubc9, a protein that has already been introduced as the sole E2 enzyme responsible for conjugating SUMO onto a specific target (Introduction, Chapter 1B).

To verify this interaction, *S.cerevisiae* strain PJ69-4A was transformed with plasmids expressing the GAL4 activation domain (AD) fused to Ubc9, the GAL4 binding domain (BD) fused to TRAF3 or with control empty vectors in all possible combinations. Cells were grown in standard medium (SD-leu-trp) in the presence or absence of X gal, in medium lacking adenine (SD-leu-trp-ade) to detect expression of the *GAL-ADE2* reporter gene or in medium lacking histidine (SD-leu-trp-his + 3-AT) to detect expression of the *GAL-HIS3* reporter gene. The growth patterns of the transformed cells and the expression levels of β-galactosidase activity (Figure 3.1) confirmed that TRAF3 and Ubc9 interact in yeast.

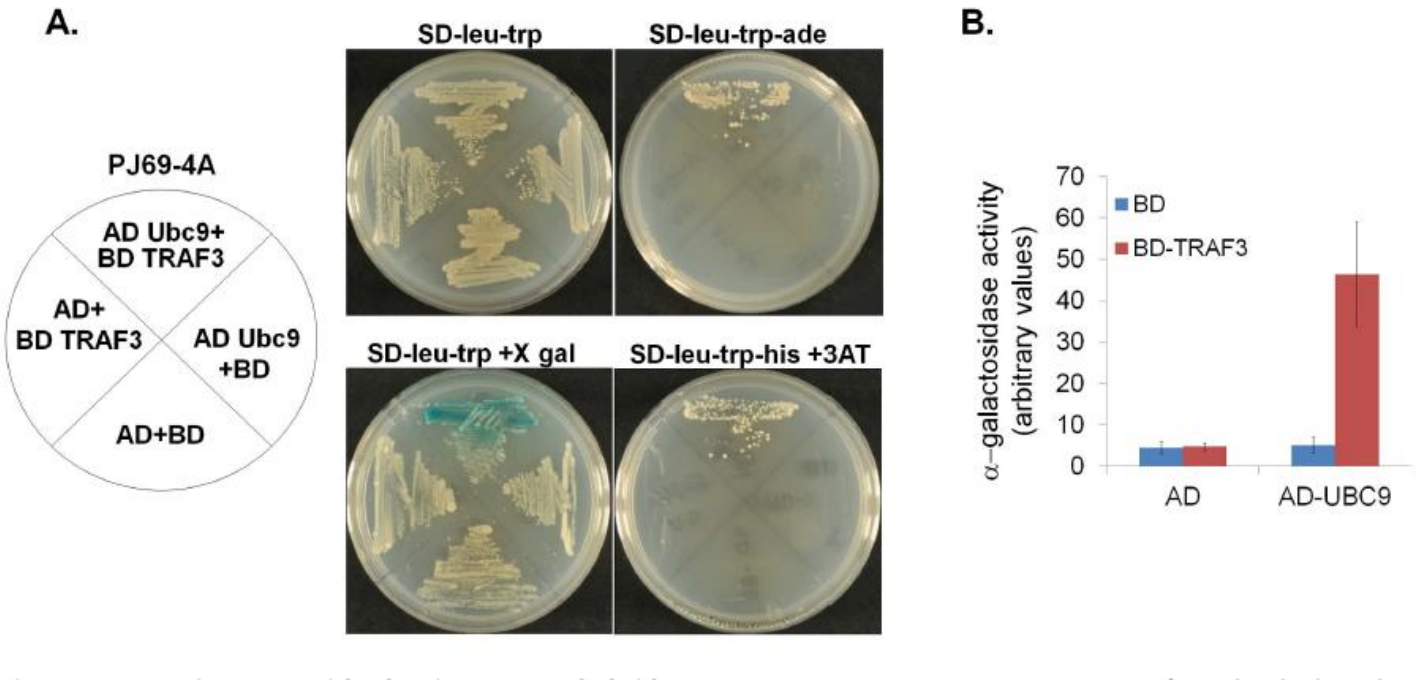


Figure 3.1: TRAF3 interacts with Ubc9 in a yeast 2-hybrid assay.

(A) *S.cerevisiae strain PJ69-4A was transformed with plasmids expressing the GAL4 activation domain (AD) fused to Ubc9, the GAL4 binding domain (BD) fused to TRAF3 or with control empty vectors in all possible combinations. Cells were grown on standard medium (SD-leu-trp) in the presence or absence of X gal (SD-leu-trp+X gal), medium lacking adenine (SD-leu-trp-ade) to detect expression of the GAL-ADE2 reporter gene or medium lacking histidine (SD-leu-trp-his + 5 mM 3-AT) to detect expression of the GAL-HIS3 reporter gene.*

(B) *Quantitation of galactosidase reporter activity in yeast transformed with the vectors described in (A). Results are representative of 3 independent experiments.*

3.2 Ubc9 associates with TRAF3 *in vitro* and *in vivo*

In order to validate the interaction between TRAF3 and Ubc9, co-immunoprecipitation assays were performed. The full-length Ubc9, expressed as an N-terminal fusion protein tagged with GST was examined for its ability to interact with FLAG-tagged TRAF3, ectopically expressed in HEK293 cells. As seen in Figure 3.2A, GST-Ubc9 strongly associated with FLAG-TRAF3 whereas control GST did not. SMAD4, which is known to interact with Ubc9 (Lin et al., 2003) was used as a positive control. The levels of expression of GST and GST-Ubc9 are comparable as shown in Figure 3.2B.

The interaction was further analyzed in mammalian cells using co-immunoprecipitation assays. Full-length Ubc9, containing a N-terminal Myc tag was transiently co-expressed in HEK293 cells with FLAG-tagged TRAF3 or positive control FLAG-SMAD4. Cell lysates were immunoprecipitated with monoclonal antibody against the FLAG epitope and co-precipitating Ubc9 was detected using a polyclonal Myc antibody. Figure 3.2C demonstrates that FLAG-TRAF3 interacted with myc-Ubc9.

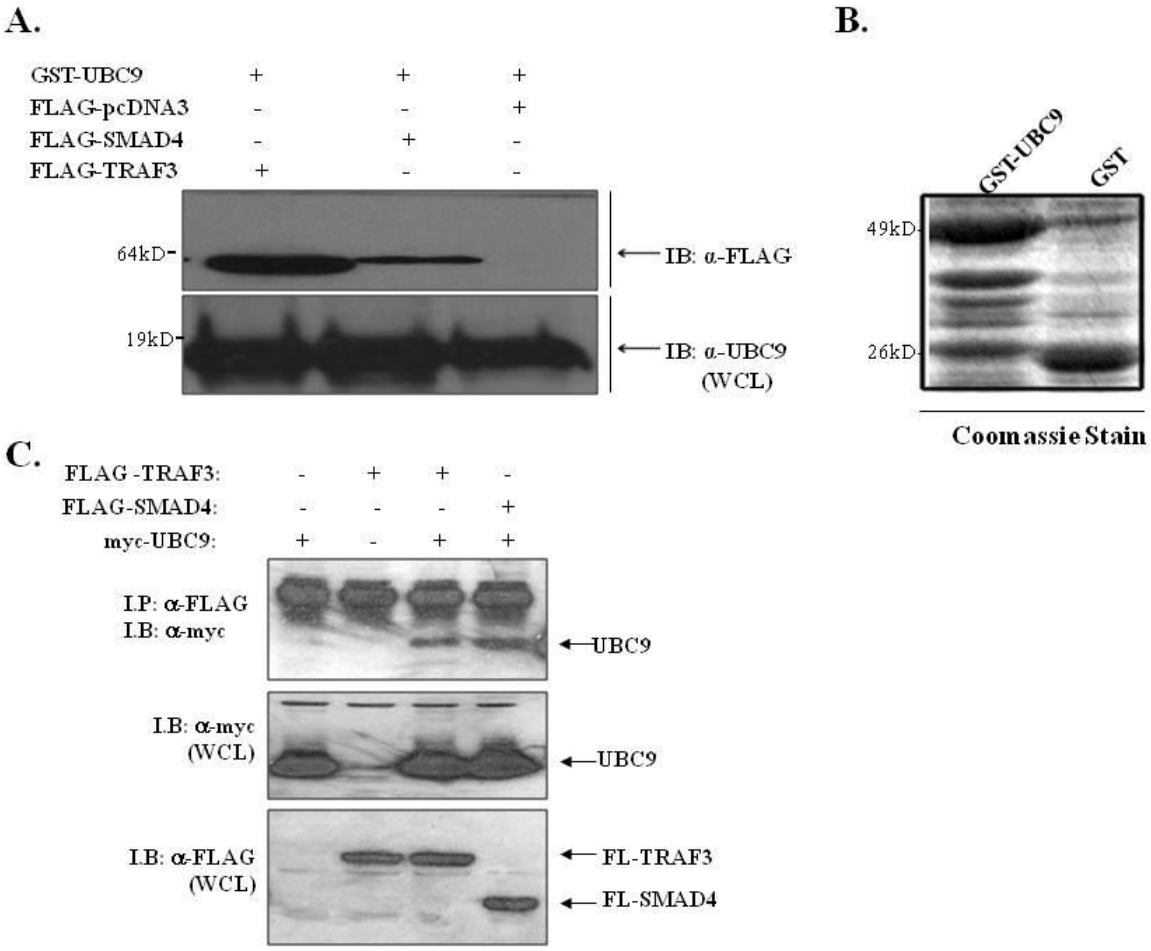


Figure 3.2: TRAF3 interacts with UBC9 in vivo and in vitro

(A) *In vitro GST-pulldown assay. Extracts from HEK293 cells with over-expressed FLAG-TRAF3 were incubated with GST or GST-Ubc9 fusion proteins and processed as described in "Materials and Methods." Bound proteins were resolved by SDS-PAGE and analyzed by immunoblotting.*

(B) *Coomassie staining of bacterially- produced GST-Ubc9 and GST control protein.*

(C) *Myc-Ubc9 and FLAG-TRAF3 interact in vivo. HEK293 cells were transiently transfected with expression vectors encoding Myc epitope-tagged Ubc9 (full-length) and the FLAG-tagged TRAF3 or FLAG-tagged SMAD4 (Lin et al, 2003). After 36 h extracts were prepared and immunoprecipitated (IP) with anti-FLAG monoclonal antibody. Co-precipitating Myc-Ubc9 was detected by immunoblotting (IB) analysis using the anti-Myc monoclonal antibody (top panel). The amount of TRAF3 or SMAD4 immunoprecipitated and the expression level of Myc-Ubc9 in total cell extracts were determined by immunoblotting with anti-Myc monoclonal antibody (middle panel) and anti-FLAG monoclonal antibody (bottom panel).*

3.3 The TRAF domain of TRAF3 is the domain responsible for the interaction between TRAF3 and Ubc9

TRAF3 contains an N-terminal ring finger domain followed by several zinc fingers. The C-terminal half of TRAF3 is the TRAF domain, which is conserved among the members of the TRAF family and can be further subdivided into the TRAF-N and TRAF-C domain. To determine which regions of TRAF3 contribute to Ubc9 binding, various FLAG-tagged TRAF3 deletion mutants were generated and assayed for association with Myc-tagged Ubc9.

Four TRAF3 mutants were generated (Figure 3.3A) using the polymerase chain reaction: one lacking the TRAF domain (aa1-266), one lacking the TRAF-C domain of TRAF (aa1-376), one lacking the N-terminal ring domain and zinc fingers (267-568) and one lacking the N-terminal ring domain and zinc fingers as well as most of the TRAF-N domain (aa346-568). All the TRAF3 deletion mutants were expressed as fusion proteins containing a N-terminal FLAG-epitope tag using the pcDNA3 vector (Invitrogen). The mutants were verified by sequencing and Western Blotting (Figure 3.3B)

To determine which regions of TRAF3 contribute to the Ubc9 binding, we utilized the TRAF3 deletion mutants. The TRAF3 mutants were transiently transfected into HEK293 cells and assayed for interaction with GST-Ubc9 or GST (Figure 3.3C). The domain in TRAF3 responsible for the Ubc9 binding is located between the aa267-568 which comprises the TRAF-N domain.

