TRADD MEDIATES INFLAMMATORY RESPONSES IN THE CYTOPLASM AND TUMOR SUPPRESSION IN THE NUCLEUS

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Medical Biophysics
University of Toronto

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Thesis Abstract

**TRADD mediates inflammatory responses in the cytoplasm and tumor suppression in the nucleus**

Iok In Christine Chio, Doctor of Philosophy 2012, Department of Medical Biophysics, University of Toronto

TNF is a proinflammatory cytokine whose pleiotropic biological properties are signaled through the receptor TNFR1. Activation of this signaling pathway has been implicated in a broad range of biological functions, including host defense, inflammation, apoptosis, autoimmunity, and cancer. TRADD is an adaptor protein that is recruited to TNFR1 upon receptor engagement. Using a Tradd-deficient murine model, we demonstrated that TRADD is essential for both TNF-mediated apoptosis and inflammatory responses. In addition to refining the role of TRADD in TNFR1 signaling, we have also identified a novel function of TRADD in TLR3 and TLR4 pathways, which are key drivers of the innate immune response. We showed that TRADD is involved in NF-κB activation upon TLR3 and TLR4 stimulation, and Tradd-deficient macrophages showed impaired inflammatory cytokine production in response to TLR ligands *in vitro*. These data reveal the multifaceted functions of TRADD in immune signaling pathways.

Beyond its role in the immune response, TNF has also been shown to play a crucial, cell-non-autonomous role in driving tumor growth in various models of cancer. We initially sought to determine whether TRADD is essential for this aspect of TNF function by employing the use of a chemical induced skin carcinogenesis model in which the tumor-promoting role of TNF is very well established. In this model, H-Ras is the major driving oncogene. We found that Tradd deficiency accelerated tumor formation in mouse skin, in
strong contrast to what was observed in Tnfr1-deficient mice. Further in vitro analyses revealed that upon expression of oncogenic H-Ras, Tradd-deficient murine fibroblasts displayed both reduced cell cycle arrest and repression of Ras induced cellular senescence. Importantly, the level of p19Arf induced by H-Ras expression was reduced in Tradd-deficient fibroblasts in a post-translational manner. Our biochemical evidence suggests that TRADD can shuttle dynamically between the cytoplasm and the nucleus; in doing so, nuclear TRADD interacts with ULF, a newly identified E3 ubiquitin ligase for p19Arf. Interaction between nuclear TRADD and ULF sequesters ULF away from p19Arf, leading to p19Arf stabilization and tumor suppression. Together, these data demonstrate the functional diversity of TRADD in different compartments of the cell.
For my parents, Lan Sio Lei and Peng Sek Chio
Acknowledgements

I feel greatly indebted to my PhD supervisor, Dr. Tak Mak, for giving me the opportunity to complete my graduate studies in his laboratory. During the five years I have spent in his laboratory, I have acquired not only solid scientific knowledge and skills but also the right attitude to do science. Tak constantly reminds us that if curiosity is the basis of science, then open-mindedness is the recipe for success. I would like to thank Tak, for making my graduate experience truly memorable and exceptionally fruitful. I will not be who I am without his guidance. In this regard, I would also like to thank my supervisory committee members, Dr. Benjamin Neel and Dr. Rama Khokha, for their scientific guidance and support throughout my graduate studies. I must also thank Dr. Andrew Wilde and Dr. Lloyd Berger, my undergraduate lab mentors, for they have brought me into science and have initiated my love for this fascinating endeavor.

In addition to an enlightening supervisor, I was also fortunate enough to have encountered lots of friends and support from the Mak lab. In particular, I would like to thank Dr. Nien Jung Chen, for being a very patient mentor to me during the early stages of my graduate studies. Without him, immunology would still have been a bunch of unintelligible dot-plots to me. Above all, I must thank him for inviting me on board the exciting quest to analyze TRADD. His generosity has given me a challenging and highly rewarding graduate experience.

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<th>Description</th>
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<tbody>
<tr>
<td>18S rRNA</td>
<td>18S ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>2-ME</td>
<td>2-Mercaptoethanol</td>
</tr>
<tr>
<td>7AAD</td>
<td>7-amino-actinomycin D</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ACK</td>
<td>Ammonium chloride potassium hydrocarbonate</td>
</tr>
<tr>
<td>AICD</td>
<td>Activation induced cell death</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
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<tr>
<td>APAF1</td>
<td>Apoptotic protease activating factor-1</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>Apo</td>
<td>Apoptosis</td>
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<tr>
<td>ARC</td>
<td>Apoptosis repressor with CARD</td>
</tr>
<tr>
<td>ARE</td>
<td>Adenine and uridine rich element</td>
</tr>
<tr>
<td>ARF</td>
<td>Alternative reading frame</td>
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<tr>
<td>Arfbp</td>
<td>ARF-binding protein</td>
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<tr>
<td>ASK1</td>
<td>Apoptosis signal-regulating kinase 1</td>
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<tr>
<td>ATCC</td>
<td>American type culture collection</td>
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<tr>
<td>BAFF</td>
<td>B cell activating factor</td>
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<td>BCL</td>
<td>B cell lymphoma</td>
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<td>BMDM</td>
<td>Bone marrow derived macrophage</td>
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<tr>
<td>bp</td>
<td>Basepairs</td>
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<td>BrdU</td>
<td>5'-bromo-2'-deoxyuridine</td>
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<tr>
<td>c-IAP</td>
<td>Cellular inhibitor of apoptosis protein</td>
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<td>CARD</td>
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<td>CR1</td>
<td>Complement receptor 1</td>
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<td>CRD</td>
<td>Cysteine rich domain</td>
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<tr>
<td>CRE</td>
<td>Causes recombination</td>
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<td>CREBBP</td>
<td>Cyclic AMP-response element binding protein</td>
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<tr>
<td>CrmA</td>
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<tr>
<td>CT</td>
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<td>Carboxyl-terminal activating regions</td>
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<td>CTCF</td>
<td>CCCTC-binding factor</td>
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<td>CV</td>
<td>Crystal violet</td>
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<td>Cyt</td>
<td>Cytoplasmic</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<td>Decoy receptor</td>
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<tr>
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DED  Death effector domain
DMBA  7,12-dimethylbenz[α]anthracene
DMEM  Dulbecco’s Modified Eagle medium
DMSO  Dimethyl sulfoxide
DN  Dominant negative
DNA  Deoxyribonucleic acid
DNase  Deoxyribonuclease
DOC  Deoxycholate
DR  Death receptor
dsRNA  Double stranded RNA
EBV  Epstein Barr virus
EDAR  Anhidrotic ectodysplasin receptor
EDTA  Ethylenediaminetetraacetic acid
ELISA  Enzyme-linked immunosorbent assay
EMSA  Electrophoretic mobility shift assay
Epi  Epidermis
ERK  Extracellular signal-regulated kinase
ES  Embryonic stem
FADD  Fas associated via death domain
Fas  TNF superfamily receptor 6
Fc  Fragment, crystallizable
FCS  Fetal calf serum
FDC  Follicular dendritic cells
FP  Flanking probe
GalN  D-galactosamine
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
GAS  Interferon-gamma activated sequence
GC  Germinal centre
GFP  Green fluorescent protein
GUK  Guanylate kinase like
H-Ras  Harvey-rat sarcoma
H&E  Hematoxylin and eosin
HA  Hemagglutinin
HCl  Hydrochloric acid
HeLa  Henrietta Lacks (cervical cancer cell line)
HEPES  4-(2-hydroxyethyl)-1-piperazinediethanesulfonic acid
HIPK  Homeodomain-interacting protein kinase
hrs  Hours
HY-TCR  Male specific T-cell receptor
ICD  Intracellular domain
IFN  Interferon
IgG  Immunoglobulin G
IgM  Immunoglobulin M
IKK  IkB kinase
IL  Interleukin
INK4  Inhibitor of CDK4
<table>
<thead>
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<td>IP</td>
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</tr>
<tr>
<td>IRAK</td>
<td>Interleukin-1 receptor-associated kinase</td>
</tr>
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<td>IRF</td>
<td>Interferon regulatory factor</td>
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<tr>
<td>IkB</td>
<td>Inhibitor of kappa B</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N terminal kinase</td>
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<td>K₃Fe[CN]₆</td>
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<tr>
<td>KO</td>
<td>Knockout</td>
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<td>Nacht, leucine-rich repeat and pyrin domain containing protein</td>
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<tr>
<td>NP40</td>
<td>Nonyl phenoxypolyethoxylethanol 40</td>
</tr>
<tr>
<td>NPM</td>
<td>Nucleophosmin</td>
</tr>
<tr>
<td>NT</td>
<td>Amino terminal</td>
</tr>
<tr>
<td>Nuc</td>
<td>Nuclear</td>
</tr>
<tr>
<td>O.C.T.</td>
<td>Optimal cutting temperature</td>
</tr>
<tr>
<td>OIS</td>
<td>Oncogene induced senescence</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIDD</td>
<td>p53-induced protein with a death domain</td>
</tr>
<tr>
<td>PLAD</td>
<td>Pre-ligand-binding assembly domain</td>
</tr>
<tr>
<td>PML</td>
<td>Promyelocytic leukemia</td>
</tr>
<tr>
<td>PNA</td>
<td>Peanut agglutinin</td>
</tr>
<tr>
<td>Poly(I:C)</td>
<td>Polyinosinic:polycytidylic acid</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>PYD</td>
<td>Pyrin domain</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcription PCR</td>
</tr>
<tr>
<td>Raf</td>
<td>Rapidly accelerated fibrosarcoma</td>
</tr>
<tr>
<td>RAIDD</td>
<td>RIP associated Ich-1/CED homologous protein with death domain</td>
</tr>
<tr>
<td>RelA</td>
<td>v-rel reticuloendotheliosis viral oncogene homolog A</td>
</tr>
<tr>
<td>RIG</td>
<td>Retinoic acid inducible gene</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor interacting protein</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RNAi</td>
<td>Ribonucleic acid interference</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SA-β-Gal</td>
<td>Senescence associated-beta-galactosidase</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SODD</td>
<td>Silencer of death domain</td>
</tr>
<tr>
<td>SRBC</td>
<td>Sheep Red blood cells</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TAK1</td>
<td>Transforming growth factor β-activated kinase 1</td>
</tr>
<tr>
<td>T_H</td>
<td>Helper-T-cells</td>
</tr>
<tr>
<td>TIM</td>
<td>TRAF interacting motifs</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/interleukin-1 receptor-like domain</td>
</tr>
<tr>
<td>TK</td>
<td>Thymidine kinase</td>
</tr>
<tr>
<td>TL1a</td>
<td>TNF-like ligand 1a</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TNE</td>
<td>100 mM Tris; 0.2 M NaCl; 10 mM EDTA</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol 13-acetate</td>
</tr>
<tr>
<td>TRADD</td>
<td>Tumor necrosis factor receptor associated death domain</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumor necrosis factor associated factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumor necrosis factor related apoptosis inducing ligand</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-beta</td>
</tr>
<tr>
<td>TRIP12</td>
<td>Thyroid receptor interacting protein 12</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>TWEAK</td>
<td>TNF-like weak inducer of apoptosis</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>UFD</td>
<td>Ubiquitin fusion degradation</td>
</tr>
<tr>
<td>ULF</td>
<td>Ubiquitin ligase of ARF</td>
</tr>
<tr>
<td>UPA</td>
<td>Urokinase plasminogen activator</td>
</tr>
<tr>
<td>USF</td>
<td>Upstream transcription factor</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGI</td>
<td>Vascular endothelial growth factor inhibitor</td>
</tr>
<tr>
<td>WCL</td>
<td>Whole cell lysate</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside</td>
</tr>
</tbody>
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CHAPTER 1

INTRODUCTION
1.1 Tumor necrosis factor and tumor necrosis factor receptor superfamily

1.1.1 The players

Four decades ago, lymphotoxin (LT) and tumor necrosis factor (TNF) were identified as products of lymphocytes and macrophages that caused the lysis of certain types of cells, especially tumor cells (Carswell et al., 1975; Granger et al., 1969). The determination of the amino acid sequence (Gray et al., 1984; Pennica et al., 1984) and the cloning of the cDNAs for LT and TNF (Aggarwal et al., 1985a; Aggarwal et al., 1985b; Aggarwal et al., 1984) indicated that the two proteins were homologous, with 30% amino acid identity. The binding of TNF to its receptor and its displacement by LT further confirmed the functional homology between the two proteins (Aggarwal et al., 1985b). This was perhaps the earliest indication of the existence of a potential TNF superfamily. Soon after, mouse TNF was independently discovered as a factor that mediated lipopolysaccharide (LPS)-induced wasting (cachexia) in mice by Beutler et al. (Beutler et al., 1985), and as a myeloid differentiation factor by Takeda and co-workers (Takeda et al., 1986). These two cytokines laid the foundation for the isolation and identification of the larger family of cytokines, now known as the TNF superfamily. Not surprisingly, the receptors for these proteins also constitute a TNF receptor (TNFR)-related gene superfamily.

At present, at least 19 different ligands have been identified that belong to the TNF superfamily. The 19 ligands mediate their cellular response through 29 receptors that belong to the TNFR superfamily, and they are characterized by the presence of varying numbers (three to six) of cysteine-rich repeats in their extracellular domains (Smith et al., 1994). Based upon their cytoplasmic sequences and signaling properties, these TNF receptors can be classified into three major groups (Locksley et al., 2001). The first group, also called death receptors, including CD95 (Fas), TNFR1, death receptor (DR)-3, tumor necrosis factor
related apoptosis inducing ligand (TRAIL)-R1 (DR4), TRAIL-R2 (DR5), and DR6, contains a death domain (DD) in the cytoplasmic tail, activation of which leads to induction of apoptosis. The second group of receptors includes TNFR2, CD40, B cell activating factor (BAFF)-R, Ox40 (CD134) and others, all of which contain one or more TNF receptor associated factors (TRAF)-interacting motifs (TIMs) in their cytoplasmic tails, which is involved in activation of multiple signal transduction pathways involved in survival responses. The third group of TNF receptor family members, including TRAIL-R3, TRAIL-R4, decoy (Dc)-R3 and osteoprotegerin (OPG), does not contain functional intracellular signaling domains or motifs. Although this group of receptors cannot provide intracellular signaling, they can effectively compete with the other two signaling groups of receptors for their corresponding ligands. These “decoy receptors” therefore function by impeding the activation of signal transduction pathways by other TNF receptors. The ligands that activate the above TNF receptor family members are mostly known (Smith et al., 1994) and are listed in Figure 1.1.
Figure 1.1 Tumor necrosis factor and tumor necrosis factor receptor superfamily proteins.
Figure 1.1 TNF and TNFR superfamily proteins (Modified from Locksley et al. (Locksley et al., 2001)).

