Cellular and Genetic Etiology, Diagnosis and Treatment of Human Disease

Characterization of novel proteins interacting with TRAF3

Master Thesis

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ΚΥΤΤΑΡΙΚΗ ΚΑΙ ΓΕΝΕΤΙΚΗ ΑΙΤΙΟΛΟΓΙΑ, ΔΙΑΓΝΩΣΤΙΚΗ ΚΑΙ ΘΕΡΑΠΕΥΤΙΚΗ ΤΩΝ ΑΣΘΕΝΕΙΩΝ ΤΟΥ ΑΝΘΡΩΠΟΥ

Χαρακτηρισμός νέων πρωτεϊνών που αλληλεπιδρούν με την TRAF3

Master Thesis

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'The aim of Science is not to open the door to infinite knowledge but to set a limit for infinite error'

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My endless love and gratitude to my family and friends for always being there, for putting up with me and giving me the support and courage I need to continue. And of course, Nikolas thanks for making each day better. This one is for you.

Η παρούσα μελέτη εστιάζει στον χαρακτηρισμό νέων πρωτεϊνών που αλληλεπιδρούν με την πρωτεΐνη TRAF3. Συγκεκριμένα η TRAF3 αλλά όχι οι άλλες TRAFς αλληλεπιδρά με την πρωτεϊνική κινάση PKN1 παρουσία της ογκοπρωτεΐνης LMP1, υποδεικνύοντας ένα καινούριο ρόλο για την TRAF3, καθότι και οι δύο αυτές πρωτεΐνες εμπλέκονται στην αναδιοργάνωση του κυτταρικού σκελετού, ένα φαινόμενο το οποίο είναι απαραίτητο για πολλές κυτταρικές λειτουργίες. Με τη κατασκευή μεταλλαγμένων πρωτεϊνών αναγνωρίστηκε η περιοχή υπεύθυνη για την πρωτεϊνική αλληλεπίδραση (TRAF domain).

Μια άλλη πρωτεΐνη που βρέθηκε να αλληλεπιδρά με την TRAF3 μέσω του TRAF domain είναι το ένζυμο Ubc9. Είναι μια πρωτεΐνη απαραίτητη για πολλά κυτταρικά μονοπάτια και στην παρούσα μελέτη υποθέτουμε ότι μαζί με την TRAF3 μπορεί να εμπλέκεται στο μη-κανονικό μονοπάτι σήμανσης του NFκB (non canonical NFκB pathway).

Περαιτέρω μελέτες είναι απαραίτητες για να αναδειχθεί ο ρόλος της TRAF3 σε αυτά τα νέα σηματοδοτικά μονοπάτια.

Abbreviations used

TNF- Tumor Necrosis Factor

TNFR- TNF Family Receptors

NFκB- Nuclear Factor κB

LMP1- Latent Membrane Protein 1

TLR- Toll-like Receptor

Ubc9- Ubiquitin-Conjugating enzyme 9

ATF2- Activating Transcription Factor 2

PKN1- Protein Kinase 1

NIK- NFκB Inducing Kinase

Tab tents

1. Introduction

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). 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. Although a few TNFRs (TNFRs, Fas) signal cell death through the caspase pathway by using their death domains, most recruit a family of intracellular adapter molecules to promote cell survival by initiating complex kinase cascades and ultimately members of the NFκB and the AP-1 family. In this way, several genes are activated that promote cell proliferation, differentiation and apoptosis (reviewed by Lee & Lee, 2002).

1.1. Identification of the TRAF family of proteins

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). Subsequent studies have shown that 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* (reviewed by Chung *et al*, 2002).

The mammalian TRAFs have emerged as major signal transducers for the TNF receptor superfamily and the interleukin-1 receptor/Toll-like receptor (IL-1R/TLR)

superfamily. A wide range of biological functions such as adaptive and innate immunity, stress response and bone metabolism are mediated by TRAFs through the induction of cell survival, proliferation, differentiation and death. TRAFs are also involved in the signal transduction of the Epstein-Barr virus transforming protein LMP-1 (Mosialos *et al*, 1995).

To date, seven TRAF proteins have been described. Mammalian TRAF1 and TRAF2 were found to associate with TNFRII by Rothe and colleagues (1994); TRAF3 was described independently, as a cytoplasmic factor that interacts with CD40 and the Epstein-Barr virus transforming protein LMP1 (Cheng *et al*, 1995; Mosialos *et al*, 1995); TRAF4 was identified by its over-expression in breast carcinoma cells (Regnier *et al*, 1995);TRAF5 by its interaction with CD40 and LTβR (Ishida *et al*, 1996; 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*, 1996). TRAF7 was described recently and 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).

1.2. Structure of TRAF proteins

TRAF proteins have been characterized on the basis of a conserved domain (TRAF domain) at the C-terminus which has been shown to be required for the binding of TRAF proteins to their associated receptors. The TRAF domain has been divided into two subregions (Rothe *et al*, 1994). The carboxy-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 1). The TRAF domain has an important role in TRAF function

as is responsible for self- association as well as for the upstream, direct or indirect interactions with their cognate surface receptors and other signaling molecules (Takeuchi *et al*, 1996).

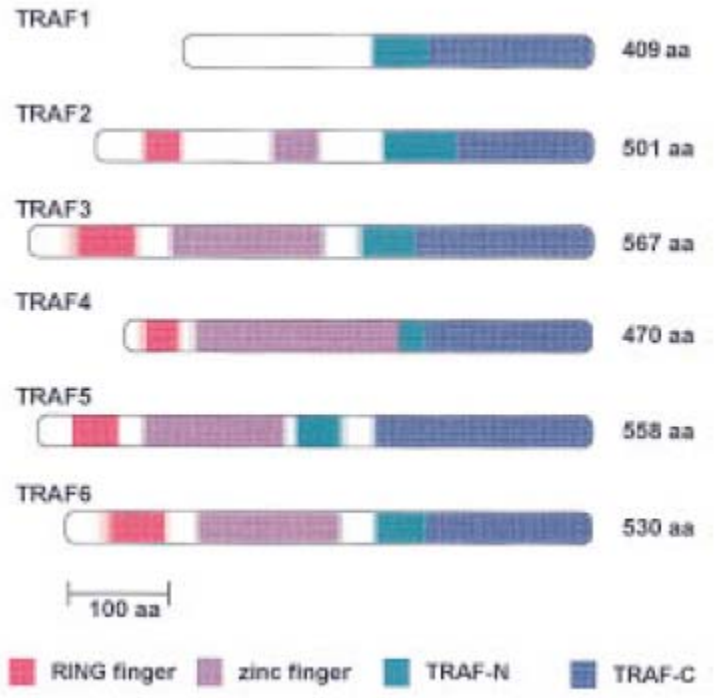

Figure 1: Domain structure of the TRAF protein family. The TRAF domain comprises the coiled-coil domain (TRAF-N) and the highly conserved TRAF-C domain.

The structural differences among the TRAFs influence the range of receptors, heterodimerization partners, adapter molecules and signal transducers that each TRAF interacts with (Kaufman *et al*, 1999). All of the 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).

1.3. Specific biological functions of the mammalian TRAF proteins

As it has already be 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 *et al*, 1998). Extensive studies have shown that the specific biological function of each TRAF protein is not necessary 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).

This study focuses primarily on TRAF3 however the roles of the rest of the TRAF proteins are mentioned here and summarized in Table 1. More specifically, TRAF2 is found in most tissues and therefore is considered 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 of severe runting and are more sensitive to TNF-induced death (Yeh *et al*, 1997). TRAF2 is also important in NFκB activation but this role may be partially compensated by TRAF5 (Nakano *et al*, 2000). 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).