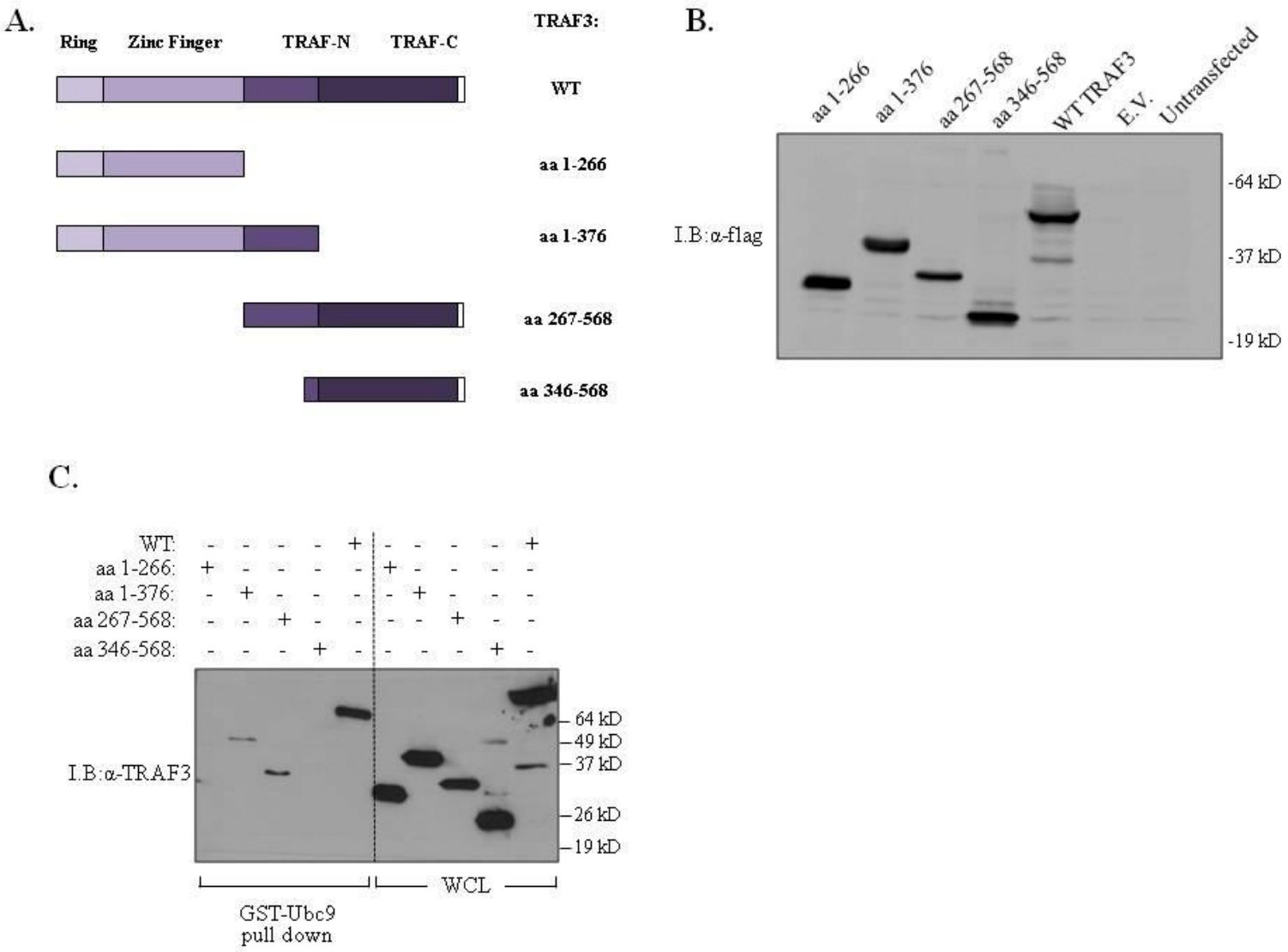


Figure 3.3: Identification of the TRAF-domain of TRAF3 as the domain responsible for the interaction with Ubc9

(A) *Generation of TRAF3 deletion mutants.*

(B) *Expression of TRAF3 deletion mutants in HEK293.*

(C) *HEK293 cells were transiently transfected with the FLAG-tagged TRAF3 deletion mutants. Each lane represents 20μg of total protein . After 36 hours the extracts were incubated with purified GST-Ubc9 bound to glutathione-Sepharose as described in the Materials & Methods Chapter. Bound proteins were fractionated by SDS-PAGE and exposed to x-ray film. For each reaction, 300μg of total protein for each TRAF3 deletion mutant was used whereas the amount of purified GST or GST-Ubc9 used for each reaction was empirically calculated from analyzing the proteins by SDS-PAGE.*

3.4 TRAF3 can be SUMOylated by SUMO-1 and SUMO-2/3 *in vivo* but not *in vitro*

It has previously been mentioned that Ubc9 is the sole known E2 enzyme participating in the SUMO pathway. Therefore, because of the association of TRAF3 with Ubc9 we postulated that TRAF3 could be a potential SUMO substrate. Utilizing the SUMOplot (Abgent, http://www.abgent.com/tools/) and various others online tools including SUmoMOtif finder (http://cbg.garvan.unsw.edu.au/sumofi/form.do) we identified that TRAF3 contains several consensus motifs for SUMOylation (Fig 3.4A).

To verify the *in silico* evidence, lysates from HEK293 cells, EJ bladder carcinoma cells, BJAB lymphoma cells, mouse splenocytes and HeLa cells stably expressing SUMO-1 were lyzed under normal conditions or in the presence of N-ethylmaleimide (NEM). NEM acts to stabilize SUMO conjugates by covalently modifying the sulfhydryl group of the catalytic cysteine on SUMO-specific proteases (Gregory, 1955). The lysates were immunoprecipitated with TRAF3 antibody and precipitates were immunoblotted with either SUMO-1 or SUMO-2 antibody (Figure 3.4B, right panel). The results indicated that in the presence of NEM, upper bands that correspond to SUMO-conjugated TRAF3 were readily detected (Figure 3.4B, lanes 1, 3, 5, 7) whereas less or none such bands were visible in the absence of the inhibitor (Figure 3.4B, lanes 2, 4, 6, 8).

To further validate these results, HeLa parental cells or HeLa cells stably expressing His-tagged SUMO-1 or SUMO-2 were lyzed under denaturing conditions and purification of His-SUMO conjugates was performed with Nickel beads. Using TRAF3 antibody, it was noted that TRAF3 was co-purified with His-SUMO1 and His-SUMO-2 (Figure 3.4C). Similar results were obtained when we transiently expressed His-SUMO-1 or His-SUMO-2 in EJ cells and carried out Nickel-NTA pull-downs (Figure 3.4D).

Although detection of TRAF3 SUMOylation was a fairly straightforward *in vivo*, the same cannot be said about the *in vitro* SUMOylation assays. TRAF3 was *in vitro*

transcribed and translated as ^{32}S tagged protein and then incubated in a reaction containing the SUMO E1 (SAE1/2) and E2 enzymes and SUMO-1 or SUMO-2 as His-tagged or GST-tagged purified proteins. The reactions were analyzed by SDS-PAGE and autoradiography to detect lower mobility bands that could correspond to SUMO-modified forms of TRAF3. However, no such bands were detected either for SUMO-1 or SUMO-2 adding a perplexing side to this hypothesis (Supplementary Figures 3.4E). Addition of E3 SUMO ligases PIAS3 or PIAS4 did not affect the results (Figure 3.4F).

A.

No.	Pos.	Group	Score
1	K550	ENGTY **IKDD** TIFIK	0.94
2	K138	HLLVH **LKND** CHFEE	0.91
3	K513	HLGDA **FKPD** PNSSS	0.85
4	K522	PNSSS **FKKP** TGEMN	0.74

No.	Pos.	Group	Score
5	K191	PMIAL **QKHE** DTDCP	0.50
6	K6	MESS **KKMD** SPGAL	0.48
7	K429	RDYKR **RKQE** AVMGK	0.44
8	K99	ESIVK **DKVF** KDNCC	0.15

1 MESSKKMDSP GALQTNPPLK LHTDRSAGTP VFVPEQGGYK EKFVKTVEDK
51 YKCEKCHLVL CSPKQTECGH RFCESCMAAL LSSSSPKCTA CQESIVKDKV
101 FKDNCCKREI LALQIYCRNE SRGCAEQLML GHLLVHLKND CHFEELPCVR
151 PDCKEKVLRK DLRDHVEKAC KYREATCSHC KSQVPMIALQ KHEDTDCPCV
201 VVSCPHKCSV QTLLRSELSA HLSECVNAPS TCSFKRYGCV FQGTNQQIKA
251 HEASSAVQHV NLLKEWSNSL EKKVSLLQNE SVEKNKSIQS LHNQICSFEI
301 EIERQKEMLR NNESKILHLQ RVIDSQAEKL KELDKEIRPF RQNWEEADSM
351 KSSVESLQNR VTELESVDKS AGQVARNTGL LESQLSRHDQ MLSVHDIRLA
401 DMDLRFQVLE TASYNGVLIW KIRDYKRRKQ EAVMGKTLSL YSQPFYTGYF
451 GYKMCARVYL NGDGMGKGTH LSLFFVIMRG EYDALLPWPF KQKVTLMLMD
501 QGSSRRHLGD AFKPDPNSSS FKKPTGEMNI ASGCPVFVAQ TVLENGTYIK
551 DDTIFIKVIV DTSDLPDP

Motifs with high probability

Motifs with low probability

Figure 3.4: (A) *SUMOplot for TRAF3 (PubMED NP: 663777). The probable sites are predicted based on the consensus motif Ψ-K-x-D/E where Ψ is a hydrophobic residue, K is the lysine conjugated to SUMO, x is any amino acid (aa), D or E is an acidic residue. The SUMOplot score system is based on two criteria: 1) direct amino acid match to the SUMO-CS observed and shown to bind Ubc9, and 2) substitution of the consensus amino acid residues with amino acid residues exhibiting similar hydrophobicity (Fig 3.4 continues overleaf).*

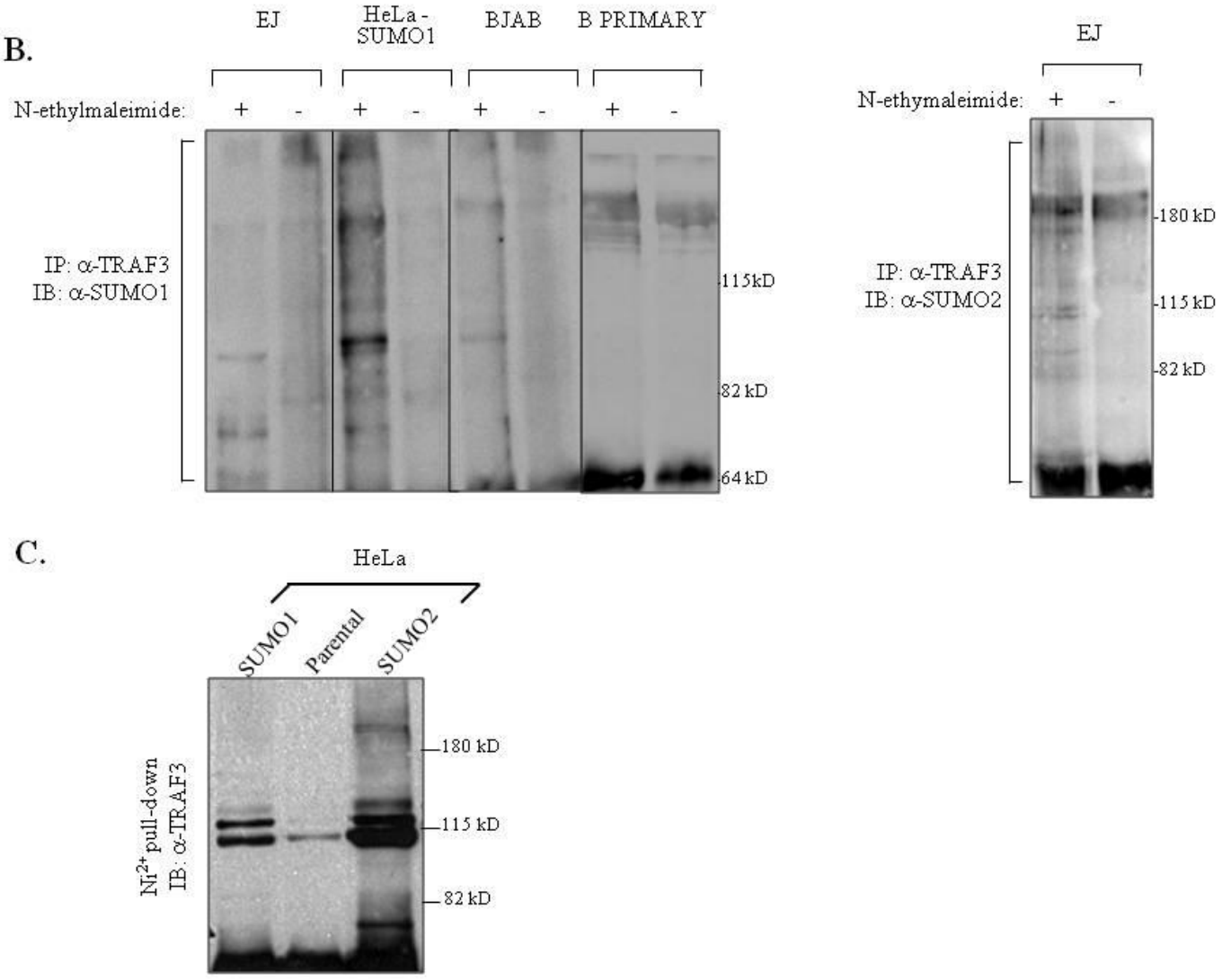


Figure 3.4: TRAF3 is SUMOylated in vivo but not in vitro (continued)

(B) *EJ, HeLa SUMO1, BJAB cells and mouse primary B cells extracted from splenocytes were lyzed in the presence or absence of inhibitor NEM (25mM), immunoprecipitated with α-TRAF3 antibody and immunoblotted with α-SUMO1. Similarly, EJ lysates ± NEM were immunoprecipitated with α-TRAF3 and immunoblotted with α-SUMO2. Equal amounts of total protein (4mg) were loaded per IP reaction.*

(C) *HeLa cells stably expressing His-tagged SUMO1 or SUMO2 and parental HeLa cells were lysed in a protein-denaturing buffer and lysates were subjected to enrichment of SUMOylated proteins on nickel-nitrilotriacetic acid (Ni-NTA) columns. Eluates were immunoblotted with a-TRAF3 polyclonal antibody.*

