A Novel Role for the TRAFs as Co-Activators and Co-Repressors of Transcriptional Activity

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A NOVEL ROLE FOR THE TRAFS AS CO-ACTIVATORS AND CO-REPRESSORS OF TRANSCRIPTIONAL ACTIVITY

By

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A NOVEL ROLE FOR THE TRAFS AS CO-ACTIVATORS AND CO-REPRESSORS OF TRANSCRIPTIONAL ACTIVITY

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The tumor necrosis factor (TNF) receptor-associated factors (TRAFs) were initially discovered as proteins that inducibly interact with the intracellular region of TNF receptors (TNFRs). Because the TNFRs lack intrinsic catalytic activity, the TRAFs are hypothesized to orchestrate signaling activation downstream of the TNFR superfamily, however their mechanism of activation remains unclear (Inoue et al., 2000; Bishop, 2004). Originally, the TRAFs were compared to the signal transducers and activators of transcription (STAT) protein family, due to their sequence homology, and the presence of multiple RING- and zinc-finger domains, suggesting that their function may be to regulate transcriptional activity (Hu et al., 1994; Rothe et al., 1994; Cheng et al. 1995; Sato et al., 1995). However, subsequent research focused predominantly on their cytoplasmic functions, and more recently on their function as E3 ubiquitin ligases (Pineda et al., 2007).

In my research, I analyzed the subcellular localizations of the TRAFs following CD40 ligand (CD40L)-stimulation, and found that TRAF2 and 3 rapidly translocate into the nucleus of primary neurons and Neuro2a cells. Interestingly, similar analysis conducted in pre-B lymphocytes (Daudi cells) revealed a different
response to CD40L-stimulation, with TRAF2 and 3 being rapidly degraded within 5-minutes of stimulation. These findings are significant because they demonstrate for the first time that the TRAFs translocate into the nucleus and suggest that they may function within the nucleus in a cell-specific manner.

I next analyzed the ability of TRAF2 and 3 to bind to DNA, and found that they both bind to chromatin and the NF-κB consensus element in Neuro2a cells, following CD40L-stimulation. Similar analyses of the chromatin binding of TRAF2 and 3 in Daudi cells revealed that they were rapidly degraded, similar to the results from my analysis of their subcellular localization. These findings show for the first time that the TRAFs interact with DNA, and therefore support the hypothesis that the TRAFs may function within the nucleus as transcriptional regulators.

Finally, I analyzed the ability of the TRAFs to regulate transcriptional activity by luciferase assay. Previous studies showed that overexpression of TRAF2 and 6 could induce NF-κB transcriptional activity; however researchers have not been able to determine the mechanism by which they do so. In my studies, I found that every TRAF can directly regulate transcriptional activity either as co-activators or co-repressors of transcription, in a cell- and target protein-specific manner. Additionally, I found that TRAF2 can act as a transcriptional activator, and that its ability to regulate transcription is largely dependent upon the presence of its RING-finger domain.

In conclusion, these studies have revealed an entirely novel function for the TRAFs as immediate-early transcriptional regulators. Future research into
the genes that are regulated by the specific TRAF complexes will further elucidate how the TRAFs regulate TNFR signaling, as well as whether dysfunctions in TRAF signaling may be associated with known disorders. If specific TRAF complexes are found to regulate specific genes, then pharmacological targeting of the individual TRAF complexes may allow for the highly specific inhibition of signaling events downstream of the TNFRs, without compromising overall receptor signaling, transcription factor pathways, or cellular systems.
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Chapter 1

INTRODUCTION

The tumor necrosis factor receptor-associated factors (TRAFs) are a family of adaptor proteins that act as intermediate intracellular signaling molecules downstream of the TNF receptor (TNFR) superfamily. The TNFR superfamily is a diverse, ever-expanding family of cytokine receptors that mediate a wide range of physiological functions, including regulation of the immune system, apoptosis, growth, survival, differentiation, osteoclastogenesis (Lee & Lee, 2002; Li & Lin 2008), and even the induction of long term potentiation (Beattie et al., 2002). As the TNFRs lack intrinsic catalytic activity, downstream activation has been hypothesized to be orchestrated predominantly by the TRAF proteins, although the exact mechanisms by which the TRAFs regulate such activity remain to be elucidated (Inoue et al., 2000). The goal of this study was to determine whether the TRAFs may function as transcriptional regulators downstream of the TNFR superfamily. Background information relevant to the TNFRs, the TRAFs, the NF-κB signaling pathways, the CD40 receptor, and E3-ubiquitin ligases, will be reviewed in this section.

The TNFR Superfamily

The TNFR superfamily includes: TNFR1 (p55), TNFR2 (p75), TNFR3 (TNF-RP), Fas, DR3, DR4, DR5, PV-T2, PV-A53R, CAR1, p75(NTR), CD27, CD30, CD40, LTβR, 4-1BB, OX-40, ATAR, RANK, LMP-1, BAFFR, BCMA, and TACI (Baker & Reddy, 1998; Ni et al., 2000; Bishop, 2004). Each of these
receptors are type I transmembrane proteins that are characterized by the presence of conserved cysteine rich domains (CRDs) in the extracellular ligand-binding domain, which function to stabilize multimeric TNFR complexes through the formation of intracysteine disulfide bonds (Baker & Reddy, 1998). The cytoplasmic domains of these receptors vary in length between 40 and 200 amino acid residues, share very little sequence homology, and lack intrinsic catalytic activity (Ni et al., 2000).

Two types of conserved motifs have been identified within the intracellular domain of TNFRs: the death domain and the TRAF binding motifs. The death domain is conserved within multiple TNFRs and can bind soluble intracellular proteins that mediate the induction of apoptosis through activation of the ASK1-JNK pathway, a transcription factor pathway that regulates cell survival. The TRAF binding motifs are conserved amino-acid sequences that bind to specific TRAFs and are variably present within the intracellular domains of the different TNFRs (Baker & Reddy, 1998).

Upon ligand binding, the TNFRs multimerize into active, hetero- and homotrimeric receptor complexes. This multimerization is believed to activate downstream signaling pathways by bringing the requisite signaling components into close proximity with one another (such as the TRAFs, which are active in homo- and heterotrimeric form), however the exact mechanisms remain to be elucidated (Pullen et al., 1999). Genetic ablation experiments have shown that deletion of various TRAF proteins that interact with specific TNFRs can block the ability of those TNFRs to activate downstream signaling pathways (Yeh et al.,
1997; Lomaga et al., 1999; Naito et al., 1999; Tada et al., 2001; Kobayashi et al., 2003). For this reason, the functions of each particular TNFR are believed to be determined by the specific combination of TRAFs that it interacts with (Bishop, 2004).

The TRAFs

The TRAF proteins are ubiquitously expressed, and are almost exclusively cytoplasmic (Zapata et al., 2000). However, while most literature regarding the TRAFs indicate that they are cytoplasmic proteins, some studies have also found individual TRAFs to be localized within the nucleus of particular cell types, such as TRAF3 being localized predominantly within the nucleus of Purkinje neurons (Figure 1A) (Krajewski et al., 1997). There have also been several instances where overexpression of either full-length or domain-deletion isoforms altered the subcellular distribution of the proteins (Min et al., 1998; Arch et al., 2000; Glauner et al., 2002; Morita et al., 2005), such as TRAF2 being localized within both the cytoplasm and nucleus of human umbilical vein endothelial cells (HUVEC), while deletion of the RING-finger domain abolished its nuclear localization, and deletion of the C-terminal TRAF domains resulted in its constitutive nuclear localization (Figure 1B) (Min et al., 1998). Additionally, while nuclear localization sequences (NLSs) have not been identified for the TRAFs, TRAF3 has been found to interact directly with p62 nucleoporin, which binds to classical NLS-containing import complexes during activity-dependent nuclear translocation (Gamper et al., 2000).
**Figure 1. Previous findings of TRAF2 and TRAF3 nuclear localization.**

A) TRAF3 immunostaining of mouse cerebellum sections, showing TRAF3 in Purkinje cell neurons (large cells) and granule cells (small cells) (methyl green counterstain). In Purkinje cells, TRAF3 shows moderate cytoplasmic staining and intense nuclear staining, as can clearly be seen at higher magnification (upper panel = 150x; lower panel = 1000x) (Krajewski et al., 1997). In the upper panel, the arrow points to a Purkinje cell that does not have nuclear staining, thus exemplifying that there is variability of nuclear expression between Purkinje cells. GCL = granular cell layer.

B) Western blot analyses of the subcellular localization of TRAF2 in primary human umbilical vein endothelial cells (HUVEC), and what effect domain deletion or TNFα stimulation has on its subcellular distribution. In the first panel, wild-type TRAF2 is localized predominantly in the cytoplasm of HUVEC, and, to a lesser degree, within the nucleus. Stimulation with TNFα for 15 minutes results in the degradation of TRAF2. In the second panel, deletion of the RING-finger domain completely abolishes the nuclear localization of TRAF2. In the third panel, elimination of the C-terminal TRAF domains results in the constitutive nuclear localization of the N-terminal TRAF mutant (Min et al., 1998).
Currently, seven TRAFs have been identified (TRAF1-7), but the exact functions of these proteins have not yet been elucidated. Part of the reason for this is that there has been considerable variability and contradiction between previous studies into their functions because most of them were limited to overexpression systems. Since protein expression levels are an important component of endogenous TRAF signaling, overexpression of the TRAFs can lead to either activation or inhibition of multiple signaling pathways and may also alter their subcellular localization. To further complicate the situation, functional studies performed in TRAF knockout (KO) models have been difficult or even impossible because most of the TRAF KOs are lethal within the embryonic or early postnatal periods (Bishop, 2004). For example, TRAF2\(^{−/−}\) mice are born extremely runted, with an atrophic thymus and spleen, as well as depletion of their B-cell precursors, and they die within the early postnatal period (Yeh et al., 1997). TRAF3\(^{−/−}\) mice are born normal, but have functionally defective T-cells and become progressively runted, in direct correlation with progressive hypoglycemia and depletion of their peripheral white cells, culminating in their death within 10-days of birth (Xu et al., 1996). While the gross biological impairments of these KO mice have been characterized rather extensively, the effects that such KOs have on the central nervous system (CNS) have not yet been characterized.