TNFR- and TNF-related proteins are shown on the left and right of the figure, respectively, with arrows connecting ligand-receptor pairs. Cysteine rich domains (CRDs) are shown as blue ovals and red boxes mark the locations of DD-related sequences in the cytoplasmic regions of the TNFR-related proteins. All ligands, except LTα and VEGI, which are secreted, are type II transmembrane proteins with a carboxyl-terminal extracellular domain, an amino-terminal intracellular domain and a single transmembrane domain. The C-terminal extracellular domain, known as the TNF homology domain, has 20–30% amino-acid identity between the superfamily members and is responsible for binding to the receptor. TRAIL and CD95L have the highest homology among the TNF-superfamily members, in parallel with their common ability to induce apoptosis. Most members of the TNF superfamily are released from the cell surface by proteolysis through distinct proteases (furin, metalloproteinase or matrilysin). Unlike the ligands, the TNFRs are characterized as type I transmembrane proteins (extracellular N-terminus and intracellular C-terminus). Because they lack a signal peptide sequence, B cell maturation antigen (BCMA), transmembrane activator and cyclophilin ligand interactor (TACI), BAFF receptor (BAFFR) and X-linked EDA receptor (XEDAR) belong to the type III transmembrane protein group. Osteoprotegerin (OPG) and DcR3 lack a transmembrane domain and are therefore secreted as soluble proteins. Until now, eight different death-domain-containing-receptors have been identified: death receptor 1 (DR1, also known as TNFR1), DR2 (CD95/Fas), DR3, DR4 (TRAILR1), DR5 (TRAILR2), DR6, EDAR and low-affinity nerve growth factor receptor.
(NGFR). The numbers on the left represent the number of amino acids in the cytoplasmic domain of the receptor.

Abbreviations: GITR, glucocorticoid-induced TNFR family receptor; HVEM, herpes-virus entry mediator; RELT, receptor expressed in lymphoid tissues; TRAF, TNFR-associated factor.
1.1.2 Characteristic features of TNF superfamily ligands and receptors

The normal function of TNF/TNFR superfamily proteins depends on the obligatory 3-fold symmetry that defines the essential signaling stoichiometry and structure. The ligands are type II transmembrane proteins that can have both membrane-embedded “immature” as well as cleaved, soluble “mature” forms. Both forms are active as self-assembling noncovalent trimers, whose individual chains fold as compact “jellyroll” β sandwiches and interact at hydrophobic interfaces (Fesik, 2000). The 20-30% amino acid similarity between TNF-like ligands is largely confined to internal aromatic residues responsible for trimer assembly. The external surfaces of ligand trimers show little sequence similarity, which accounts for receptor selectivity. The ligand shape is that of an inverted bell that is embraced on three sides at the base by elongated receptor chains forming a 3:3 symmetric complex (Idriss and Naismith, 2000). Certain ligands and receptors in the TNF/TNFR superfamily can bind more than one partner (Figure 1.1), thereby enhancing regulatory flexibility and complexity (Fesik, 2000).

TNFR-like receptors are type I transmembrane proteins that adopt elongated structures by a scaffold of disulfide bridges. The disulphide bonds form “cysteine-rich domains” that are the hallmark of the TNFR superfamily (Banner et al., 1993; Eck and Sprang, 1989). These 40 amino acid pseudorepeats are typically defined by 3 intrachain disulfides generated by 6 highly conserved cysteines (Smith et al., 1994). The elongated receptor chains fit in the grooves between protomers within the ligand trimer (Idriss and Naismith, 2000).

The cytoplasmic domains of TNFRs are modest in length and function as docking sites for signaling molecules. Signaling occurs through two principal classes of cytoplasmic adaptor proteins: TRAFs and DD molecules. The signaling adaptor is selected by whether the
cytoplasmic domain of the receptor harbors either a DD or a TIM (Wilson et al., 2009). The DD is a roughly 80 amino acid globular bundle of 6 conserved α helices found in the receptor tail and the adaptor that promotes homotypic interaction (Ashkenazi and Dixit, 1998). It was first identified independently by two different groups in the cytoplasmic domain of CD95/Fas and TNFRs; their deletion abolishes ligand-induced apoptosis (Itoh and Nagata, 1993; Tartaglia et al., 1993). By contrast, the TIM is a stretch of amino acids (less than a dozen contact residues) in the receptor tail that is clutched by a pocket in the globular head group of the adaptor through charged residues (Ye et al., 1999).

In addition to intracellular signaling domains, a conserved domain in the extracellular region of TNFR1 and TNFR2 has been identified that mediates specific ligand-independent assembly of receptors trimers (Chan et al., 2000). This pre-ligand-binding assembly domain (PLAD) is physically distinct from the domain that forms the main contacts with the ligand, but is required for the assembly of TNFR complexes that bind TNF and mediate signaling. Other members of the TNFR superfamily, including TRAIL-R1 and CD40, show similar homotypic associations (Chan et al., 2000). So, TNFRs and related receptors seem to function as preformed complexes rather than as individual receptor subunits that oligomerize after ligand binding.

Almost all of the TNF ligands are expressed by cells of the immune system, including B cells, T cells, NK cells, monocytes and dendritic cells, the only exception is the vascular endothelial growth factor inhibitor (VEGI), which is expressed mainly by endothelial cells (Zhai et al., 1999). The TNFRs, however, are expressed by a wide variety of cells. No cell type in the body has yet been found to not express TNFR1, whereas expression of TNFR2 is mainly restricted to immune and endothelial cells. Numerous activities are assigned to TNF,
perhaps because its receptors are expressed ubiquitously. This might also account for the non-specific toxicity of TNF.

1.2 The death domain superfamily

1.2.1 The players

The death domain superfamily is one of the largest and most studied domain superfamilies (McEntyre and Gibson, 2004). It currently comprises four subfamilies: the death domain (DD) subfamily, the death effector domain (DED) subfamily, the caspase recruitment domain (CARD) subfamily, and the pyrin domain (PYD) subfamily (Reed et al., 2004). These proteins are evolutionarily conserved in many multicellular organisms, including mammals, arthropods and nematodes. In the human genome, there are 32 DDs, 7 DEDs, 28 CARDs and 19 PYDs (Kohl and Grutter, 2004; Reed et al., 2004). A shortlist of the commonly studied members of this family is shown in Figure 1.2. Perhaps as evidence of integration with host apoptotic and inflammatory processes, some viruses have acquired DD superfamily sequences. For example, herpes viruses have DED-containing sequences that inhibit cell death, and poxviruses have both DED-containing and PYD-containing sequences that interfere with host apoptotic and inflammatory responses to viral infection (Bertin et al., 1997; Hu et al., 1997; Johnston et al., 2005; Thome et al., 1997). In some cases, the DD superfamily domain is the only motif present in these proteins. In most cases, however, the DD superfamily domain is combined with domains of other subfamilies or domains outside the DD superfamily.

A subfamily of the TNF receptor superfamily containing the DD is referred to as death receptors (Ashkenazi and Dixit, 1998). There are six human death receptors (DR), and
their cognate ligands have been identified. CD95 ligand/Fas ligand (CD95L/FasL) binds to CD95/Fas (Suda et al., 1993); TNF and LT bind to TNFR1 (Pennica et al., 1984); Apo3 ligand (Apo3L, also called TWEAK and TL1a) (Chicheportiche et al., 1997; Marsters et al., 1998) binds to DR3 (Marsters et al., 1998) and Apo2 ligand (Apo2L, also called TRAIL) (Wiley et al., 1995) binds to DR4 (Pan et al., 1997) and DR5 (Schneider et al., 1997). The native ligand for DR6 (Pan et al., 1998a) is currently unknown. Mice have orthologs of all human DRs and ligands, with the exception of mouse DR5, which acts as a single ortholog of human DR4 and DR5 and shares 76% and 79% amino acid identity, respectively, with the human receptors (Ashkenazi and Dixit, 1998). Death receptors can be divided into two categories based upon the primary adaptor protein to which they bind. CD95/Fas, DR4 and DR5 bind FADD and show mainly proapoptotic function. In contrast, TNFR1 and DR3 bind TRADD and mediate mainly proinflammatory and immune-stimulatory activity (Ashkenazi, 2002). DR6 can bind TRADD when overexpressed (Pan et al., 1998a), although its primary physiological adaptor has yet to be determined.

A central paradigm common to activation of the above receptor pathways is the assembly of oligomeric signaling complexes in response to external stimuli. The DD plays a critical role in this assembly by participating in both self-association and other protein-protein interactions.
**Figure 1.2 Death domain superfamily proteins.**

Domain organization of selected proteins containing the DD superfamily domains. Abbreviations: CARD, caspase recruitment domain; DD, death domain; DED, death effector domain; PYD, pyrin domain; GUK, guanylate kinase like; LRR, leucine rich repeat; NOD, nucleotide binding oligomerization domain; TLR, Toll/Interleukin 1-receptor; CRD, cysteine rich domain; TM, transmembrane domain; ZU5, domain present in ZO-1 and Unc5-like netrin receptors). Figure modified from Park *et al.* (Park et al., 2007).
1.2.2 Characteristic features of the death domain superfamily proteins

Despite only 10-20% amino acid sequence identity among the DD, DED, CARD, and PYD, the unifying feature of the DD superfamily is the antiparallel six helical bundle in the Greek key topology (Liang and Fesik, 1997), thereby indicating a common evolutionary lineage. In addition to this conserved structural fold, individual subfamilies also exhibit distinct structural and sequence characteristics not shared with other subfamilies. This suggests a common origin from a prototype molecule that became specialized for roles at different points in the signaling pathway. For example, whereas DD and DED play essential associative roles in death receptor pathways and Toll-like receptor (TLR)-mediated innate immune responses (Barton and Medzhitov, 2003), the CARD has been diversified for mitochondrial death pathways and inflammatory responses (Fesik, 2000; Humke et al., 2000). The DD also resembles the “ankyrin” repeat, an oligomerization domain common for other signaling systems (Feinstein et al., 1995).

The structures of at least eleven death domain superfamily members have been solved to atomic resolution either by X-ray crystallography or by nuclear magnetic resonance (NMR). All structures consist of an isolated death domain superfamily domain. For the DD subfamily, this includes, Fas DD, FADD DD, TNFR1 DD, TRADD DD, the p75 NGFR (nerve growth factor receptor) DD, IRAK4 DD and RAIDD DD (Berglund et al., 2000; Huang et al., 1996; Jeong et al., 1999; Lasker et al., 2005; Liepinsh et al., 1997; Park and Wu, 2006; Sukits et al., 2001; Tsao et al., 2007). For the DED subfamily, structure for FADD DED is known (Eberstadt et al., 1998), and for the CARD subfamily, the APAF-1 CARD and pro-caspase 9 CARD structures have both been solved (Zhou et al., 1999). As for the PYD subfamily, the NALP1 PYD has also been analyzed (Hiller et al., 2003). Whereas all DD subfamilies exhibit the six-helical bundle fold, variations exist in the length and direction
of the helices. Structurally, the DD and the CARD subfamilies are most dissimilar to each other. This is illustrated by the relative orientation of the six helices. Members of the CARD family tend to contain six helices aligned almost parallel to each other, whereas those of the DD family can be described as two mutually perpendicular three-helix bundles (Liang and Fesik, 1997) (Figure 1.3). Because of low sequence homology among DDs, the surface features of these DDs are also very different, which may be responsible for their specificity in protein-protein interactions (Weber and Vincenz, 2001).

Despite the substantial database of solved DD structures, it has been difficult to identify functional interaction surfaces by structural analysis of a monomer alone. This can only be achieved in combination with mutational analysis and by solving the structures of death domain superfamily complexes. Consequently, the structural basis of DD:DD homotypic interactions involved in death signaling (such as those in the Fas DD:FADD DD complex, the TRADD DD: FADD DD complex, and the PIDD DD: RAIDD DD complex) remains largely unresolved. In the case of the Fas DD:FADD DD complex, it is believed to be trimeric, most likely with Fas possessing the self-oligomerization surface. Therefore, the complex might comprise at least two interfaces, a self-oligomerization surface and a Fas DD: FADD DD interaction surface. This assumption of multiple interaction surfaces in these complexes is supported by mutational data, which showed wide spreads of residues important for binding and/or function on the surfaces of Fas (Martin et al., 1999), FADD (Hill et al., 2004), TRADD (Park and Baichwal, 1996a) and TNFR1 (Telliez et al., 2000).

Given the high structural similarity among the subfamilies of the DD superfamily, it is interesting that almost all known interactions in the DD superfamily are homotypic interactions between members of the same subfamily. Currently, only two cases of potential
heterotypic interactions have been reported, and it is not known whether these interactions are just a few rare examples or represent a major functional aspect of the DD superfamily. One case of heterotypic interaction is present in the phosphoprotein enriched in astrocytes (PEA)-15 DED protein. PEA-15 is unusual because it may interact homotypically with DED-containing proteins such as FADD and caspase 8 and also heterotypically with non-DED proteins such as the extracellular signal-regulated kinase (ERK) (Hill et al., 2002). Another case of heterotypic interaction is present in a CARD containing protein called apoptosis repressor with CARD (ARC), which appears to interact heterotypically with both the DDs of Fas and FADD, and the C-terminus of the B cell lymphoma (Bcl) 2-associated X protein (BAX) (Nam et al., 2004). These interactions underlie ARC’s ability to inhibit both the death receptor and the mitochondria cell death pathway.
Figure 1.3 Representative ribbon structures of DD superfamily members.

(a) Fas death domain (DD), (b) FADD death effector domain (DED), (c) RAIDD caspase recruitment domain (CARD), and (d) NALP1 pyrin domain (PYD). Adapted from Park et al. (Park et al., 2007). Abbreviations: N, amino terminus; C, carboxy terminus; H, helix.
1.3 Physiological functions of TNF

The discovery that cachectin, a protein known to cause fever and wasting, was identical to TNF provided an early illustration of the importance of members of this family in human disease (Beutler and Cerami, 1986). Though systemic toxicity dashed early hopes of using LT and TNF as anti-tumor agents, research during the past two decades has shown that TNF can have important contributions in host defense and in development. Aberrant production of TNF, however, can be pathogenic, resulting in multiple organ inflammation, autoimmunity and cancer.

1.3.1 Host defence

TNF secretion can be induced by conserved structural elements common to microbial pathogens, including cell wall moieties such as peptidoglycan, LPS, and bacterial DNA CpG motifs, that are bound by Toll-like receptors (TLRs) (Aderem and Ulevitch, 2000). TLRs, conserved in protein sequence from Drosophila to humans, decorate epithelial cells, tissue macrophages, and dendritic cells, of which the latter two are the sentinel phagocytic and antigen processing cells of the immune system, respectively. TLRs transcriptionally induce proinflammatory cytokines, including TNF, through the convergence of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the nuclear factor of activated T-cells (NF-AT) activating pathways, and enhance translational efficiency by a mechanism targeting consensus 3’-untranslated AU-rich elements (ARE) present in the messenger ribonucleic acid (mRNA) (Dumitru et al., 2000). Interleukin (IL)-1R, a receptor that shares cytoplasmic homology with TLRs, activate similar pathways, thus amplifying the response (de Fougerolles et al., 2000; Shornick et al., 1996). The result is a highly complex biological
cascade—involving chemokines, cytokines, and the induction of endothelial adhesins—that recruits and activates granulocytes, monocyte/macrophages, and lymphocytes at the damaged or infected tissue sites. Local injection of TNF recapitulates these events, and Tnf and Tnfr1 deficient mice show attenuated contact hypersensitivity to irritants and susceptibility to diverse microbial pathogens (Erickson et al., 1994; Pasparakis et al., 1996a; Pfeffer et al., 1993; Rothe et al., 1993), indicating an important role of TNF in non-specific immunity (Marino et al., 1997; Pfeffer et al., 1993; Rothe et al., 1993). These mice are also protected from hepatitis induced by LPS following D-galactosamine treatment and have a deficiency in granuloma development (Pfeffer et al., 1993; Roach et al., 2002). Mice deficient of Tnf and Tnfr1 have also been reported to exhibit defective splenic architecture and fail to initiate a germinal centre reaction, which is a critical step in mounting a humoral immune response (Matsumoto et al., 1997; Pasparakis et al., 1996b).