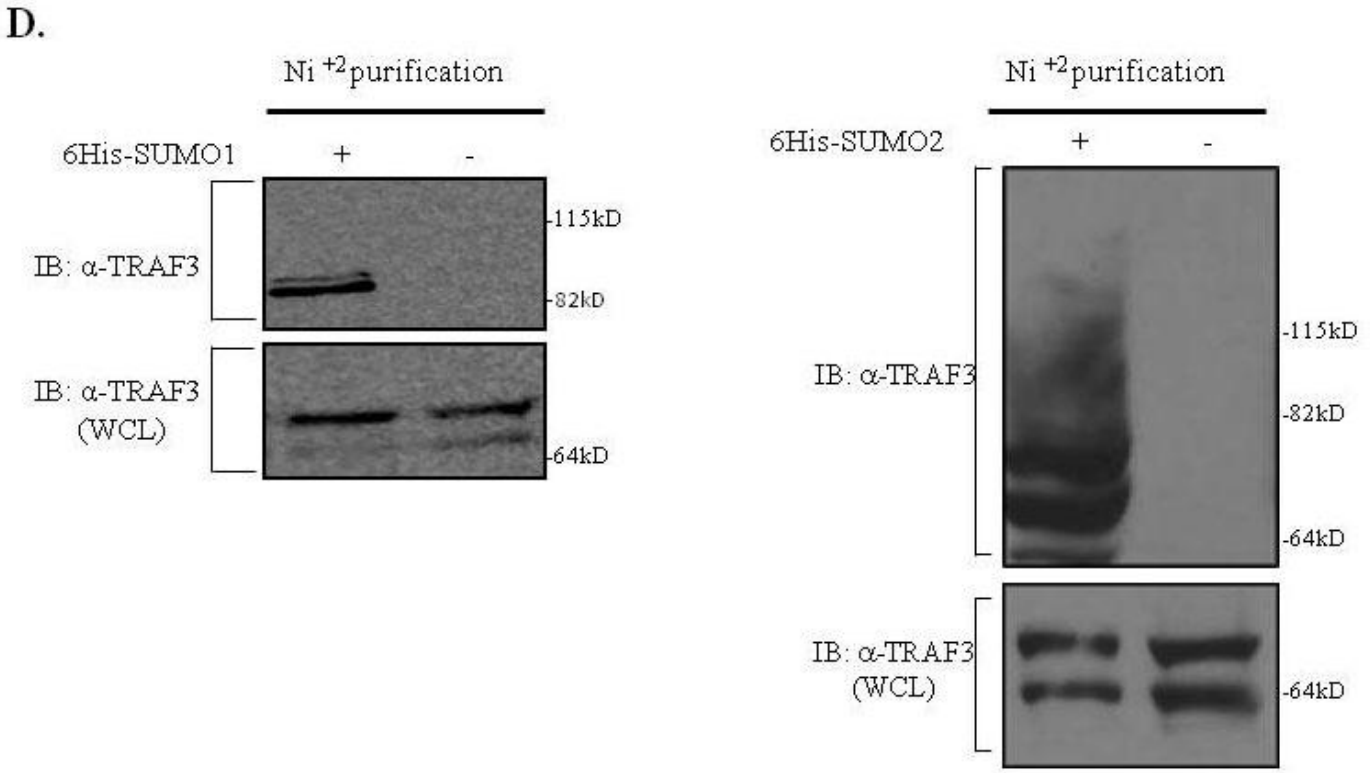
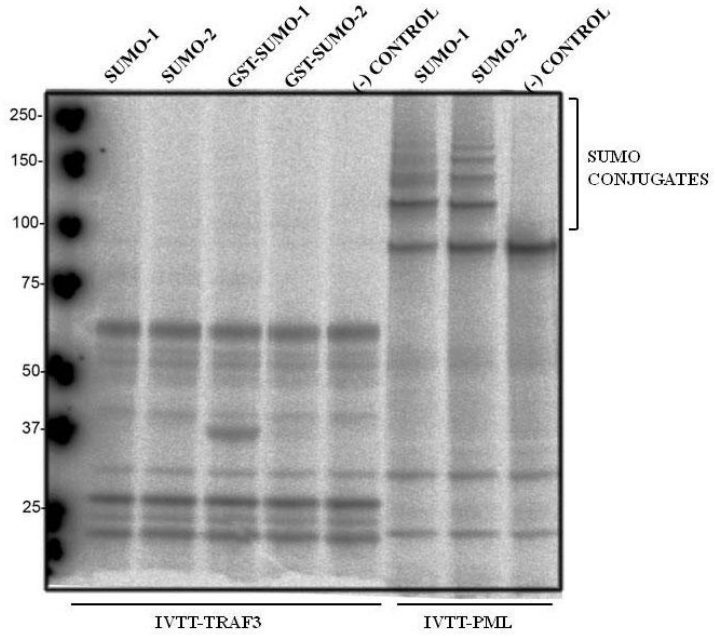


Figure 3.4: TRAF3 is SUMOylated in vivo but not in vitro (continued)

(D) *EJ cells were transiently transfected with His-SUMO-1 or His-SUMO-2 plasmids and lyzed in a protein-denaturing buffer. Lysates were subjected to enrichment of SUMOylated proteins on nickel-nitriotriacetic acid columns. Eluates were immunoblotted with α-TRAF3.*

E.



F.

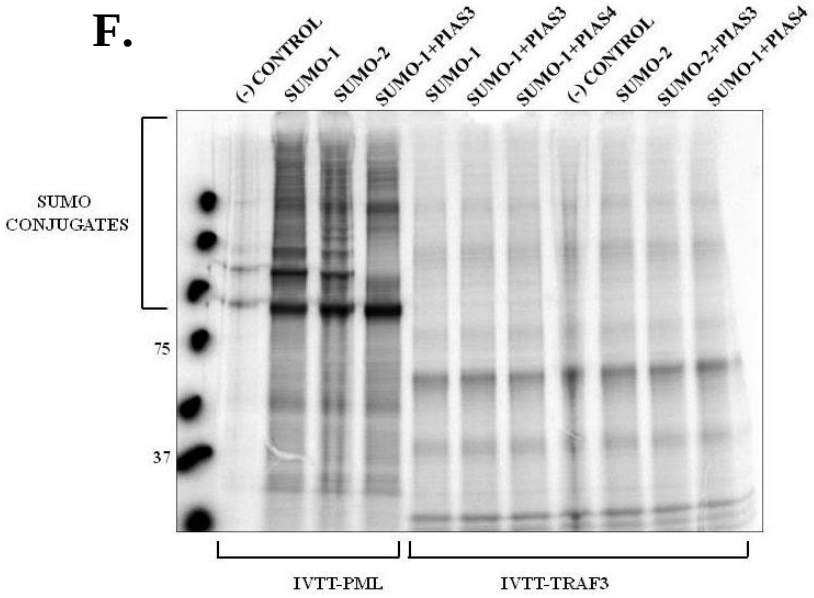


Figure 3.4: TRAF3 can be SUMOylated in vivo but not in vitro (continued)

(E) *In vitro translated ^{32}S –labeled TRAF3 was incubated either in the presence (lanes 1-4) or absence (lane 5) of an assay mix containing recombinant E1 (SAE1/2), Ubc9 and SUMO-1 or SUMO-2. SUMO modification of PML served as a positive control. Proteins were resolved by SDS-PAGE and visualized by autoradiography. The Figure is representative of at least 4 independent experiments.*

(F) *As before, only purified E3 SUMO enzymes PIAS3 or PIAS4 were added to the mix as indicated. The Figure is representative of at least 3 independent experiments.*

3.5 Ubc9 is essential for the modification of TRAF3 by SUMO1 and SUMO2/3

In order to further explore the link between TRAF3 SUMOylation and Ubc9, we performed either over-expression experiments using a Ubc9 dominant-negative mutant or RNAi-mediated knockdown of Ubc9, in HEK293 or HeLa SUMO-1 cells. The dominant-negative (DN) Ubc9 carries a mutation on Cys^{93} which is changed to Ser. This mutation prevents Ubc9 from conjugating SUMO moieties. Over-expression of Ubc9 DN in HEK293 cells showed a marked decrease in SUMO-1 and SUMO-2 SUMOylation compared to over-expression of an empty vector (Fig 3.5A). Similar results were observed when over-expressing Ubc9 DN or EV in HeLa stably expressing SUMO-1 (Fig 3.5B). When Ubc9 is silenced with siRNA in HEK293 cells we detected a comparable result (Fig 3.5C). This further underlines the importance of Ubc9 in the SUMOylation process but also establishes a functional link between TRAF3 and Ubc9.

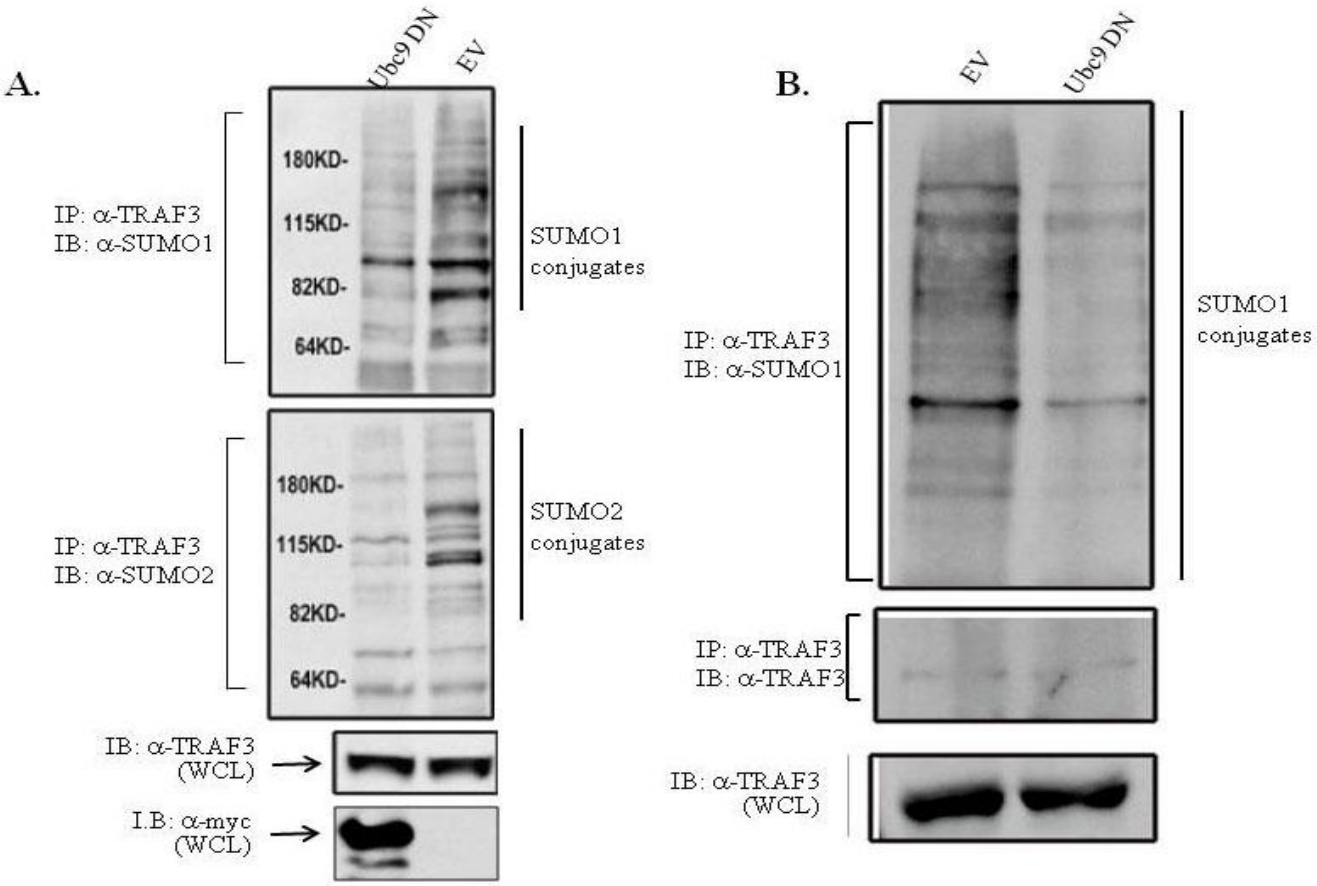


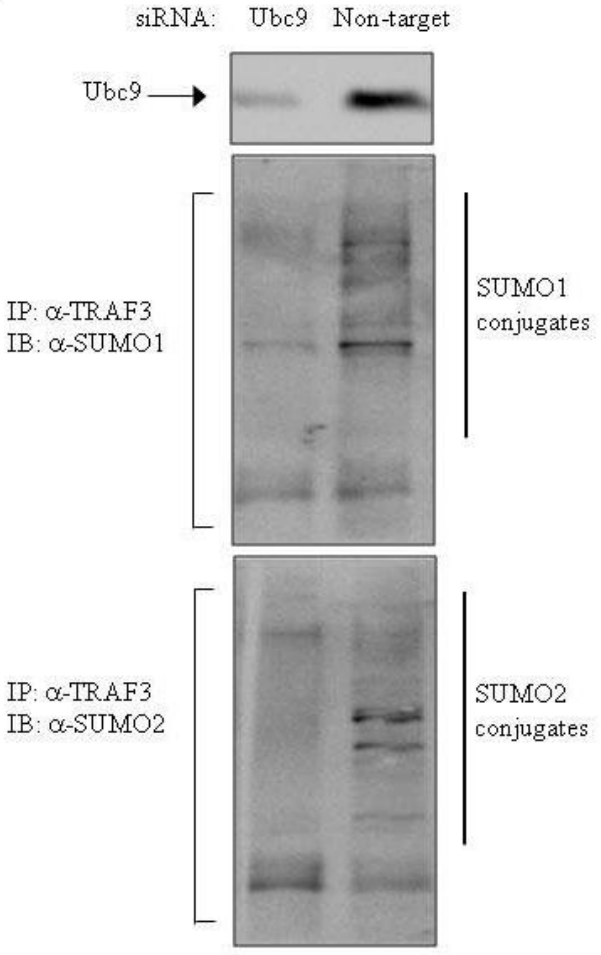
Figure 3.5: Ubc9 is essential for TRAF3 modification by SUMO

(A) *Ubc9 DN or EV (2μg of DNA per reaction) was transiently expressed in 293T cells. After 48 hours, lysates were immunoprecipitated with α-TRAF3 polyclonal antibody (3μg per sample) and immunoblotted with either SUMO-1 or SUMO-2 antibody to detect immunoreactive bands that correspond to SUMO conjugates. The figure is representative of at least 3 independent experiments.*

(B) *Ubc9 DN or EV (3 μg per reaction) was transiently transfected in HeLa SUMO1 cells. After 48 hours lysates were immunoprecipitated with α-TRAF3 antibody and immunoblotted with anti-SUMO-1.*

(Figure 5 continues overleaf)

C.