TRAFs	Implicated functions
TRAF1	Apoptotic protection Feedback regulation of receptor signaling
TRAF2	Anti-apoptotic signaling JNK activation Perinatal survival
TRAF3	T-cell-dependent antigen response Perinatal survival
TRAF4	Tracheal formation
TRAF5	CD27 and CD40 signaling
TRAF2 and 5	NF-κB activation
TRAF6	Bone metabolism CD40 signaling IL-1 signaling LPS signaling Perinatal survival
TRAF7	AP-1 & CHOP activation c-Myb regulation

Table 1: Summary of TRAF proteins' functions

TRAF5 is considered to be a close functional and structural homologue of TRAF2 and over-expression of TRAF5 can also activate NFκB and AP-1 transcription factors (Ishida *et al*, 1996; 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. TRAF6 possesses a unique receptor binding specificity which explains its crucial role as a signaling mediator for both the TNF receptor and the IL-1R/TLR superfamily. Lack of TRAF6 leads to defective signaling by IL-1 and IL-18, as well as hyporesponsiveness to bacteria liposaccharides (LPS) which signals through TLR4. These observations place TRAF6 as an important player in innate immunity against pathogens (Lomaga *et al*, 1999; Naito *et al*, 1999). In epithelial cells and fibroblasts, TRAF6 is critical for CD40-mediated signal transduction on the p38 and JNK MAPK, NFκB and the PI3-kinase/Akt axis (Davies et al 2005). TRAF7 has been found to specifically interact with MEKK3 and potentiated MEKK3-mediated AP-1 and CHOP activation. It is also found to be a negative regulator of c-Myb, sequestering it to the

cytoplasm via sumoylation (Xu *et al*, 2004; Morita *et al*, 2005). However, as it lacks the C-terminal domain, conserved in TRAF1-6, further insights are needed to elucidate the way TRAF7 interacts with members of the TNF family.

1.4. TRAF3- a protein involved in many pathways

TRAF3 was the first identified TRAF family member by virtue of its ability to bind directly to both the cytoplasmic domains of CD40 and the oncogenic Epstein-Barr virus LMP1. Subsequently, it was shown to interact with the cytoplasmic tails of many TNFR family members including lymphotoxin β-receptor (LTβR), CD27, CD30, OX40, HEPES virus entry mediator and receptor activator of NFκB (Mosialos *et al*, 1995; Cheng *et al*, 1995; Sato *et al*, 1995; Arch *et al*, 1998).

1.4.1. TRAF3 has a role in the immune system

Whilst the precise role of TRAF3 in signal transduction is still unclear, null mutations in TRAF3 are unable to support the long-term survival of the mouse models, in part as a result of impaired T-cell dependent immune responses. TRAF3 deficient mice appear normal at birth but become progressively runted, correlating with progressive hypoglycemia and depletion of peripheral white cells (Xu *et al*, 1996). T-cells that were isolated from TRAF3 deficient mice had intrinsic defects and were impaired in their ability to respond to antigens. Consequently, it is evident that TRAF3 is important to signaling cascades that promote the activation and survival of T cells (Lee & Lee, 2002).

1.4.2. TRAF3 and the NFκB pathway

TRAF2 and TRAF5 have been known to activate the NFκB pathway whereas studies have shown that TRAF3 is not able to activate NFκB (Rothe *et al*, 1995). In contrast, it was reported that TRAF3 recruitment to LTβR led to cell death

(Force *et al*, 1997), and that both N- and C-terminal domains of TRAF3 negatively regulate NFκB activation induced by OX40 (Takaori-Kondo *et al*, 2000). However, it has also been shown that there are a variety of mRNA species of TRAF3 and that some splice variants do induce NFκB activation (van Eyndhoven *et al*, 1999).

Recent evidence indicates that TRAF3 is a negative regulator of the so called 'non-canonical pathway' of NFκB (see Figure 2) which involves the processing of p100 NFκB2 to the transcriptionally active p52 NFκB subunit. This effect is mediated through the ability of TRAF3 to physically interact with and promote the proteasomal degradation of NIK (NFκB-inducing- kinase), a kinase which plays a critical role in the pathway. (Liao *et al*, 2004).

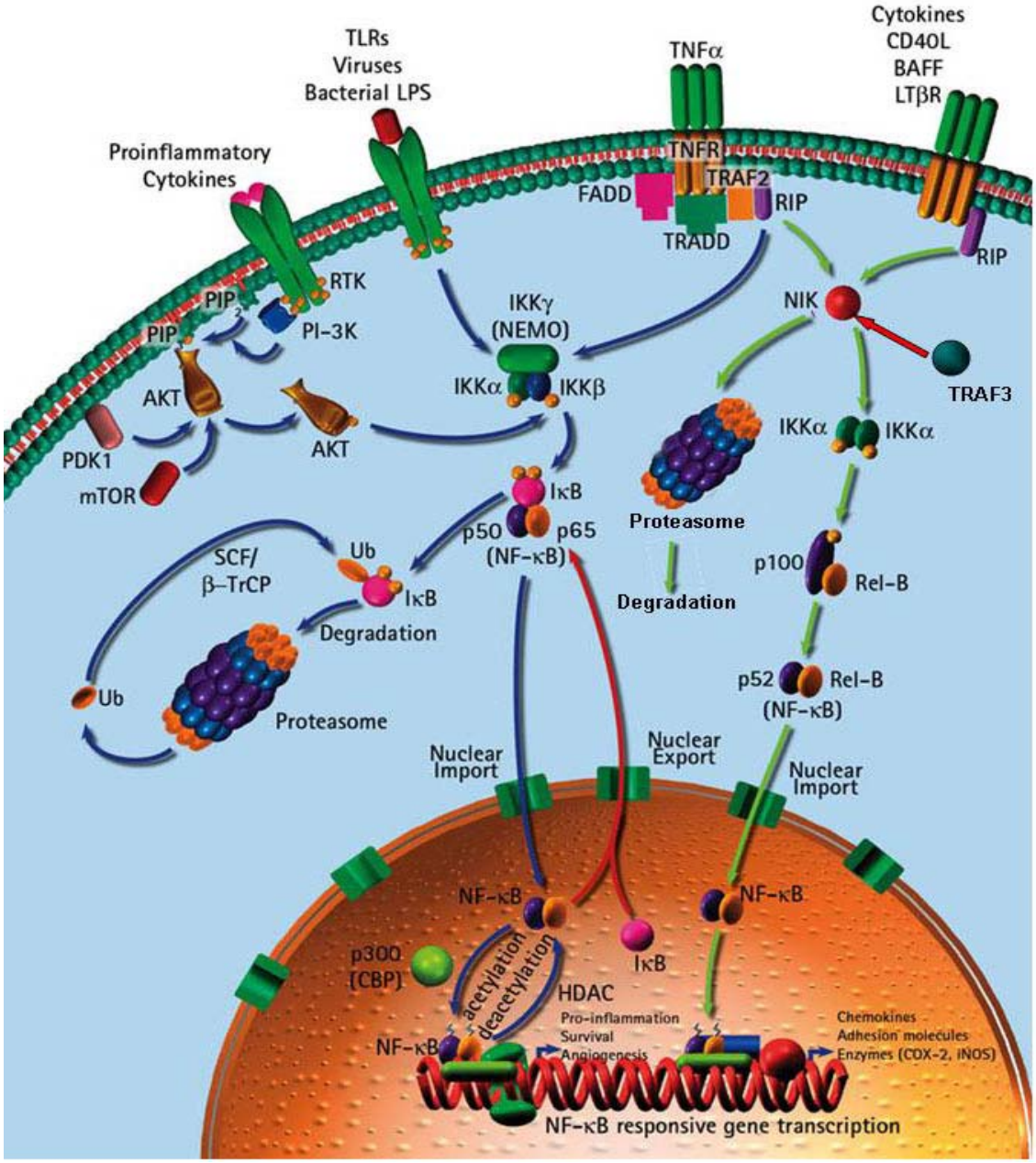

Figure 2: Overview of the NFκB pathway. The canonical pathway of NFκB activation (left side) requires activation of the trimeric IκB kinase (IKK) complex (IKKα, IKKβ and IKKγ or NEMO) which mediates phosphorylation and degradation of IκBα and the release of p50:RelA and p50:cRel dimers. The non-canonical pathway (left side) requires activation of NIK which in association with IKKα binds to the C-terminus of p100, leading to p100 processing to p52 and the preferential release of p52:RelB dimers. TRAF3 acts as a negative regulator of NIK and targets it for continuous degradation through the proteasome.

1.4.3. Involvement of TRAF3 in novel pathways?

Unpublished results from Dr Eliopoulos' laboratory highlight a previously undisclosed function of TRAF3 in promoting actin polymerization in response to LMP1 expression. Yeast-two hybrid assays, using TRAF3 as bait and a HeLa cDNA library as prey has identified novel TRAF3-interacting proteins, namely the human protein kinase N (PKN1;also known as protein kinase C-related kinase 1, PRK1) and Ubc9, an E2 enzyme. The aim of this thesis is to characterize in detail the nature of these' proteins interactions with TRAF3.

1.5. PKN1- a key player in the actin remodeling

PKN1 is a lipid-activated serine/threonine protein kinase and a member of the Protein Kinase C superfamily. It has a catalytic domain highly homologous to those of protein kinase C (PKC) family members in the carboxy-terminal and a unique regulatory sequence in the amino-terminal part (Palmer *et al*, 1995; Kitagawa *et al*, 1995; Watanabe *et al*, 1996). PKN1 was originally identified in the liver as a protease activated serine/threonine kinase via conventional protein chemistry (Gabrielli *et al*, 1984; Wettenhall *et al*, 1991).