1.3.2 Inflammation and autoimmunity
According to the classic proinflammatory scenario, failure to regulate the production of TNF at a site of immunological injury may lead to chronic activation of innate immune cells and to chronic inflammatory responses, which may consequently lead to organ specific inflammatory pathology and tissue damage. The temporal and spatial deregulation of TNF production in transgenic, non-autoimmune-prone mice, promotes TNFR-dependent pathologies, such as multi-organ inflammation (Higuchi et al., 1992; Picarella et al., 1993; Probert et al., 1993), rheumatoid arthritis (Keffer et al., 1991), multiple sclerosis (Akassoglou et al., 1998; Akassoglou et al., 1997; Probert et al., 1995) and inflammatory bowel disease (Kontoyiannis et al., 1999). Multiple studies have also implicated the TNF/TNFR system in
systemic lupus erythematosus (SLE) pathogenesis and perpetuation. However, the mode of action of TNF in SLE remains puzzling, since there is evidence both for its pathogenic activity and for its beneficial requirement against the disease (Jacob et al., 1991; Jacob et al., 1996).

1.3.3 Cancer

Although initially thought to be a potent anticancer agent, it is now generally believed that TNF has limited activity in the suppression of cancer, mainly because of its systemic toxicity (Feinberg et al., 1988). Conversely, it is now a general consensus that TNF, produced mainly by tumor associated macrophages, can actually contribute to tumorigenesis by modifying the tumor stroma and by mediating the proliferation, invasion and metastasis of tumor cells (Babbar and Casero, 2006; Kawai et al., 2002; Sethi et al., 2008). TNF has been shown to be an autocrine and paracrine growth factor for a wide variety of tumors. Through the activation of NF-κB, TNF induces the expression of various genes that are involved in invasion and metastasis, including adhesion molecules, urokinase plasminogen activator (UPA) (Bechtel et al., 1996; Hashizume et al., 2008), matrix metalloproteinase 9 (MMP9) (Montesano et al., 2005; Yang et al., 2004), cyclo-oxygenase 2 (COX2) (Surh et al., 2001; Yan et al., 2006) and vascular endothelial growth factor (VEGF) (Paleolog et al., 1998; Ryuto et al., 1996; Xiao et al., 2011). In addition, activation of NF-κB can suppress apoptosis, which further contributes to tumorigenesis.
1.3.4 Others
TNF also exhibits a wide range of other functions in areas such as wound healing (Feiken et al., 1995), cell migration (Dekaris et al., 1999; Suto et al., 2006; Wang et al., 2001c), hematopoiesis (Zhang et al., 1995), osteoclastogenesis (Hase et al., 2006) and obesity (Schreyer et al., 1998).

1.4 TNF signaling
Given its pleiotropic functions, inappropriate production of TNF or sustained activation of TNF signaling has been implicated in the pathogenesis of a wide spectrum of human diseases, including sepsis, cerebral malaria, diabetes, cancer, osteoporosis, allograft rejection, and autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and inflammatory bowel diseases (Aggarwal, 2003). Spatial and temporal constraints on inflammation by TNF are imposed by processing of membrane-tethered ligand and receptor into soluble forms (Blobel, 1997), regulated expression of receptors and most importantly, feedback inhibition (Carballo et al., 1998; Hoffmann et al., 2002). A more complete understanding of the signaling pathways activated upon TNF stimulation is imperative to fully comprehend the relevant mechanisms of disease induction and progression in humans.

TNF is a homotrimer of 157 amino acid subunits primarily produced by activated macrophages. It signals through two distinct cell surface receptors, TNFR1 and TNFR2. Expression of TNFR1 is found on the surface of all cell types, whereas expression of TNFR2 is mainly by immune and endothelial cells (Aggarwal, 2003). Numerous activities are assigned to TNF, perhaps because its receptors are expressed ubiquitously. This might also account for the non-specific toxicity of TNF. In the past, it has been suggested that TNFR1 might be a receptor for the soluble ligand, whereas TNFR2 might be a receptor for the
membrane bound ligand (Grell et al., 1995). Also, some reports have suggested that TNFR1 mediates apoptosis whereas TNFR2 mediates proliferation, while other reports have suggested that the two TNFRs transduce their signals cooperatively (Mukhopadhyay et al., 2001; Weiss et al., 1998). Currently, it is believed that TNFR1 initiates the majority of TNF’s biological activities.

1.4.1 TNFR1: a pathway of split personality

The initial step in TNF signaling involves the binding of the TNF trimer to the extracellular domain of TNFR1 and the release of the inhibitory protein silencer of death domains (SODD) from TNFR1’s intracellular domain (ICD) (Jiang et al., 1999; Takada and Aggarwal, 2003). Ligand-induced reorganization of the preassembled receptor complex now enables TNFR1 to recruit the adapter protein TNF receptor-associated protein with a death domain (TRADD) and the serine–threonine kinase receptor-interacting protein 1 (RIP1) (Chan, 2007). TRADD and RIP1 both contain a C-terminal DD, which can mediate binding to the DD of TNFR1. There are contradictory reports claiming competitive, but also cooperative, effects in the recruitment of TRADD and RIP1 to TNFR1, but it is generally accepted that RIP1 and TRADD are capable of interacting independently with TNFR1 (Devin et al., 2000; Jin and El-Deiry, 2006; Zheng et al., 2006a). In addition, TRADD and RIP1 also interact strongly with each other by virtue of their DDs. Upon association with ligand engaged TNFR1, TRADD is believed to further recruit the adapter protein TNF receptor associated factor 2 (TRAF2) via its N-terminal TRAF-binding domain. TRAF2 consists of an N-terminal RING domain followed by five zinc fingers and a C-terminal TRAF domain, which mediates homotrimerization and interaction with TRADD (Hsu et al.,
1996a; Hsu et al., 1996b). TRAF2 also forms complexes with cellular inhibitor of apoptosis protein (cIAP) 1 and cIAP2 with high efficacy (Wang et al., 1998) and therefore all these proteins contribute to the TNF-induced TNFR1 signaling complex I, (Figure 1.4) which is considered to activate the NF-κB pathway via the transforming growth factor β–activated kinase 1 (TAK1) and the mitogen activated protein (MAP) kinase kinase kinase-3 (MEKK-3), leading to phosphorylation of the inhibitor of κB kinase (IKK) which in turn phosphorylates the inhibitor of κB (IκB). This phosphorylation step causes ubiquitination-dependent degradation of IκB allowing NF-κB to enter the nucleus and to initiate gene transcription (Ghosh and Karin, 2002). NF-κB is a well-described key regulatory transcription factor regulating numerous processes such as inflammation, development, oncogenesis and cellular stress. About 200 physiological stimuli are known to activate NF-κB. These include bacterial and viral products, cellular receptors and ligands, mitogens and growth factors and physical and biochemical stress inducers. The active transcription factor controls a plethora of genes such as cytokines, chemokines, stress response genes, regulators of apoptosis, immune receptors and adhesion molecules, but also growth and cell cycle proteins (Karin and Lin, 2002).

The second signaling pathway elicits cell death. By a largely unknown mechanism receptor complex I is internalised and a complex consisting of TRADD, RIP1 and TRAF2 is released from TNFR1 (Schutze et al., 1999). Subsequently, Fas-associated death domain protein (FADD) binding to TRADD and pro-caspase 8 are recruited to form complex II (Figure 1.4). This signaling step results in activation of caspase 8 and subsequently that of caspase 3, leading to the initiation of apoptosis (Wang et al., 2008).

In a third signaling pathway another protein is recruited to the TRADD-RIP1-TRAF2
complex, the apoptosis-signaling kinase-1 (ASK-1), which is a member of the MEKK family. This protein complex is thought to activate the MAP kinase kinases MEK-4 and MEK-6 (Ichijo et al., 1997) (Figure 1.4). These enzymes phosphorylate and activate c-Jun N-terminal kinases (JNKs) and p38 MAPKs. JNKs phosphorylate c-Jun, a subunit of the transcription factor activating protein-1 (AP-1). Similar to NF-κB, AP-1 is considered to promote inflammation and cell survival. However, TNF signaling through ASK-1 may also provoke cell death since overexpression of dominant-negative ASK-1 or knock-down of ASK-1 inhibits TNF-induced apoptosis (Ichijo et al., 1997; Tobiume et al., 2001).

Besides pathways modulating cell survival, TNF has also been shown to promote growth through the activation of ERK and AKT (also known as protein kinase B) (Rivas et al., 2008). In summary, TNF acting through TNFR1 induces numerous signaling pathways provoking a variety of cellular effects. The complexity and crosstalk of TNF signaling pathways are still not fully understood. In this apparent dichotomy of activation where TNFR1 assembles a signaling complex that can activate both pro-apoptotic and anti-apoptotic pathways, TRADD is believed to play a central role. However, owing to the lack of a gene deficient model for this molecule, the precise role of this crucial adaptor protein and its contribution to the complexity of the TNFR1 signaling pathway has yet to be fully elucidated.
Figure 1.4 Model of TNFR1 signaling pathway.

See text for details. T bar represents inhibition; arrows denote activation; dashed lines indicate unclear recruitment sequence to the receptor complex.
1.5 The TRADD adaptor protein

1.5.1 Discovery and Structure

The human TRADD gene is mapped to chromosome 16q22 (Scheuerpflug et al., 2001), whereas the murine counterpart maps to the distal region of mouse chromosome 8 (Pan et al., 1995). TRADD was initially isolated as a protein that interacts with the cytoplasmic DD of TNFR1 in a yeast two-hybrid system (Hsu et al., 1995). Overexpression of TRADD was found to lead to 2 major TNF-induced responses, apoptosis and activation of NF-κB. TRADD-mediated cell death could be suppressed by expression of the Cowpox virus crmA gene, which encodes an inhibitor of caspase 1, a cysteine protease involved in TNF-induced apoptosis. However, crmA expression did not inhibit NF-κB activation by TRADD, demonstrating that the two signaling pathways emanating from TRADD are distinct (Hsu et al., 1995).

TRADD is a 34 kDa protein that is expressed ubiquitously. The human and mouse TRADD protein consists of 312 and 310 amino acids (aa), respectively, and are 75% identical (Pan et al., 1995). TRADD contains two functionally separate domains, which allow the protein to couple to at least two distinct signaling pathways (Hsu et al., 1996b). The C-terminal region of the protein (aa 170–301) contains a DD with the characteristic six-helical bundle topology (Figure 1.5). This domain is believed to mediate TRADD oligomerization, and interaction with the DD of TNFR1, FADD, and RIP1 through homotypic DD interactions (Boldin et al., 1995; Hsu et al., 1996b). The N-terminal region of TRADD spanning from residues 51-144 appears to be a domain which has been reported to mediate interaction with TRAF2, possibly leading to its recruitment to the TNFR1 complex (Hsu et al., 1996b; Tsao et al., 2000). Interestingly, this region in TRADD has also been reported to
be responsible for interaction with signal transducers and activators of transcription (STAT) 1-α, and although it remains controversial, this is believed to influence interferon (IFN) γ signaling (Wesemann et al., 2004). There is also evidence that the C-terminus of TRADD can interact with keratins 8/18, which are major components of intermediate filament proteins of simple or single-layered epithelia (Figure 1.6). Although this interaction is believed to attenuate the interaction between TRADD and activated TNFR1, it is still speculative whether TRADD may exhibit influence over the cytoskeletal structure through this interaction (Inada et al., 2001).

Although generally perceived as a cytoplasmic protein, there is evidence suggesting that the cellular localization of TRADD is dynamic (Morgan et al., 2002). In fact, the primary structure of TRADD contains both a putative nuclear localization signal (NLS) at aa 229-242 and a putative nuclear export signal (NES) at aa 147-163, which might allow TRADD to shuttle dynamically between the cytoplasm and the nucleus (Figure 1.6).
Figure 1.5 Structure of the death domain of TRADD.

(A) Representative ribbon structure of TRADD DD. (b) Stereo view showing the backbone and side chains of 25 superimposed structures (residues 214–304). The unstructured amino- and carboxyl-termini are not shown. Adapted from Tsao et al. (Tsao et al., 2007).
**Figure 1.6 Known protein binding domains and motifs in TRADD.**

The 312 aa long TRADD protein has two distinct domains: the C-terminal DD and the N-terminal TRAF2 binding domain. In addition to DD-interactions, the DD has also been demonstrated to interact with keratins, whereas the N-terminus of TRADD has been demonstrated to bind to STAT1-α. The primary structure of TRADD also contains a putative NES and NLS.
1.5.2 Predicted functions of TRADD outside the TNFR signaling pathway

1.5.2.1 TRADD in death receptor signaling

Through homotypic DD interactions, it has been shown that TRADD can be recruited to death receptors other than TNFR1.

(i) Death receptor 3 (DR3)

DR3 is one of the eight DD-containing TNFR family members and is the one most closely related to TNFR1. DR3, like TNFR1, can bind to TRADD and FADD (Chinnaiyan et al., 1996; Kitson et al., 1996), and this is believed to mediate apoptotic responses. DR3 also recruits TRAF2, likely through TRADD as an adaptor and thus activates the transcription factor, NF-κB, which induces the transcription of a number of immune genes (Marsters et al., 1996). Mice deficient in Dr3 exhibit significant impairment in thymocyte negative selection and in anti-CD3-induced apoptosis (Wang et al., 2001a). Such defects have not been reported in Tnfr1 deficient mice. This reveals a non-redundant in vivo role for this TNF receptor family member in the removal of self-reactive T cells in the thymus.

(ii) TNF-related apoptosis-inducing ligand receptor (TRAIL-R)

TRAIL has been shown to induce apoptosis in cancer cells specifically through binding to its receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5). Like other death receptors, TRAIL-R1 and TRAIL-R2 contain a cytoplasmic DD that can associate with TRADD, RIP1 and FADD, to trigger multiple cellular signals, including the activation of caspases, MAPKs and NF-κB (Johnstone et al., 2008). It is believed that TRADD plays a pro-survival role in these signaling pathways, by limiting FADD recruitment to the death inducing complex (Cao et al., 2011).
(iii) Death receptor 6 (DR6)

DR6 is another DD-containing receptor within the TNFR superfamily (Pan et al., 1998a). The expression of human DR6 is observed in a number of tissues, including lymphoid tissues (Pan et al., 1998a). DR6 was reported to interact with TRADD and ectopic expression of this protein was shown to induce apoptosis and activation of both NF-κB and JNK (Pan et al., 1998a). Mice deficient of Dr6 exhibit enhanced CD4⁺ T cell proliferation and T helper type 2 (Th2) cytokine production (Liu et al., 2001; Schmidt et al., 2003; Zhao et al., 2001), consequently contributing to an enhancement of B cell expansion, survival, and humoral responses (Liu et al., 2001; Schmidt et al., 2003; Zhao et al., 2001). Again, these phenotypes have not been reported in Tnfr1-deficient mice.