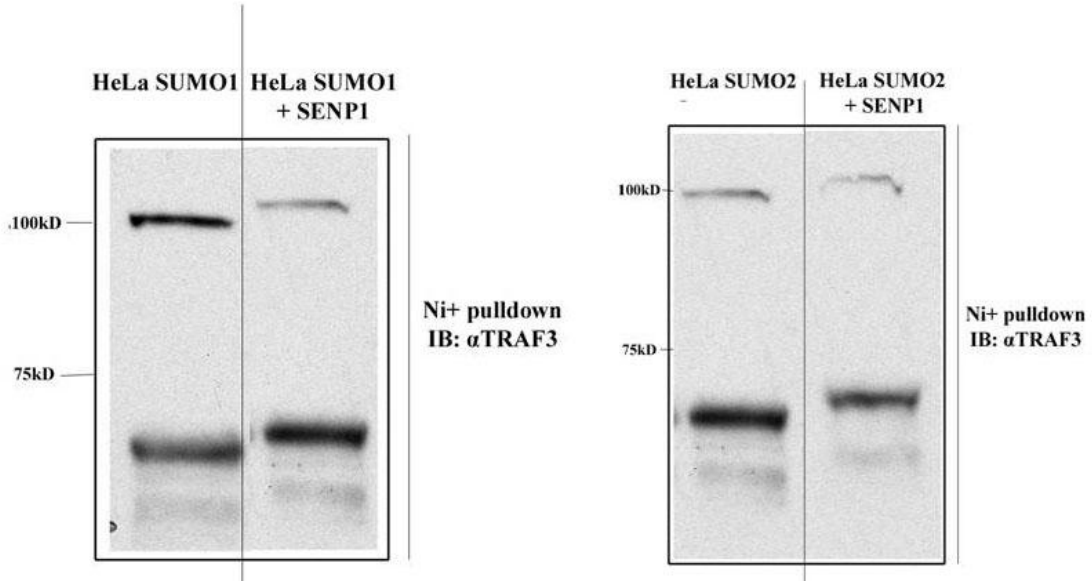
(C) *siRNA knockdown was performed for Ubc9 or non-target siRNA for 72 hours in HEK293 cells. The lysates were immunoprecipitated with α-TRAF3 and immunoblotted with SUMO-1 or SUMO-2. The figure is representative of at least 3 independent experiments.*

** Unless otherwise indicated cell lysis was performed in the presence of iodoacetamide.*

3.6 SENP1 is involved in TRAF3 de-SUMOylation

SENP1, as has been described in chapter 1B, is a SUMO protease that can remove SUMO-1 and SUMO-2/3 from the target protein (Hang and Dasso, 2002; Zhang et al., 2002; Gong et al., 2000). Furthermore, SENP1 is localized in the cytoplasm (Table 1.2, Chapter 1B) as is the majority of TRAF3, making it a good candidate for the TRAF3-specific de-SUMOylating enzyme. HeLa SUMO-1 and HeLa SUMO-2 cells were lyzed under denaturing conditions to preserve SUMOylation and subsequently subjected to purification through Nickel columns. The SUMO-enriched conjugates were treated with purified, recombinant SENP1 in a mild alkaline environment (a kind gift from Dr Linnan Shen) (Shen et al., 2006) and analyzed by Western Blotting. As seen in Figure 3.6A, treatment with the SUMO protease led to a marked reduction of TRAF3 SUMOylation and an increase in free SUMO (Figure 3.6B). De-SUMOylated TRAF3 was detected in the supernatant of the protease buffer (data not shown due to poor quality of image).

A.



B.

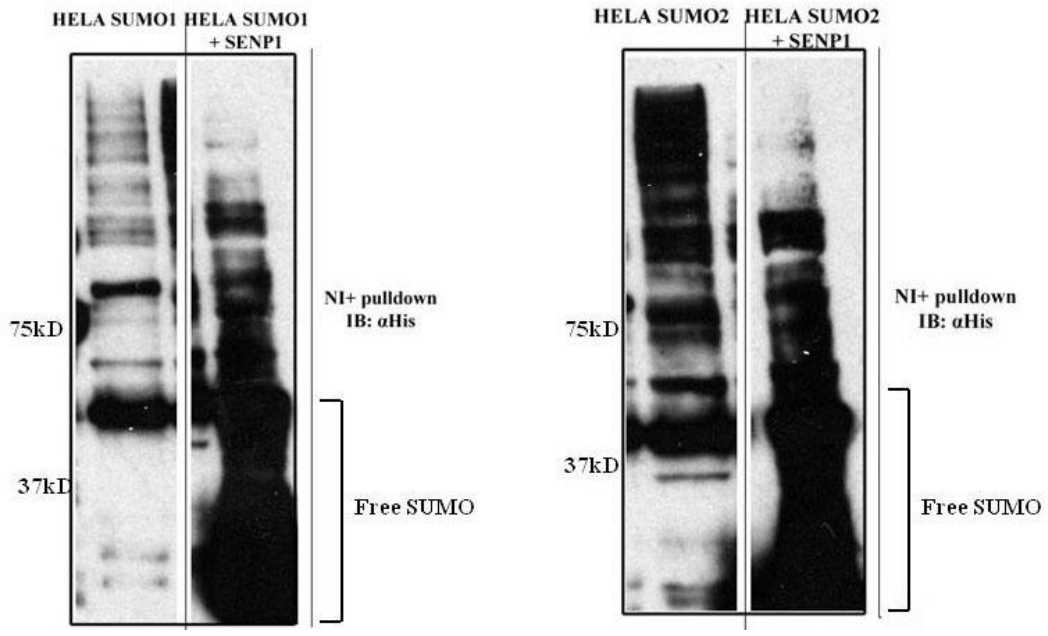


Figure 3.6: SENP1 is involved in TRAF3 de-SUMOylation

(A) *HeLa SUMO-1 and HeLA SUMO-2 were lyzed in a protein-denaturing buffer and subjected to* Ni^{2+} *bead pull-down. The pull-downs (lane 2 of each panel) were subjected to treatment with purified, recombinant SENP1 (5μg per sample) for a total of 15 minutes. The total lysates and pull-downs were immunoblotted with α-TRAF3 polyclonal antibody.*

(B) *Approximately 1% of each pull-down was immunoblotted with α-His monoclonal antibody to detect the efficiency of de-SUMOylation by recombinant SENP1.*

3.7 TRAF3 is a direct target of SUMO

TRAF3, as has been extensively analyzed in Chapter 1, is localized in the cytoplasm as part of protein complexes but also as a heterodimer with other TRAF proteins. In line to verify whether TRAF3 is a direct target of SUMO-1 and SUMO-2 and that is not co-immunoprecipitated in native IPs as part of other complexes, we performed siRNA knockdown of TRAF3 and immunoprecipitation with α-TRAF3 polyclonal antibody. As seen in Figure 3.7B, the bands that correspond to SUMO conjugates are decreased when TRAF3 is knocked down, which implies that these bands may well correspond to SUMOylated forms of TRAF3.

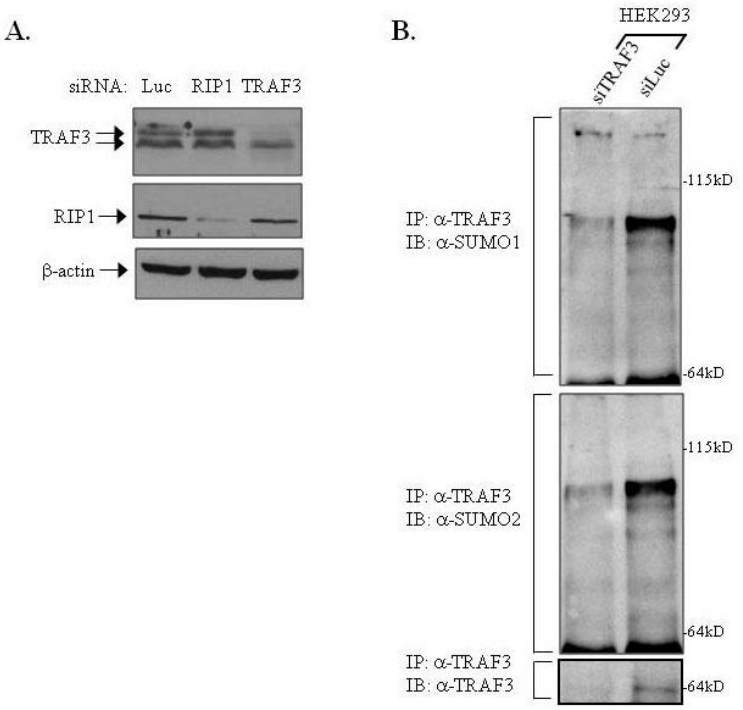


Figure 3.7: TRAF3 is a direct SUMO target.

(A) *Validation of siRNA targeting TRAF3. EJ bladder carcinoma cells were transfected with siRNAs against TRAF3, RIP1 or the unrelated luciferase gene and knock-down efficacy and specificity were determined by immunoblotting cell lysates with anti-TRAF3, RIP1 or β-actin antibodies.*

(B) *Specificity of anti-SUMO-1/2 immunoreactivity in TRAF3 precipitates. EJ cells were transfected with siRNAs targeting TRAF3 or the unrelated luciferase gene, lyzed in the presence of iodoacetamide and TRAF3 was immunoprecipitated in a non-denaturing buffer. Immunoprecipitates were then immunoblotted with either α-SUMO-1 or SUMO-2 or TRAF3 antibodies, as indicated.*

3.8 TRAF3:Ubc9 interaction is diminished following stimulation with CD40 ligand

TRAF3 has an important role in CD40-mediated pathways where it acts as a negative regulator of downstream signaling. We wanted to determine whether the interaction between TRAF3 and Ubc9 and ultimately, TRAF3 SUMOylation, are implicated in CD40 signaling. To this end, EJ cells were stimulated with CD40 ligand for 30 minutes and lysates were immunoprecipitated with α-TRAF3 antibody followed by immunoblotting with either anti-Ubc9 or anti-TRAF3. The interaction between TRAF3 and Ubc9, although evident at steady state, is diminished at 30 minutes following the kinetics of TRAF3 degradation (Figure 3.8 panel 1 and 3).

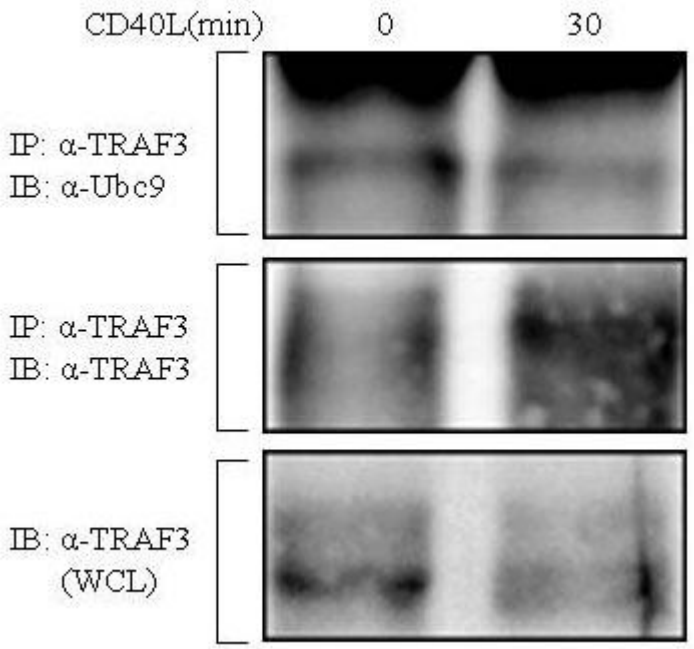


Figure 3.8: TRAF3: Ubc9 interaction is diminished following CD40 receptor stimulation.

EJ cells were stimulated for 30 minutes with 500ng/ml CD40 ligand followed by lysis and immunoprecipitation with α-TRAF3 C20 (1μg) antibody along with unstimulated cells. The amount of total protein loaded per each immunoprecipitation reaction was 1mg. The immunoprecipitation reactions were subjected to Western Analysis by α-Ubc9 and α-TRAF3.

3.9 CD40 receptor stimulation leads to decreased TRAF3 SUMOylation

TRAF3 exerts a negative role in the CD40-mediated NF-κB activation (Cheng et al., 1995; Nakano et al., 1996) through the assembly of multi-unit signaling complex at the cytoplasmic domain of the receptor. The signaling complex assembles at the plasma membrane but translocates to the cytoplasm upon TRAF3 ubiquitination and subsequent degradation mediated by TRAF2 and cIAPs (Matsuzawa et al., 2008; Vallabhapurapu et al., 2008) (Figure 1.4b). In order to determine whether SUMO-TRAF3 has a role in this pathway, we examined whether CD40 receptor stimulation had any effect on the SUMOylation status of TRAF3.

EJ cells were stimulated with CD40 ligand for 5, 15 or 30 minutes and lyzed in the presence of NEM. The lysates were immunoprecipitated with anti-TRAF3 antibody and immunoblotted with anti-SUMO1. In Figure 3.9A, it is clear that SUMOylation of TRAF3 diminishes over time. The same effect was seen at even later time-points, when EJ cells had been lyzed in denaturing buffer to preserve modifications, boiled and immediately analyzed by SDS-PAGE (Figure 3.9B).