Structurally, TRAFs 1-6 have similar features, including a carboxy-terminal receptor binding domain (TRAF-C) and a coiled-coil, leucine-zipper domain (TRAF-N). Additionally, every TRAF, except for TRAF1, is also characterized by
the presence of a variable number of zinc-finger domains and a RING-finger domain (Bishop, 2004). TRAF7 was more recently identified as a member of the family based upon the similarity of its RING- and zinc-finger domains to those of the TRAFs, however it lacks the C-terminal TRAF domains (Figure 2) (Xu et al., 2004).

Functionally, the TRAF domains mediate homo- and heterotrimerization of the TRAFs, while the zinc-finger domains mediate interaction of the TRAFs with other proteins. Additionally, the RING-finger domain exhibits E3-ubiquitin ligase activity, and the ability of particular TRAFs to activate downstream signaling cascades depends on the presence of a functional RING-finger domain (Min et al., 1998; Bishop, 2004; Habelhah et al., 2004). While the exact mechanisms by which the different TRAF molecules activate downstream signaling cascades are yet unknown, multiple TRAFs are required for downstream activation of the NF-κB pathways (Au & Yen, 2007; He et al., 2007), and are involved in the activation of several additional pathways, including the p38, ERK1/2, ASK/JNK, and PI3K pathways (Dempsey et al., 2003).

The TRAFs in the Central Nervous System

TRAF1

In the normal rat cortex, TRAF1 associates with the TNFR1 receptor complex within lipid raft microdomains. Following moderate traumatic brain injury (TBI), both TRAF1 and TNFR1 are polyubiquitinated and recruited to lipid rafts, where they activate caspase-8 and induce apoptosis (Lotoki et al., 2004). In
Figure 2. The structure of the TRAF proteins. TRAFs 1-6 are each characterized by the presence of an N- and C-terminal TRAF domain. Additionally, TRAFs 2-7 are characterized by the presence of a variable number of N-terminal zinc-finger domains, as well as a RING-finger domain (RING). TRAF1 lacks both RING- and zinc-finger domains, and TRAF7 lacks both the N- and C-terminal TRAF domains. Figure adapted from Bishop, 2004.
gene expression analyses between primary CNS lymphomas and nonmalignant germinal center centroblasts, *TRAF1* is among the genes with significantly reduced expression in primary CNS lymphomas, which suggests that TRAF1 may have a negative regulatory role on the progression of such lymphomas (Courts et al., 2007).

**TRAF2**

In the normal rat cortex, TRAF2 is also found within lipid raft microdomains in association with TNFR1 (Lotoki et al., 2004). In differentiated PC12 cells, TNF$_{\alpha}$ induces apoptosis in tandem with the increased gene expression of *TRAF2* and *TNFR1*, the protein products of which have been suggested to mediate such apoptosis (Mielke & Herdegen, 2002). In primary astrocytes stimulated with beta-amyloid, TRAF2 inhibition prevents reactive gliosis and iNOS expression (Akama & Van Eldik, 2000). Infection of astrocytes by *E. coli* induces brain inflammation dependent on TRAF2-mediated NF-$\kappa$B activation (Kim et al., 2005). In mouse models of Huntington’s disease, the polyQ-induced neuronal cell death depends upon endoplasmic reticulum (ER) stress-induced activation of ASK1, through the formation of a TRAF2-ASK1 complex (Nishitoh et al., 2002). And, in mouse models of Parkinson’s disease, wild-type parkin is neuroprotective by activating NF-$\kappa$B and promoting the degradation-independent ubiquitylation of TRAF2 and IKK$_{\gamma}$; while the pathogenic parkin mutants are impaired in their ability to activate NF-$\kappa$B and thus are impaired in neuroprotection (Henn et al., 2007).
**TRAF4**

Genetic ablation of TRAF4 is embryonic lethal, resulting in axial skeletal malformations, tracheal ring disruptions, and impaired closure of the neural tube, which leads to spina bifida (Régnier et al., 2002). In 1-day and 4-week-old wild-type mice injected intracerebrally with a recombinant strain of the prototype alphavirus, Sindbis virus, a decreased mortality rate in the older mouse population correlates with the developmental downregulation of TRAF4 (Labrada et al., 2002). And, in microarray analyses on postmortem temporal cortices from human patients with schizophrenia, TRAF4 is among the genes with the most consistently altered gene expression between the control and schizophrenic test groups, suggesting that TRAF4 may play a role in the pathogenesis of schizophrenia (Aston et al., 2004).

**TRAF5**

In a mouse model of HIV-induced neuronal injury, ethanol (EtOH) strongly potentiates HIV-1 gp120-induced neuronal injury, which occurs in direct correlation with a significant increase in TRAF5 gene expression. For this reason, TRAF5 has been suggested to play an essential role in mediating the potentiation of HIV-1 gp120-induced neuronal apoptosis by EtOH (Chen et al., 2005). And, similar to TRAF2, the infection of astrocytes by *E. coli* also induces brain inflammation dependent upon TRAF5-mediated NF-κB activation (Kim et al., 2005).
**TRAFT6**

In TRAF6<sup>−/−</sup> mice, an increase in the frequency of impaired neural tube closure and exencephaly has been found, suggesting that TRAF6 may regulate the levels of programmed cell death within specific regions of the developing CNS (Lomaga et al., 2000). In primary astrocytes stimulated with beta-amyloid, TRAF6 inhibition prevents reactive gliosis and iNOS expression (Akama & Van Eldik, 2000). In PC12 cells, nerve growth factor (NGF) induces NF-κB activation through both p75(NTR) and the TrkA receptor, with p75(NTR) signaling dependent upon TRAF6, and TrkA dependent upon Shc. Interestingly, blocking p75(NTR)-mediated NF-κB activation significantly enhances apoptosis, while blocking TrkA-mediated NF-κB activation significantly inhibits neurite process formation. This suggests that, although both receptors activate NF-κB, their mechanisms of activation proceed through distinct signaling intermediates, thus resulting in different cellular outcomes (Foehr et al., 2000). Finally, activation of p75(NTR) in neurons results in the nuclear translocation of the neurotrophin receptor interacting factor (NRIF), which depends upon the polyubiquitination of NRIF by TRAF6 (Geetha et al., 2005).

**TRAFT3 and TRAF7**

There have been no studies regarding the functions of TRAF3 or 7 in the CNS, either direct or implied.
The NF-κB Signaling Pathways

The NF-κB signaling pathways regulate diverse biological processes, including cell growth, survival, differentiation, proliferation, immune and inflammatory responses, and apoptosis. The NF-κB family of transcription factors consist of homo- and heterodimeric complexes composed of NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB, and c-Rel (Liao et al., 2004).

There are currently two known NF-κB pathways: the classical pathway and the alternative pathway (Figure 3). In general, the classical pathway leads to the activation of homo- and heterodimers composed of p50, RelA, RelB, and c-Rel, while the alternative pathway leads to the activation of heterodimers composed of p52 and RelB. Activation of the classical pathway is mediated primarily by the inhibitor of κB (IκB) kinase (IKK) complex, composed primarily of the two catalytic subunits, IKKα (IKK1) and IKKβ (IKK2), in addition to the regulatory subunit, IKKγ (NEMO). Under non-stimulated conditions, transcriptional complexes of the classical pathway (e.g. p50/RelA) are retained within the cytoplasm by IκBα. Upon stimulation, the IKK complex phosphorylates IκBα and signals it for proteasomal-dependent degradation, releasing the classical NF-κB complexes for nuclear translocation and subsequent transcriptional activation. Activation of the alternative pathway is mediated primarily by NF-κB inducing kinase (NIK) (Xiao et al., 2001). Under non-stimulated conditions, transcription factor complexes of the alternative pathway (e.g. p100/RelB) are retained in the cytoplasm due to the C-terminal tail of p100, which is homologous to IκBα. Upon stimulation, NIK activates IKKα homodimers that phosphorylate p100,
Figure 3. The NF-κB signaling pathways. There are two known NF-κB signaling pathways: the classical and the alternative. The classical pathway is characterized by the activation of the IKK complex, which phosphorylates IκBα, signaling it for proteasomal-dependent degradation, thus releasing the p50 and RelA homo- and heterodimers for nuclear translocation and transcriptional activation. The alternative pathway is characterized by the activation of NIK, which in turn activates IKKα (IKK1), which then phosphorylates p100, signaling it for proteasomal-dependent processing, thus releasing the p52 and RelB homo- and heterodimers for nuclear translocation and transcriptional activation. Figure adapted from Chen & Greene, 2004.
leading to proteasomal-dependent processing of p100 into p52. The processing of p100 releases the NF-κB complexes of the alternative pathway, allowing for nuclear translocation and subsequent transcriptional activation (Liao et al., 2004).