1.5.1. Structure of PKN1

There are at least two other gene products identified that belong to the same family as PKN1; PKN2 and PKN3;all have similar structural organization as seen in Figure 3. The amino-terminal region of PKNs contains three repeats of the anti-parallel coiled-coil (ACC) domain and a C2-like region and is assumed to restrict the protein kinase activity of the catalytic domain in the absence of activators (Takahashi *et al*, 2003). ACC domains were reported to function as binding interfaces to a variety of proteins including small GTPase RhoA (Shibata *et al*, 1996). Recent evidence however, demonstrate that the very C-terminus

beyond the hydrophobic motif in PKN1 is essential for the activation of this kinase by RhoA (Lim *et al*, 2006).

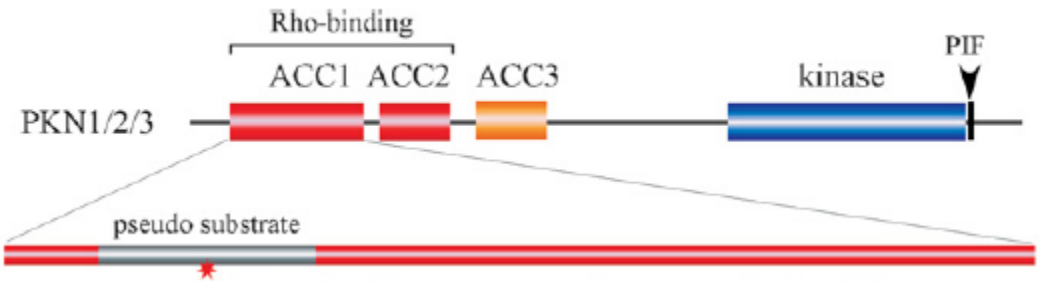

Figure 3: Domain structures of the PKN family kinases. PKNs contain three ACC structures at their N-termini and a catalytic domain at the C-terminus (blue). The first two ACCs form the RhoA-binding domain (shown in red) that overlaps a pseudo-substrate region which is indicated in grey.

The importance of PKN1 in development is underscored by the finding that a loss-of-function mutation in the Drosophila *Prk1* gene results in a dorsal closure defect during embryogenesis that is lethal to the developing fly embryo (Lu & Settleman, 1999). In mammalian cells, PKN1 has been shown to act downstream of Rho GTPase to regulate the intracellular trafficking of the epidermal growth factor receptor in HeLa carcinoma cells (Gampel *et al*, 1999); to modulate stress-induced gene expression in NIH3T3 and HEK293T cells (Marinissen *et al*, 2001) and to mediate the formation of cortical actin in mouse embryonic fibroblasts.

1.5.1. PKN1 and the actin cytoskeleton

The actin cytoskeleton is predominantly regulated by members of the Rho family GTPases. Rho activates many proteins including Rho-kinases (ROCKs), mDia and PKN (Burridge & Wennerberg, 2004). Stress fiber formation is promoted when ROCK phosphorylates and activates LIM-kinase (LIMK) which subsequently phosphorylates and inactivates cofilin, an actin-depolymerizing factor, resulting in net polymerization of actin filaments (Figure 4) (Riento & Ridley, 2003).

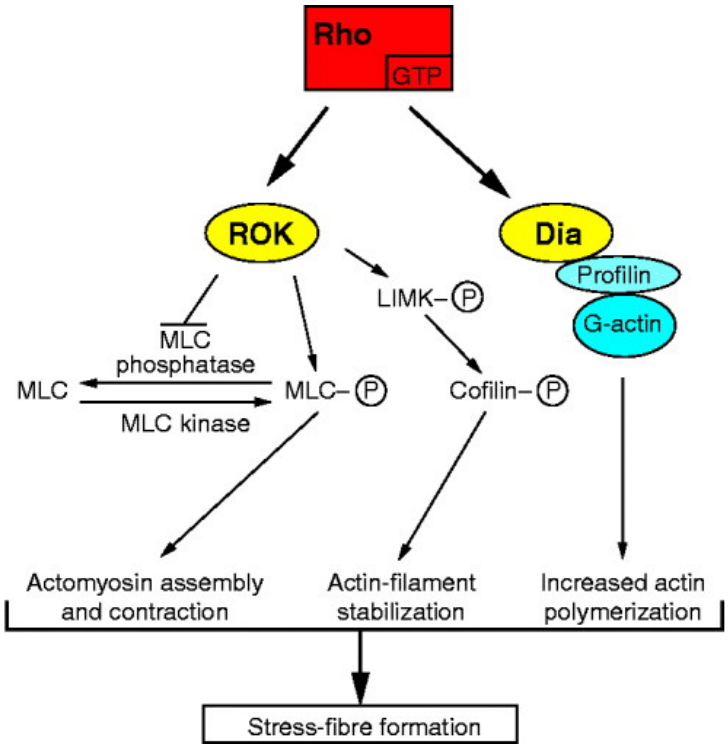

Figure 4: Overview of the Rho-actin pathway.

Another pathway that regulates the actin cytoskeleton has recently been described. In this pathway, insulin-induced loss of actin stress fibers and accumulation of cortical actin are mediated by Phosphoinositide-Dependent Protein Kinase 1 (PDK1) phosphorylation and activation of PKN1. PKN1 acts further downstream of PI-3 kinase and is activated by either RhoA binding or phosphorylation by PDK1 (Amano *et al*, 1996; Watanabe *et al*, 1996; Shibata *et al*, 1996; Dong *et al*, 2000; Flynn *et al*, 2000). Given the functional link between PKN1 and RhoA, in-depth analysis into the role of PKN1 in TRAF3-dependent signal transduction and stimulus-mediated re-organization of actin cytoskeleton is warranted.

1.6. Ubc9

1.6.1. Ubc9 and the sumoylation pathway

Post-translational modifications play an important role for the *in vivo* functions of proteins through the regulation of protein activity, turnover and localization and/or interactions. Such modifications include phosphorylation, methylation, glycosylation and ubiquitination (Mo *et al*, 2005).

Another type of modification, recently described is sumoylation, or small ubiquitin (Ub)-related modifier (SUMO) conjugation (Muller *et al*, 2001). SUMO-1 is a member of the Ub and Ub-like superfamily. Like ubiquitination, sumoylation modulates protein function through post-translational covalent attachment to lysine residues within targeted proteins (Figure 5) (Muller *et al*, 2001).

The sumoylation is a multi-step process that involves maturation, activation, conjugation and deconjugation (Muller *et al*, 2001). Ubc9 is an E2 enzyme that transfers the activated SUMO to the target protein (Figure 5), (Johnson & Blobel, 1997). Ubc9 is the only known E2 enzyme required for sumoylation although several others are involved in the ubiquitination pathway. Various proteomic analyses in both lower eukaryotic and mammalian cells (Zhou *et al*, 2005) have revealed that a significant number of sumoylated proteins are involved

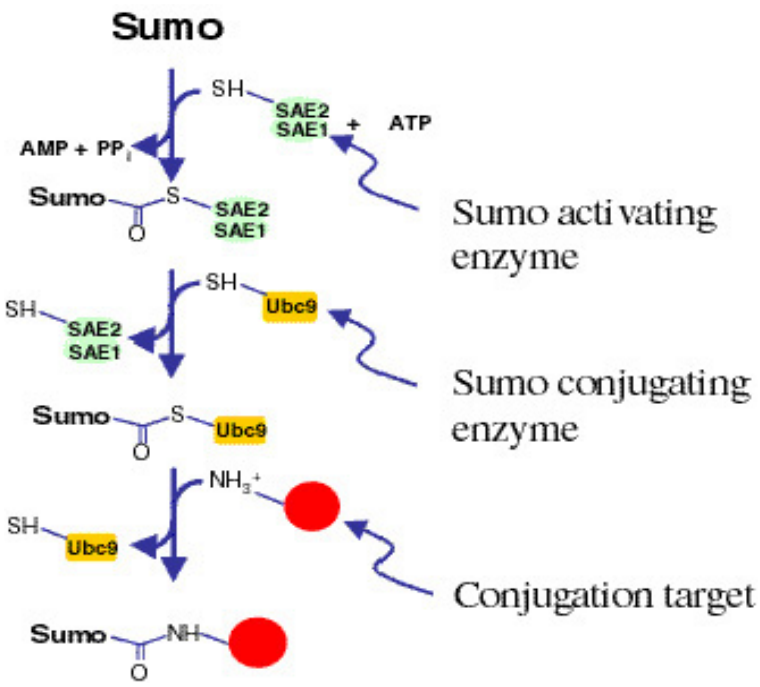

Figure 5: Simplified overview of the SUMO conjugation pathway. Figure adapted from http://www.biochemistry.ucla.edu/biochem/Faculty/Courey/images/image002.jpeg

in important nuclear functions, such as chromosome aggregation, DNA replication, transcription and damage repair. This outlines a role for Ubc9 in at least one critical pathway.