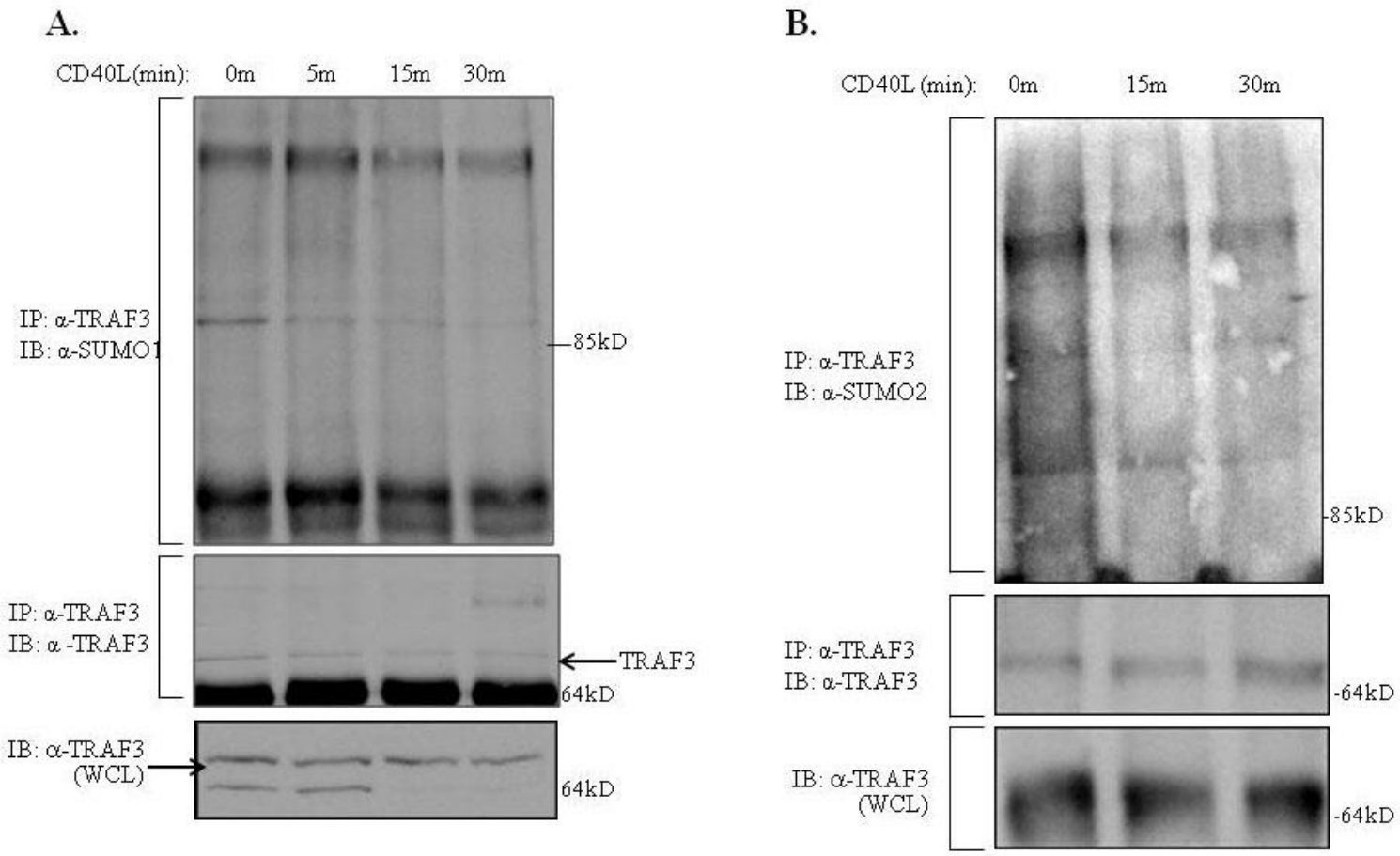


Figure 3.9: CD40 affects SUMOylation.

(A)(B) *EJ cells were treated with CD40L for 5m, 15m or 30m and lyzed in the presence of iodoacetamide (25mM). Lysates were measured with Bradford assay to determine protein content and 4mg of total protein was used per immunoprecipitation reaction. The lysates were immunoprecipitated with α-TRAF3 polyclonal antibody and immunoblotted with α-SUMO-1 or α-SUMO-2. The figures are representative of at least 4 independent experiments.*

(Figure continues overleaf)

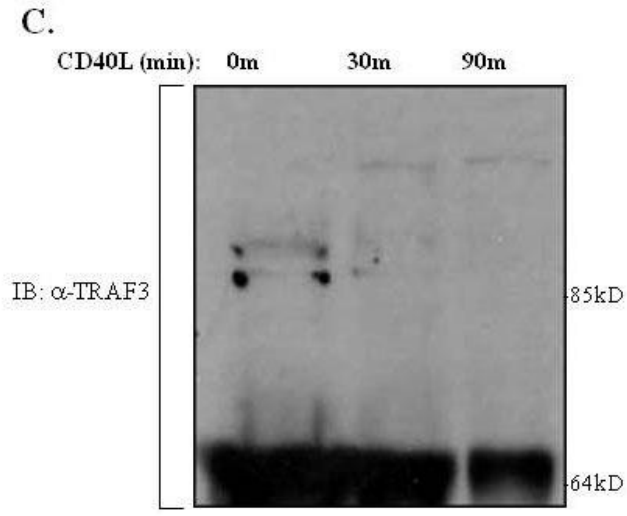


Figure 3.9: CD40 affects SUMOylation (continued)

(C) *EJ cells (4x10^6 per time point) were stimulated with CD40L for 30m or 90m and immediately lyzed in Lysis Buffer (as described before) in the presence of N-ethylmaleimide (25mM). The lysates were sonicated briefly (40sec at medium power), analyzed by SDS-PAGE and immunoblotted with α-TRAF3.*

3.10 Ubc9 knockdown affects the stability of TRAF3 and delays NF-κB2 activation but does not affect other downstream signaling events

TRAF3 degradation contributes to the induction of signaling downstream of CD40 activation. In order to investigate the effect of Ubc9 on CD40-mediated pathways, we performed siRNA studies in EJ cells. Ubc9 was knocked down in EJ cells (bladder carcinoma cell line) which were subsequently stimulated with CD40 ligand. It was determined that TRAF3 degradation is significantly delayed in the cells transfected with Ubc9 compared to those transfected with control siRNA (Figure 3.10A top panel). TRAF2 also follows similar kinetics with TRAF3 (Figure 3.10A, third panel). This further leads to delay in the induction of the alternative NF-κB pathway (Figure 3.10B). Analysis of cIAP2, a known inducible gene of CD40 ligand did not show any differences between the siUbc9 and control transfected cells (Figure 3.10A, second panel). Furthermore, no effect was shown in the phospho-JNK pathway which is induced following TRAF3 degradation after CD40 ligation (Georgopoulos et al., 2006; Matsuzawa et al., 2008; Cheng et al., 2010) (Figure 3.10C). Activation of the canonical NF-κB pathway, as determined by the extent of nuclear translocation of the p65/RelA NF-κB subunit following CD40 activation was similar between Ubc9 and control siRNA-transfected cells (Fig 3.10D)

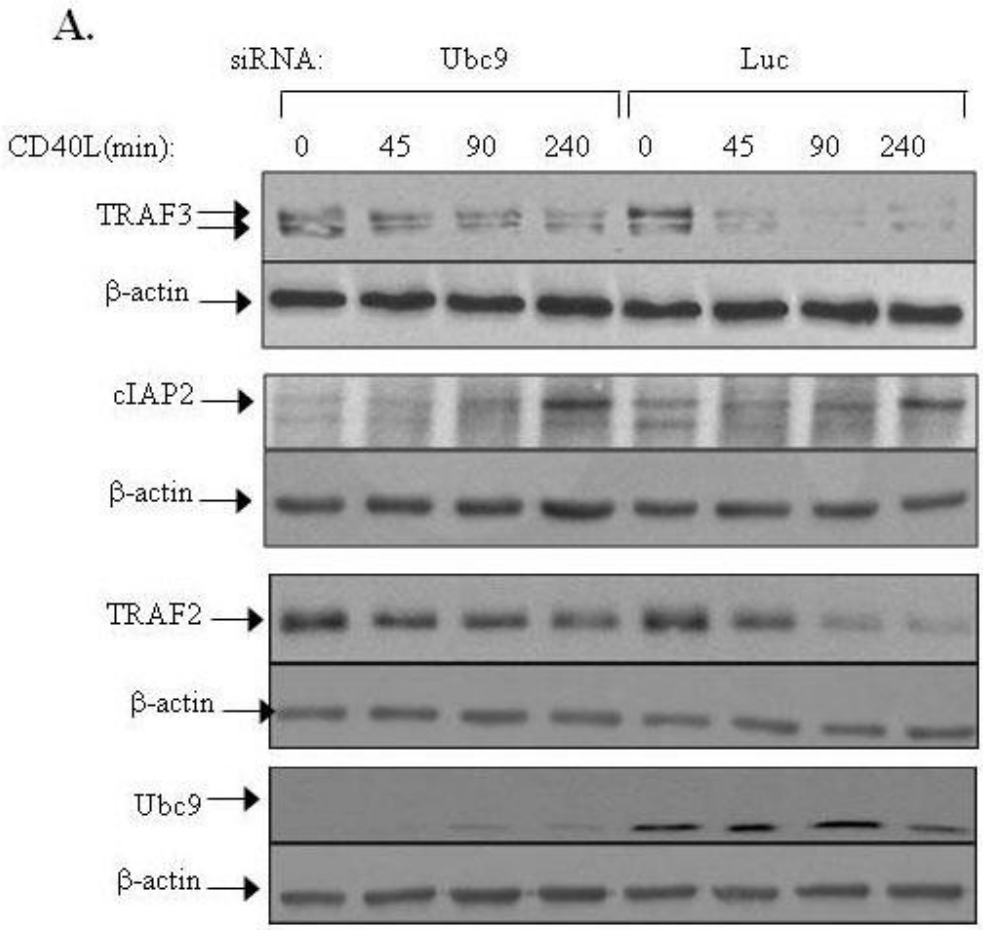
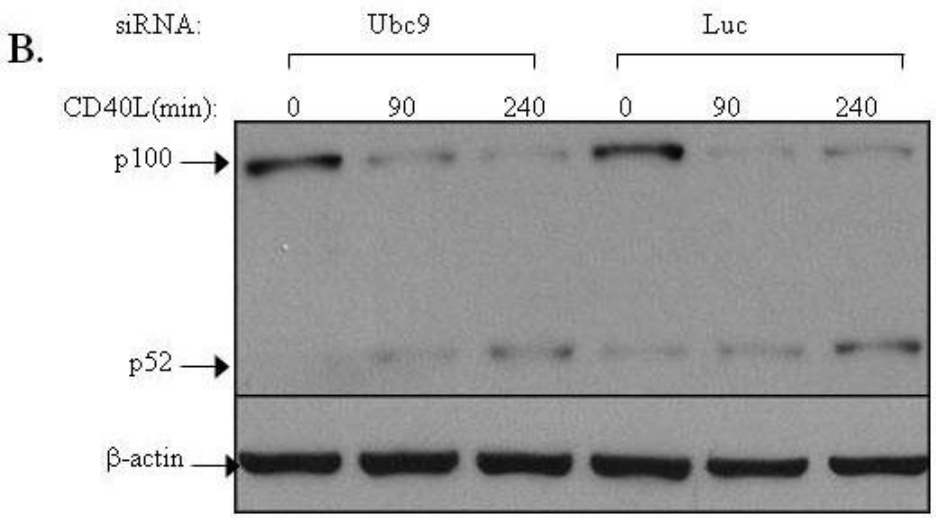


Figure 3.10: Ubc9 knockdown delays CD40-induced TRAF3 degradation and NFκB2 activation but does not affect other downstream events

(A) *EJ cells were transfected with Ubc9 siRNA (5nmol) or an unrelated siRNA (5nmol) targeting luciferase (Luc) prior to stimulation with 0.5 μg/ml CD40L. Lysates (15μg or 20μg) were immunoblotted with α-TRAF3, α-cIAP2 and α-TRAF2. β-actin was used as a loading control. Results are representative of at least 5 independent experiments.*

(Figure continues overleaf)



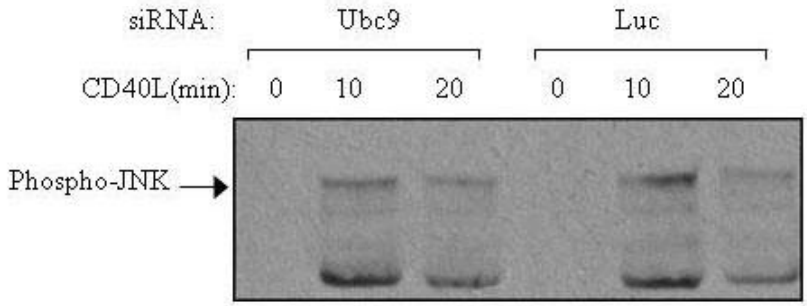


Figure 3.10: Ubc9 knockdown delays CD40-induced TRAF3 degradation and NFκB2 activation but does not affect other downstream events

(B) *EJ cells were transfected with Ubc9 siRNA (5nmol) or an unrelated siRNA (5nmol) targeting luciferase (Luc) prior to stimulation with 0.5 μg/ml CD40L. Lysates (20μg) were immunoblotted with α-NF-κB2 and β-actin was used as a loading control. Results are representative of at least 4 independent experiments.*

(C) *EJ cells were transfected with Ubc9 siRNA (5nmol) or an unrelated siRNA for Luc (5nmol) prior to stimulation with 0.5μg/ml CD40L. Lysates (40μg) were immunoblotted with α-phospho-JNK.*

D.