1.5.2.2 TRADD outside death receptor signaling

(i) Epstein-Barr virus (EBV) latent infection membrane protein 1 (LMP1)

LMP1 is essential for EBV-mediated growth transformation of resting primary human B lymphocytes into indefinitely proliferating lymphoblastoid cell lines (LCLs) (Kaye et al., 1993). Genetic and biochemical evidence indicates that the signal transduction pathway through which LMP1 mediates B lymphocyte growth transformation resembles those of activated TNFR (Izumi et al., 1997). Mimicry of an activated TNFR is concordant with similar effects of LMP1 and activated CD40 on B lymphocyte growth and gene activation (Kilger et al., 1998). TRADD was found to interact with one of the carboxyl-terminal activating region (CTAR2) in the cytoplasmic tail of LMP1 and was found to constitutively associate with LMP1 in EBV-transformed cells. Upon overexpression, TRADD and CTAR2 synergistically mediated high level NF-κB activation (Izumi et al., 1997). It is now believed
that LMP1 appropriates TRADD to enable efficient long-term lymphoblastoid cell outgrowth by forging a “CD40-like” activation in B lymphocytes (Kilger et al., 1998).

(ii) Retinoic acid inducible gene-I (RIG-I) helicase antiviral pathway

Upon detection of viral dsRNA, the helicase retinoic acid inducible gene-I (RIG-I), via CARD adaptor inducing interferon-β (Cardif), initiates activation of the transcription factors NF-κB and interferon regulatory factor (IRF) 3, which collaborate to induce antiviral type I IFN response (Sun et al., 2006). A recent study demonstrated that TRADD is crucial for this signaling pathway, through its recruitment to Cardif (Michallet et al., 2008).

(iii) Interferon gamma (IFNγ) signaling pathway

A well known physiological function of IFNγ is the activation and priming of macrophages for a T_{H1} immune response (Stout and Bottomly, 1989). Binding of IFNγ to its receptor induces a series of events that ultimately result in the tyrosine phosphorylation, dimerization and subsequent nuclear translocation of STAT1-α. STAT1-α homodimers then induce transcription through binding to the γ-activation site (GAS) in IFNγ inducible promoters. In a screen for STAT1-α binding proteins, TRADD was found to interact with STAT1-α in a TNF-dependent manner in HeLa cells (Wang et al., 2000c). Extending from this, a more recent study suggested that TRADD-STAT1-α complex could in fact directly influence pathways that use STAT1-α as a primary signaling transducer, namely the IFNγ pathway (Wesemann et al., 2004). Specifically, this study suggested that TRADD functions as a negative regulator of IFNγ induced STAT1-α activation.
1.5.2.3 TRADD in the nucleus

(i) Interaction with promyelocytic leukemia (PML) nuclear bodies

There is evidence suggesting that the cellular distribution of TRADD is dynamic. Whereas cytoplasmic TRADD serves as an adaptor protein, in the nucleus, TRADD has been found in association with PML-nuclear bodies (Morgan et al., 2002). The DD of TRADD is believed to be important for localization to PML-nuclear bodies and has been demonstrated to trigger a caspase-independent form of cell death upon over-expression. Apoptosis through TRADD-DD overexpression is in part inhibited by Bcl-X\textsubscript{L}, a mitochondria-localized antiapoptotic member of the Bcl-2 protein family, and relies on p53 and PML expression (Morgan et al., 2002).

(ii) Interaction with homeodomain-interacting protein kinase (HIPK) 2

HIPKs belong to a novel family of predominantly nuclear serine/threonine kinases (Hofmann et al., 2000) and function in transcriptional regulation (Choi et al., 1999; Moilanen et al., 1998), growth suppression (Pierantoni et al., 2001) and apoptosis (D'Orazi et al., 2002; Hofmann et al., 2002). In humans and mice the family comprises three members, HIPK1-3, which were originally identified as corepressors for homeodomain transcription factors (Kim et al., 1998). HIPK2 was found to interact with TRADD when overexpressed in 293T cells (Li et al., 2000). Whether this interaction is of importance for the TRADD or TRADD-DD-induced cell death remains to be determined in the future.
1.6 Project rationale

*In vitro* biochemical studies have reflected the numerous functions that TRADD might be involved in. In the realm of TNFR signaling, efforts to overexpress and knockdown TRADD have produced mixed results. While most studies suggest that TRADD is essential for TNFR1 induced cell death and inflammatory responses, some data based on depletion of *TRADD* by ribonucleic acid interference (RNAi) have suggested the possibility that TRADD may not be required for TNFR1-induced apoptosis, thus complicating the scenario (Jin and El-Deiry, 2006). This underscores the need for genetic analyses to depict the precise role of TRADD in the TNFR1 pathway. Through genetic deletion of *Tradd* in mice, we hope to gain useful insights not only for TNFR1 signaling, but also for other TNFR-unrelated functions of TRADD.
1.7 Thesis objectives

1. **Investigate the role of TRADD in inflammatory and immune signaling pathways**
   a. Characterize the role of TRADD in TNFR1 signaling
   b. Investigate the role of TRADD in signaling events mediated by other death receptors shown previously to interact with TRADD
   c. Explore the role of TRADD in other immune signaling cascades

2. **Investigate the role of TRADD in tumorigenesis**
   a. Evaluate the effect of Tradd deficiency in a murine tumor model
   b. Dissect the TNF dependence of TRADD in regulating tumorigenesis
1.8 Summary and hypotheses

(i) Based on existing biochemical evidence, we speculate that TRADD is essential for both TNFR1 mediated cell death as well as inflammatory responses. Evaluation at the molecular and physiological level will serve to clarify the extent of TRADD’s involvement in TNFR signaling.

(ii) Given the prominent role of DD containing proteins such as RIP1, myeloid differentiation primary-response protein (MyD)-88, IL-1R associated kinases (IRAKs), etc., in Toll-like receptor (TLR) signaling, we speculate that the DD containing protein TRADD will also participate in TLR signaling.

(iii) TNF has an important role in tumor promotion. Since TRADD is predicted to be the core mediator of TNF induced cellular responses, we speculate that TRADD is also essential for TNF-mediated tumor promotion.

Characterization of Tradd deficient mice in the above aspects is described and discussed in the following chapters of this dissertation.
1.9 List of publications and contributions


Chen NJ, **Chio II**, Lin WJ, Duncan G, Chau H, Katz D, Huang HL, Pike KA, Hao Z, Su YW, Yamamoto K, de Pooter RF, Zúñiga-Pflücker JC, Wakeham A, Yeh WC, Mak TW.

2. First author publication: TRADD contributes to tumor suppression by regulating ULF-dependent p19Arf ubiquitination. Manuscript under revision for *Nature.* October 2011

**Chio II**, Sasaki M, Ghazarian D, Ueda T, Chang YL, Chen NJ and Mak TW.

*Tradd*−/− mice were generated by Dr Nien Jung Chen

Processing, histological and immunohistochemical staining of tissues for data presented in chapters 2 and 3 were performed by lab members of the Pathology Department (PRP) at Toronto General Hospital and myself. Detailed histopathological analyses of tissues were performed by Drs Danny Ghazarian, Masato Sasaki and myself. All other experiments including animal husbandry, monitoring of animals for gross phenotypes, dissections, tissue collection, photomicrography, molecular biology, cell culture, FACS analysis, measurements and statistics were carried out by myself with some assistance from members of the Mak lab.
CHAPTER 2

TRADD IS ESSENTIAL FOR TNFR1 SIGNALING AND IS INVOLVED IN TLR SIGNALING CASCADES

A version of this chapter is published in *PNAS* 2008, 105(34):12429-34. (Chen and Chio et al. 2008).
2.1 Introduction

TNF is a pleiotropic cytokine involved in a broad range of biological activities, including inflammation and cell differentiation, survival and death (Aggarwal, 2003). TNF mediates these activities by engaging two distinct cell surface receptors: TNFR1 and TNFR2. Studies of Tnfr1- and Tnfr2 deficient mice have demonstrated that TNFR1 is the major receptor mediating most of the biological functions of TNF (Mukhopadhyay et al., 2001).

Activation of TNFR1 leads to the recruitment of TRADD, through homotypic interaction with the DD of TNFR1. TRADD was originally discovered in a yeast two-hybrid screen performed to identify TNFR1-interacting proteins (Hsu et al., 1995). Although TRADD is the first adaptor protein identified that binds directly to the DD of TNFR1, its precise role in this pathway has remained elusive. It is generally believed that TRADD transduces signals resulting in either apoptosis or NF-κB activation upon TNFR1 engagement (Chen and Goeddel, 2002; Hsu et al., 1995; Mak and Yeh, 2002). While TRADD overexpression in 293T cells has been shown to activate both apoptotic and cell survival signaling pathways (Hsu et al., 1995), some data based on depletion of TRADD by RNAi have suggested the possibility that TRADD may not be required for TNFR1-induced apoptosis (Jin and El-Deiry, 2006).

In addition to TNFR1, TRADD might also mediate signaling downstream of other death receptors in the TNFR superfamily, such as DR3 and DR6 (Chinnaiyan et al., 1996; Pan et al., 1998b). Recent knockdown studies for TRADD have also indicated that TRADD may be involved in signaling mediated by receptors unrelated to the TNFR superfamily (Sethi et al., 2007; Wesemann et al., 2004; Zheng et al., 2006b). Notably, in RAW246.7 cells (a murine macrophage cell line), Tradd knockdown resulted in increased IFNγ-induced STAT1-α DNA-binding activity and transcription potential (Wesemann et al., 2004),
suggesting that TRADD can interact with STAT1-α and regulate IFNγ-mediated signaling. These findings give tantalizing hints of the potential breadth of TRADD functions in immune signaling cascades.

Like the TNFR superfamily of proteins, another prominent family of evolutionarily conserved receptors that are also important for the immune response is the Toll-like receptor (TLR) family, the members of which are responsible for the recognition of pathogen-associated molecular patterns (PAMPs) expressed by a wide spectrum of infectious agents (Beutler and Rietschel, 2003; Janeway and Medzhitov, 2002). To date, over thirteen TLRs have been reported in human and in mouse. TLRs activate the NF-κB pathway, which regulates cytokine expression, through several adaptor molecules including myeloid differentiation primary-response protein (MyD)-88, MyD88 adaptor-like (MAL) and TIR-domain-containing adapter-inducing interferon-beta (TRIF). Activation of the NF-κB pathway links innate and adaptive immune response by production of inflammatory cytokines such as IL-1, IL-6, IL-8, TNF, IL-12, chemokines and induction of costimulatory molecules such as CD80, CD86, and CD40 (Beutler and Rietschel, 2003; Janeway and Medzhitov, 2002).

The discovery of the TLR family began with the identification of Toll, a receptor that is expressed by Drosophila and was found to be essential for establishing dorsoventral polarity during embryogenesis (Anderson et al., 1985; Hashimoto et al., 1988). Subsequent studies revealed that Toll has also an essential role in the insect innate immune response against fungal infection (Lemaitre et al., 1996). The TLRs are type I integral membrane glycoproteins, and on the basis of considerable homology in the cytoplasmic region, they are members of a larger superfamily that includes the interleukin-1 receptors (IL-1Rs). By
contrast, the extracellular regions of the TLRs and IL-1Rs differ markedly: the extracellular region of TLRs contains leucine-rich repeat (LRR) motifs, whereas that of IL-1Rs contains three immunoglobulin-like domains. TLRs and IL-1Rs have a conserved region of ~200 amino acids in their cytoplasmic tails, which is known as the Toll/IL-1R (TIR) domain. Within the TIR domain, the regions of homology are comprised of three conserved boxes, which are crucial for signaling (Slack et al., 2000).

TLR3 and TLR4 are two well-known family members of the TLR family. TLR3 recognizes double-stranded RNA (dsRNA), which is produced by most RNA viruses during their replication (Alexopoulou et al., 2001). TLR4 recognizes lipopolysaccharide from Gram-negative bacteria and is the mammalian homolog of the Drosphilia Toll (Hoshino et al., 1999). Engagement of the TLR3 and TLR4 with their respective ligands elicits potent activation of the NF-κB transcription factor and the induction of type I interferons (IFN) (Doyle et al., 2002). This is achieved through the recruitment of adaptor molecules such as MyD88, IRAKs, MAL and TRIF (Dunne and O'Neill, 2003; Takeda et al., 2003) (Figure 2.1).

MyD88 is a common player downstream of most TLRs. It has an amino-terminal DD, which is separated from its carboxyl-terminal TIR domain by a short linker sequence. (Burns et al., 1998; Muzio et al., 1997; Wesche et al., 1997). MyD88 functions as an adaptor linking TLRs/IL-1Rs with downstream signaling molecules that have DDs, such as IRAKs (Dunne et al., 2003). The binding of MyD88 to IRAK4 facilitates IRAK4-mediated phosphorylation of a crucial residue or residues in the kinase-activation loop of IRAK1, which induces the kinase activity of IRAK1. This ultimately results in the activation of NF-κB and MAPK, and the production of inflammatory cytokines (Medzhitov et al., 1998) (Fig 2.1)

In MyD88-deficient macrophages, TLR4 ligand-induced production of inflammatory
cytokines is severely reduced; however, activation of NF-κB is observed with delayed kinetics (Kawai et al., 1999). This indicates that although TLR4-mediated production of inflammatory cytokines depends mostly on the MyD88-dependent pathway, a MyD88-independent component exists in TLR4 signaling. Subsequent studies have demonstrated that TLR4 stimulation leads to activation IRF3, as well as the late phase of NF-κB activation in a MyD88-independent manner (Kawai et al., 2001). TLR4-induced activation of IRF-3 leads to production of IFN-β, which in turn activates STAT1-α and induces several IFN-inducible genes (Doyle et al., 2002). Viral infection or dsRNA was found to activate IRF-3 (Yoneyama et al., 1998). Accordingly, the TLR3-mediated pathway also activates IRF-3 and thereby induces IFN-β in a MyD88-independent manner. Hence, TLR3 and TLR4 utilize the MyD88-independent component to induce inflammatory cytokines (Fig 2.1).

TRIF was identified as the TIR domain-containing adaptor that is crucial for mediating MyD88-independent IFN-β production (Yamamoto et al., 2002). Trif-deficient mice generated by gene targeting showed no activation of IRF-3 and had impaired expression of IFN-β and IFN-inducible genes in response to TLR3 and TLR4 ligands. They also showed impaired ligand-induced inflammatory cytokine production (Diebold et al., 2003). Therefore, activation of both the MyD88-dependent and MyD88-independent/TRIF-dependent components is required for the TLR4-induced inflammatory cytokine production (Fig 2.1).

A recent study indicates that TRIF-dependent NF-κB activation requires RIP1, the DD containing protein that is crucial for TNFR1 induced NF-κB activation (Meylan et al., 2004b) (Fig 2.1). The carboxyl terminus of TRIF contains a RIP homotypic-interaction motif, which is required for association with RIP1. A dominant-negative form of RIP1 inhibits TRIF-mediated NF-κB activation, and embryonic fibroblasts from Rip1-deficient mice
showed impaired TLR3-mediated NF-κB activation (Meylan et al., 2004b). Given the prominence of DD containing proteins in TLR signaling, as well as the known interaction between TRADD and RIP1, it was speculated that in addition to a role in TNFR1 signaling, TRADD may also participate in signaling downstream of TLR3 and TLR4.