1.6.2. Ubc9 and genome integrity

Conversely, the sumoylation pathway is not the only pathway that Ubc9 is implicated in. It was originally described as an important protein for normal mitosis and cell cycle progression in lower eukaryotes because its absence was associated with cell cycle arrest and abortive mitosis (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). 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).

1.6.3. A role for Ubc9 in tumorigenesis

Ubc9 and by extension sumoylation have already been established as important players in the maintenance of genome integrity as described above. However, Ubc9 has been found to be over-expressed in several malignancies such as lung adenocarcinoma (McDoniels-Silvers *et al*, 2002), ovarian carcinoma (Mo *et al*, 2005) and melanoma (Moschos *et al*, 2005). There is evidence for its involvement in tumorigenesis through regulation of bcl-2 expression (Mo *et al*, 2005) whereas inactivating mutations of Ubc9's SUMO conjugating activity enhances sensitivity to DNA damaging agents (Mo *et al*, 2004).

All these evidence suggest that Ubc9 may act as an inhibitor of the apoptotic pathways and therefore contributing to tumor progression. Recent evidence have demonstrated that over-expression of Ubc9-DN is associated with increased drug sensitivity. Furthermore, Ubc9-DN has a negative effect on tumor growth in a xenograft mouse model. In yeast, a defect in the *ubc9* gene causes increased sensitivity to genotoxic drugs (Jacquiau *et al*, 2005) Therefore, alterations of Ubc9 expression or function can impact such cellular events as drug responsiveness and tumorigenesis. However, the underlying mechanism by which Ubc9 affects these aspects is not fully understood. It is necessary that the interaction of TRAF3 and Ubc9 must examined to possibly identify a role in the above-mentioned pathways; however it is entirely possible that Ubc9 may be revealed to play a role in other important cellular pathways.

2. Objectives

1. To characterize in detail the nature of the novel TRAF3:PKN1 interaction.
2. To characterize in detail the nature of the novel TRAF3:Ubc9 interaction.
3. To determine the role of these interactions in TRAF3-mediated signal transduction.

3. Materials and methods

3.1. Tissue culture techniques

3.1.1. Maintenance of cell lines

HEK 293T 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) and kept in a Forma Series II Water Jacketed CO_2 (5%) Incubator (Thermo Electron Corporation, Ohio, USA). They were routinely passaged when they reached 90% cofluency.

3.1.2. Transfection of cells

3.1.2a. Transient 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 media was removed from the dishes and the cells were washed once with Serum Free D-MEM. 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 buffer A (Tris base pH 7.5 20mM, 150mM NaCI, 1% Triton X-100, 1mM EDTA)

3.1.2b. Transient transfection in 12-well plates using Lipofectamine™

HEK 293T 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 100n μl OptiMEM (Gibco, Paisley, UK) (were n is the no of samples) was aliquoted in eppendorfs (100μl/well) containing 100μl OptiMEM and 2.5μl Lipofectamine™ (Invitrogen, CA, USA) and incubated for 15 minutes at room temperature. 200μl of the transfection cocktail and 500μl OptiMEM were added to the cells and the plate was transferred in 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 (30μg per sample).

3.1.2c. Transient transfection using Calcium Phosphate

HEK 293T 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).

3.2. Molecular Biology Techniques

3.2.1. PCR amplification

The Ubc9 fragment was amplified using polymerase chain reaction (PCR). The primers 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).

3.2.2. cDNA cloning of 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 (G˅GATCC) and EcoRI (G˅AATTC). The ligation reaction was performed at 16ºC using the T4 DNA ligase (Roche, Germany).

3.2.3. Formation 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 3.1.):

	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 3.1. Primers used in the construction of 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).

3.3. Protein biochemistry techniques

3.3.1. Determination of protein concentration

The protein concentration of the samples was determined by the BioRad Bradford Assay (BioRad Lab Ltd, U.K.). In standard test tubes, 1.2ml of sterile water, 300μl of the BioRad Bradford Assay reagent and 2.5μl of protein sample were mixed. The OD was measured at 595nm in a Jenway 6405 UV/Vis Spectrophotometer using 1.5ml cuvettes (Plastibrand®, Germany). The protein concentration was calculated according to a standard curve which corresponds to the equation y=0.01655x (y is the OD, x is μg/5μl).

3.3.2. Co-Immunoprecipitation Studies

Cell lysates were cleared and incubated with anti-flag M2 antibody or anti-myc 9E10 antibody (3μg/sample) and G-Sepharose Beads (20μl) for 12-18 hours at 4ºC on a rotor. The beads were then washed extensively 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)].

3.3.3. Western Analysis

Samples were analyzed by SDS-page on 10% or 12% polyacrylamide gels run at 100V in a BioRad Mini-PROTEAN 3 cell Apparatus (BioRad Laboratories Inc, Hertfordshire, UK) and electrophoretically transferred to BioTrace^{TM} NT (O.2μm) nitrocellulose membrane (PALL Life Sciences, USA) for 2 hours at 400 mA. Blocking was performed for 1 hour in 5% non-fat milk (Regilait, France)–TBS/Tween 0.1%. The membranes were incubated with various polyclonal antibodies (Table 3.1.) overnight at 4ºC 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 reagents and analysis system (Amersham Biosciences Ltd, Buckinghamshire, UK), exposed in autoradography films (HyperfilmTM, Amersham Biosciences Ltd, Buckinhamshire, UK & Fujifilm, Japan) and developed in a Kodak X-OMAT 1000 Processor (Kodak Int, Rochester USA).

ANTIBODY	COMPANY	DILUTION
a-myc 9E10 (mouse)	Cancer Research UK Antibody Production Fcl	1:500
a-flag M2 (mouse)	Sigma Cooperations	1:500
a-TRAF3 C20 (goat)	Santa Cruz Biotech	1:500
a-PKN1 (goat)	Transduction Labs	1:500

Table 3.2. Primary antibodies

ANTIBODY	COMPANY	DILUTION
Anti-mouse HRP-congugated	Chemicon	1:8000
Anti-goat HRP-congugated	Santa Cruz Biotech	1:8000

Table 3.3. Secondary antibodies

3.3.4. Reprobing of membranes

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 new primary antibody on a Stuart Roller Mixer SRT1.

4. Results

4.1.1. PKN1 specifically interacts with TRAF3

Unpublished results from Dr Eliopoulos' laboratory from a yeast-two-hybrid assay using TRAF3 as bait and a HeLa cDNA library as prey, the human protein kinase N (PKN1 also known as protein kinase C-related kinase 1, PRK1) has been identified as a novel TRAF3-interacting protein. We performed co-immunoprecipitation assays in order to confirm the interaction between TRAF3 and PKN1 observed in the yeast two-hybrid system. The full length PKN1 construct containing a N-terminal Myc-epitope tag was transiently expressed in HEK-293 cells with Flag epitope-tagged TRAFs (TRAF2, TRAF3, TRAF6 and TRAF5). Cell lysates were immunoprecipitated using a monoclonal antibody against the Flag epitope and co-precipitating myc-PKN1 was detected by immunoblotting analyses with anti-myc polyclonal antibody (Fig. 4.1A). In this assay, PKN1 specifically co-precipitates TRAF3 but not TRAF2, TRAF6 (Fig 4.1.1A) or TRAF5 (Figure 4.1.1B).

The reverse experiment was also carried out, where cell lysates were immunoprecipitated using a polyclonal antibody against the Myc epitope and co-precipitating Flag-TRAF3 was detected in a Western Blot using anti-TRAF3 antibody (recognizing an epitope of 20 amino acids at the C-terminus, see Materials & Methods). The results for this assay are shown in Fig. 4.1.1C.