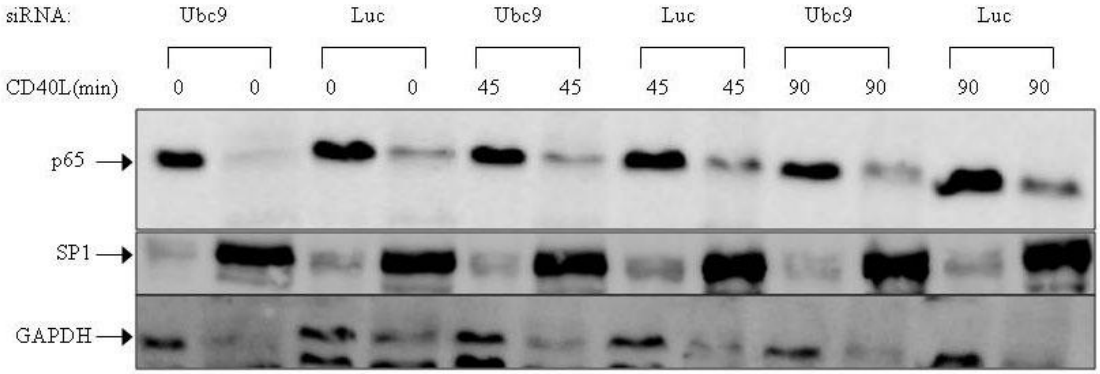
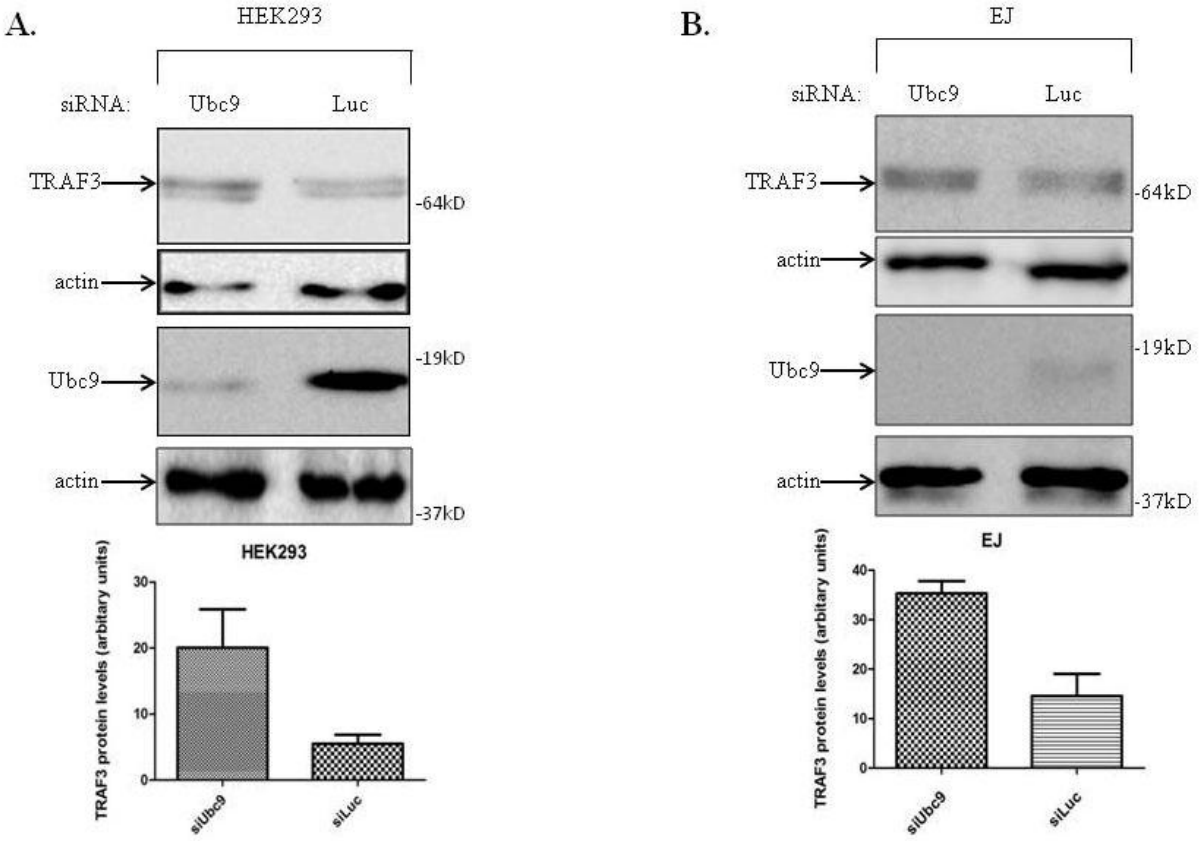


Figure 3.10: Ubc9 knockdown delays CD40-induced TRAF3 degradation and NFκB2 activation but does not affect other downstream events (continued).

(D) *EJ cells were stimulated with CD40L (0.5μg/ml) and separated into nuclear and cytoplasmic fractions according to the method described in Chapter 2. Lysates (20μg) were immunoblotted with a-p65 and SP1 and GAPDH were used as nuclear and cytoplasmic markers/loading controls respectively.*

3.11 SUMOylation of TRAF3 may affect basal TRAF3 turnover

It has already been established that TRAF3 is SUMOylated even in the absence of stimulus. It is therefore possible that SUMOylation of TRAF3 may also affect basal TRAF3 turnover. Using semi-quantitative analysis of TRAF3 levels in HEK293 and EJ cells that had previously been transfected with siRNA targeting Ubc9 or unrelated siRNA Luc, we demonstrated elevated basal levels of TRAF3 in cells transfected with UBc9 siRNA (Figure 3.11).



(A) *HEK293 or EJ cells were transfected with siRNA (5nmol) targeting Ubc9 or an unrelated target (Luc). EJ cells were transfected in two rounds. Lysates were supplemented with 25mM iodoacetamide and immunoblotted (10μg) with α-TRAF3 or Ubc9. B-actin was used as a loading control. Semi-quantitative analysis was carried out using ImageJ (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012).*

3.12 SUMOylation of TRAF3 leads to increased binding to the CD40 receptor

Based on previous results, it is evident that absence of SUMOylation leads to stabilization of TRAF3 following CD40 receptor stimulation. Given that in CD40 induced-pathways, degradation of TRAF3 is important for downstream events, it was necessary to establish whether TRAF3 binding to the receptor is affected by the absence of SUMOylation. To this end, the cytoplasmic tail of CD40 (CT) was produced as fusion protein with GST in bacteria and incubated with lysates from HEK293T cells, in the presence or absence of inhibitor of de-SUMOylation (iodoacetamide). As control, a mutant GST-CD40 fusion protein which carries a $Thr^{254}Ala$ mutation (CTA) was used which abolishes the binding site for TRAF3 and TRAF2 (Figure 3.12A). As is evident from Figure 3.12B, CD40 strongly binds TRAF3 in the presence of iodoacetamide but the association is weaker when no inhibitor is added.

Furthermore, we over-expressed Ubc9 DN or E.V. in HEK293T cells and lyzed them in the presence of iodoacetamide. The lysates were incubated with GST-CD40 or GST-CD40 mutant bound to G-Glutathione beads and analyzed by Western Blotting. The results demonstrated that in the Ubc9 DN-transfected samples the binding of TRAF3 to CD40 is reduced (Figure 3.12C).

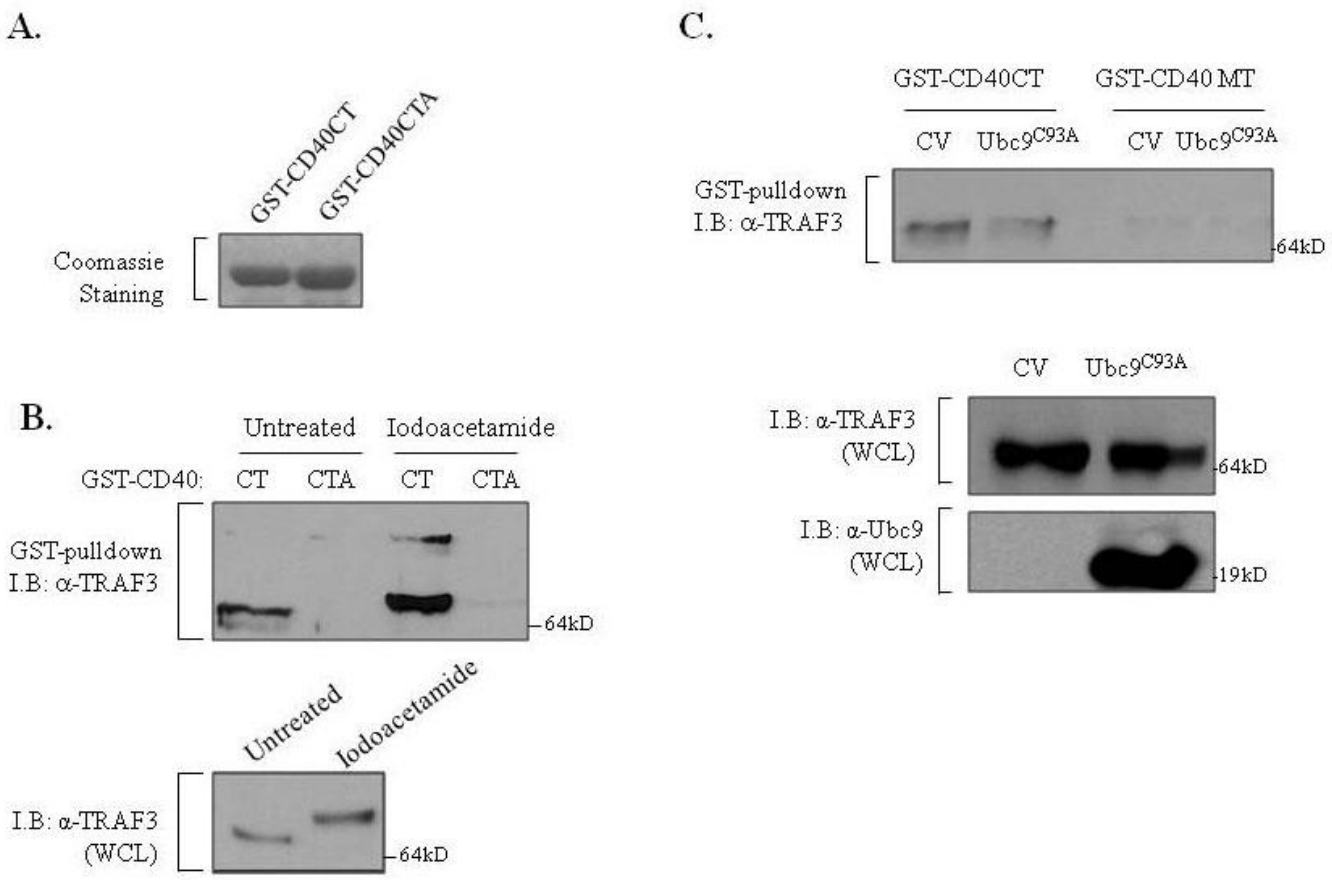


Figure 3.11: SUMOylation of TRAF3 leads to increased binding to the CD40 receptor

(A) *Coomassie-stained gel showing the levels of bacterially produced GST-CD40 C-terminus (CT) and GST-CD40CT carrying a $T^{254}\rightarrow A$ mutation (GST-CD40CTA) that abolishes interaction with TRAF3 and TRAF2.*

(B) *The interaction of TRAF3 with bacterially produced CD40CT increases when SUMO modification is maintained. HEK293 cells were lysed in the presence or absence of Iodoacetamide and lysates were incubated with GST-CD40CT or, as control GST-CD40CTA, bound to glutathione sepharose beads. Interacting proteins were fractionated by SDS-PAGE and immunoblotted with anti-TRAF3 Ab. Whole cell lysates (WCL; 30μg) were analyzed by immunoblot for TRAF3 expression levels.*

(C) *Over-expression of dominant-negative $Ubc9^{C93A}$ reduces binding of TRAF3 to CD40. HEK293 cells were transfected with $Ubc9^{C93A}$ or control vector (CV), lysates were obtained using RIPA lysis buffer supplemented with iodoacetamide and incubated with GST-CD40CT or GST-CD40CTA bound to glutathione sepharose beads. Interacting proteins were fractionated by SDS-PAGE and immunoblotted (I.B.) with anti-TRAF3. Whole cell lysates (WCL; 30μg) were analyzed for TRAF3 and $Ubc9^{C93A}$ expression levels by immunoblotting using anti-TRAF3 and anti-Ubc9, respectively. Results in (B) & (C) are representative of 3 independent experiments.*

3.13 SUMOylation affects TRAF3 heterodimers with TRAF2

TRAF3, TRAF2 and cIAP1-cIAP2 are all involved in rapid NIK turnover in un-stimulated cells and their inactivation or deletion results in NIK accumulation and p100 processing (He et al., 2006; Vince et al., 2007; Keats et al., 2007; Varfolomeev et al., 2007). Analyses of TRAF3 mutants have suggested that TRAF3 binds to NIK and TRAF2, which is associated with cIAPs (Rothe et al., 1995) therefore suggesting that TRAF3 acts as adaptor that links an E3 ligase complex (containing TRAF2 and cIAPs) to NIK in un-stimulated cells. SUMOylation regulates diverse biological processes, such as protein-protein interaction, protein ubiquitination, protein phosphorylation, and gene transcription. In this case, we wanted to examine whether TRAF3 SUMOylation had any effect on TRAF3 protein interactions at steady state. The TRAF2:TRAF3 interaction acts essentially as a molecular bridge that brings together the E3 ligase of the TRAF2:cIAP complex and NIK, targeting NIK for continuous ubiquitination and thus, keeping NF-κB2 in check. By over-expressing the Ubc9 DN mutant or empty vector in HEK293 cells, and performing immunoprecipitaion experiments followed by Western Analysis, we were able to show that the TRAF2:TRAF3 interaction is reduced when TRAF3 is not SUMOylated (Fig 3.13A left upper panel).