In this study, we generated Tradd deficient mice using gene targeting and showed that not only is TRADD indispensable for TNF-induced apoptosis and inflammatory responses in vitro and in vivo, but also that this molecule is involved in the TRIF-dependent arm of TLR3 and 4 signaling. This establishes new avenues for the role of TRADD in inflammatory and immune signaling cascades, revealing the functional diversity of this adaptor protein.
Figure 2.1 TLR3 and TLR4 signaling pathways.
See text for details. Solid arrows denote activation; dashed arrows indicate unclear mechanisms. P denotes phosphorylation events. Modified from Kawai and Akira. (Kawai and Akira, 2006).
2.2 Materials and Methods

2.2.1 Genotype analysis
Genomic DNA was isolated from mouse tail for polymerase chain reaction (PCR) analysis by digesting tissue in proteinase K (150 µg/ml), sodium dodecyl sulfate (SDS) (1%, w/v) and TNE buffer (10 mM Tris, pH 8.0, 1 mM EDTA and 0.1 M NaCl at 55°C overnight and precipitating DNA in 1.5 volumes of ethanol. DNA was dried and resuspended in water. Genotyping primers are listed in Table 2.1. PCR product for the Tradd wildtype allele is 230 bp, obtained using the following PCR program: 1 cycle at 95°C for 5 min; 35 cycles at 94°C for 45 sec, 62°C for 45 sec, and 72°C for 1 min 20 sec; 1 cycle at 72°C for 10 min for the last extension. PCR product for the Tradd knockout allele is a 600-bp fragment obtained by using the following program: 1 cycle at 95°C for 5 min; 40 cycles at 94°C for 45 sec, 60°C for 45 sec, and 72°C for 1 min 20 sec; 1 cycle at 72°C for the last 10 min extension.

Southern blot analyses presented in this data chapter were performed by Dr. Nien Jung Chen. Briefly, genomic DNA was isolated by phenol-chloroform extraction, digested overnight with BglII/KpnI, precipitated and resuspended in 10 mM Tris (pH 8.5). Digested DNA was electrophoresed on a 1% agarose gel in TAE buffer for 18-24 hours. Depurination of DNA was performed using 0.25 M HCl for 15 minutes. DNA was incubated in denaturing buffer (0.5 M NaOH, 1.5 M NaCl) for 30 minutes followed by a neutralization step in a solution of 1 M Tris (pH 7.0) and 1.5 M NaCl for 30 minutes. Digested and electrophoresed DNA was transferred to a positively charged nylon membrane (Amersham, Hybond N+) by upward capillary transfer using a high salt buffer (0.3 M sodium citrate, 3 M NaCl) for 48 hours. DNA was then immobilized onto the membrane by UV-crosslinking. Prehybridization of the membrane in SDS (7% w/v), sodium phosphate buffer pH 7.2 (0.5 M) and EDTA (10 mM) was performed at 65°C for 2 hours prior to addition of the Tradd probe. The Tradd probe was
prepared and separated using the Rediprime II random prime labeling system and NICK columns as per manufacturer’s instructions (Amersham and Pharmacia, respectively). Denatured salmon sperm DNA was boiled for 5 minutes, snap-cooled and added with the radiolabelled probed to the hybridization buffer at a concentration of 100 µg/ml. After overnight hybridization at 65°C, blots were washed twice in 1.5 mM sodium citrate, 15 mM NaCl, 0.1% (w/v) SDS (pH 7.0), wrapped in saran wrap and exposed to phosphor screens for autoradiography (Molecular Dynamics Phosphorimager v4.1, Storm 869, Amersham).

Mice were maintained at the Ontario Cancer Institute Animal Facility in compliance with the regulations of the Animal Ethics and Animal Care Committees at the Princess Margaret Hospital. These bodies approved all experiments.

2.2.2 Preparation and culture of mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) were derived from of Tradd<sup>+/+</sup> and Tradd<sup>−/−</sup> mouse embryos at day 14.5 of gestation. After removing the head and all internal organs, embryos were chopped into 1.5 mm<sup>3</sup> pieces and incubated for 20 min in 2 ml trypsin solution (Invitrogen) in 6 well plates. Two mls fresh trypsin solution were added to each well and incubation was continued for another 20-30 min, mixing the samples every 10 min using a 5 ml pipette. The trypsinization was stopped by adding 4 ml serum. The cells were then washed and transferred into 10 cm plates for further culture in DMEM containing 10% FBS at 37°C in 5% CO<sub>2</sub>.
2.2.3 Preparation of lung fibroblasts

Lung fibroblasts are prepared from of Tradd\textsuperscript{+/+} and Tradd\textsuperscript{−/−} mice. Lung tissues were harvested from mice of age 8-12 weeks, minced into small pieces, and digested for 30 min at 37°C in a 0.25% trypsin solution containing 400 nM EDTA. Cells were cultured in DMEM supplemented with 10% FCS, L-glutamine (2 mM), 2-mercaptoethanol (2-ME; 50 μM), penicillin/streptomycin (100 g/ml). For experiments, lung fibroblasts were used 10 days after harvesting.

2.2.4 Preparation and culture of peritoneal macrophages

Peritoneal macrophages of Tradd\textsuperscript{+/+} and Tradd\textsuperscript{−/−} mice were harvested from mice injected intraperitoneally with 1ml of 4% thioglycolate. Five days later, cells were harvested from the peritoneal cavity by lavage and cultured in RPMI medium containing 10% FBS and 50 μM 2-ME.

2.2.5 Preparation and culture of bone marrow-derived macrophages (BMDM)

Single cell suspensions were prepared from the bone marrow of Tradd\textsuperscript{+/+} and Tradd\textsuperscript{−/−} mice. Red blood cells were removed from bone marrow cells by incubating suspended cells in sterile ACK buffer (0.155 mM ammonium chloride, 0.1 mM EDTA, 10 mM potassium bicarbonate) for 5 min on ice. After washing in RPMI medium containing 10% FBS and 50 mM 2-ME, cells were counted and plated in 6-well tissue culture plates (2x10\textsuperscript{6} cells/well) in 2 ml RPMI medium containing 10% FBS, 2-ME and M-CSF (25 ng/ml). One ml of fresh medium containing M-CSF was added to each well every other day for 5-7 days. Prior to
stimulation, cells were washed and rested in serum-free medium for 2 hrs. Cells were then treated with 10 ng/ml TNF or 100 U/ml IFNg as indicated in the Figures.

2.2.6 Preparation and culture of CD4⁺ T and B220⁺ B cells
Single cell suspensions of spleen and lymph node cells from Tradd⁺/+ and Tradd⁻/⁻ mice were obtained by passing these tissues through a 70 µm mesh. Red blood cells were removed from spleens by incubating suspended cells in sterile ACK buffer for 5 min on ice. Cell suspensions were mixed with MACS magnetic beads (conjugated to anti-CD4 or anti-B220 antibodies) according to the manufacturer’s instructions (Miltenyi Biotec). Purified CD4⁺ T cells and B200⁺ B cells were counted, resuspended in RPMI medium containing 10% FBS and 50 µM 2-ME, and cultured at 37°C in 5% CO₂.

2.2.7 Stimuli and reagents
Mouse TNF, human TNF, mouse IL-1β, mouse IFNg and mouse IL-2 were all from R&D Systems. Polyinosine-polycytidylic acid (poly I:C) was from Invivogen. Mouse TL1a was the kind gift of Dr. Ping Wei of Human Genome Science (Rockville, Maryland, U.S.A.). LPS, 7-amino-actinomycin-D (7AAD), cyclohexamide (CHX), sorbitol, etoposide, doxorubicin and anisomycin, were all from Sigma. The anti-CD3 and anti-CD28 antibodies used for T cell stimulation were from BD Bioscience.
2.2.8 $^3$H-thymidine incorporation

For T cell proliferation studies, 96 well round-bottom plates were pre-coated with either anti-CD3 antibody (0.3 µg/ml, BD Biosciences) or with anti-CD3 plus anti-CD28 (5 µg/ml, BD Biosciences). Purified T cells were seeded in these plates at $10^5$ cells/well in a total volume of 200 µl RPMI containing 10% FBS and 50 µM 2-ME. Stimulation was allowed to proceed for 48 hrs prior to the addition of a pulse of 1 µCi $^3$H-thymidine per well followed by incubation for an additional 8 hrs. The amount of $^3$H-thymidine incorporated by the proliferating T cells was measured using a Topcount (Perkin Elmer).

2.2.9 Cell death assay

MEFs were seeded at $10^5$ cells/well in 12-well plates and were either left untreated or treated with 10 ng/ml TNF, 1-10 µg/ml CHX, or TNF plus CHX for 24 hrs. Cells were trypsinized and subjected to 7-AAD staining followed by flow cytometry to evaluate cell viability.

2.2.10 In vivo cytotoxicity assay

For liver toxicity induced by TNF and D-galactosamine (GalN), wild-type and Tradd-deficient mice were injected intraperitoneally with a single dose of GalN (20 mg per mouse) followed by intraperitoneal injection of TNF (0.4 mg per 20 g body weight). Mice were monitored and were euthanized when moribund.

2.2.11 In vivo TNF injection

Wildtype, Tradd$^{-/-}$ and Tnfr$^{-/-}$ mice were injected intravenously in the tail vein with 3.0 mg murine TNF in 100 ml PBS. Serum samples were collected from blood in the tail vein of
each mouse just before TNF injection and at 6 and 24 hrs post-injection, and IL-6 concentrations were evaluated by enzyme-linked immunosorbent assay (ELISA).

### 2.2.12 Cytokine enzyme-linked immunosorbent assay (ELISA)
Quantitation of cytokines [IL-6, IL-4, TNF ELISA (eBioscience and BD Pharmingen), and IFNγ (eBioscience)] in cell culture supernatants after stimulation was performed by a sandwich ELISA according to the manufacturers’ instructions.

### 2.2.13 TH1 and TH2 Differentiation
TH1 and TH2 cell differentiation was performed according to the method described in (McKenzie et al., 1998). In brief, purified CD4+ cells were cultured on anti-CD3- and anti-CD28-coated plates (1 µg/ml) in the presence of exogenous cytokines or anti-cytokine antibodies. IL-2 (10 ng/ml) was added to all cultures. For TH1 differentiation, IL-12 (5 ng/ml; Peprotech) and anti-IL-4 antibody (2 µg/ml; R&D Systems) were added to the culture. For TH2 differentiation, IL-4 (100 ng/ml; Peprotech) and anti-IFN-γ (2 µg/ml; R&D Systems) were added to the culture.

### 2.2.14 HY-TCR transgenic model for thymocyte negative selection
Tradd−/− HY+ mice were generated by crossing Tradd−/− mice to HY-TCR+ transgenic mice, which were obtained from Dr. Pamela Ohashi (Toronto, Ontario, Canada). Genotyping primers are listed on Table 2.1. Thymocytes from these mice were analyzed between 6-8 weeks of age, by flow cytometry using anti-CD4, anti-CD8 and anti-HY TCR antibodies from Ebioscience.
2.2.15 Immunoblot analysis

MEFs and BMDM were rested in serum-free DMEM or RPMI, respectively, for 2 hrs prior to stimulation with 10 ng/ml TNF, 10 U/ml IFNγ, 100 ng/ml LPS or 100 μg/ml polyinosinic:polycytidylic acid (poly(I:C)). Cells were lysed in 50 μl RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% NP40, 0.5% DOC, 0.1% SDS and complete proteinase inhibitor cocktail from Roche) and protein concentrations were determined using the BCA protein assay (Thermo Scientific). Samples containing equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (4-12% precast, Invitrogen) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Roche). TRADD levels were determined by incubating the blot with rabbit anti-TRADD polyclonal antibody (Santa Cruz). For signaling studies, rabbit anti-pIkB, anti-pIRF3, anti-pERK1/2, anti-pp38, anti-pAKT, and anti-pp65 (from Cell Signaling), anti-pJNK (from Santa Cruz), and anti-pSTAT1-α (from Upstate) were used as primary antibodies to determine levels of phosphorylated proteins. Anti-IkB (from Cell Signaling) was used to determine IkB degradation. To visualize proteins, blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (GE Health), and processed with the ECL Plus system (GE Health).

2.2.16 Immunoprecipitation (IP)

Cells cultured on 15 cm tissue culture plates were treated with TNF (10 ng/ml) or LPS (100 ng/ml). Cells were washed, harvested and lysed in IP buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.2% NP40, 10% glycerol, with complete proteinase inhibitor cocktail). Each IP binding sample contained 500-1000 μg total lysate protein. Anti-TNFR1 or anti-TLR4
antibody (BD Biosciences) was mixed first with protein G beads (GE Health) and then with the lysates and incubated overnight. The beads plus the antibodies and associated proteins were washed 5 times in IP buffer and resuspended in SDS-PAGE sample buffer. Samples were separated by SDS-PAGE and protein components of receptor complexes were determined by immunoblot analysis.

2.2.17 Real-time quantitative reverse transcription (qRT)-PCR
MEFs (2x10^6 cells) were plated in 10 cm plates and cultured for 24 hrs before stimulation with 10 ng/ml TNF in 4 ml RPMI medium for 1 or 4 hrs. Stimulated cells were washed, lysed with 1 ml Trizol (Invitrogen) solution, and transferred into 1.5 ml eppendorf tubes for total RNA purification according to the manufacturer’s protocol. Total RNA was reverse-transcribed and assayed by real-time qRT-PCR. All reactions were performed in an ABI-7900HT Fast Real-Time PCR system using Power SYBR Green PCR reagents according to the manufacturer’s instructions (Applied Biosystems). Primer sequences are listed in Table 2.1.

2.2.18 Electrophoretic mobility shift assay (EMSA)
Lysates from MEFs were separated into nuclear and cytoplasmic membrane fractions using a nuclear/cytosol protein extraction kit (BioVision) according to the manufacturer’s instructions. Nuclear extracts were then utilized in standard gel mobility shift assays. The NF-κB oligonucleotide probes for EMSA were end-labeled with ^32P-ATP using T4 polynucleotide kinase (New England BioLabs). Nuclear extracts (10 µg) were prepared in EMSA buffer (10 mM HEPES pH 7.9, 6% (v/v) glycerol, 2% (v/v) Ficoll, 100 mM KCl, 0.5
mM EDTA, 2.5 mM MgCl₂, and 1 mM dithiothreitol) and incubated with the DNA probes for 15 min at room temperature. Protein-DNA complexes were analyzed on a 5% native polyacrylamide gel.

2.2.19 RelA/p65 immunofluorescent staining
MEFs (2x10⁴ cells) were plated on cover slips overnight and stimulated with TNF (10 ng/ml), IL-1β (10 ng/ml) or LPS (500 ng/ml) for 30 min. Stimulated cells were fixed in methanol and stained with mouse anti-NF-κB (RelA/p65, Santa Cruz) followed by Cy3-conjugated sheep F(ab’)₂ anti-mouse IgG (Sigma). The stained slides were mounted in DAPI-containing mounting solution and observed under a fluorescence microscope (Leica Microsystems).