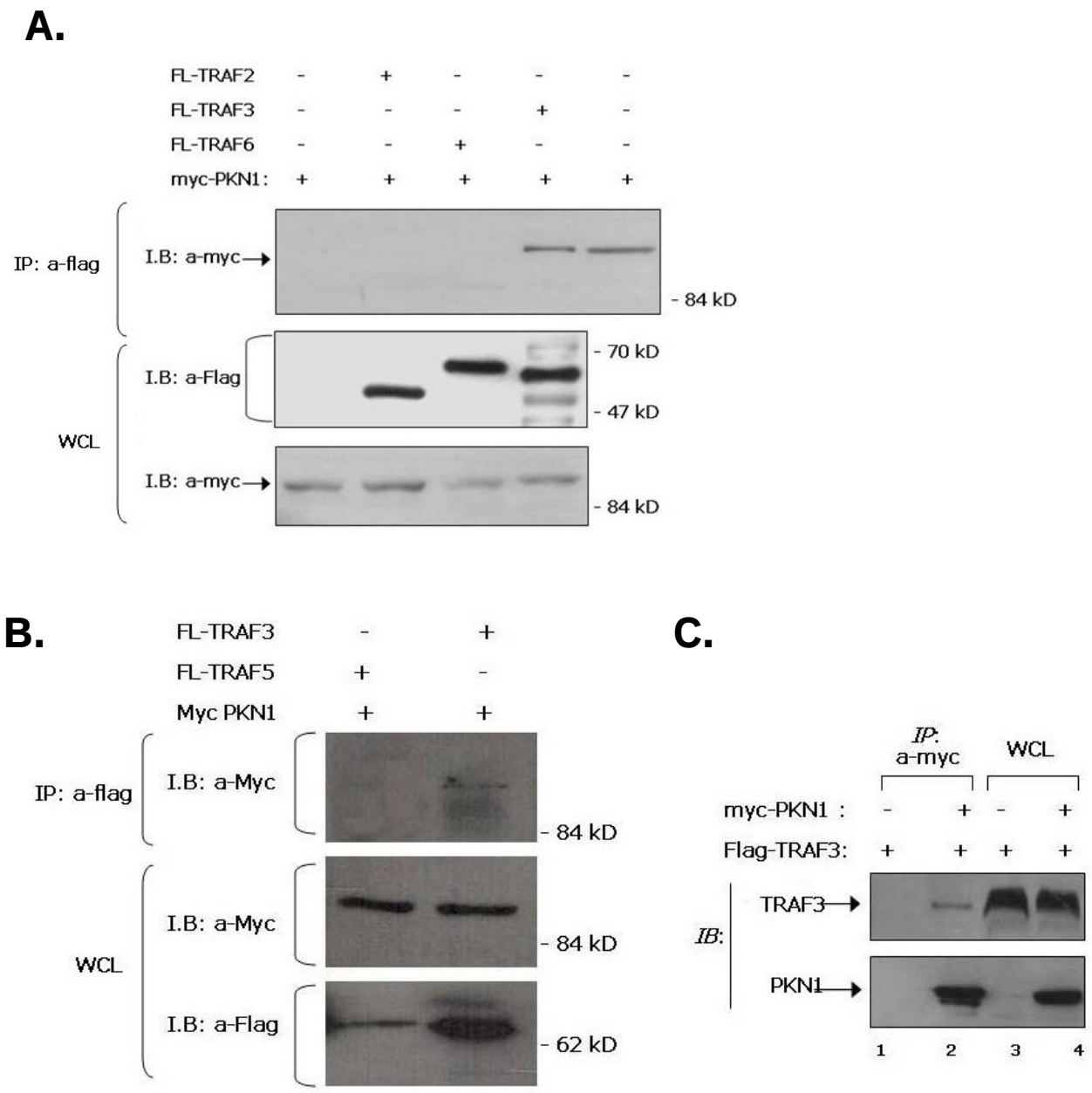

Figure4.1.1. ***Interaction of PKN1 with TRAF3 in vitro and in vivo****. In-vitro interaction of PKN1 with TRAF3. 293 cells were transiently transfected with expression vectors encoding myc epitope tagged PKN1 (full length) and Flag-tagged TRAF2,3,5,6. After 36 hours, extracts were prepared and immunoprecipitated (IP) with anti-Flag monoclonal antibody (A,B) or anti-Myc polyclonal antibody (C). Co-precipitating myc-PKN1 was detected by immunoblotting (IB) analysis using the anti-Myc (A,B) or anti TRAF3 C20 or anti-PKN1 antibody (C). The amount of TRAFs immunoprecipitated represented the 95% of the total cell lysate whereas the expression levels of myc-PKN1 and TRAFs represented the 1.5% of the total cell lysate.*

4.1.2. TRAF3 and PKN1 interact in the presence of LMP1in vitro and in vivo

LMP1 (Latent Membrane Protein 1) is expressed in lymphoblastoid cell lines and could contribute to Epstein-Barr virus mediated carcinogenesis. Most importantly however, it has the ability to induce actin stress-fiber formation, a Rho-GTPase mediated phenomenon (Eliopoulos & Young, 2001) and it has the ability to bind to TRAFs, specifically to TRAF3 (Mosialos *et al*, 1995). Given the functional link between PKN1 and RhoA, and that of PKN1 and TRAF3, investigation of stimulus-mediated re-organization of actin cytoskeleton is necessary to elucidate the role of TRAF3 in this process.

Full length PKN1, tagged with a Myc epitope was ectopically expressed in 293 cells along with Flag-tagged TRAF3 at two different concentrations (2μg and 5 μg, lanes 3 and 4 respectively) and Flag-tagged LMP1. As seen in Figure 4.1.2A, TRAF3 interacts with PKN1 in the presence of LMP1 (lanes 3 and 4, bottom panel). To verify this, we attempted to examine the endogenous interaction, if any, between TRAF3 and PKN1. We ectopically expressed LMP1 in 293 cells and immunoprecipitated the cell lysates with anti-PKN1. Following Western Analysis with anti-TRAF3, it was indeed concluded that endogenous PKN1 and TRAF3 interact in the presence of the transforming protein LMP1 (Figure 4.1.2B).