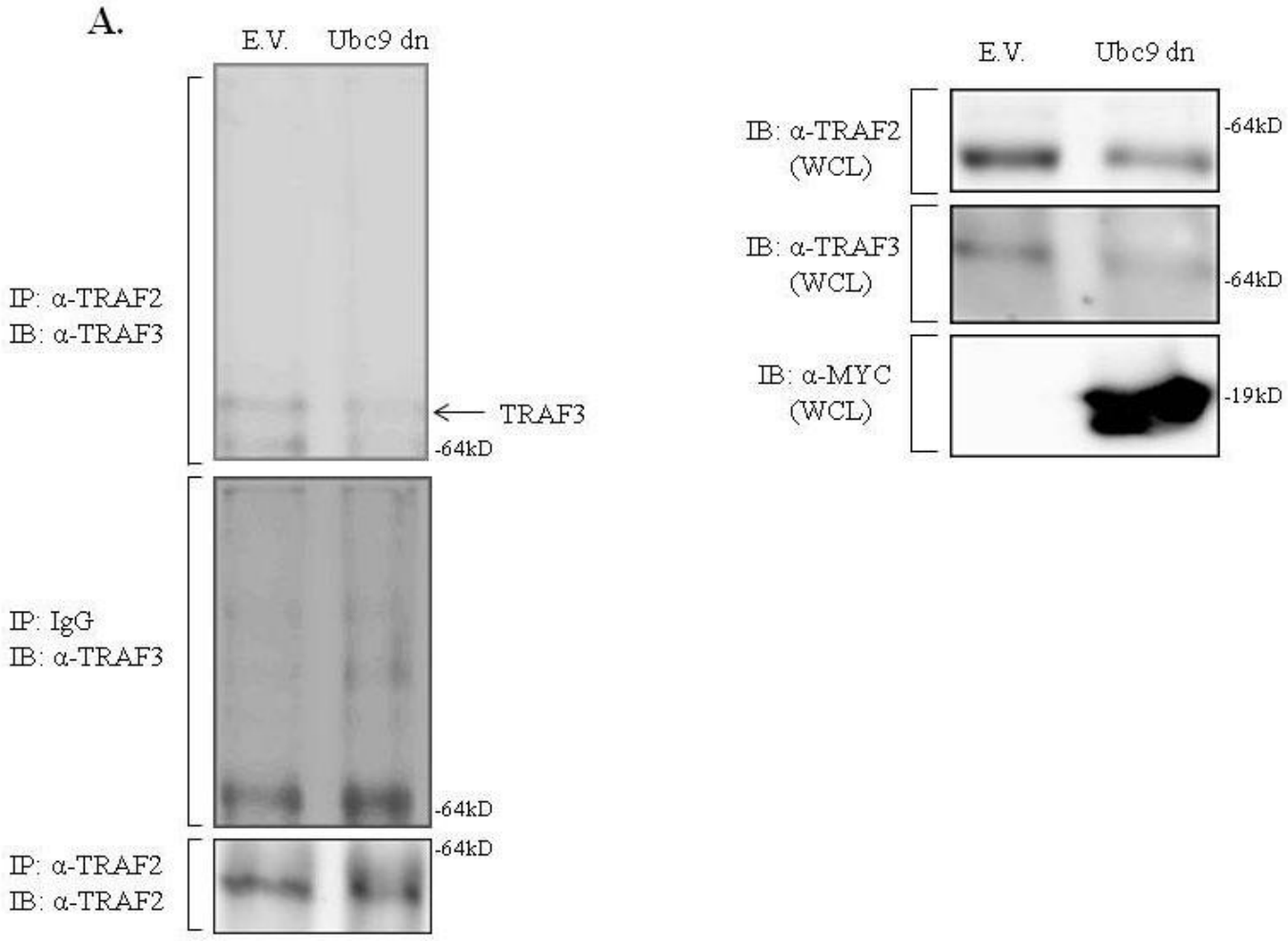


Figure 3.13: SUMOylation of TRAF3 affects the ability of TRAF3 to form heterodimers with TRAF2 at steady state

(A) *HEK293T cells were transfected with Ubc9 dominant negative plasmid or Empty Vector and lyzed in RIPA buffer supplemented with 25mM iodoacetamide. The lysates were immunoprecipitated with α-TRAF2 and immunoblotted with α-TRAF3 or α-TRAF2 as control. The total amount of protein per IP reaction was 2mg. Whole cell lysates (WCL: 30μg) were analyzed for TRAF3, TRAF2 and Ubc9^{C93A} expression levels by immunoblotting using anti-TRAF3, TRAF2 and anti-MYC, respectively. Results are representative of 3 independent experiments.*

3.14 TRAF2 may have a role in TRAF3 SUMOylation

Although ligases that have dual functionality in both the SUMO and the Ubiquitin system (Rajendra et al., 2004; Weger et al., 2005; Pungaliya et al., 2007) are reported but not extensively studied, a case of a large multi-subunit complex with both SUMO and potential Ubiquitin ligase activity has been described (Zhao and Blobel, 2005). In line with this, TRAF2 and TRAF3 interact directly with each other as part of a large multi-subunit complex and even though both proteins share structural similarities, only TRAF2 is known to possess Ubiquitin E3 ligase properties (Tewari and Dixit, 1996; Ely et al., 2007). TRAF2 can therefore be considered a good candidate to be the SUMO E3 ligase in TRAF3 SUMOylation with potent implication in the regulation of the alternative NF-kB pathway, where both proteins play a pivotal role (Vince et al., 2009).

To test this hypothesis, we over-expressed TRAF2, TRAF2 DN (N-terminal deleted obtained from Dr Elliot Kieff) or E.V in HEK293 cells. Endogenous TRAF3 was immunoprecipitated from cell lysates and immunoblotted with α-SUMO-1. Results (Figure 3.14A) demonstrate that although bands corresponding to TRAF3-SUMO are readily detected in all three cases, their intensity is markedly increased in the presence of TRAF2. When TRAF2 is targeted by siRNA and immunoprecipitation of the endogenous TRAF3 is performed, followed by immunoblot by α-SUMO-1, we observe that SUMOylation of TRAF3 is reduced (Fig 3.14B). Furthermore, TRAF2 itself is SUMOylated (Figure 3.14C), another indication that it could act as an E3 SUMO ligase as often the enzymes involved in the SUMO pathway are subject to auto-SUMOylation.

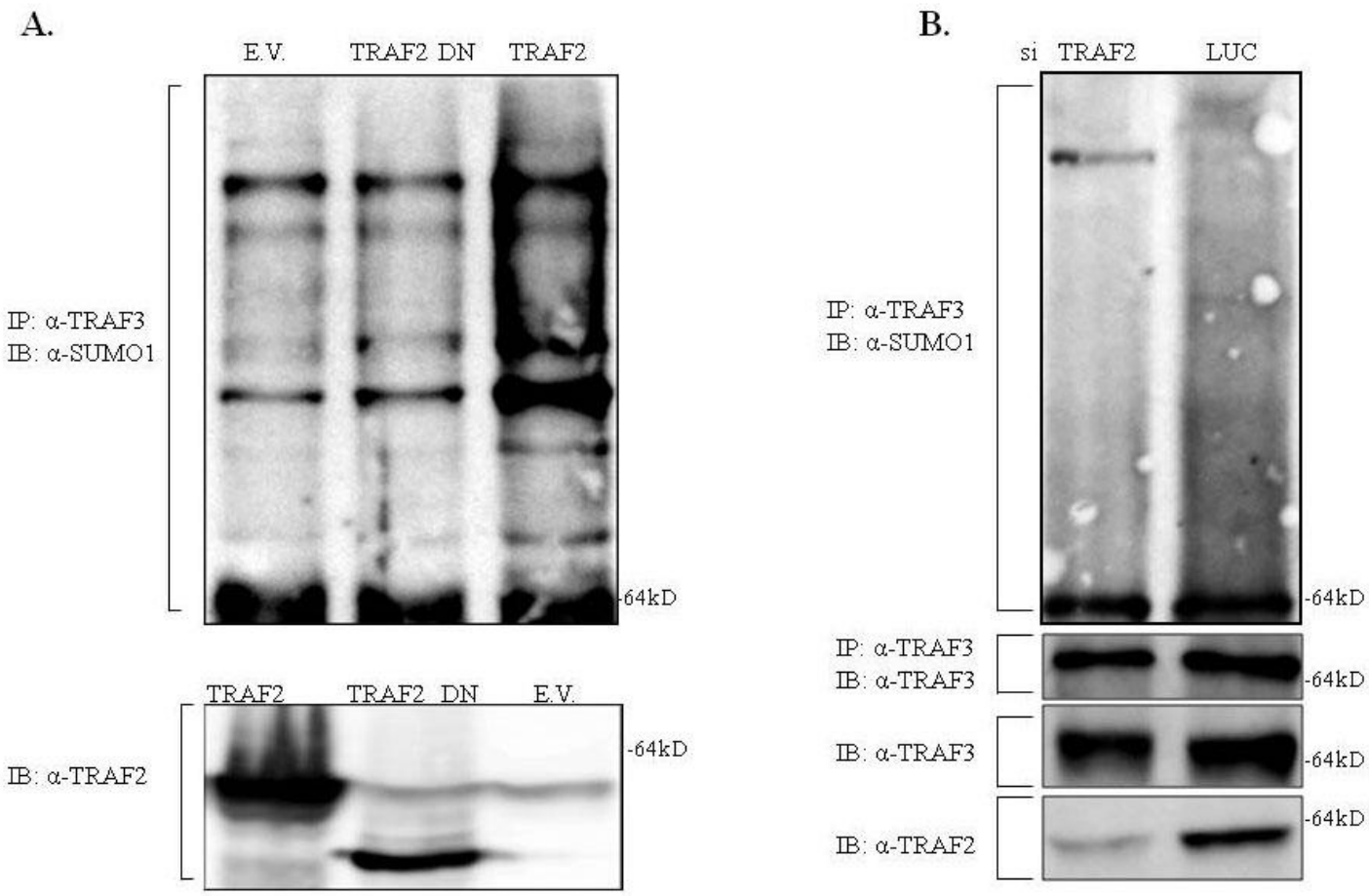


Figure 3.14 TRAF2 has a role in TRAF3 SUMOylation.

(A) *HEK293 cells were transfected with Empty Vector, TRAF2 dominant negative plasmid, or TRAF2 wt and lyzed in RIPA buffer supplemented with 25mM N-ethymaleimide. The lysates were immunoprecipitated with α-TRAF3 and immunoblotted with α-SUMO-1. The total amount of protein per IP reaction was 2mg. Whole cell lysates (WCL: 30μg) were analyzed for TRAF2 expression levels by immunoblotting using anti-TRAF2 polyclonal antibody.*

(B) *EJ cells were transfected with TRAF2 siRNA (5nmol) or an unrelated siRNA (5nmol) targeting luciferase (Luc) in 2 rounds of transfection. Lysates were immunoprecipitated in the presence of iodoacetamide with α-TRAF3 and immunoblotted with α-SUMO-1, or TRAF3. Whole cell lysates (30μg) were analyzed for TRAF2 and TRAF3 expression levels by immunoblotting using α-TRAF2 or TRAF3 polyclonal antibodies.*

(Figure continues overleaf)

C.

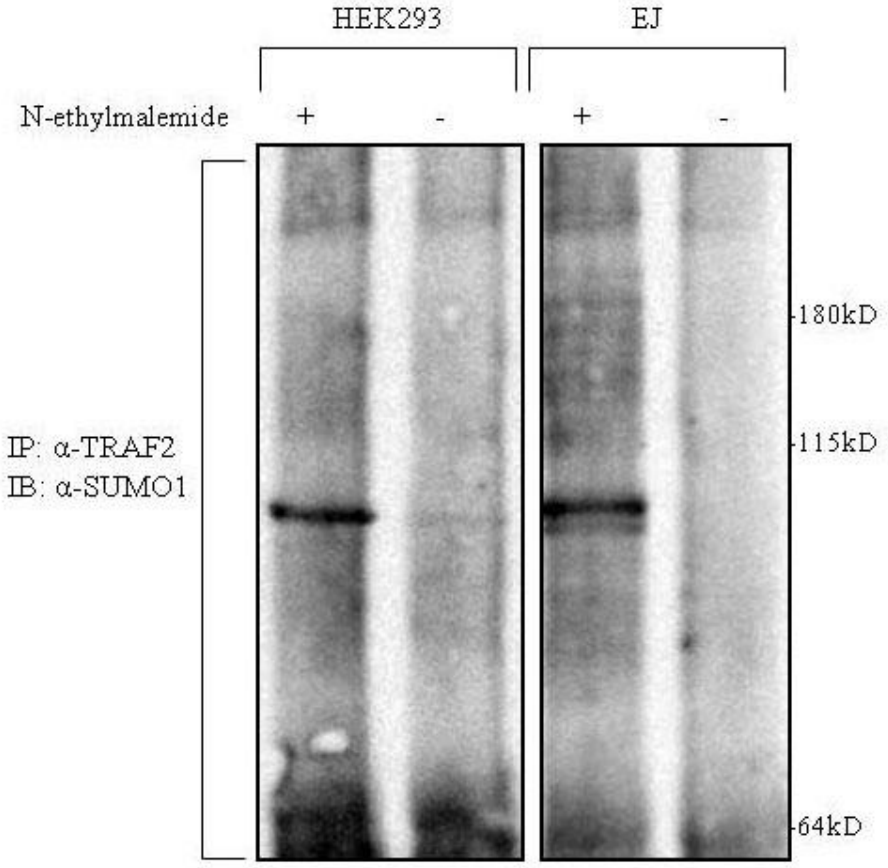


Figure 3.14 TRAF2 has a role in TRAF3 SUMOylation. (continued)

(C) *Lysates from HEK293 or EJ cells were immunoprecipitated in the presence or absence of N-ethylmaleimide (25mM) using α-TRAF2 polyclonal antibody and immunoblotted using α-SUMO-1. The total amount of protein per IP reaction was 4mg.*

Chapter 5: Discussion

5.1 Discussion of data

In this study we report the identification of a novel interaction between the TNF-associated-factor 3 (TRAF3) and Ubc9, the sole identified E2 enzyme in the SUMO pathway. This interaction, originally identified by yeast two-hybrid (Figure 3.2A), was further validated utilizing *in vitro* GST-pull-downs (Figure 3.2B) and co-immunoprecipitation studies with over-expressed proteins in HEK293 cells. TRAF3, similar to other TRAF family members, contains a conserved TRAF domain which is further subdivided into TRAF-C and TRAF-N domain. By generating four deletion mutants (Figure 3.3A) we were able to identify that the domain contributing to Ubc9 binding is the TRAF-N domain (Figure 3.3C). Past studies have shown that the TRAF-N domain of TRAF3 is important for TRAF3 self association and efficient recruitment to receptors (Force et al., 1997) but most importantly, for association with other proteins (TRAF-interacting partners) (Ling and Goeddel, 2000) as is the case here. As the association is not comparable between the wild-type TRAF3 and the deletion mutants we can further deduce that efficient association between TRAF3 and Ubc9 is probably dependent on conformation.

The aforementioned observations indicated that TRAF3 could be a putative SUMO target. Although at least four isoforms of SUMO are present in humans only three of these (SUMO-1, SUMO-2 and SUMO-3) can be processed *in vivo* to bear the C-terminal di-glycine motif required for post-translational conjugation. SUMO-2 and SUMO-3 are nearly identical and are assumed to be largely redundant in their functions (Praefcke et al., 2012). Initially, *in silico* analysis suggested that TRAF3 has several lysines that could be potential SUMO targets (Figure 3.4A). The modified lysines are often part of a (I/V/L)Kx(D/E) motif which provides direct binding site for Ubc9 (Johnson, 2004; Hay, 2005) although, some examples of SUMO-modified proteins were found to have sites that did not match the consensus motif, suggesting alternative sequence features could specify a particular lysine for SUMO modification (Kamitani et al., 1998; Rangasamy et al., 2000; Hoege et al., 2002; Castillo-Lluva et al., 2010). For TRAF3 at least eight lysines are within the indicated motif but several others exist in the protein which cannot

be excluded as possible targets. Indeed, the high number of lysines in TRAF3 renders it difficult to identify the sites not only for SUMOylation but also for ubiquitination as has been evident for the literature so far (Matsuzawa et al., 2008; Vallabhapurapu et al., 2008; Razani et al., 2010).