Among the most commonly studied TRAF proteins that regulate the NF-κB pathways, TRAF2 has been hypothesized to primarily regulate the classical pathway, and TRAF3 to regulate the alternative pathway. These hypotheses are based on the fact that specific TNFRs that selectively activate a particular NF-κB pathway, have been found to interact preferentially or specifically with only one of the two TRAFs. For example, TNFR1 and 2, which mainly activate the classical pathway, both interact with TRAF2, but not TRAF3, while the BAFF receptor, which primarily activates the alternative pathway, interacts only with TRAF3. Interestingly, the CD40 receptor (CD40R), which activates both the classical and alternative pathways, coordinately interacts with both TRAF2 and 3. Surprisingly, overexpression studies have suggested that TRAF2 functions as a positive regulator of NF-κB activation, while TRAF3 functions as a negative regulator. In particular, TRAF2 overexpression activates the classical pathway (Au & Yen, 2007), while TRAF3 overexpression inhibits NF-κB activation (He et al., 2007), both dependent upon the presence of their RING-finger domain and potentially their ubiquitin ligase activity. Research into the mechanism for TRAF3 activating the alternative pathway through negative regulation has revealed that overexpression of TRAF3 results in the degradation of NIK, the upstream kinase in the alternative pathway. Therefore, stimulation-induced degradation of TRAF3 has been hypothesized to relieve such repression, in turn resulting in the
accumulation of NIK, thus activating the alternative pathway (Liao et al., 2004). More recent research has suggested that TRAF3 may actually function as an adaptor molecule for NIK and TRAF2, while TRAF2 functions to recruit the cellular inhibitors of apoptosis 1 and 2 (cIAP1 and 2) as E3 ubiquitin ligases, in order to constitutively degrade NIK and thus inhibit the alternative pathway (Vallabhapurapu et al., 2008; Zarnegar et al., 2008).

The CD40 Receptor

CD40R is unique among the TNFRs that activate the NF-κB signaling pathways because it can activate both the classical and the alternative pathways. CD40R also interacts with a majority of the TRAF proteins: TRAF1, 2, 3, 5 and 6 (Inoue et al., 2000; Wajant et al., 2001; Moore & Bishop, 2005; Bishop et al., 2007), and is both present and functional in primary neurons (Tan et al., 2002 (A)). Activation of CD40R by its cognate ligand, CD40 ligand (CD40L), regulates the induction of proliferation, differentiation, isotype switching, cytokine production, and surface molecule upregulation; protection from apoptosis; and promotion of humoral memory (Bishop, 2004). And, although the functions of CD40R in the CNS have not been fully characterized, CD40R was found to regulate the induction of the amyloidogenic processing of the amyloid precursor protein (APP) (Tan et al., 2002 (B)).

While the specific functions of the individual TRAFs have not yet been determined, the mechanism of TRAF1, 2, and 3 signaling downstream of CD40R has been characterized fairly extensively. In particular, TRAF1 regulates the
association and dissociation of TRAF2 and 3 to the receptor. And, as previously mentioned, TRAF2 and 3 regulate the activation of the classical and alternative NF-κB pathways, respectively (Bishop, 2004). Following receptor activation, TRAF2 induces the degradation of TRAF3, along with its own degradation after longer stimulation intervals (Figure 4) (Brown et al., 2001; Brown et al., 2002; He et al., 2004; Moore & Bishop, 2005), which may occur indirectly through its recruitment of cIAP1 and 2 (Vallabhapurapu et al., 2008; Zarnegar et al., 2008).

**TRAF E3 Ubiquitin Ligase Activity**

E3 ubiquitin ligases function to conjugate ubiquitin from E2 ubiquitin ligase carrier molecules to target proteins (Figure 5). Conjugation of ubiquitin to target proteins serves a variety of functions, dependent upon the nature of the covalent linkages that connect the individual ubiquitin moities or polyubiquitin chains to the target proteins. In particular, Lys-48-linked polyubiquitination targets proteins for proteasomal-dependent degradation, while Lys-63-linked mono- or polyubiquitination targets proteins to specific subcellular compartments or activates them by structural modification or proteasomal-processing (Krappman & Scheidereit, 2005). In the case of the TRAFs, TRAF2 and 6 function as E3 ubiquitin ligases, dependent upon the presence of their RING-finger domains. And, both TRAF2 and 6 predominantly catalyze Lys-63-linked polyubiquitination. While other TRAFs also display ubiquitin ligase activity, the exact nature of their ubiquitin ligase activity has yet to be determined (Xia & Chen, 2005; Lamothe et al., 2007; Pineda et al., 2007).
Figure 4. The CD40 receptor. The CD40 receptor is a member of the TNFR superfamily that is active in trimeric form, with CD40L as its cognate ligand. The primary TRAFs that were studied downstream of CD40 receptor activation are TRAFs 1, 2 and 3. TRAF1 was found to regulate the association and dissociation of TRAF2 and 3 to the receptor, thus regulating their functions. TRAF2 positively regulates NF-κB signaling, while TRAF3 negatively regulates NF-κB signaling. TRAF2 can induce the degradation of both TRAF3 and itself. Figure adapted from Xie et al., 2006.
Figure 5. Two models of TRAF2 as an E3 ubiquitin ligase. TRAF2 recruits specific E2 ubiquitin ligases in order to catalyze the Lys-63-polyubiquitination of target proteins. This activity is dependent upon its RING-finger domain. In this figure TRAF2 is shown to interact with two different E2 ubiquitin ligases, Ubc5 and Ubc13-Uev1A. ZnF = zinc-finger domain. Figure adapted from Xia & Chen, 2005.
Rationale

In my preliminary studies, I routinely found TRAF2 and 3 to be variably present within the nucleus of Neuro2a cells (Figure 6), similar to previous findings (see Figure 1 for examples). Additionally, TRAF2 and 3 each contain multiple RING- and zinc-finger domains, which are generally considered DNA-binding domains, and, although the TRAFs have since been defined as cytoplasmic proteins, they were originally compared to the signal transducers and activators of transcription (STAT) family of transcription factors due to their high level of sequence homology (Hu et al., 1994; Rothe et al., 1994; Cheng et al. 1995; Sato et al., 1995). For these reasons, I hypothesized that TRAF2 and 3 may actively translocate into the nucleus and may function within the nucleus as direct regulators of transcriptional activity.

In order to test these hypotheses, I first analyzed the ability of TRAF2 and 3 to translocate into the nucleus following CD40L-stimulation. Next, I analyzed the ability of TRAF2 and 3 to bind to both chromatin and the NF-κB consensus element. And finally, I analyzed the ability of the TRAFs to regulate transcriptional activity using a variety of luciferase assays.
Figure 6. Immunocytochemical analysis of TRAF2 and TRAF3 localization in Neuro2a cells. Confocal immunocytochemical analysis of TRAF2 and 3 in Neuro2a cells shows both cytoplasmic and nuclear localization (subnuclear puncta; 63x magnification). The lower panels are close-up images of the areas depicted as A and B within the upper two panels.
Dissertation Overview

Chapter 2 describes the Materials and Methods that were utilized in this study.

In Chapter 3.1, I analyze the subcellular distribution of TRAF2 and 3 by Western blotting of subfractionated protein extracts from Neuro2a cells, primary cortical neurons, and Daudi cells that have been stimulated for 5, 10, or 30-minutes with CD40L, in order to determine whether the TRAFs inducibly translocate into the nucleus following CD40L-stimulation. I show, for the first time, that TRAF2 and 3 translocate into the nucleus following CD40L-stimulation, in a cell-specific manner.

In Chapter 3.2, I analyze the binding of TRAF2 and 3 to chromatin and to the NF-κB consensus element in Neuro2a cells by chromatin binding assay, NF-κB electromobility shift assay (EMSA), and NF-κB oligoprecipitation assay, in order to determine whether the TRAFs bind to DNA once in the nucleus. I show, for the first time, that TRAF2 and 3 both constitutively and inducibly bind to the chromatin and the NF-κB consensus element. Additionally, I analyze the chromatin binding of TRAF2 and 3 in Daudi cells, and find that the TRAFs also bind to DNA in a cell-specific manner.

In Chapter 3.3, I analyze the transcriptional regulatory potential of the TRAFs by a variety of luciferase assays, including NF-κB promoter-driven luciferase assay, 5x Gal4 promoter-driven luciferase assay, and 4x Gal4-minimal thymidine kinase (TK) promoter-driven luciferase assay. Using these assays, I show that each of the TRAF proteins have the capacity to function as a co-
activator, co-repressor, or even a transcription factor, in a cell- and target protein-specific manner. Additionally, I show that the transcriptional regulatory potential of TRAF2 is dependent upon the presence of its RING-finger domain.

Chapter 4 reviews the major conclusions of Chapter 3. Discussion is provided regarding insights into the nuclear translocation, DNA-binding, and transcriptional regulatory potentials of the TRAF proteins.
Chapter 2

MATERIALS AND METHODS

Cell Culture

Neuroblastoma cells were cultured at 37°C in 1:1 Opti-MEM:DMEM, supplemented with 5% fetal bovine serum (FBS), 100 IU/mL Penicillin/Streptomycin (P/S), and 2 mM L-Glutamine (L-Glut) (Gibco) (Borchelt et al., 1996). Daudi cells were cultured at 37°C in RPMI 1640 (Gibco) supplemented with 10% FBS, 100 IU/mL P/S, 2 mM L-Glut, 55 μM 2-mercaptoethanol (Fisher Biotech), 20 μg/mL Gentamycin, and 1 mM Na-pyruvate (Gibco) according to the American Type Culture Collection (ATCC) protocol. HEK 293 cells were cultured at 37°C in DMEM supplemented with 10% FBS and 100 IU/mL P/S (Hu et al., 2005). Primary cortical neurons were cultured at 37°C in Eagle’s basal medium (BME) (Gibco), supplemented with 10% FBS, 100 IU/mL P/S, 2 mM L-Glut, 25 mM KCl, and 10 μM cytosine β-D-arabinofuranoside (Sigma) (Brambilla et al., 2005).