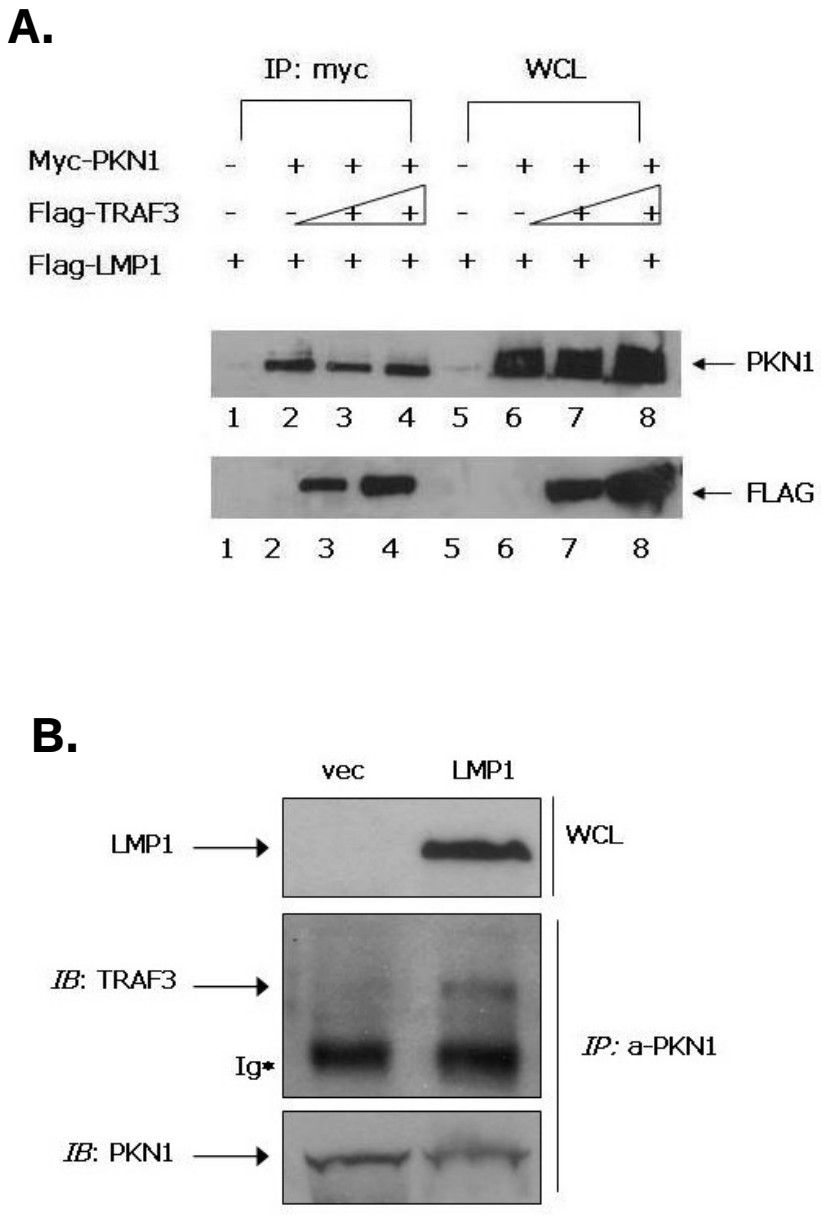

Figure4.1.2. ***Interaction of PKN1 and TRAF3 in the presence of LMP1****. A: 293 cells were transfected with myc-PKN1, Flag-LMP1 and 2μg or 5μg TRAF3 expression plasmids (lanes 3 & 4, 7 & 8 respectively). After 36 hours cell extracts were prepared and immunoprecipited with anti-Myc. The co-precipitating TRAF3 was detected with the use of anti-Flag antibody by immunoblotting. B: Lysates prepared from 293 cells transfected with LMP1 or empty vector were immunoprecipitated with anti-PKN1. Co-precipitated endogenous TRAF3 was detected by immunoblotting (IB) with anti-TRAF3 polyclonal antibody. The amount of lysate immunoprecipitated represented the 95% of the total cell lysate.*

4.1.3. The TRAF domain contributes to the interaction between TRAF3 and PKN1

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 PKN1 binding, various Flag-tagged TRAF3 deletion mutants were generated and assayed for association with myc-tagged PKN1.

Four TRAF3 mutants were generated 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 and zinc fingers (267-568) and one lacking the N-terminal ring and zinc fingers as well as most of the TRAF-N domain (aa346-568) (Figure 4.1.3B). All the TRAF3 deletion mutants were expressed as fusion proteins containing a N-terminal Flag-epitope tag using the pcDNA3 vector (Invitrogen).

The region of TRAF3 that appears critical for the interaction with PKN1 is aa267-568 which corresponds to the TRAF domain. From these experiments it could not be determined whether the TRAF3 mutant aa346-568 also interacts with PKN1 as this deletion mutant co-migrates with the Ig under these conditions (lane 4, bottom panel). We therefore, performed the reverse experiment where cell lysates transfected with TRAF3 mutants were immunoprecipitated with anti-Flag antibody and the co-immunoprecipitated PKN1 was detected with anti-Myc antibody. However, this immunoprecipitation experiment failed to show any interactions between any of the TRAF3 mutants and PKN1 (data not shown). This may indicate hindrance of association as a result of the small size of immunoprecipitated TRAF3 mutants and the large size of PKN1. The binding

results are shown in Figure 4.1.3B. In summary, it appears that TRAF3 is interacting with PKN1 through its conserved TRAF domain.

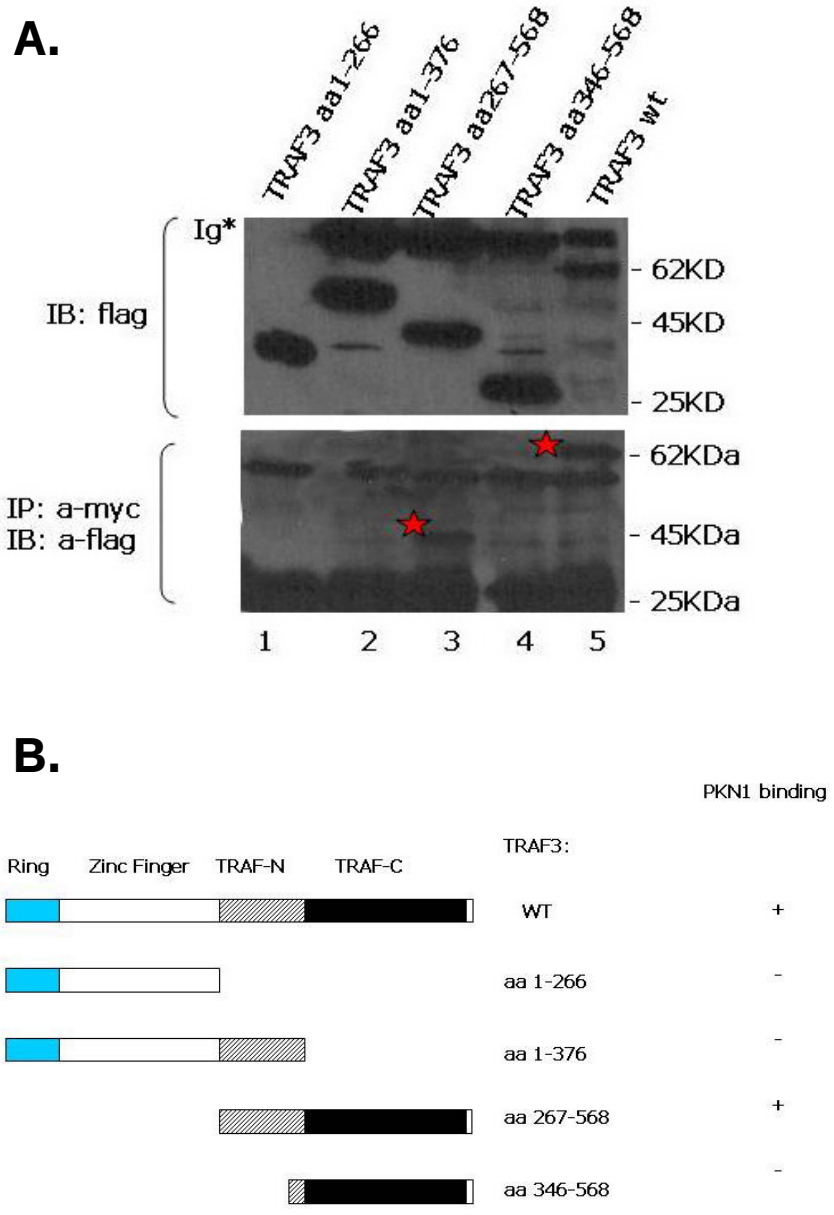

Figure4.1.3. ***Interaction of TRAF3 deletion mutants with PKN1*** *A: Co-immunoprecipitation studies. 293T cells were transiently transfected with the Myc epitope-tagged PKN1 and the Flag-tagged TRAF3 deletion mutants. After 36 hours, extracts were prepared and immunoprecipitated (IP) with anti-Myc monoclonal antibody. Co-precipitating TRAF3 and TRAF3 mutants were detected by immunoblotting (IB) using the anti-Flag antibody (bottom panel). The amount of lysate immunoprecipitated represented the 95% of the total cell lysate. Red asterisks denote co-immunoprecipitated proteins B: summary of association data.*

4.2.1. Interaction of TRAF3 with Ubc9 in vitro

Previously undisclosed data from Dr Eliopoulos' laboratory has identified another novel interacting protein for TRAF3 through a yeast two hybrid system, using TRAF3 as bait. This protein is the human Ubc9, an E2 enzyme required for sumoylation as well as for several other critical pathways.

To confirm this interaction between Ubc9 and TRAF3 we performed *in vitro* co-immunoprecipitation assays. The full-length Ubc9 expressed as an N-terminal fusion protein tagged with myc-epitope was transiently expressed in 293 cells with flag-tagged SMAD4, which is known to interact with Ubc9 (Lin *et al*, 2003), and flag-tagged TRAF3 (Figure 4.2.1.)