In vivo experiments, in which a number of cell lines were processed in the presence or absence of N-ethylmaleimide, an alkylating agent that inhibits the action of de-SUMOylating enzymes, showed that TRAF3 is modified by both SUMO-1 and SUMO-2/3 at steady state. This was evident by the presence of lower motility bands that corresponded to modified TRAF3 (Figure 3.4B). When using HeLa cells, stably expressing SUMO-1 or SUMO-2 or EJ cells transiently transfected with His-SUMO-1 or SUMO-2 that were lyzed in denaturing conditions and subjected to enrichment of SUMOylated proteins in NTA-columns, we were able to identify TRAF3 SUMO-conjugates. Similar results were obtained when we analyzed mouse splenocytes which suggests that TRAF3-SUMO modification is not phenomenon restricted to transformed cells. These results combined are indicative of TRAF3 being poly-SUMOylated and bearing large poly-SUMO-chains consisting of SUMO-2/3 molecules as SUMO-2/3 have the ability to such chains by covalently binding to themselves via the lysine residue at the N-terminus motif ψKxE (Ulrich, 2009). SUMO-1 moieties only act as chain-terminators, since they lack the intrinsic motif that is recognizable by other SUMO moieties and they are themselves poor SUMOylation substrates (Matic et al., 2008). The existence of SUMO-1 modification on TRAF3 is probably due to SUMO-1 'capping' and terminating the SUMO-2/3 chains. The observation that TRAF3 is poly-SUMOylated combined with the fact that the SUMOylation state of target proteins is not static but instead reflects a dynamic equilibrium between the forward process of SUMO and its removal by cellular deSUMOylating enzymes, may further explain the variability of SUMO-conjugates observed across the experiments performed in this thesis.

Conversely, TRAF3 SUMOylation was not detected in *in vitro* SUMOylation assays under any of the conditions tested (Figure 3.4E). The absence of an E3 enzyme was considered as a factor although E3s are not routinely included in this assay. This is due to

the fact that in the SUMO pathway, E3s are often dispensable, although in some instances they promote specificity or enhance conjugation by stimulating the ability of Ubc9 to discharge SUMO to substrates (Wilkinson and Henley, 2010; Gareau and Lima, 2010). The *in vitro* SUMOylation assay was repeated with the addition of either PIAS3 or PIAS4, members of the well-characterized families of SUMO E3 ligases (Hochstrasser, 2001). Nonetheless, once more no SUMOylated TRAF3 was detected for either SUMO-1 or SUMO-2 (Figure 3.4F) and therefore concluded that PIAS could not be the E3 ligases responsible for TRAF3-SUMO modification. A number of interesting questions were raised, as it is quite common for potential SUMO targets to be easily SUMOylated *in vitro* but not *in vivo* whereas the opposite is not. To explain the conundrum, one must take into account that the *in vitro* conditions utilized, are far from the physiological *in vivo* setting. Thus, one can argue that aspects such as protein folding and conformation are important in order to be recognized by SUMO or indeed Ubc9. It could also indicate that TRAF3 can only be SUMOylated as part of a larger protein complex or the quaternary structure of the protein is conducive to post-translational modifications. The former of these stipulations will be further discussed below. Furthermore, the required E3 enzyme in this case could either be a yet-to-be un-identified protein or one of the various TRAF3 binding partners.

To establish a functional link between TRAF3 SUMOylation and Ubc9 we over-expressed a dominant negative form of Ubc9, which carries a $Cys^{93}Ser$ mutation that renders the mutant unable to bind SUMO moieties (Tashiro et al., 1997), in HEK293 cells. Bands corresponding to SUMOylated forms of TRAF3 were readily detected in the empty-vector transfected lysates but were significantly reduced in the Ubc9 $Cys^{93}Ser$ transfected lysates (Figure 3.5A). Similar results were observed when Ubc9 was knocked down (Figure 3.5B). These observations further verify the importance of Ubc9 in the SUMO pathway but also underline the functional significance of the TRAF3:Ubc9 interaction.

SENP1, is a member of the SENP family of SUMO proteases and, similarly to SENP2, can process and deconjugate both SUMO-1 and SUMO-2 (Gong and Yeh, 2006; Hang

and Dasso, 2002; Zhang et al., 2002) but to date, it has not been implicated in the deconjugation of other ubiquitin-like modifiers (Kim and Baek, 2009). Its localization is mainly in the cytoplasm (Table 1.2) and as TRAF3 is mainly cytoplasmic, it is a good candidate to examine in the context of TRAF3-SUMO de-conjugation. Following purification through Nickel-columns and treatment with purified SENP1, the bands corresponding to SUMO-conjugated TRAF3 were relatively reduced whereas the amount of un-modified substrate increased in the case of SENP1-treated samples (Figure 6A). Un-conjugated TRAF3 was also detected in the supernatant of the protease buffer although only at very high exposure.

The efficiency of the purified SENP1, was not thorough as can been seen from Figure 6B, where the immunoblot demonstrates the amount of free versus conjugated His-SUMO-1 and -2. During this protocol, the proteins were denatured during the lysis step and then re-natured in subsequent wash-steps. As the procedure can never be 100% efficient, it is possible that SENP1 cannot properly de-conjugate all SUMO from the substrates. However, taken together, these findings further validate the hypothesis that the upper TRAF3 bands observed are SUMO-related modifications.

TRAF3 is an important component in the negative regulation of CD40-activated alternative NF-κB signaling (Liao et al., 2004). In the absence of stimulus, TRAF3 regulates the levels of NIK by constantly targeting it for degradation through the proteasome (Liao et al., 2004) (Figure 1.4a). This activity depends on cIAP-mediated ubiquitination (which is orchestrated by TRAF2) and results in low levels of NIK which prevents p100 processing in unstimulated cells. Upon CD40 engagement, TRAF3 is targeted for degradation through K48 ubiquitination by the cIAPs/TRAF2/Ubc13 complex which allows NIK to accumulate and p100 to be processed to p52 (Xiao et al., 2001; Xie et al., 2007; Gardam et al., 2008) (Figure 1.4b). TRAF3 SUMOylation may represent a new level of regulation in the CD40 pathway as surmised by the present findings. Upon CD40L stimulation, the interaction between Ubc9 and TRAF3 is reduced within 30 minutes of CD40 receptor engagement which follows the kinetics of TRAF3 degradation (Figure 8, panel 3). Combined with the observation that immunoprecipitated

TRAF3 from EJ lysates that had been stimulated with CD40L and immunoblotted with α-SUMO-1 or α-SUMO-2, exhibited a decrease in the TRAF3-SUMO conjugates, one can postulate that the TRAF3:Ubc9 interaction deteriorates upon TRAF3 engagement to the CD40 receptor (Figure 3.9). These results suggest that SUMO-modified TRAF3 is targeted for degradation following CD40 signaling.

When targeting Ubc9 with siRNA, we observed that TRAF3 but also TRAF2 degradation kinetics are delayed after stimulation with CD40 ligand compared to the control siRNA-transfected samples. cIAP2, a CD40-inducible gene shows no significant difference between Ubc9 and control siRNA-transfected samples. Given these results, it is possible that a crosstalk between SUMOylation and ubiquitination of TRAF3 (and possibly TRAF2) exists, a notion which has been described in the literature already for other proteins (Denuc and Marfany, 2010; Praefcke et al., 2012; Jackson and Durocher, 2013). In fact, in addition to being structurally related, SUMO and Ubiquitin share a number of functional inter-relations such as the targeting of the same attachment sites in certain substrates or SUMO-dependent ubiquitination in others (Praefcke et al., 2012). The recent discovery of SUMO-targeted Ubiquitin ligases (ULSs E3s) has underlined a novel interplay between the SUMO and the Ubiquitin system: ULS-mediated ubiquitination of SUMOylated proteins and eventual degradation by the proteasome (Uzunova et al., 2007; Tatham et al., 2008). Data in the present study, provide evidence of an association between SUMOylation and Ubiquitination by demonstrating that Ubc9 knockdown significantly reduces CD40 mediated TRAF3 degradation and NF-κB2 processing (Figure 3.10A & B). As the target lysine residues for both ubiquitination and SUMOylation of TRAF3 remain unknown, further studies are required to elucidate whether absence of TRAF3 SUMOylation stabilizes the protein by affecting proteasomal recognition.

The observed delay in the p100 processing after Ubc9 knockdown is entirely expected and in line with the existing literature, given the negative-regulatory role of TRAF3 and TRAF2 in the CD40-induced NF-κB2 pathway. In fact, the Ubc9:SUMO:TRAF3 interaction/modification may be conducive to the previously-reported p100

SUMOylation. It appears that basal SUMOylation of p100 is required for its phosphorylation both *in vivo* and *in vitro* (Vatsyayan et al., 2008) and since protein SUMOylation may create binding sites for protein-protein interactions it would be intriguing to examine whether TRAF3 SUMOylation facilitates a possible transfer of SUMO onto p100 upon TRAF3 degradation.

The physiological functions of CD40 can be mediated by various signal transducers including JNK, p38 and ERK. It has been shown that the TRAF molecules required for CD40 signaling have separate, non-redundant functions. Specifically, TRAF2 has been shown to be the primary mediator of JNK and p38 activation (Lee et al., 1997; Yeh et al., 1997; Xie et al., 2006; Hostager et al., 2003). Since TRAF2 exhibits the same delayed kinetics as TRAF3 after Ubc9 knockdown (Figure 3.10A, third panel) and it is also SUMOylated (Figure 3.14C) we examined whether the downstream events are affected. However, no effect was noted on JNK (Figure 3.10B, bottom panel). Similarly, no significant effect is observed for the canonical NFκB pathway as demonstrated by p65/RelB migration to the nucleus. On the basis of these observations, one can hypothesize that the TRAF-SUMO modification exerts its role early in the signaling cascade and becomes redundant, leaving downstream events impervious to its effects.

In fact, the present findings demonstrate that SUMO modification of TRAF3 impact on its capacity to bind CD40. More specifically, as seen on Figure 3.12A & B, SUMOylated TRAF3 can bind the CD40 receptor more efficiently. Based on these observations, it is suggested that the SUMO-modified TRAF3 may represent a separate TRAF3 pool which is primed for CD40 binding and stimulus-dependent ubiquitination and subsequent proteasomal degradation. SUMOylation may affect how TRAF3 interacts with other proteins; our observations support that SUMO-modified TRAF3 forms stronger heterodimers with TRAF2, thus allowing NIK ubiquitination and degradation to take place by acting as a molecular platform that brings the cIAPs-TRAF2 and NIK together (Figure 3.13) (Zarnegar et al., 2008; Vallabhapurapu et al., 2008).

TRAF2 may indeed have a more important role in this: it could act as an E3 SUMO ligase in TRAF3 SUMOylation. It has already been characterized as a ubiquitin ligase although this has not been extensively analyzed. Our results, demonstrate that over-expression of wild-type TRAF2 has a positive effect on TRAF3 SUMOylation (Figure 3.14A) and that abrogation of TRAF2 by siRNA leads to attenuation of TRAF3 SUMOylation (Figure 3.14B). These findings, coupled with the fact that TRAF2 is SUMOylated itself, a characteristic of enzymes participating in the SUMO pathway, provide theoretical support for TRAF2 acting as a TRAF3 E3 SUMO ligase. Nonetheless, further experiments would be needed to validate this hypothesis.

5.2 Future prospects

The present study has revealed the novel interaction between TRAF3 and the E2 enzyme Ubc9. These proteins were shown to interact *in vivo* and *in vitro* in a number of cell lines. It has been demonstrated that TRAF3 SUMOylation is important, to some degree, for the stability of the protein and affects the way TRAF3 binds to the CD40 receptor and forms heterodimers with TRAF2 in the absence of stimulus.

The most important question that remains to be answered is the modification site(s) of SUMO on TRAF3. The results shown here imply that more than one site for SUMO binding exist on TRAF3, as there are multiple bands visible in the SUMO blots (Fig 3.4). Direct detection is possible with mass spectrometry (MS) by the identification of the SUMO–SUMO branched peptide remnant after proteolytic digestion. Alternatively, site-directed mutagenesis of all possible target lysines and their combinations within TRAF3 should be carried out as indicated by SUMOplot (Figure 3.4A) and other bioinformatics tools. However, while the indicated consensus motif has proven valuable for predicting potential SUMOylated lysines to characterize by site-directed mutagenesis, the mutagenic approach is more problematic in cases such as TRAF3 where substrates have multiple possible SUMOylation sites. Furthermore, it may be difficult in mutational studies to distinguish between lysines that are actual sites for SUMOylation and lysines whose

mutation simply changes the substrate and results in loss of SUMOylation at a distal lysine.

The role of TRAF2 in this situation is worth expanding upon. Results from this study have hinted a role for TRAF2 in TRAF3 SUMOylation, possibly as an E3 enzyme. TRAF2 is SUMOylated itself (Figure 3.14C), something that is often the case with E3 SUMO enzymes. *In vitro* SUMOylation of TRAF3 with purified TRAF2 as an E3 ligase could demonstrate a potential role for TRAF2 . However, due to the limitations of the *in vitro* approach in this particular case, that have already been discussed, an *in vivo* approach utilizing TRAF2 silencing with siRNA may be more efficient.

Finally, TRAF3 SUMOylation must be examined under different stimuli to examine the global nature of the phenomenon. As has already been discussed in the Introduction Chapter, TRAF3 has been described to have an important role in a variety of signaling cascades such as TLR, LMP1 and LTβ signaling. SUMOylation may play an important role in the outcome of these cascades and warrants further investigation.

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