Preparation of Primary Cortical Neurons

Primary cortical neurons were prepared from the cortices of E15 wild-type C57BL6 mouse embryos. The brains were removed from the embryos, and the cortices were carefully dissected from the brain under a stage microscope and were pooled together. The dissected cortices were then chopped up with a sterile razor blade. The diced cortical material was pipetted into a 15 mL tube and centrifuged at 4°C in order to pellet the cortical material. The media was
then carefully aspirated from the tube, and the cortical material was digested in 1 mL of 0.25% Trypsin-EDTA for 10-minutes at 37°C, with agitation following 5-minutes of incubation. After digestion, 1 mL of cortical neuron culture media was added to stop the reaction, and the cortical material was triturated 5-times with a fire-polished glass pipette to further dissociate the digested brain matter. The sample was then centrifuged at 4°C in order to pellet the digested brain material, and the media was carefully aspirated from the tube and replaced with 1 mL of culture media. The digested brain material was again triturated 5-times with a fresh fire-polished glass pipette, and the solution was allowed to stand for 10-minutes at room temperature (RT) in order to allow any undigested brain material to settle at the bottom of the tube. The solution containing the digested cortices was carefully removed from the tube, leaving the undigested material behind, and was diluted into culture media. Finally, the culture media containing the isolated cortical neurons was distributed into poly-L-lysine-coated 6-well culture dishes, at a ratio of approximately 2 embryonic cortices per dish. The neurons were then cultured for 3-days prior to use, as described above (Bethea laboratory protocol; Brambilla et al., 2005).

**Expression Vectors and Transfections**

The Gal4-TRAF and RelA fusion protein constructs were generated by cloning the individual TRAF and RelA cDNAs into the pBind vector (Promega). The TRAF constructs that were used for co-transfection with Gal4-tagged RelA were generated by cloning the individual TRAF cDNAs into the pCMV-Tag2B
vector (Stratagene). Each of the cDNAs were amplified from the whole genome cDNA library (Promega) by polymerase chain reaction (PCR) and sequenced. The TRAF2 N-terminal RING-finger deletion constructs were generated by removing a 261-bp DNA fragment corresponding to the N-terminal 87 amino acids of TRAF2 (Habelhah et al., 2004). Cells were transfected using Lipofectamine 2000, according to the manufacturer's instructions (Invitrogen).

**Immunostaining**

Cells were plated onto poly-L-lysine-coated 8-well glass slides, and cultured until 70-80% confluency. The cells were then fixed with 4% paraformaldehyde, washed, and permeabilized for 15-minutes in 0.2% Triton X-100 in PBS supplemented with 1% normal serum. Nonspecific staining was blocked by incubation in 10% normal serum in PBS for 1-hour at RT. Cells were then incubated overnight at 4°C with the primary antibody diluted in blocking solution. The following day, the cells were washed 3-times and incubated for 1-hour in the dark at RT with the fluorochrome-conjugated secondary antibody (Invitrogen) diluted in PBS supplemented with 1% BSA. Finally, the cells were washed 3-times with PBS, coverslipped with Vectashield + DAPI (Vector Laboratories), and visualized using confocal microscopy.

**Protein Extraction**

Whole cell extracts were prepared by lysing the cells in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1
mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol (DTT), 1x Complete protease inhibitor (Roche), and 1x Phosphatase Inhibitor Cocktail I & II (Sigma)) (Liao et al., 2004). To prepare cytoplasmic and nuclear extracts, the cells were first incubated for 30-minutes with Buffer A (10 mM HEPES, pH 7.9; 1.5 mM MgCl\(_2\); 10 mM KCl; 0.5 mM DTT; 1x Complete protease inhibitor; 1x Phosphatase Inhibitor Cocktail I & II; 0.8% NP-40) and centrifuged at 4,600 x g for 10-minutes. Following centrifugation, the supernatants were collected as cytoplasmic extracts, and the isolated nuclei pellets were washed 3-times with phosphate-buffered saline (PBS), and then incubated for 30-minutes with Buffer B (50 mM HEPES, pH 7.9; 0.4 M KCl; 0.75 mM MgCl\(_2\); 0.5 mM EDTA; 12.5% glycerol; 0.5 mM DTT; 1x Complete; 1x Phosphatase Inhibitor Cocktail I & II). After 30-minutes, the samples were centrifuged at 18,300 x g for 10-minutes, and the nuclear extracts were collected (Bethea et al., 1998; Brambilla et al., 2005). All steps were carried out at 4°C.

**Chromatin Extraction**

For chromatin binding assays, the residual chromatin pellets following soluble nuclear protein extractions were washed with PBS, and then incubated in Buffer B supplemented with Deoxyribonuclease I (Sigma) for 2-hours on ice. After 2-hours, an equivalent volume of 2x High Saline Solution (300 mM NaCl; 2% Triton X-100; 100 mM HEPES, pH 7.5; 20% glycerol; 1x Complete; 1x Phosphatase Inhibitor Cocktail I & II) was added to the samples, followed by an additional hour incubation on ice. The samples were then centrifuged at 18,300
x g for 15-minutes, and the chromatin extracts were collected (Fujita et al., 2002). All steps were carried out at 4°C.

**Western Blotting**

Protein extracts were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membranes, and the membranes were blocked for 1-hour in Tris-buffered saline-0.1% Tween 20 (TBS-t) containing 5% bovine serum albumin (BSA). Following overnight incubation with the primary antibodies in blocking solution at 4°C, the membranes were washed 3-times and then probed with HRP-conjugated secondary antibodies for 1-hour at RT. The antibodies were visualized using a chemiluminescent kit (ECL; GE Healthcare). The primary antibodies that were used were anti-TRAF2, anti-TRAF3, anti-Glut3, anti-HSP60 (Santa Cruz Biotechnologies), anti-RelA, anti-Lamin A/C, and anti-HDAC1 (Cell Signaling Technologies). The specificities of the TRAF antibodies were verified by peptide blocking (Figure 7). After probing, the membranes were stripped in Stripping Buffer (62.5 mM Tris, pH 6.8; 0.4% SDS; 100 mM 2-mercaptoethanol) for 40-minutes at 65°C, washed in TBS-t, and then reblocked.

**Electrophoretic Mobility Shift Assay**

Oligonucleotide consensus sequences for transcription factor binding sites (Santa Cruz; Promega) were \(^{32}\)P-labeled with T4 polynucleotide kinase (Promega). Equal amounts of nuclear extracts were either untreated or pre-
Figure 7. Verification of the specificity of the TRAF antibodies. A Western blot was performed with unstimulated Neuro2a whole cell extract (30 μg). The membrane was probed with an antibody to TRAF2 or TRAF3, either with or without preadsorption to their respective blocking peptide. Preadsorption was performed by incubating the antibodies at 4°C overnight with 10-fold excess blocking peptide. β-actin was used as a loading control. Ab = antibody.
incubated with 200 ng of anti-TRAF2 or anti-TRAF3 antibody, or with 100-fold excess of unlabeled NF-κB or NFAT consensus elements. The samples were then incubated with the radiolabeled consensus sequence for 30-minutes at RT in DNA Binding Buffer (10 mM Tris, pH 7.5; 50 mM NaCl; 1 mM MgCl2; 0.5 mM EDTA; 0.5 mM DTT; 4% glycerol) supplemented with 125 μg/ml poly(dI-dC):poly(dI-dC) (Sigma). After 30-minutes, the samples were loaded onto a pre-electrophoresed 6% polyacrylamide gel in 0.5x Tris-borate EDTA buffer, and separated at 150V for ~1.5-hours. Gels were then fixed and dried, and autoradiograms were obtained.

NF-κB Consensus Oligoprecipitation Assay

NF-κB consensus oligo-coated agarose beads were obtained from Santa Cruz Biotechnologies. Nuclear extracts were incubated with the beads overnight in DNA Binding Buffer (Hu et al., 2005). The following day, the beads were centrifuged at 12,000 x g for 10-minutes at 4°C. The samples were then washed 3-times with DNA Binding Buffer and then incubated at 100°C for 5-minutes in 2x SDS sample buffer (125 mM Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 0.2M DTT; 0.02 mg/mL bromophenol blue). Finally, Western blots were performed on the fractionated samples, according to the protocol described above.