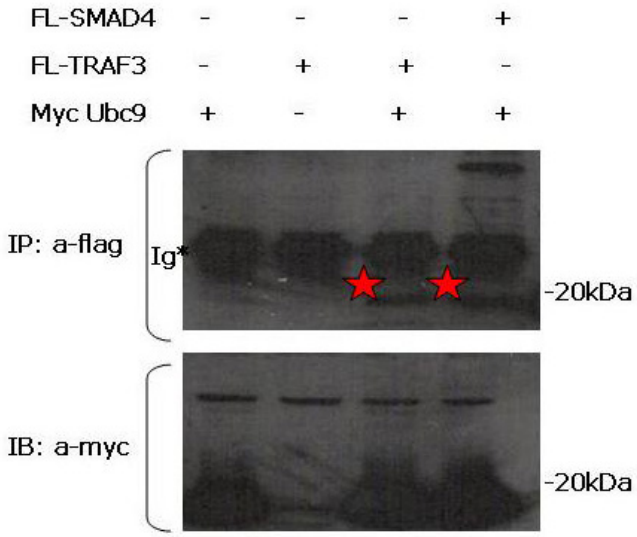

Figure4.2.1. ***Interaction of TRAF3 with Ubc9****. Lysates prepared from 293T cells which were transfected with 5µg of Flag-tagged TRAF3 or Flag-tagged SMAD4 and myc-tagged Ubc9, were immunoprecipitated with anti-flag antibody. Co-immunoprecipitated Ubc9 was detected by immunoblotting with the anti-Myc antibody. Red stars denote co-immunoprecipitated proteins.*

Immunoprecipitation was carried out using anti-flag monoclonal antibody and the immunoprecipitates were separated by SDS-page. The results demonstrate that TRAF3 does indeed interact with Ubc9 *in vitro.*

4.2.2. Identification of the TRAF-domain of TRAF3 as the domain responsible for the interaction with Ubc9

To determine which regions of TRAF3 contribute to the Ubc9 binding, we utilized the TRAF3 deletion mutants mentioned in 4.1.3. The TRAF3 mutants and myc-tagged Ubc9 were transfected into 293T cells and the co-immunoprecipitation experiments were carried out using anti-flag antibody (Figure 4.2.2A). The

domain in TRAF3 responsible for the Ubc9 binding is located between the aa 267-568 which comprises the TRAF domain (Figure 4.2.2B).

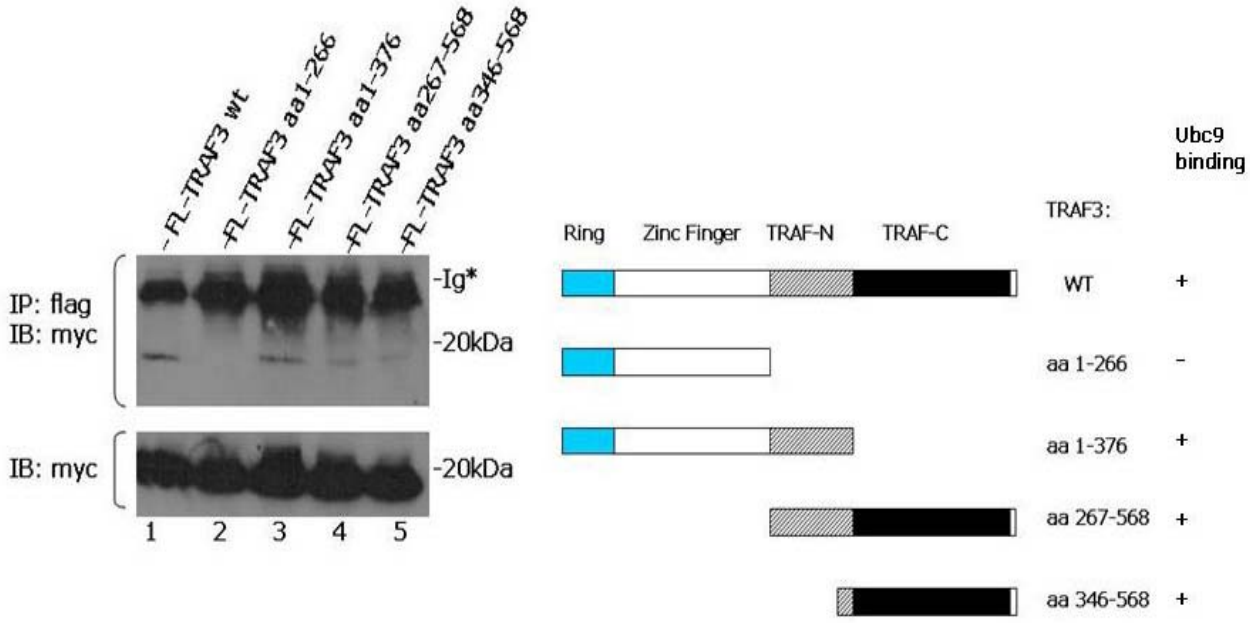

Figure4.2.2. ***Interaction of TRAF3 deletion mutants with PKN1*** *A: Co-immunoprecipitation studies. 293T cells were transiently transfected with the Myc epitope-tagged Ubc9 and the Flag-tagged TRAF3 deletion mutants. After 36 hours, extracts were prepared and immunoprecipitated (IP) with anti-flag polyclonal antibody. Co-precipitating TRAF3 and TRAF3 mutants were detected by immunoblotting (IB) using the anti-myc antibody (top panel). The amount of lysate immunoprecipitated represented the 95% of the total cell lysate. The amount of cell lysate represented the 1.5% of the WCL B: summary of association data.*

5. Discussion

In the present study we report the identification of two novel TRAF3-interacting proteins PKN1 and Ubc9, which are implicated in diverse pathways. However, TRAF3, as most TRAFs, can act as a molecular link between signaling pathways and the TNFRs as well as a branching point between pathways mediating contradicting functions (Hauer *et al*, 2005). Here we examined both proteins separately in relation to TRAF3.

5.1.1. TRAF3 but not other TRAF proteins interacts with PKN1

PKN1 belongs to a family that includes at least two other kinases, PKN2 and PKN3, which share similar structure (Gotoh *et al*, 2003). Interestingly however, it is PKN1 that appears to directly interact with TRAF3 through its TRAF domain. It is noteworthy, that although the TRAF domain is conserved amongst all TRAF proteins, TRAF3 is the only one that shows evidence of association with PKN1 (Figure 4.1.1.). This could be due to the existence of a unique sequence motif in TRAF3, absent from the TRAF domain of other TRAFs. This could only be verified by performing extensive bioinformatics analyses among the seven TRAF proteins. Additionally, in order to pinpoint the exact sequence in TRAF3 that interacts with PKN1, more fine deletion or point mutants must be generated.

5.1.2. PKN1 interacts with TRAF3 in the presence of LMP1

PKN1 is known to be activated by Rho-GTPases (Amano *et al*, 1996; Watanabe *et al*, 1996) which have been identified as important regulators of the actin cytoskeleton. The precise role of PKN1 in the regulation and reorganization of the actin cytoskeleton is not yet fully understood. Dong and colleagues (2000) have shown that the kinase-dead PKN1 prevents insulin-induced actin stress fiber breakdown and membrane ruffling suggesting that PKN1 is involved in insulin-induced actin cytoskeleton reorganization.

On the other hand, EBV-encoded LMP1, which interacts with TRAF1, TRAF2 and TRAF3 through its CTAR1 effector domain in a ligand-independent manner (Eliopoulos & Young, 2001), also induces stress fiber, lamellipodia and filopodia formation in model fibroblast cell lines (Puls *et al*, 1999; Dawson *et al*, 2003). These cytoskeletal rearrangements are known to be regulated by the small GTPases Rho, Rac and Cdc42 but how TNFR family members and LMP1 engage these molecules to promote actin cytoskeleton remodeling is currently unclear.

Meanwhile, unpublished data highlight a previously undisclosed function of TRAF3 in regulating actin polymerization in response to LMP1 expression. Subsequently, an N-terminus deleted 'dominant-negative' TRAF3 but not equivalent mutants of TRAF1 or TRAF2, appear to selectively abolish LMP1-induced actin stress fiber formation in fibroblasts (A. Eliopoulos, unpublished observations). Given the importance of actin cytoskeleton remodeling in many key biological phenomena and the emerging novel role of TRAF3 in this process, a detailed in-depth analysis for the physiological role of TRAF3 and PKN1 in the signaling pathways that result in the actin cytoskeleton reorganization is warranted. It is possible that TRAF3 acts a mediator for Rho-dependent activation of PKN1, resulting in actin cytoskeleton reorganization or that the TRAF3-PKN1 complex may comprise a regulatory switch that links TNF family receptors and viral proteins to the remodelling of the actin cytoskeleton and the induction of cell motility. Similarly, the activation of certain TNF family receptors by their ligands is also known to promote remodeling of the actin cytoskeleton (reviewed by Hall, 1998) so it would be noteworthy to examine the TRAF3-PKN1 association in relation to these stimuli.

5.2.1. TRAF3 interacts specifically with Ubc9

Ubc9 is known to be important for genome integrity and overall cell survival (Moschos & Mo, 2006) as well as being the sole E2 enzyme known required for sumoylation (Seufert *et al*, 1995; Hayashi *et al*, 2002). Here we have identified it as a novel TRAF3-interacting protein through co-immunoprecipitation experiments, demonstrating that the domain important for this interaction is the TRAF domain of TRAF3. It is vital to identify whether this interaction is specific for TRAF3 or whether this interaction is true for other TRAFs as the TRAF domain is highly conserved among them. This can occur by performing co-

immunoprecipitation experiments with the other TRAF proteins or yeast two-hybrid systems using Ubc9 as bait.