2.2.20 Immunization and FDC/GC detection
Male mice (6–12 weeks of age) of the Tradd⁺/⁺ and Tradd⁻/⁻ genotypes were injected intraperitoneally on day 0 with 10⁸ washed sheep red blood cells (SRBC) (HemoStat Laboratories). At 10 days post-immunization, spleens were isolated from immunized mice, fixed for 24 hrs in zinc-buffered formalin (Fisher Scientific), and transferred to 70% ethanol before processing through paraffin. For frozen sections, spleens were frozen in optimal cutting temperature (O.C.T.) embedding medium (Sakura Finetek) and sections (6 µm thick) were cut using a cryostat microtome (Leica). The sections were mounted onto slides coated with poly-L-lysine and air-dried. Detection of FDCs was carried out by incubating frozen spleen sections with biotinylated anti-CR1 (clone 8C12) (BD Biosciences). Detection of GCs was carried out by incubating formalin-fixed spleen sections with biotinylated peanut agglutinin (PNA; Vector Labs) and biotinylated anti-B220 (BD Biosciences).
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<td><strong>Mouse screening</strong></td>
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2.3 Results

2.3.1 Generation of Tradd knockout mice

The Tradd knockout mouse was generated by a post-doctoral fellow in the Mak laboratory. Briefly, Tradd-deficient embryonic stem (ES) cells were generated using a gene targeting strategy described in Figure 2.2A. ES cells bearing a wild type (WT) Tradd allele and a 3lox Tradd allele were microinjected into C57BL/6 mouse blastocysts to generate heterozygous mice (Tradd^+/3lox). These mice were then crossed to deleter CRE mice (Schwenk et al., 1995) to remove Tradd coding exons 3-5 and the neomycin resistance and HSV thymidine kinase genes (TK/Neo), generating TRADD heterozygous mice (Tradd^+/−). Deletion of exons 3-5 does not remove the entire coding region but the known functional domain of TRADD (death domain) is eliminated. The remaining sequences only encode a very short fragment of the TRADD N-terminus. Tradd^+/− mice were then intercrossed to generate Tradd-deficient (Tradd^−/) mice. Southern blotting confirmed homologous recombination in ES cells (Figure 2.2B) and the successful cre/loxP recombination in Tradd^−/− mice (Figure 2.2C). TRADD protein depletion in Tradd^−/− cells was confirmed by immunoblot analysis (Figure 2.2D). All mice were backcrossed at least five times into C57BL/6 prior to analysis. Tradd^−/− mice were born at expected Mendelian ratios. They were healthy and showed no obvious abnormalities, suggesting that TRADD plays little or no exclusive role in embryogenesis.
Figure 2.2 Targeting strategy and germline confirmation.
Figure 2.2 Targeting strategy and germline confirmation.

(A) Tradd coding exons 3-5 and a TK/Neo selection cassette were flanked by loxP sequences and subcloned into the targeting vector. K, KpnI; B, BglII; triangle, loxP sequences. (B) Genomic Southern blotting performed using the flanking probe (FP) to detect recombinant ES clones. 3 different clones are shown. (C) Genomic Southern blotting performed using the long arm probe (LAP) and tail DNA from mice of the indicated genotypes to identify Tradd^{+/+} and Tradd^{-/-} mice. For (B-C), DNA was digested with BglII and KpnI. (D) Immunoblot analysis of primary murine embryonic fibroblasts (MEF), bone marrow derived macrophages (BMDM) and T cells showing successful deletion of Tradd in Tradd^{-/-} cells.
2.3.2 TRADD is indispensable for TNF-mediated cell death

To dissect the role of TRADD in cell death, we generated Tradd\textsuperscript{+/+} and Tradd\textsuperscript{−/−} murine embryonic fibroblasts (MEFs), and treated them with a variety of death inducing agents. Cell death was assessed by 7-AAD staining and flow cytometry. MEFs exhibited equivalent sensitivity to a variety of cell death-inducing agents, such as sorbitol (induction of hyperosmotic stress), etoposide (induction of DNA damage), doxorubicin (induction of DNA damage) and anisomycin (inhibition of protein and DNA synthesis) (Figure 2.3A). Similarly, Tradd deficiency did not alter the susceptibility of CD4\textsuperscript{+} T cells to Fas-mediated activation-induced cell death (AICD) (Ju et al., 1995) (Figure 2.3B), indicating that TRADD plays no role in Fas-mediated apoptosis. In contrast to the above death-inducing agents, treatment of MEFs with TNF plus the translational inhibitor cycloheximide (CHX) for 24 hrs resulted in cell death in greater than 70% of Tradd\textsuperscript{+/+} MEFs whereas Tradd\textsuperscript{−/−} MEFs were completely resistant to the same treatment (Figure 2.3C, D). A similar experiment in vivo also shows that while Tradd\textsuperscript{+/+} mice succumbed to treatment with TNF plus a liver specific transcriptional inhibitor, GalN (D-galactosamine), Tradd\textsuperscript{−/−} mice were completely resistant to this treatment (Figure 2.3E). Taken together, these results show that TRADD is indispensable for the cell death arm of TNFR1 signaling but is not involved in the death triggered by many other agents.
Figure 2.3 TRADD is indispensable for TNF-induced cell death but not for other forms of death inducing agents.
Figure 2.3 TRADD is indispensable for TNF-induced cell death but not for other forms of death-inducing agents.

(A-D) *In vitro* cytotoxicity assays. Viability was assessed by 7-AAD staining and flow cytometry. (A) Primary MEFs from *Tradd*+/+ and *Tradd*−/− mice were treated for 24 hrs with the indicated chemicals. Bar graph summary of flow cytometric data from chemical-treated MEFs is shown. (B) CD4+ T cells *Tradd*+/+ and *Tradd*−/− were stimulated with anti-CD3 plus anti-CD28 and IL-2 for 5 days. The cells were rested for 24 hrs and live cells were purified and then restimulated in 24 well plates pre-coated with anti-CD3 for 24 hrs, stained with 7-AAD, and analyzed by flow cytometry for cell viability. (C, D) Primary MEFs from *Tradd*+/+ and *Tradd*−/− mice were treated with TNF alone (10 ng/ml), CHX alone, (1-10µg/ml) or TNF plus CHX. (C) Representative flow cytometric data with 1 µg/ml CHX. (D) Bar graph summary of flow cytometric data of MEFs treated at different doses of CHX. For (A), (B) and (D), results shown are the mean viability ± S.D. of triplicate determinations. ** p< 0.01, Student’s *t*-test. All data are representative of at least three independent experiments. (E) *In vivo* cytotoxicity assay. *Tradd*−/− mice are resistant to TNF cytotoxicity *in vivo*. *Tradd*+/+ (n=8) and *Tradd*−/− (n=8) mice were treated with 700 mg/kg GalN and 20 mg/kg TNF. Survival of mice was monitored over 10 hours post injection.
2.3.3 TRADD is essential for TNF-mediated NF-κB activation

NF-κB activation, marked by its nuclear entry, is a key signaling event required for TNF-induced production of inflammatory cytokines (Aggarwal, 2003; Chen and Goeddel, 2002; Hsu et al., 1995). Electrophoretic mobility shift assay (EMSA) demonstrated that, in the absence of TRADD, TNF-induced accumulation of NF-κB in the nucleus was almost completely abolished (Figure 2.4A). Immunofluorescent staining confirmed that RelA/p65 (a major subunit of the NF-κB complex) was not translocated into the nucleus upon TNF stimulation of Tradd+/− MEFs, whereas translocation occurred normally in the mutant cells after LPS or IL-1β stimulation (Figure 2.4B). Thus, the machinery of NF-κB activation is intact in Tradd+/− MEFs but TRADD is specifically required for TNF-stimulated NF-κB activation.

Several endogenous NF-κB inhibitors, including IκB and A20, reportedly deliver a negative feedback signal that downregulates NF-κB activation (Beg et al., 1995; Heyninck et al., 1999; Klement et al., 1996; Song et al., 1996). To rule out the possibility that the abrogation of NF-κB activation observed in TNF-stimulated Tradd+/− cells was due to upregulation of NF-κB inhibitors, we used real-time quantitative reverse transcription (qRT)-PCR to monitor IκB and A20 expression in TNF-stimulated Tradd+/+ and Tradd+/− MEFs. A strong induction of A20 and IκB was observed in Tradd+/+ MEFs after 1 hr of TNF stimulation but only a very weak induction of these inhibitors could be detected in similarly-treated Tradd+/− MEFs (Figure 2.4C, center and right). Comparable results were seen after 4 hrs of TNF stimulation. This is consistent with the fact that these NF-κB inhibitors are transcriptional targets of NF-κB itself (Karin and Lin, 2002). Thus, the abrogation of TNF-
induced NF-κB activation in Tradd-deficient cells is not due to upregulation of the NF-κB inhibitors studied and TRADD is therefore essential for TNF-induced NF-κB activation.

NF-κB activation is a key signaling event required for TNF-induced inflammatory cytokine production (Aggarwal, 2003; Chen and Goeddel, 2002; Hsu et al., 1995). To strengthen our previous findings, we evaluated IL-6 production in TNF-stimulated Tradd\textsuperscript{+/+} and Tradd\textsuperscript{−/−} MEFs by real-time qRT-PCR (Figure 2.4C, left). IL-6 mRNA was clearly not induced in the absence of Tradd. Confirming these results, cytokine secretion into culture medium, as quantitated by enzyme-linked immunosorbent assay (ELISA), showed that IL-6 was not produced in the absence of Tradd. (Figure 2.4D, left). In an in vivo corollary of this experiment, we injected 3.0 µg recombinant TNF intravenously into wildtype, Tradd\textsuperscript{−/−} and Tnfr1\textsuperscript{−/−} mice, and measured the level of IL-6 production in the serum 6 hrs after injection. Reminiscent of Tnfr1\textsuperscript{−/−} animals, Tradd\textsuperscript{−/−} mice did not exhibit an induction of IL-6 in the serum upon TNF stimulation, in contrast to what was observed in wildtype counterparts (Figure 2.4E).

Our results thus far confirm that TRADD is essential for TNF induced cell death, as well as TNF induced NF-κB activation and subsequent inflammatory cytokine production.
Figure 2.4 TRADD is essential for TNF-mediated NF-κB activation.
Figure 2.4 TRADD is essential for TNF-mediated NF-κB activation.

(A-D) *In vitro* analyses of TNF induced NF-κB responses. (A) Electrophoretic mobility shift assay (EMSA). MEFs from *Tradd*<sup>+/+</sup> and *Tradd*<sup>−/−</sup> mice were stimulated with 10 ng/ml TNF for the indicated times and NF-κB activation was evaluated by EMSA. (B) RelA/p65 translocation. The MEFs in (A) were subjected to immunofluorescent staining to detect RelA/p65 translocation into the nucleus. MEFs were also treated with LPS (500 ng/ml) or IL-1β (10 ng/ml) and RelA/p65 translocation was assessed. (C) Inhibited IL-6 production and normal endogenous NF-κB inhibitors. MEFs from *Tradd*<sup>+/+</sup> and *Tradd*<sup>−/−</sup> mice were treated for 1 or 4 hrs with 10 ng/ml TNF and the induction of IL-6 (positive control) and the NF-κB inhibitors A20 and IκB was evaluated by real-time qRT-PCR. Results shown are the average fold induction over background. For (A-C), data are representative of at least three independent experiments. (D) MEFs from *Tradd*<sup>+/+</sup> and *Tradd*<sup>−/−</sup> mice were treated for 24hrs with TNF (10 ng/ml), IFNγ (100 U/ml) or TNF+IFNγ. IL-6 in the culture supernatant was measured by ELISA. The results shown are the mean IL-6 level and SD of triplicate determinations. **p<0.01, Student’s *t*-test. C’, control (no stimulus). (E) *In vivo* analysis of TNFα induced NF-κB mediated IL-6 production. Wildtype (n=4), *Tradd*<sup>−/−</sup> (n=5) and *Tnfr1*<sup>−/−</sup> (n=3) mice were injected in the tail vein with TNF (3 mg per mouse) and serum IL-6 levels were determined by ELISA 6 hrs later. Horizontal bar, mean value. *, p<0.05, Student’s *t*-test.
2.3.4 TRADD is essential for upstream signaling events upon TNF stimulation

We next examined cytoplasmic signaling events upstream of NF-κB activation in TNF-stimulated Tradd+/+ and Tradd−/− MEFs and bone marrow derived macrophages (BMDM). The key step in NF-κB activation is the release of the NF-κB dimers from their inhibitory proteins, achieved via degradation of the IκBs. This irreversible signaling step constitutes a commitment to transcriptional activation. The signal is eventually terminated through nuclear expulsion of NF-κB, the outcome of a negative feedback loop based on IκBα transcription, synthesis, and IκBα-dependent nuclear export of NF-κB (Ghosh and Karin, 2002). We used immunoblot analysis to show that TNF treatment failed to induce significant degradation of IκB in Tradd−/− MEFs (Figure 2.5A) or BMDM (Figure 2.5B), in contrast to the situation in Tradd+/+ cells. Thus, TRADD is required for TNF induced IκB degradation in both MEFs and BMDMs.

Mitogen-activated protein kinase (MAPK) activation is reportedly involved in transducing TNF signaling (Kalb et al., 1996). We therefore analyzed the effect of Tradd deficiency on TNF-induced extracellular signal-regulated kinases (ERK) and c-Jun N-terminal kinases (JNK) activation. Surprisingly, Tradd−/− MEFs still exhibited some degree of JNK and ERK activation in response to murine TNF stimulation, albeit with altered kinetics (Figure 2.5A, B). To eliminate potential confounding effects due to MAPK activation downstream of TNFR2, we stimulated Tradd+/+ and Tradd−/− cells with human TNF (huTNF), which is thought to preferentially activate TNFR1 in murine cells (Ameloot et al., 2001). Under these conditions, MAPK activation was completely abolished in the absence of TRADD (Figure 2.5C), confirming that TRADD is essential for TNFR1-mediated MAPK activation.
2.3.5 The composition of the TNFR1 complex is altered in TNF-stimulated *Tradd*-deficient cells

TRADD is believed to be the core adaptor that recruits RIP1, TRAF2 and FADD into the TNFR1 complex in response to TNF stimulation (Chen and Goeddel, 2002; Mak and Yeh, 2002). To determine whether deletion of *Tradd* would affect TNFR1 complex formation, we treated *Tradd*+/+ and *Tradd*−/− MEFs with TNF and carried out immunoprecipitation (IP) experiments using an antibody specific for TNFR1. Peak binding of TRAF2 to TNFR1 was detected in the *Tradd*+/+ MEFs after 5 min of TNF stimulation, and RIP1 exhibited a ladder-like pattern of ubiquitination at the same timepoint. Such ubiquitination was not observed in *Tradd*−/− counterparts (Figure 2.5D). RIP1 polyubiquitination has been shown before to be important for IKK activation after TNF binds to TNFR1 (Ea et al., 2006). This is consistent with our data showing that NF-κB activation is defective in the absence of *Tradd*. Surprisingly, the association of RIP1 with TNFR1 occurs even without receptor triggering, and RIP1 (but not its ubiquitinated counterpart) remained associated with TNFR1 in TNF-stimulated *Tradd*+/− cells. Recruitment of TRAF2 to the TNFR1 complex after TNF stimulation was abolished in the absence of TRADD (Figure 2.5D). Thus, *Tradd* deficiency alters the components that associate with TNFR1 upon TNF stimulation. Most importantly, our data collectively show that the main function of TRADD in TNFR1 mediated proinflammatory signaling is to recruit TRAF2 to the receptor-proximal complex, which then triggers the ubiquitination of RIP1 (Lee et al., 2004) and possibly other components of the signaling complex to induce efficient activation of the NF-κB and MAPK cascades.
Figure 2.5 Alterations to TNFR1 signaling and complex formation in the absence of TRADD.
Figure 2.5 Alterations to TNFR1 signaling and complex formation in the absence of TRADD.