Gal4 Luciferase Assays

The Gal4-TRAF and RelA fusion protein constructs were cotransfected into Neuro2a and HEK 293 cells along with either the 4x Gal4-minimal TK
promoter-driven luciferase plasmid (R. Evans, Salk Institute), the 5x Gal4 promoter-driven luciferase plasmid (Promega), or the NF-κB promoter-driven luciferase plasmid (Clontech) (see Figure 8 for a comparison of the three luciferase reporter plasmids). The Gal4-RelA fusion protein constructs were additionally transfected either with or without the flag-tagged TRAF protein constructs. At 24-hours post-transfection, lysates were collected and luciferase assays were performed according to the Promega Dual-Luciferase Reporter Assay System Technical Manual (Promega).
Figure 8. A comparison of the NF-κB, 5x Gal4, and 4x Gal4-minimal TK promoter-driven luciferase plasmids. The NF-κB promoter-driven plasmid (NF-κB luc) contains an NF-κB promoter. Coexpression of the NF-κB luc plasmid along with the TRAFs allows for the determination of whether the TRAFs activate or inhibit NF-κB activity. The 5x Gal4 promoter-driven plasmid (pG5-luc) has five Gal4 sites, allowing for the determination of transactivation potentials for Gal4-tagged proteins. However, the pG5-luc vector does not allow for the analysis of transcriptional repression. The 4x Gal4-minimal TK promoter-driven plasmid (pG4-mTK-luc) has four Gal4 sites in tandem with a minimal TK promoter, which provides a basal level of expression, and thus allows for the analysis of transcriptional repression. Abbreviations: (+) = activation, (-) = repression, 0 = no effect.
Chapter 3

RESULTS

3.1 TRAF2 and TRAF3 Inducibly Translocate into the Nucleus

In order to test my hypothesis that TRAF2 and 3 actively translocate into the nucleus, I first analyzed the subcompartmental localization of TRAF2 and 3 in mouse neuroblastoma cells (Neuro2a cells) following CD40L-stimulation. It has previously been shown that both TRAF2 and 3 are degraded following several hours of CD40L-stimulation (Moore & Bishop, 2005). In addition, if the TRAFs were to function as transcriptional regulators, as I hypothesized, then I would expect them to translocate into the nucleus relatively rapidly. For these reasons, I analyzed the subcompartmental localization of TRAF2 and 3 following 5, 10 and 30-minutes of CD40L-stimulation. Immediately following stimulation, the cells were subfractionated into cytoplasmic and nuclear extracts, and Western blots were performed. I found that both TRAF2 and 3 translocate into the nucleus as early as 5-minutes after CD40L-stimulation, the translocation peaked at 10-minutes, and decreased to control levels by 30-minutes of stimulation (Figure 9). Interestingly, this pattern of nuclear translocation differed from RelA, which did not increase within the nucleus until 30-minutes of CD40L-stimulation (Figure 9). Quantification of three independent experiments showed a statistically significant increase of both TRAF2 and 3 in the nucleus after 10-minutes of stimulation (Figure 10). These results clearly show nuclear translocation of TRAF2 and 3 following CD40L-stimulation, independently of RelA.
To determine whether my findings in Neuro2a cells were retained in primary cells, I repeated these experiments in cortical neurons isolated from mouse embryos (Figure 11). Following stimulation with CD40L, I found that TRAF2 translocates into the nucleus by 5-minutes of stimulation, decreasing by 10-minutes, while TRAF3 was present at constitutively high levels in the nucleus, and did not appear to be affected by CD40L-stimulation (Figure 11). Moreover, CD40L did not induce the nuclear translocation of RelA by 30-minutes of stimulation in primary cortical neurons (Figure 11), which is consistent with previous research that has suggested that NF-κB may not be activated in neurons (Massa et al., 2006). Overall, these results differed from those obtained with the Neuro2a cell line, with nuclear TRAF3 levels not changing following CD40L-stimulation. However, these results also showed that TRAF2 and 3 nuclear protein levels are different and that TRAF2 translocates into the nucleus following CD40L-stimulation in both Neuro2a cells and primary cortical neurons.

I next analyzed nuclear translocation of TRAF2 and 3 in a non-CNS cell-line, using pre-B lymphocytes (Daudi cells) as a model. My results in Daudi cells differed drastically from neuronal cell-types (Figure 12). Indeed, CD40L-stimulation induced a strong reduction in the levels of both TRAF2 and 3 in the nucleus by 5-minutes, and these levels remained low at longer stimulation time points. In contrast, CD40L-stimulation strongly induced the nuclear translocation of RelA in these cells by 5-minutes, which remained elevated by 30-minutes (Figure 12). These results demonstrate that the nuclear translocation of TRAF2 and 3 in response to CD40L-stimulation occurs in a cell-specific manner.
FIGURES

Figure 9. Analysis of the nuclear translocation of TRAF2 and TRAF3 in Neuro2a cells following CD40L-stimulation. Neuro2a cells were either untreated (control) or stimulated with mouse CD40L for 5, 10, or 30-minutes (100 ng/mL), cytoplasmic and nuclear extracts were then prepared, and a Western blot performed. TRAF2 runs as a doublet in Neuro2a cells, and both bands can be seen to change with equal intensities in all stimulation conditions. As controls for both the loading and the relative purity of the cytoplasmic and nuclear extracts, heat shock protein 60 (HSP60) was used as a cytoplasmic marker (Cheng et al., 1990), and Lamin A/C was used as a nuclear marker (Dagenais et al., 1984). C = control.
Figure 10. Quantification of the nuclear translocation of TRAF2 and TRAF3 in Neuro2a cells following CD40L-stimulation. The data represent the means of three independent experiments conducted as in Figure 9 ± SEM, normalized to Lamin A/C. In the case of TRAF2, both bands were used for quantification purposes. One way ANOVA was performed, followed by Tukey’s test. * \( p \leq 0.05 \).
Figure 11. Analysis of the nuclear translocation of TRAF2 and TRAF3 in primary cortical neurons following CD40L-stimulation. Mouse cortical neurons were either untreated (control) or stimulated with mouse CD40L for 5, 10, or 30-minutes (100 ng/mL), cytoplasmic and nuclear extracts were then prepared, and a Western blot performed. As controls for both the loading and the relative purity of the extracts, glucose transporter 3 (Glut3) was used as a cytoplasmic marker (Suzuki et al., 1999; Choeiri et al., 2002), and histone deacetylase 1 (HDAC1) was used as a nuclear marker (Hendzel et al., 1991). C = control.

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Figure 12. Analysis of the nuclear translocation of TRAF2 and TRAF3 in Daudi cells following CD40L-stimulation. Daudi cells were either untreated (control) or stimulated with human CD40L for 5, 10 or 30-minutes (100 ng/mL), cytoplasmic and nuclear extracts were then prepared, and a Western blot was performed. As controls for both the loading and the relative purity of the cytoplasmic and nuclear extracts, HSP60 and HDAC1 were used. C = control.
3.2 TRAF2 and TRAF3 Bind to Chromatin and the NF-κB Consensus Element

I next evaluated whether, following nuclear translocation, TRAF2 and 3 were able to bind to chromatin, and more specifically to the NF-κB consensus element. For this purpose, I first prepared chromatin extracts, and analyzed chromatin binding by Western blotting. I found that both TRAF2 and 3 were minimally bound to chromatin in unstimulated Neuro2a cells, with TRAF3 bound to a greater degree than TRAF2 (Figure 13). Surprisingly, CD40L induced the chromatin binding of both TRAF2 and 3 by 5-minutes of stimulation, reducing thereafter. These results show for the first time that TRAF2 and 3 constitutively and inducibly bind to chromatin, suggesting that the TRAFs may be direct regulators of transcriptional activity.

I next evaluated the possible binding of TRAF2 and 3 to the NF-κB consensus element by electromobility shift assay (EMSA). NF-κB DNA-binding activity was constitutively present in unstimulated Neuro2a nuclear extracts, in the form of four differentially-sized bands (Figure 14). The intensity of the upper three bands progressively increased following 5, 10, and 30-minutes of CD40L-stimulation. However, preincubation of control and 10-minute nuclear extracts with antibodies to either TRAF2 or 3 greatly attenuated their DNA-binding activity, with TRAF3 binding decreased to a greater degree than TRAF2. This suggests that TRAF3 is bound more strongly to the NF-κB consensus element, in agreement with the results from my chromatin binding assay. These results show that TRAF2 and 3 constitutively and inducibly bind to the NF-κB promoter element in a time-dependent manner following CD40L-stimulation. Furthermore,
both proteins are essential components of their respective DNA-binding complexes because blocking their interactions by antibody preincubation attenuated the DNA-binding activity.

To further demonstrate the inducible binding of TRAF2 and 3 to the NF-κB promoter element, I conducted an NF-κB promoter element oligoprecipitation assay. Similarly to the EMSA experiment, both TRAF2 and 3 bound to the NF-κB consensus element in unstimulated extracts (Figure 15). CD40L-stimulation induced the binding of both TRAF2 and 3 to the NF-κB consensus element in a time-dependent manner, with maximum binding after 30-minutes of stimulation. In addition, RelA bound to the NF-κB consensus element following 30-minutes of CD40L-stimulation, in direct correlation to the increased levels of RelA in CD40L-stimulated nuclear extracts (Figure 9), but to a lesser degree than both TRAF2 and 3. Collectively, these results support my EMSA analyses, and show that TRAF2 and 3 both constitutively and inducibly bind to the NF-κB promoter element.

Considering the differences between the nuclear translocation of TRAF2 and 3 between Neuro2a and Daudi cells, I next analyzed the chromatin binding of these proteins in Daudi cells. The levels of TRAF2 and 3 binding to chromatin correlated with their nuclear levels (compare Figure 16 to Figure 12). Particularly, both TRAF2 and 3 were constitutively bound to chromatin at high levels, and this binding greatly diminished following CD40L-stimulation. These results further show that the translocation and chromatin binding of TRAF2 and 3
may function in a cell specific manner, but also suggest that the TRAFs may have a role in regulating transcriptional activity in Daudi cells as well.
FIGURES