5.2.2. Speculation on the functional importance of the TRAF3:Ubc9 association

TRAF3 has been involved in a variety of pathways including the so called non-canonical NFκB pathway. More precisely, Liao and colleagues (2004) have shown that TRAF3 physically associates with NIK (Figure 2) and targets it for degradation through the proteasome. However, although that study demonstrates that the TRAF3/NIK association triggers NIK ubiquitination, the authors were unable to show that TRAF3 possessed any significant E3 activity (Liao *et al*, 2004, He *et al*, 2006). This leads us to believe that TRAF3 itself may not be sufficient to act as ubiquitin ligase. Moreover, TRAF proteins in general have only been shown to mediate lysine 63 ubiquitin linkages, which are not associated with protein degradation but rather with the formation of positive signaling complexes (Deng *et al*, 2000; Sun *et al*, 2004). This postulates the existence of additional players to complete our understanding of TRAF3-dependent suppression of NIK.

Here we hypothesize a role for Ubc9 in the TRAF3-dependent NIK degradation and consequently to the non-canonical NFκB pathway suppression. Previous studies, have demonstrated that Ubc9 can facilitate the ubiquitination of the ATF2 (Activating Trancription Factor 2) in T cells (Firestein & Feuerstein, 1998). Furthermore, structural data reveal that Ubc9 is capable of forming complexes with structure suitable for several interactions, crucial for E3-assisted E2 conjugation (Reverter & Lima, 2005). Subsequently, we could say that TRAF3 may provide an interface upon which NIK is targeted for degradation through the proteasome either by Ubc9 or, more likely, by a yet unidentified E3 enzyme that is sequestered to the complex by Ubc9.

Already, preliminary experiments where we over-expressed Ubc9 and TRAF3 in 293T cells provide evidence for increased p52 processing due to increased NIK processing (data not shown). To test the hypothesis more effectively however, we will need to utilize techniques such as siRNA to knock down Ubc9 in order to observe the effects it may have on the non-canonical NFκB pathway. Another useful method of investigation could be the utilization of the dominant-negative Ubc9 to test the effect on the NFκB2 processing.

5.3. Conclusion

Preliminary data suggest a novel role for TRAF3 in actin polymerization, therefore creating the need for further characterization of the significance of this newly identified interaction with PKN1 in signal transduction and phenotypic effects. Further work is required to provide new insight into the mechanism by which TNF family receptors and oncogenic viral proteins induce actin cytoskeleton remodeling and affect cell motility. We have further provided evidence that may shed light in a different pathway involving TRAF3; the suppression of the non-canonical NFκB pathway may require the E2 enzyme Ubc9, another novel TRAF3-interacting protein. The clarification of the importance of these associations will shed new light in the TRAF3 signaling pathway and will provide important evidence for its role in the cell.

6. References

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APPENDIX A: Lab-made solutions

A1. Molecular Biology Solutions

L-AGAR (500 ml)

Tryptone	5gr	Scharlau (Spain)
Yeast Extracts	2.5gr	Scharlau (Spain)
NaCI	5gr	Scharlau (Spain)
Agar bacteriological	6gr	Scharlau (Spain)

L-BROTH (500 ml)

Tryptone	5gr	Scharlau (Spain)
Yeast Extracts	2.5gr	Scharlau (Spain)
NaCI	5gr	Scharlau (Spain)

Tris Borate Saline (TBE) 5x (500 ml)

Tris base	27 gr	Roth GmbH, KG

Boric Acid	13.75 gr	Sharlau (Spain)
EDTA 0.5 M	10 ml	Allied Signal (Germany)
Adjust pH to 8		

DNA Loading Buffer

Bromophenol blue		Fluka (Switzerland)
Xylene Cyanol EF		
Glycerol		MERCK (Germany)

A2- Protein Biochemistry Solutions

Phosphate Buffered Saline (PBS) 1x (1lt)

NaCI	8 gr	Scharlau (Spain)
KCI	0.2 gr	Scharlau (Spain)
Na_2HPO_4	1.44 gr	Scharlau (Spain)
KH_2PO_4	0.24 gr	Scharlau (Spain)

Adjust pH to 7.4 with HCI

Lysis Buffer A

Tris base 1M pH 7.5	5 ml	Roth GmbH KG
NaCI 5M	7.5 ml	Scharlau (Spain)
Triton X-100	2.5 ml	Scharlau (Spain)
EDTA 0.5 M	0.5 ml	Allied Signal (Germany)

Tris-Buffered-Saline (TBS) 10x (1000ml)

Tris-base	24.2gr	Roth GmbH KG
NaCI	80gr	Scharlau (Spain)

Adjust pH to 7.6 with HCI

Protein Gel Sample Buffer (Loading Buffer)

Tris base 1M	1ml	Roth GmbH KG
SDS 10% (w/v)	8 ml	Scharlau (Spain)
Glycerol	2 ml	MERCK (Germany)
B-mercaptoethanol	1 ml	Sigma (Germany)
Bromophenol blue	0.001gr	Fluka (Switzerland)

Running Buffer 10x

Tris base	0.25M	Roth GmbH KG
Glycine	1.92M	Scharlau (Spain)
1% SDS (w/v)		Sigma-Aldrich (Germany)

Transfer Buffer 1x

Tris-base 25mM	3.94g/lt	Roth GmbH KG
Glycine 0.2M	15g/lt	Scharlau (Spain)
Methanol	200ml	Scharlau (Spain)

A3. Solutions for mini preparation of DNA (Alkaline method)

Lysis Buffer

NAOH 10N	0.1ml	Merck (Germany)
SDS 10%	0.5ml	Scharlau (Spain)
Sterile Water	4.4ml	Demo S.A. (Hellas)

GTE

Glucose 20%	0.5ml	Scharlau (Spain)
Tris base 1M pH 7.5	0.25ml	Roth GmbH KG
EDTA 0.5M	0.2ml	Allied Signal (Germany)
Water for Injection	9.05ml	Demo S.A.

Solution III

Potassium Acetate 5M	60ml	Scharlau (Spain)
Acetic Acid	11.5ml	Scharlau (Spain)
Water for Injection	28.5ml	Demo S.A.

A4. Transfection solutions ($CaPO_4$ method)

1x HBS

NaCI	1.63gr	Scharlau (Spain)
Hepes	1.19gr	Sigma-Aldrich (Germany)
$Na_2HPO_42H_2O$	0.026gr	Scharlau (Spain)

Adjust pH to 7.1 with 0.5N NaOH, filter 0.22μM

TE

Tris-HCI	10mM	Scharlau (Spain)
EDTA pH 8.0	1mM	Allied Signal (Germany)

APPENDIX B- Commercial Solutions

B1.MidiPrep Buffers (QIAGEN, UK)

P1- Resupension Buffer

50 mM Tris-CI, pH 8.0
10 mM EDTA
100 µg/ml RNase A

P2- Lysis Buffer

200 mM NaOH
1% SDS (w/v)

P3- Neutralisation Buffer

3.0 M potassium acetate
pH 5.5

Buffer QBT (Equilibration Buffer)

750 mM NaCI
50 mM MOPs, pH 7.0
15% isopropanol (v/v)
0.15% Triton® X-100 (v/v)

Buffer QC (Wash Buffer)

1.0 M NaCI
50 mM MOPS, pH 7.0
15% isopropanol (v/v)

Buffer QF (Elution Buffer)

1.25 M NaCI
50 mM MOPS, pH 8.5
15% isopropanol (v/v)

B2- Cell culture Solutions (Gibco, Paisley UK)

Dulbeco's Modified Eagle Medium (D-MEM)

1gr/lt D-glucose
Puruvate
10% Fetal Calf Serum (Gibco)

F-12

L-glutamine 1mM
10% fetal bovine serum

OptiMEM

Hypoxanthine

Thymidine
Sodium Puruvate
L-Glutamine
HEPES/Sodium bicarbonate buffered

B3. Molecular Biology Buffers

Expand High Fidelity PCR system Buffer with $MgCl_2$ 10x (Roche, Germany)

100 mM Tris-HCI pH 8.3
500 mM KCI
15 mM $MgCI_2$
0.01% gelatin (w/v) pH 8.3

TA Cloning® Reagents

10x Ligation Buffer (Roche, Germany) & 10x Ligation Buffer (Invitrogen, CA, USA)

60mM Tris-HCI, pH 7.5
60mm MgCI2
50mM NaCI
1mg/ml bovine serum albumin
70mM β-mercaptoethanol
1mM ATP
20 mM dithiothreitol
10 mM spermidine