(A, B, C) ERK, JNK and IκB signaling responses. Tradd<sup>+/+</sup> and Tradd<sup>−/−</sup> MEFs (A) and BMDMs (B) were stimulated with 10 ng/ml murine TNF (A, B) or 10 ng/ml human TNF (C) for the indicated times and ERK and JNK phosphorylation and IκB degradation were assessed by immunoblot analysis. Tubulin and actin, loading controls. (D) TNFR1 complex composition. Tradd<sup>+/+</sup> and Tradd<sup>−/−</sup> MEFs were stimulated with 10 ng/ml TNF for the indicated times and the TNFR1 complex was isolated by IP. TRAF2, RIP and ubiquitinated RIP (Ub-RIP) within the TNFR1 complex were detected by immunoblotting. For (A-D), results are representative of at least three independent experiments.
2.3.6 TRADD is required for FDC clusters and germinal center formation

Superimposed on the architecture of lymphoid organs is the coalescence of follicles and germinal center (GC) reactions facilitated by TNF/TNFR superfamily proteins during active immune responses (Matsumoto et al., 1996). These are cellular aggregates comprised mostly of B cells but also T cells and follicular dendritic cells (FDC) (Cyster et al., 2000). FDCs are specialized mesenchymal cells that collect antigens in draining lymph nodes, interact with clonally expanding B cells, and form networks in the follicle under the influence of TNF, LTα, LTβ, TNFR1 and LTβR (Fu and Chaplin, 1999). In GCs, B cells are stimulated and their antigen receptor genes are somatically hypermutated; those with better antigen avidity are selected and can undergo heavy chain class switching to produce different antibody subclasses. Mice deficient of Tnf and Tnfr1 have severe defects in FDC structure and in GC formation (Fu and Chaplin, 1999). To determine whether TRADD is important for these features of splenic architecture, we first used anti-CR1 staining to analyze the distribution of FDCs in primary splenic follicles of unimmunized wildtype, Tradd+/− and Tnfr1−/− mice. In contrast to wildtype mice, Tradd-deficient mice and Tnfr1-deficient mice showed no organized FDC clusters in the spleen (Figure 2.6A). We next moved on to assess GC formation by immunizing Tradd+/+ and Tradd−/− mice with sheep red blood cells (SRBC) and detected GC in splenic follicles by peanut agglutinin (PNA) and anti-B220 staining. SRBC immunization clearly induced GC formation at the junction of the T cell and B cell regions in splenic follicles of Tradd+/+ mice but not in Tradd−/− mice (Figure 2.6B). These results demonstrate that the TNFR1-dependent development of normal FDC clusters and GC formation in the spleen require TRADD, further reinforcing the indispensable role of TRADD in TNFR1 mediated cellular events.
Figure 2.6 Abolition of TNF-induced inflammatory responses and absence of FDC clusters and SRBC-induced GCs in $Tradd^{-/-}$ mice.

Decreased FDC and impaired GC formation. (A), FDC clusters in non-immunized mice of the indicated genotypes were determined by anti-CR1 staining. (B), GC formation in splenic follicles of SRBC-immunized $Tradd^{+/+}$ and $Tradd^{-/-}$ mice was determined by anti-B220 and PNA staining. Results shown are representative of three mice examined per group.
2.3.7 TRADD is required for TL1a/DR3-mediated T cell costimulation but not for DR3 mediated thymocyte negative selection

In addition to TNFR1, TRADD may also participate in signaling events downstream of the TNF receptor superfamily member death receptor (DR)-3 (Chinnaiyan et al., 1996; Pan et al., 1998b), which is thought to mediate thymocyte negative selection (Wang EC, 2001), and to provide costimulatory signals during T cell activation (Chinnaiyan et al., 1996), attributes not found downstream of TNFR1. This reveals a nonredundant *in vivo* role for this TNF receptor family member in the removal of self-reactive T cells in the thymus and the activation of T cells in the periphery. To evaluate the role of TRADD in DR3 signaling, we began by looking at the effect of *Tradd* deficiency on thymocyte negative selection using T-cell receptor transgenic mice expressing a receptor reactive with the male antigen HY presented by class I MHC H-2Db molecules (Kisielow et al., 1988). The life or death of a developing thymocyte rests on the affinity of its T-cell receptor (TCR) for peptide–MHC ligands, such that a high-affinity interaction with an agonist ligand leads to death (negative selection) and an intermediate-affinity interaction with a partial agonist ligand promotes survival (positive selection) (Hogquist et al., 2005). In the HY model, HY-specific thymocytes are positively selected for in female mice but negatively selected against in male mice, which express the agonist ligand male antigen HY (Kisielow et al., 1988). Deletion of *Tradd* in female mice did not seem to promote positive selection of HY TCR\(^+\)CD8\(^+\) T cells, as similar percentages of CD8\(^+\) and HY TCR\(^+\)CD8\(^+\) thymocytes were found in these mice compared to *Tradd*-proficient counterparts (Figure 2.7A, left). As well, both *Tradd\(^{+/+}\)* and *Tradd\(^{-/-}\)* HY TCR-transgenic male mice exhibited almost no HY TCR\(^+\)CD8\(^+\) T cells (Figure 2.7A, right), demonstrating efficient negative selection regardless of the status of *Tradd*. This is in contradiction to *Dr3* deficient mice, which exhibit defects in negative selection in the
same model (Wang EC, 2001). These data suggest that TRADD is dispensable for DR3 mediated thymocyte negative selection.

The processes of positive and negative selection shape the population of thymocytes into a peripheral pool of T cells that are able to respond to foreign pathogens. Through its CD3 T cell receptor, a T cell recognizes a specific antigen presented by an antigen presenting cell (APC), constituting the first activation signal. In order to be fully activated, however, a second, APC-derived costimulatory signal transduced by the CD28 receptor is also required. This requirement represents a key point in the control of T cell activation as well as clonal proliferation (Janeway, 2007). Like the CD28 receptor, DR3 has been demonstrated to mediate T cell costimulatory signals through engagement with its native ligand, TL1a (Migone et al., 2002). To evaluate TRADD’s role in DR3 mediated costimulation of T cells in the periphery, we treated Tradd+/+ and Tradd−/− CD4+ T cells with anti-CD3 plus TL1a and assessed T cell proliferation by ³H-thymidine incorporation. Stimulation with anti-CD3 alone, anti-CD3 plus anti-CD28, or anti-CD3 plus IL-2 induced similar levels of T cell activation in cells of both genotypes (Figure 2.7B). In contrast, only Tradd+/+ CD4+ T cells, but not Tradd-deficient CD4+ T cells, exhibited increased proliferation after anti-CD3 plus TL1a stimulation. Thus, TRADD is indispensable for DR3-mediated T cell costimulation. These data suggest that TRADD is required for selected aspects of DR3 functions. The mechanism behind this selectivity, however, warrants future investigation.
Figure 2.7 TRADD is required for TL1a/DR3-induced CD4^+ T cell costimulation but not for DR3 mediated thymocyte negative selection.
Figure 2.7 TRADD is required for TL1a/DR3-induced CD4⁺ T cell costimulation but not for DR3 mediated thymocyte negative selection.

(A) Representative CD8 versus CD4 expression profiles (top) and HY versus CD8 profiles (bottom) of thymocytes from Tradd⁺/⁺ and Tradd⁻/⁻ female (left) and male (right) HY TCR transgenic mice. Percentage of cells in each subset is denoted in its respective quadrant.

(B) CD4⁺ T cells were purified from Tradd⁺/⁺ and Tradd⁻/⁻ mice and stimulated for 56 hrs with plate-bound anti-CD3 (0.3 µg/ml) plus anti-CD28 (5 µg/ml), IL-2 (10 ng/ml) and/or mouse TL1a (3 µg/ml). Costimulation was assessed in terms of increased T cell proliferation as measured by ³H-thymidine incorporation. Results shown are the mean fold induction (over anti-CD3 stimulation alone) ± S.D. of triplicate determinations. ** p< 0.01, Student’s t-test.

For (A and B), data shown are representative of at least three independent experiments.
2.3.8 *Tradd* deficiency does not recapitulate phenotypes observed in mice deficient of DR6

Mice deficient for Dr6 exhibit enhanced CD4\(^+\) T cell proliferation and T\(_{H2}\) cytokine production (Liu et al., 2001; Schmidt et al., 2003; Zhao et al., 2001), consequently contributing to an enhancement of B cell expansion, survival, and humoral responses (Liu et al., 2001; Schmidt et al., 2003; Zhao et al., 2001). Since TRADD has been shown to interact with DR6 in an overexpression setting (Pan et al., 1998a), we sought to determine if these physiological activities mediated through DR6 require TRADD. Purified T cells from spleens and lymph nodes of wildtype and *Tradd* deficient mice were cultured in microtiter plates coated with different doses of anti-CD3 and anti-CD28 antibodies, and T cell proliferation was determined by \(^{3}\)H-thymidine incorporation after a 48 hrs incubation. The proliferation of T cells from *Tradd*-deficient mice in response to anti-CD3/anti-CD28 was comparable to that of wild-type T cells (Figure 2.8A).

When activated by antigen presenting cells, naïve CD4\(^+\) T cells undergo clonal proliferation and produce IL-2. The activated CD4\(^+\) T cells may then become T\(_{H1}\) or T\(_{H2}\) effector cells, which are involved in inflammatory and humoral responses, respectively (Langrish et al., 2005). Animals deficient in DR6 display a profound polarization toward the T\(_{H2}\) response (Liu et al., 2001; Schmidt et al., 2003; Zhao et al., 2001), so we also investigated the role of TRADD in effector T cell differentiation, by evaluating T\(_{H1}\) and T\(_{H2}\) lineage differentiation *in vitro*. Different T\(_{H}\) populations are defined by their cytokine secretion profile. T\(_{H1}\) cells secrete IL-2 and mainly IFN\(_{\gamma}\) whereas T\(_{H2}\) cells secrete IL-5, IL-10, IL-13 and mainly IL-4 (Mosmann TR, 1989). CD4\(^+\) T cells from wild-type and *Tradd*-deficient mice were cultured under T\(_{H1}\) and T\(_{H2}\) conditions and the levels of cytokines secreted were measured by ELISA. When differentiated into either T\(_{H1}\) or T\(_{H2}\) cells, *Tradd*-
deficient lymphocytes produced levels of IFNγ and IL-4 comparable to littermate controls (Figure 2.8B).

In parallel to studying T lymphocyte proliferation and differentiation upon *in vitro* stimulation, *Tradd* deficient B lymphocytes were also evaluated for proliferative changes post anti-IgM, anti-CD40 and LPS stimulation, as DR6 has also been implicated in B cell expansion (Liu et al., 2001; Schmidt et al., 2003; Zhao et al., 2001). Analogous to T cells, no difference was observed in proliferation between *Tradd*+/+ and *Tradd*−/− B cells, as assessed by the ³H-thymidine incorporation assay (Figure 2.8C).

The above observations argue against a role of TRADD in DR6 mediated lymphocyte proliferation and differentiation. However, given that the native ligand for DR6 is unknown, it remains possible that TRADD may still participate in some DR6 signaling events under specific cellular contexts.
Figure 2.8 TRADD is dispensable for anti-CD3 induced proliferation, *in vitro* induction of T\(_{H}\) cell differentiation and *in vitro* stimulation of B cell proliferation.
Figure 2.8 TRADD is dispensable for anti-CD3 induced proliferation, *in vitro* induction of T<sub>H</sub> cell differentiation and *in vitro* stimulation of B cell proliferation.

(A) CD<sup>4</sup><sup>+</sup> T cell proliferation of Tradd<sup>−/−</sup> and littermate control after anti-CD3 (0.3 µg/ml) plus anti-CD28 (1 µg/ml) and IL-2 (10 ng/ml). Cells were stimulated for 48 hrs and pulsed for an additional 8 hrs with 1 µCi <sup>3</sup>H-thymidine per well. (B) CD<sup>4</sup><sup>+</sup> T cells purified from spleens and lymph nodes were differentiated into T<sub>H</sub>1 or T<sub>H</sub>2 cells with anti-CD3 and anti-CD28 plus IL-12 (20 ng/ml) and 5 µg/ml anti-IL-4 antibody to promote T<sub>H</sub>1 differentiation or IL-4 (20 ng/ml) and 5 µg/ml anti-IFNγ for T<sub>H</sub>2 differentiation. Production of IFNγ and IL-4 was determined by ELISA. Data is representative of three individual experiments. (C) B220<sup>+</sup> cell proliferation of Tradd<sup>−/−</sup> and littermate control after anti-IgM (20 µg/ml), anti-CD40 (5 µg/ml) and LPS (1-10 µg/ml) stimulation with/without IL-4 (20 ng/ml) costimulation. Cells were stimulated for 48 hrs and pulsed for an additional 8 hrs with 1 µCi <sup>3</sup>H-thymidine per well. For (A-C), data are representative of three individual experiments. pn conc, protein concentration.
2.3.9 *Tradd* deficiency does not enhance IFNγ-mediated STAT1-α phosphorylation

Recently, TRADD was implicated in the crosstalk between TNFR1 signaling and IFNγ signaling. In RAW264.7 cells and HeLa cells, interaction of STAT1-α and TRADD promotes the TNFR1-induced apoptotic pathway but suppresses TNFR1-induced NF-κB activation (Wang et al., 2000b; Wesemann and Benveniste, 2003). In RAW246.7 cells, *Tradd* knockdown increases IFNγ-induced STAT1-α activation and function (Wesemann et al., 2004). To validate these observations in primary *Tradd*-deficient cells, we used immunoblot analysis to examine IFNγ-induced STAT1-α phosphorylation in *Tradd*+/+ and *Tradd*−/− MEFs (Figure 2.9A), BMDM (Figure 2.9B) and CD4+ T cells (Figure 2.9C). While RIP1 and IκB levels appeared normal in mutant MEFs, no enhancement of IFNγ-induced STAT1-α activation was detected in any *Tradd* deficient cell type. In fact, if anything, the *Tradd*-deficient cells of a tested pair sometimes showed a slight reduction in STAT1-α activation following IFNγ stimulation. Further analysis of IFNγ signaling needs to be performed both in primary and transformed *Tradd*-deficient cells to resolve this controversy.
Figure 2.9 TRADD deficiency does not enhance IFNγ signaling.
Figure 2.9 TRADD deficiency does not enhance IFNγ signaling.