Figure 13. Analysis of the chromatin binding of TRAF2 and TRAF3 in Neuro2a cells following CD40L-stimulation. Neuro2a cells were either untreated (control) or stimulated with CD40L for 5, 10, or 30-minutes (100 ng/mL), chromatin extracts were then prepared, and a Western blot was performed. Histone H3 was used as a loading control, and remained constant in all four conditions. C = control.
Figure 14. EMSA analysis of NF-κB DNA-binding activity in Neuro2a extracts following CD40L-stimulation, and the effect of TRAF2 or 3 antibody preincubation on such activity. Neuro2a cells were either untreated (control) or stimulated with CD40L for 5, 10, or 30-minutes (100 ng/mL), nuclear extracts were then prepared, and an EMSA was performed. The extracts (5 µg) were then either untreated or preincubated with antibody to TRAF2 or 3, or with 100-fold excess of unlabelled NF-κB or NFAT consensus elements, as controls for the specificity of the NF-κB DNA-binding complexes. Incubation with a 100-fold excess of unlabelled NF-κB promoter element greatly attenuated the DNA-binding of all four bands, while incubation with an excess of unlabelled NFAT element had no effect on any of the four bands, demonstrating that all four bands were specific. Abbreviations: C = control, T2 = TRAF2, T3 = TRAF3, and Ab = antibody.
Figure 15. Oligoprecipitation analysis of the binding of TRAF2, TRAF3, and RelA to the NF-κB consensus element following CD40L-stimulation. Neuro2a cells were either untreated (control) or stimulated with CD40L for 5, 10, or 30-minutes (100 ng/mL), and nuclear extracts were prepared. The extracts (100 µg) were then incubated overnight with NF-κB-crosslinked agarose beads in DNA-binding buffer. The following day, the beads were collected and washed, and a Western blot was performed. C = control.
Figure 16. Analysis of the chromatin binding of TRAF2 and TRAF3 in Daudi cells following CD40L-stimulation. Daudi cells were either untreated (control) or stimulated with CD40L for 5, 10, or 30-minutes (100 ng/mL), chromatin extracts were then prepared, and a Western blot was performed. TRAF3 bound to chromatin in Daudi cells runs as a triplet, potentially due to alternative splicing or phosphorylation, although all three bands can be seen to change with equal intensities in all stimulation conditions. Histone H3 was used as a loading control, and remained constant in all four conditions. C = control.
3.3 TRAFs 1-7 Function as Co-Activators and Co-Repressors of Transcriptional Activity

Previous studies have indicated that overexpression of TRAF2 and 6 can induce NF-κB transcriptional activity (Rothe et al., 1995; Ishida et al., 1996; Min et al., 1998). Since I demonstrated that TRAF2 and 3 bind to chromatin, I then analyzed whether the TRAF proteins could activate transcription. First, I demonstrated that TRAF2 induces NF-κB transcriptional activity in Neuro2a (2.7-fold) and HEK 293 cells (2.2-fold) (Figure 17A), similarly to previous studies (Min et al., 1998). In these and subsequent experiments, HEK 293 cells were used in place of Daudi cells because the transfection of Daudi cells proved inconsistent. In consideration of the previous findings that TRAF2 functions as an E3-ubiquitin ligase, via the RING-finger domain, and that deletion of the RING-finger domain can attenuate its ability to induce transcriptional activity (Min et al., 1998; Habelhah, 2004), I repeated this experiment with an N-terminal deletion construct of TRAF2 (ΔN-TRAF2). In agreement with those studies, I also found that deletion of the RING-finger domain attenuates the ability of TRAF2 to induce NF-κB transcriptional activity (Figure 17A). However, deletion of the RING-finger domain did not completely abolish the induction of NF-κB transcriptional activity, suggesting that TRAF2 may induce transcriptional activity through additional domains, or that it may induce such activity through interaction with endogenous TRAF2.

In order to determine the direct transactivation potential of TRAF2, I next analyzed the transcriptional activity induced by TRAF2 coexpressed with a 5x Gal4 promoter-driven luciferase reporter plasmid. As a result, overexpression of
TRAF2 induced a 14.6-fold increase in luciferase activity in Neuro2a cells, and a 7.2-fold increase in HEK 293 cells (Figure 17B). In this experiment, deletion of the RING-finger domain completely abolished the transactivation potential of TRAF2 in both Neuro2a and HEK 293 cells (Figure 17B). These results suggest that TRAF2 can function as a transcription factor, although the level of transcriptional activity was relatively low in comparison to that expected for a typical transcription factor.

Based on the finding that TRAF2 functions weakly as a direct transcriptional activator, I further explored the possibility that the TRAFs could function as co-activators or co-repressors. In order to determine the co-activation/co-repression potential of the TRAFs, I coexpressed Gal4-tagged isoforms of TRAF1-7 along with a 4x Gal4-minimal TK promoter-driven luciferase reporter plasmid. This reporter plasmid maintains a basal level of transcriptional activity, allowing for the analysis of transcriptional repression (Chen & Evans, 1995; Sardi et al., 2006). Each of the TRAFs functions as a co-activator or co-repressor for the minimal TK promoter activity in both Neuro2a and HEK 293 cells, with the exception of TRAF4 and 5, which had no effect in either cell-type (Figure 18A). In Neuro2a cells, TRAF2, 6, and 7 were found to co-activate the minimal TK promoter activity, while TRAF1 and 3 were found to co-repress it. In HEK 293 cells, TRAF1, 2, 3, 6, and 7 all had a co-activator function. Surprisingly, while the majority of the TRAF proteins maintained similar co-activation or co-repression patterns between both Neuro2a and HEK 293 cells, TRAF1 and 3 had very different functions between the two cells-types,
suggesting that particular TRAF proteins may have cell-specific functions. In order to determine the role of the RING-finger domain in the co-activation activity of TRAF2 in both Neuro2a and HEK 293 cells, I next repeated the experiment with the ΔN-TRAF2 construct, and found that deletion of the RING-finger domain abolished the co-activation potential of TRAF2 in both cell-types (Figure 18B). Overall, these results show that TRAF1, 2, 3, 6, and 7 can function as co-activators or co-repressors in both Neuro2a and HEK 293 cells.

Since the TRAFs can function as co-activators and co-repressors, I evaluated whether coexpression of the TRAFs could have an effect on co-activating or co-repressing Gal4-tagged RelA-induced transcriptional activity (Figure 19A). In Neuro2a cells, TRAF2, 6, and 7 enhanced, while TRAF1, 4, and 5 repressed RelA-induced transcriptional activity. In HEK 293 cells, TRAF1, 2, 4, 5, 6 and 7 all repressed RelA-induced transcriptional activity. In both cell-types, TRAF3 had no effect on RelA-induced transcriptional activity. Additionally, deletion of the RING-finger domain from TRAF2 attenuated the co-activation or co-repression activity of TRAF2 for RelA-induced transcriptional activity in Neuro2a and HEK 293 cells, respectively (Figure 19B). Taken together, these results show that the TRAF proteins can function as co-activators or co-repressors of a known transcription factor, RelA, dependent upon the cell-type, and that their co-activation or co-repression potential may be due in part to the E3 ubiquitin-ligase activity of their RING-finger domains. Furthermore, the finding that TRAF4 and 5 functioned as co-repressors for RelA-induced transcriptional activity in both Neuro2a and HEK 293 cells, while neither had any
effect on minimal TK-induced activity, suggests that the transcriptional regulatory activity of the TRAFs is determined by the transcription factor itself in addition to the cell-type.
FIGURES

Figure 17. Analysis of the transcriptional regulatory potential of TRAF2 and the role of its RING-finger. 

A) NF-κB dual-luciferase assay performed on Neuro2a (white) and HEK 293 (gray) cell extracts, with the overexpression of the pBind vector, pBind-TRAF2 or pBind-ΔN-TRAF2. Neuro2a and HEK 293 cells were transfected either with the control pBind vector, or with pBind-TRAF2 or pBind-ΔN-TRAF2, together with the NF-κB promoter-driven luciferase reporter plasmid (1 µg each). After 24-hours, the cells were lysed and dual-luciferase assays were performed. 

B) 5x Gal4 promoter-driven dual luciferase assay performed on Neuro2a (white) and HEK 293 (gray) cell extracts, with the overexpression of the pBind vector, pBind-TRAF2 or pBind-ΔN-TRAF2, together with the 5x Gal4 promoter-driven luciferase reporter plasmid. Each of these experiments was repeated at least 3-times, and the data represent the mean ± SEM of at least four independent sample readings from one representative experiment, normalized to the pBind Renilla luciferase control readings. One way ANOVA was performed, followed by Tukey’s test. * ≤ 0.05; ** ≤ 0.001.
Figure 18. Analysis of the ability of TRAFs 1-7 to function as co-activators or co-repressors of the minimal TK promoter. A) 4x Gal4-minimal TK promoter-driven dual-luciferase assay performed on Neuro2a (white) and HEK 293 (gray) cell extracts, with the overexpression of the pBind vector or pBind-TRAF1-7, together with the 4x Gal4-minimal TK promoter-driven luciferase reporter plasmid. B) 4x Gal4-minimal TK promoter-driven dual-luciferase assay performed on Neuro2a (white) and HEK 293 (gray) cell extracts, with the overexpression of the pBind vector, pBind-TRAF2 or pBind-ΔN-TRAF2, together with the 4x Gal4-minimal TK promoter-driven luciferase reporter plasmid. Each of these experiments was repeated at least 3-times, and the data represent the mean ± SEM of at least four independent sample readings from one representative experiment, normalized to the pBind Renilla luciferase control readings. One way ANOVA was performed, followed by Tukey’s test. * ≤ 0.05; ** ≤ 0.001. Abbreviations: VC = vector control, T1 = TRAF1, T2 = TRAF2, T3 = TRAF3, T4 = TRAF4, T5 = TRAF5, T6 = TRAF6, and T7 = TRAF7.
Figure 19. Analysis of the ability of TRAFs 1-7 to function as co-activators or co-repressors of RelA-induced transcriptional activity.  

A) 5x Gal4 promoter-driven dual luciferase assay performed on Neuro2a (white) and HEK 293 (gray) cell extracts, with the overexpression of the pBind vector or pBind-RelA, in turn with either the pCMV-Tag2C vector or pCMV-Tag2B-TRAF1-7, together with the 5x Gal4 promoter-driven luciferase reporter plasmid.  

B) 5x Gal4 promoter-driven dual luciferase assay performed on Neuro2a (white) and HEK 293 (gray) cell extracts overexpressed with pBind RelA, in turn coexpressed with the pCMV-Tag2B vector, pCMV-Tag2B-TRAF2 or pCMV-Tag2B-ΔN-TRAF2.  

Each of these experiments was repeated at least 3-times, and the data represent the mean ± SEM of at least four independent sample readings from one representative experiment, normalized to the pBind Renilla luciferase control readings. One way ANOVA was performed, followed by Tukey’s test.  * ≤ 0.05; ** ≤ 0.001.  Abbreviations: VC = vector control, T1 = TRAF1, T2 = TRAF2, T3 = TRAF3, T4 = TRAF4, T5 = TRAF5, T6 = TRAF6, and T7 = TRAF7.
Chapter 4

GENERAL DISCUSSION

Nuclear Translocation of the TRAFs

Previous research has focused exclusively on the cytoplasmic role of the TRAF proteins proximal to the TNFR superfamily. However, several studies have found particular TRAFs to be localized within the nucleus of certain cell-types, such as Purkinje neurons, and others have shown various domain-deletion isoforms to have alternate subcellular distributions (see Chapter 1 for discussion) (Krajewski et al., 1997; Min et al., 1998; Arch et al., 2000; Glauner et al., 2002; Morita et al., 2005). In Chapter 3.1, my goal was to determine whether TRAF2 and 3 are present within the nucleus, as well as whether stimulation with CD40L could induce their nuclear translocation. In Neuro2a cells, I found that 5 to 10-minutes of CD40L-stimulation induced the nuclear translocation of both TRAF2 and 3, while RelA, a known effector that is rapidly induced downstream of the CD40 receptor, did not translocate until 30-minutes of stimulation. These findings are novel, and clearly show, for the first time, that the TRAFs have the capacity to translocate into the nucleus following cytokine stimulation.

Because the use of cell lines can sometimes lead to aberrant results that may not be physiologically relevant, I next analyzed whether the TRAFs inducibly translocate into the nucleus of primary cortical neurons. I found that 5-minutes of CD40L-stimulation induced the nuclear translocation of TRAF2, while TRAF3 was present in the nucleus at high levels, but was not affected by CD40L. Additionally, RelA was not induced to translocate by CD40L-stimulation. These
results support my findings in Neuro2a cells, showing that both TRAF2 and 3 were present within the nucleus at different levels, and that TRAF2 was induced to translocate following 5-minutes of CD40L-stimulation. However, they differed in that TRAF3 was not affected by CD40L. I hypothesize that this discrepancy is most likely due to the particular nature of the two different cell-types. In fact, TRAF3 nuclear translocation appears to be constitutively activated in cortical neurons.

Finally, I sought to determine whether there were any differences between my results in neuronal cell cultures and a non-CNS cell line, using Daudi cells as a model. I found that both TRAF2 and 3 were present in the nucleus at high levels in unstimulated conditions, and that both were rapidly induced to degrade following 5-minutes of CD40L-stimulation. Conversely, RelA was strongly induced to translocate into the nucleus by 5-minutes of stimulation. These findings are very interesting because they show that the TRAFs indeed function in a cell-specific manner, with very marked differences between neuronal cells and immune cells.

These differences are significant because most of the models for intracellular signaling pathways have been devised from data accumulated in immune cells, and all research in, as well as all therapies for, systems outside of immune cells are potentially based upon erroneous data. In this case, based upon previous studies in immune cells, one would preliminarily conclude that the TRAFs do not translocate into the nucleus, which would thus preclude analysis of the DNA-binding activity or the transcriptional regulatory potential of the TRAFs
in such cells. For this reason, these data also suggest that the models for each of the intracellular signaling pathways may only be appropriate for the individual cell-types or tissues in which they were discovered, and additional research will need to be conducted in order to elucidate the pathways in additional cell-types.

Overall, these findings are novel and show for the first time that the TRAF proteins can inducibly translocate into the nucleus following cytokine stimulation. Additionally, they show that the mechanism for inducible translocation of the TRAFs is extremely rapid, and functions in a cell-specific manner. Given the numerous pathways and functions that the TNFRs have been, and continue to be, found to regulate, the possibility that the TRAFs may be rapidly induced to translocate into, and function within, the nucleus is very interesting. Future research into the nuclear functions of the TRAFs, and their specific target proteins within the nucleus, will open the door to further elucidating how the TRAFs orchestrate all activities downstream of the TNFR superfamily.

**TRAF DNA-Binding Activity**

In Chapter 3.2, my goal was to determine whether TRAF2 and 3 bind to chromatin and the NF-κB consensus element. In Neuro2a cells, I found by chromatin binding assay that both TRAF2 and 3 are constitutively bound to chromatin, and that their binding is further induced maximally by 5-minutes of CD40L-stimulation. By NF-κB EMSA analysis, I found that NF-κB DNA-binding activity is constitutively present, and that CD40L induced NF-κB DNA-binding activity in a time-dependent manner. I also found that preincubation of samples
from control or 10-minute stimulation conditions with antibodies to either TRAF2 or 3 greatly attenuated such DNA-binding activity. This suggests that both TRAF2 and 3 are crucial components of their respective DNA-binding complexes, as eliminating their binding abolished the DNA-binding activity of their respective complexes. Finally, by NF-κB oligoprecipitation assay, I found that TRAF2 and 3 were both constitutively bound to the NF-κB consensus element, and that CD40L-stimulation induced their binding in a time-course dependent manner, similar to, and confirming my, EMSA analysis. Conversely, RelA binding was not induced until 30-minutes of CD40L-stimulation, similar to my results for nuclear translocation, which independently confirms both the specificity of the TRAF binding and the independence of the mechanism for the nuclear translocation of the TRAFs from the machinery responsible for RelA translocation.

These findings are interesting because they show, for the first time, that the TRAFs have the capacity to bind to chromatin and, more specifically, to the NF-κB consensus element. Additionally, these findings suggest that the TRAFs may function as transcriptional regulators, possibly explaining how the TRAFs orchestrate the numerous functions that continue to be attributed to the TNFR superfamily.

Because I found such striking differences between the nuclear translocation of TRAF2 and 3 between neuronal cell cultures and Daudi cells, this prompted me to analyze whether the TRAFs have the capacity to bind to chromatin in Daudi cells following CD40L-stimulation, as well as any differences
there may be in such binding. I found by chromatin binding assay that both TRAF2 and 3 are constitutively bound to chromatin at high levels in unstimulated conditions, but that both are rapidly induced to degrade by 5-minutes of CD40L-stimulation, similarly to the results for their nuclear translocation. This finding is interesting because, with both proteins constitutively bound to chromatin in Daudi cells, it suggests that they may have a nuclear function in Daudi cells as well. It also provides additional evidence that the TRAFs function in a cell-specific manner. I cannot exclude, however, that in Daudi cells other TRAFs may translocate into the nucleus following CD40L-stimulation, or that stimulation with cytokines other than CD40L may have other effects.

**TRAF2 Can Function as a Direct Transcriptional Activator**

In Chapter 3.3, I sought to determine whether TRAF2 has the potential to function as a direct transcriptional activator. First, I repeated the previous finding that TRAF2 induces NF-κB transcriptional activation, and found that TRAF2 did in fact induce NF-κB transcriptional activity in both Neuro2a and HEK 293 cells, similar to the previous studies. In order to determine whether TRAF2 has the capacity to function as a direct transcriptional activator, I next analyzed the transcription activation potential of TRAF2 by 5x Gal4 promoter-driven luciferase assay. I found that TRAF2 has the capacity to function as a weak transcriptional activator in both Neuro2a and HEK 293 cells. This finding suggests that the induction of NF-κB transcriptional activity by TRAF2 may be due, at least in part, to the direct induction of transcriptional activity by TRAF2.
This finding is interesting because researchers have thus far been unsuccessful in determining how exactly the TRAF proteins regulate transcriptional activity. As none of the existing literature details a direct analysis of the potential ability of the TRAFs to directly induce such transcriptional activity, I can only assume that such analysis has not been conducted, which may explain why the mechanisms by which the TRAFs orchestrate all activities downstream of the TNFR superfamily has remained thus far elusive. However, the direct transcription activation potential that I found for TRAF2 was relatively weak when compared to known transcription factors, which suggests that the TRAFs may function as transcriptional co-activators or co-repressors.

In these and subsequent studies, I used Neuro2a cells and HEK 293 cells for comparison of different cell-types. As my previous analyses were conducted between Neuro2a and Daudi cells, I would have liked to continue using Daudi cells for these studies, however I found the transfection of Daudi cells to be very inefficient if it worked at all, thus resulting in widely variable data. Conversely, HEK 293 cells are among the most easily transfected cell-types, and are among the most commonly used cell-types for this very reason.

TRAFs 1, 2, 3, 6, and 7 Can Function as Transcriptional Co-Activators and Co-Repressors for the Minimal TK Promoter

I next sought to determine whether the TRAFs have the potential to function as co-activators or co-repressors. In Chapter 3.3, I analyzed the co-activation or co-repression potentials of the TRAFs by 4x Gal4-minimal TK promoter-driven luciferase assay. This assay is useful because the minimal TK
promoter provides a basal level of transcriptional activity, allowing for the additional analysis of repression (Chen & Evans, 1995; Sardi et al., 2006). Using this assay, I found that each of the TRAFs, with the exception of TRAF4 and 5, has the capacity to function as a co-activator or co-repressor. In Neuro2a cells, I found that TRAF2, 6 and 7 were co-activators of the minimal TK promoter, while TRAF1 and 3 were co-repressors. And, in HEK 293 cells, I found that TRAF1, 2, 3, 6, and 7 were all co-activators of the minimal TK promoter.

These findings are novel, and show, for the first time, that each of the TRAFs, with the exception of TRAF4 and 5, has the capacity to function directly as a transcriptional regulator, either as a co-activator or co-repressor. Interestingly, these data suggest that the co-activator or co-repressor potential of the specific TRAFs is not absolute, and depends on the intracellular milieu that the particular TRAFs are in, as TRAF1 and 3 were found to be co-repressors in Neuro2a cells and co-activators in HEK 293 cells. These differences in functions are most likely due to the different proteins and pathways that are present and activated in the different cell-types, which may affect the TRAFs either directly by altering their activation states through posttranslational modification, or indirectly by altering the presence and/or activation of potential cofactors. These findings further demonstrate that the TRAFs function in a cell-specific manner.

**TRAFs 1, 2, 4, 5, 6, and 7 Can Function as Co-Activators and Co-Repressors for RelA-Induced Transcriptional Activity**

Since I found that each of the TRAFs, with the exception of TRAF4 and 5, functioned as a transcriptional co-activator or co-repressor for the minimal TK
promoter, I next sought to determine whether they could function as co-activators or co-repressors for a known transcription factor, RelA. In Chapter 3.3, I analyzed the ability of the TRAFs to function as co-activators or co-repressors for RelA by 5x Gal4 promoter-driven luciferase assay, coexpressing the various TRAFs along with Gal4-tagged RelA. In Neuro2a cells, I found that TRAF2, 6, and 7 enhanced, while TRAF1, 4 and 5 repressed RelA-induced transcriptional activity. In HEK 293 cells, I found that TRAF1, 2, 4, 5, 6 and 7 all repressed RelA-induced transcriptional activity. In both cell-types, TRAF3 had no effect on RelA-induced transcriptional activity.

These findings are interesting because they demonstrate the ability of the TRAFs to function as co-activators or co-repressors by showing that they do in fact co-activate or co-repress RelA-induced transcriptional activity. Interestingly, in addition to the previously noted differences between the functions of particular TRAFs in different cell-types, these data also show that the functions of the TRAFs depend on the target protein that they are acting upon. Indeed, TRAF2 functions as a co-activator for the minimal TK promoter activity in HEK 293 cells, but as a co-repressor for RelA-induced activity in the same cell-type. Additionally, neither TRAF4 nor 5 functioned as a co-activator or co-repressor of the minimal TK promoter activity in either Neuro2a or HEK 293 cells, while both functioned as co-repressors for RelA-induced transcriptional activity in both cell-types. Finally, a clearer example of this is the finding that TRAF3 functions as co-repressor of the minimal TK promoter activity in Neuro2a cells, and as a co-
activator in HEK 293 cells, without having any effect on RelA-induced transcriptional activity in either cell-type.

In this manner, the TRAFs potentially function similarly to the small nuclear RING-finger (SNURF) protein. SNURF was initially identified as a co-activator for steroid receptor transcriptional activity, but was later found to function as a co-repressor for transcriptional activity when associated with a novel POZ (BTB)- and AT-hook-containing zinc-finger protein (PATZ), PATZ1, both functions dependent upon the presence of its E3 ubiquitin ligase activity (Häkli et al., 2004). Therefore, whether the TRAFs function as co-activators or co-repressors is most likely determined by the internal milieu of each individual cell-type, as defined by the specific proteins and cofactors that are present, as well as the target proteins that are being regulated. In order to elucidate this, future research will need to determine which proteins regulate the specific functions of each individual TRAF protein.

The RING-Finger Domain of TRAF2 Regulates its Transcriptional Regulatory Potential

Previous research has indicated that elimination of the RING-finger domain from TRAF2 attenuates its ability to function as a transcriptional activator (Min et al., 1998; Habelhah, 2004). For this reason, in Chapter 3.3 I also sought to determine what effect elimination of the RING-finger domain from TRAF2 would have on its transcriptional regulatory potential. By using an NF-κB promoter-driven luciferase assay, I found that deletion of the RING-finger domain attenuated its ability to induce NF-κB transcriptional activity in both Neuro2a and
HEK 293 cells. By using a 5x Gal4 promoter-driven luciferase assay, I found that elimination of the RING-finger domain completely abolished its ability to function as a direct transcriptional activator in both Neuro2a and HEK 293 cells. By using a 4x Gal4-minimal TK promoter-driven luciferase assay, I found that elimination of the RING-finger domain abolished its ability to function as a co-activator for the minimal TK promoter in both Neuro2a and HEK 293 cells. Finally, by using a 5x Gal4 promoter-driven luciferase assay, coexpressing the RING-finger domain deletion isoform of TRAF2 along with Gal4-tagged RelA, I found that eliminating the RING-finger domain attenuated its ability to co-activate or co-repress RelA-induced transcriptional activity in Neuro2a and HEK 293 cells, respectively.

These findings are interesting because they show that the RING-finger domain is greatly responsible for conferring transcriptional regulatory potential to TRAF2, whether it is functioning as a co-activator, co-repressor, or even as a transcription factor. Additionally, as previous research has indicated that deletion of the N-terminal RING-finger domain abolishes or greatly attenuates the E3 ubiquitin-ligase activity of TRAF2 (Habelhah et al., 2004), these findings suggest that the E3-ubiquitin ligase activity of TRAF2 may be required for its ability to function as a transcriptional regulator.

Interestingly, an ever increasing number of ubiquitin ligases have been found to function within the nucleus (Natoli & Chiocca, 2008). These proteins are generally localized within intranuclear promyolytic leukemia (PML) bodies, and function as co-activators or co-repressors, regulating transcription factors either by enhancing their activation or inducing their degradation (Daniels et al., 2004;
Chinnadurai, 2007; Tanaka et al., 2007). In this case, it is possible that the TRAFs may function to regulate transcriptional activity in a similar manner, as their rapid regulation of transcriptional activation could have a wide-ranging, direct regulatory effect that would explain the number of pathways and functions that the various TRAFs have been found to regulate. Additionally, since both TRAF2 and 6 have been found to catalyze Lys-63-linked polyubiquitination (Pineda et al., 2007), this suggests that the TRAFs may also directly activate proteins, rather than solely signaling proteins for degradation or proteasomal processing. However, in several cases, the transcriptional regulatory capacity of TRAF2 was not entirely abolished by deletion of the RING-finger, and TRAF1 has the capacity to function as a co-repressor without having a RING-finger domain. Therefore, this suggests that additional domains must have transcriptional regulatory capacity, and will require additional research in order to parse out such functions.

The Significance of the TRAFs Functioning as Transcriptional Co-Activators and Co-Repressors

As previously mentioned, the TRAFs are believed to orchestrate all activities downstream of TNFR activation. Given the wide range of functions that are regulated by the TNFR superfamily, and the fact that their mechanism of regulation, as well as the exact functions of the TRAFs, have remained elusive, my discovery that the TRAFs have the capacity to function as immediate-early transcriptional regulators is highly significant. In particular, my data suggest that activation of specific TNFR family members may result in the rapid nuclear
translocation of, and subsequent transcriptional regulation by, the specific combinations of TRAFs that interact with each individual TNFR. This could have a direct global effect on numerous cellular activities and functions, which, without knowledge of the ability for the TRAFs to function in such a manner, would continue to remain elusive. Additionally, without the ability for researchers and pharmacologists to directly link the TNFRs to their downstream signaling activities, it would be nearly impossible for them to accurately determine the causes of associated disorders or the appropriate pharmacological targets for the treatment of such disorders. Presently, given the novelty of my findings, it is not yet known whether dysfunctions in TRAF signaling may actually be the cause of disorders, such as Alzheimer's disease, Parkinson's disease, Huntington's chorea, etc. And, as the root causes of such disorders have remained elusive, for over 100-years in the case of Alzheimer's disease (Alzheimer, 1907; Stelzmann et al., 1995), it is very likely that their underlying cause may very well rest in the dysfunction of a previously or as yet unidentified transcriptional regulatory system, such as the one that I have presently identified. Future research into the genes that may be regulated by specific combinations of the TRAFs will help to identify whether the TRAFs may be important in the regulation of these and other disorders. Along these lines, I have initiated a chromatin immunoprecipitation on mouse genome promoter microarray (ChIP on Chip) assay, in order to determine exactly which gene promoters the TRAFs both constitutively and inducibly interact with. I am hopeful that the findings of this
study will provide important insights into the genes and pathways that are regulated by TRAF transcriptional regulation.

Another interesting possibility that I have considered is that the TRAFs may function, at least in part, to determine which of their associated promoters are activated upon cytokine signaling. In the case of the NF-κB DNA-binding sites, at least one copy can be found within nearly every promoter region in the mammalian genome. However, upon activation of the NF-κB signaling pathways, only a select subset of the NF-κB DNA-binding sites are actually activated, dependent upon the upstream receptor that is activated (Wan et al., 2007). Because there are only a select few combinations of the NF-κB transcription factors, other researchers and I have hypothesized that there must be additional factors that determine which specific promoters the NF-κB transcription factor complexes interact with. Given the large possible number of specific homo- and heterotrimeric combinations of the seven TRAFs, and their ability to both rapidly translocate into the nucleus and bind to both chromatin and the NF-κB consensus element, it is very possible that they may be the additional factors that function to select which gene promoters are activated. If so, then the ChIP on Chip analyses, in combination with analyses of the genes that are activated downstream of specific TNFRs, should also help to delineate such a function.

If the transcriptional regulatory potential of the specific homo- and heterotrimeric combinations of the seven TRAFs can be pinpointed to the regulation of specific genes or disorders, then pharmacological targeting of those
specific TRAF complexes may allow for the highly specific inhibition of specific cellular functions, without compromising overall receptor signaling, transcription factor pathways, or cellular systems. Therefore, I believe that these findings may open the door to a revolutionary paradigm shift in the treatment of human disease.
References