IFNγ signaling. Tradd+/+ and Tradd−/− MEFs (A, n=3 pairs), BMDM (B, n=7 pairs) and CD4+ T cells (C, n=3 pairs) were stimulated with 10 U/ml IFNγ for the indicated times and STAT1-α phosphorylation was assessed by immunoblot analysis. IκB and RIP1 were also examined in MEFs (A).
2.3.10 TRADD is a component of the TLR4 signaling complex

Several signaling mediators involved in TNFR downstream signaling, particularly ones containing the DD, have been reported to play roles in modulating Toll-like receptor signaling (Bannerman et al., 2004; Imtiyaz et al., 2006; Meylan et al., 2004a). We hypothesized that TRADD also participates in these pathways. To determine if TRADD plays a role in TLR signaling, we treated RAW246.7 cells with the TLR4 ligand LPS for 5 to 90 min and carried out IP with an anti-TLR4 antibody to isolate the TLR4 receptor complex. When this complex was subjected to immunoblot analysis to determine its components, we found that both TRADD and RIP1 were present (Figure 2.10A). To further assess the interaction between TRADD and components of the TLR4 complex, we cotransfected 293T cells with a TRADD-expressing vector and vectors expressing Myc-tagged TLR4 signaling mediators, including the TIR domain of TLR4 (TLR4-TIR), MAL, MyD88, IRAK4 and RIP1. After 24 hrs, TRADD was isolated using specific anti-TRADD antibody and proteins associated with TRADD were identified by immunoblot analysis using an anti-Myc antibody. The results confirmed TRADD and RIP1 interaction and demonstrated a strong direct association between TRADD and TLR4-TIR (Figure 2.10B). TRADD also bound to MAL and MyD88 under these overexpression conditions. However, no interaction could be detected between TRADD and IRAK4, a key DD-containing TLR signaling mediator (Figure 2.10C). This latter finding suggests that TRADD may either act upstream of, or in parallel to, IRAK4 in the TLR4 signaling pathway. Reciprocal IPs in which complexes associated with tagged TLR4 signaling mediators were isolated and Western-blotted to detect TRADD showed that RIP1 and TLR4-TIR, but not MAL or MyD88, can associate with TRADD (Figure 2.10D). Taken together, these data demonstrate that TRADD indeed participates in the TLR4 complex that forms upon LPS stimulation. We speculate that TRADD may do so
either directly by interacting with TLR4-TIR, or indirectly through RIP1 in the TRIF-dependent pathway (Meylan et al., 2004a).
Figure 2.10 TRADD is recruited to the TLR4 signaling complex upon LPS stimulation.
Figure 2.10 TRADD is recruited to the TLR4 signaling complex upon LPS stimulation.

(A) TRADD participates in the TLR4 complex. RAW264.7 cells were stimulated with LPS (100 ng/ml) for the indicated times and lysates were subjected to IP with anti-TLR4. TRADD and RIP1 were identified by immunoblotting to be components of the TLR4 complex. (B, C) TRADD associates with TLR4-TIR but not IRAK4. 293T cells were cotransfected with a TRADD-expressing vector and vectors expressing the indicated Myc-tagged mediators. Proteins associating with TRADD were identified by TRADD-IP and anti-Myc immunoblot analysis. pcDNA3 and pBABE, empty vector controls. (D) TLR4-TIR associates with TRADD. Reciprocal IP involving anti-Myc IP and anti-TRADD immunoblotting. For (A-D), data are representative of at least three independent experiments.
2.3.12 TRADD is involved in TLR4 signaling and cytokine production

To determine if TRADD is required for TLR-mediated cellular functions, we examined the effect of Tradd deficiency on inflammatory cytokine production in response to TLR4 engagement. We stimulated wildtype, Tradd-deficient and Tnfr1-deficient BMDM (Figure 2.11A), as well as wildtype and Tradd-deficient peritoneal macrophages (Figure 2.11B), with the TLR4 ligand LPS and analyzed TNF production 24 hrs later. In both cases, induction of TNF in response to LPS was clearly reduced in Tradd−/− macrophages compared to wildtype macrophages, whereas no such reduction was seen in Tnfr1−/− macrophages. To further dissect the role of TRADD in TLR4 signaling, we examined the impact of Tradd deficiency on LPS-stimulated NF-κB and MAPK activation in BMDMs. There were no obvious alterations in MAPK activation (Figure 2.11C), STAT1-α activation (Figure 2.11D) or IκB degradation (Figure 2.11D-E) in the absence of Tradd. However, we did observe a reduction in p65 phosphorylation status in Tradd+/− cells, particularly at later timepoints (Figure 2.11C, top). It is known that TLR4 engagement leads to activation of the MyD88-dependent pathway that mediates early NF-κB and MAPK activation, as well as the MyD88-independent, TRIF-dependent pathway that mediates late NF-κB and IRF3 activation (Sato et al., 2003; Yamamoto et al., 2003). Given the reductions in TNF production and late stage p65 phosphorylation in LPS-stimulated Tradd-deficient cells, we suspect that it is the TRIF-dependent pathway that is compromised in the absence of Tradd.
Figure 2.11 TRADD is involved in TLR4 signaling and TLR4 mediated cytokine production.
Figure 2.11  TRADD is involved in TLR4 signaling and TLR4 mediated cytokine production.

(A, B) Reduced LPS-stimulated TNF production. Tradd+/+, Tradd−/− and Tnfr1−/− BMDMs (A) and Tradd+/+ and Tradd−/− peritoneal macrophages (B) were stimulated with LPS (10, 100 or 300 ng/ml) for 24 hrs and TNF in the culture supernatants was evaluated by ELISA. * p< 0.05, Student’s t-test. (C-E) Reduced LPS-stimulated NF-κB and MAPK activation. Tradd+/+ and Tradd−/− BMDMs were stimulated with 100 ng/ml LPS for the indicated times. The phosphorylation of p65, ERK, JNK and STAT1-α and IκB degradation were assessed by immunoblotting. β-actin and β-tubulin, loading controls. For (A-E), data are representative of at least three independent experiments.
2.3.13 TRADD is involved in TRIF dependent NF-κB activation

TRIF is crucial for the MyD88-independent signaling pathway activated by engagement of TLR3 or TLR4 (Yamamoto et al., 2003). To elucidate the role of TRADD in the TRIF-dependent pathway while eliminating any potentially compensatory effects of the MyD88-dependent pathway (Covert et al., 2005), we examined the role of TRADD in TLR3 signaling, which utilizes TRIF solely for induction of inflammatory cytokines. We first examined IL-6 production in response to polyinosinic:polycytidylic acid (poly(I:C)), a synthetic TLR3 ligand. We employed Tradd/Tnf double knockout mice for these experiments to ensure that autocrine TNF could not indirectly regulate the TLR3 response. We found that BMDM and lung fibroblasts from the double mutants produced significantly less IL-6 than did wildtype and Tnf single mutant cells after 24 hrs of poly(I:C) stimulation (Figure 2.12A, B). Next, we examined TNF production in response to poly(I:C) and found that peritoneal macrophages from Tradd−/− mice produced significantly less TNF at 24 hrs post-treatment than did peritoneal macrophages from wildtype mice (Figure 2.12C). These data corroborate the deficit in TLR4 signaling we noted in LPS-treated Tradd-deficient cells and implicate TRADD in the TRIF-dependent pathway.

Signaling through the TRIF pathway results in the activation of NF-κB and IRF3 (Sato et al., 2003). Upon serine/threonine phosphorylation, the inactive cytoplasmic form of IRF3 forms a complex with cyclic AMP-response element binding protein (CREBBP). This complex translocates to the nucleus and activates the transcription of IFN α and β (Weaver et al., 1998). To further define TRADD’s role in TRIF signaling, we evaluated the levels of pIRF3 and pIkB in Tnf deficient BMDMs upon poly (I:C) stimulation. IRF3 activation was
not affected in the absence of Tradd (Figure 2.12D, top), but phosphorylation of IκB was clearly reduced in the absence of Tradd (Figure 2.12D, bottom).

In summary, the data presented in this chapter of the dissertation have significantly refined our understanding of the role of TRADD in the TNFR1 pathway, and in various TNFR related signaling pathways. Importantly, we have identified a novel function of TRADD in TLR3 and TLR4 mediated innate immune response, via its participation in TRIF-dependent NF-κB activation. Thus, TRADD is a crucial adaptor molecule in proinflammatory signaling cascades and is likely a key player in host immune responses (Figure 2.13).
Figure 2.12 TRADD is involved in TLR3 mediated NF-κB activation and cytokine production.
Figure 2.12 TRADD is involved in TLR3 mediated NF-κB activation and cytokine production.

(A, B) *Tradd* deficiency impairs poly (I:C)-induced IL-6 production. Wildtype, *Tradd*<sup>+/+</sup>*Tnf<sup>−/−</sup> and *Tradd*<sup>+/−</sup>*Tnf<sup>+/−</sup> BMDMs (A) and lung fibroblasts (B) were stimulated with 100 µg/ml poly (I:C) and IL-6 in the culture supernatants was evaluated 24 hrs later by ELISA. (C) *Tradd* deficiency impairs poly (I:C)-induced TNF production. *Tradd*<sup>+/+</sup> and *Tradd*<sup>−/−</sup> peritoneal macrophages were stimulated with 300 µg/ml poly (I:C) and TNF in the culture supernatants was evaluated 24 hrs later by ELISA. (D) Tradd deficiency impairs poly (I:C) induced activation of NF-κB but not the interferon pathway. *Tradd*<sup>+/+</sup>*Tnf<sup>−/−</sup> and *Tradd*<sup>−/−</sup>*Tnf<sup>−/−</sup> BMDMs were stimulated with 100 µg/ml poly (I:C) for the indicated times. Phosphorylation of IκB and IRF3 were assessed by immunoblot analysis. (For A-D), data shown are representative of three independent experiments.
Figure 2.13 Model of TRADD’s function in inflammatory signaling pathways involving TNFR1, TLR3 and TLR4.
Figure 2.13 Model of TRADD’s function in inflammatory signaling pathways involving TNFR1, TLR3 and TLR4.

TRADD is indispensable for TNF induced cell death. In addition, TRADD is essential for mediating TNF induced inflammatory responses, through its ability to recruit TRAF2 to the receptor complex, thereby leading to ubiquitination of RIP1 and hence activation of NF-κB. Analogous to its role in TNFR1 signaling, TRADD is also involved in TRIF dependent TLR3 and TLR4 mediated inflammatory responses, through its interaction with RIP1. TRADD is therefore a central molecule in anti-bacterial and anti-viral immune signaling pathways. Ub, ubiquitin.
2.4 Discussion

2.4.1 TRADD in death receptor signaling

Although TRADD has been identified for over a decade, definitive genetic evidence of its involvement in the TNFR1 signaling pathway, which is relevant to both immunity and disease, has been lacking. Through generating the Tradd deficient mouse, we have been able to confirm the answers to some questions while laying grounds for other interesting new possibilities in the field. Our results showed that TRADD is indispensable for TNF-induced apoptosis in both in vitro and in vivo settings. Tradd deficiency did not completely abolish murine TNF-mediated ERK and JNK activation. However, human (TNFR1-specific) TNF-mediated ERK and JNK activation was completely abolished in the absence of TRADD. This indicates that TRADD is necessary for TNFR1, but not TNFR2 mediated MAPK activation.

Our analyses of the TNFR1 signaling complex also revealed an essential role for TRADD in the ubiquitination of RIP1 that supports NF-κB activation. The most intriguing result emerging from this analysis was that the association of un-ubiquitinated RIP1 with TNFR1 is TRADD-independent. Ubiquitination of RIP1 has been proposed as an essential step during TNF-induced NF-κB activation (Li et al., 2006) and TRAF2 is believed to be an E3 ubiquitin ligase responsible for RIP1 ubiquitination (Lee et al., 2004). The absence of ubiquitinated RIP1 in the TNFR1 complex in TNF-stimulated Tradd−/− cells implies that TRADD is essential for TNF-induced NF-κB activation because it promotes TNF-induced ubiquitination of RIP1, likely through recruitment of TRAF2 to the receptor complex. Our data therefore refine the current model of TNFR1 receptor complex formation, by demarcating the association sequence of the various signaling mediators and adaptors in response to TNF stimulation.
In addition to TNFR1, TRADD has also been implicated in DR3 and DR6 signaling (Chinnaiyan et al., 1996; Pan et al., 1998b). DR3 is crucial for thymocyte development (Wang et al., 2001b) and peripheral T cell activation (Migone et al., 2002), whereas DR6 engagement has suppressive effects on T and B cell activation (Liu et al., 2001; Schmidt et al., 2003; Zhao et al., 2001). Our data confirmed that TRADD is critical for DR3-mediated T cell costimulation. However, contrary to its role in T cell costimulation, thymocyte negative selection mediated by DR3 does not seem to require TRADD. This suggests that the physiological effects of DR3 may be mediated through different signaling arms, only some of which depend on TRADD. A conjecture based on our understanding of the TNFR1 pathway is that DR3 engagement may also result in the formation of two different complexes, one that is death-inducing (possibly responsible for mediating cell death in the process of thymocyte negative selection) and one that is proliferation-inducing (possibly responsible for mediating T cell activation). Unlike the TNFR1 scenario, however, TRADD is perhaps involved only in the proliferative arm of DR3 signaling, thus resulting in its specific role in DR3 mediated T cell proliferation. Despite the general perception that TRADD is a pro-apoptotic protein, due to its possession of a death domain, it is becoming apparent that TRADD’s function may actually predominate in pro-survival/proinflammatory responses. From our study, we have clearly shown that TRADD is not essential for death mediated by various stress-inducing agents but is required specifically for TNF induced cell death. In this latter scenario, however, the role of TRADD is pro-survival by default as apoptosis occurs only upon internalization of the TNFR1 signaling complex and upon treatment of cells with a translational inhibitor. Similarly, a recent study demonstrated that, instead of promoting apoptosis, TRADD actually
serves to inhibit apoptosis upon TRAIL receptor engagement, through its ability to outcompete FADD’s interaction with the receptor (Cao et al., 2011).

With regards to DR6 signaling, we have so far been unable to clarify whether TRADD is involved. Tradd deficient cells did not exhibit any of the known defects found in Dr6 deficient mice, namely enhanced T cell proliferation, T<sub>H2</sub> cytokine production and B cell proliferation (Liu et al., 2001; Schmidt et al., 2003; Zhao et al., 2001). However, given the context dependent usage of TRADD in DR3 signaling, it remains possible that TRADD is involved in certain aspects of DR6 signaling that have not been investigated in the current study. Analysis of DR6 signaling is complicated by the fact that no native ligand(s) for DR6 have been reported. However, it has been shown that when soluble DR6-Fc protein is used to block the interaction between DR6 and its physiological ligand(s), the proliferation of anti-CD3-stimulated human T cells is enhanced (Liu et al., 2001). Future endeavors should therefore involve investigating the effect of Tradd deficiency in T cells upon treatment with human DR6-Fc.

2.4.2 TRADD in IFNγ signaling

Previous reports have suggested that TRADD interacts with STAT1-α and downregulates IFNγ-induced transcription and bioactivity. This hypothesis arises chiefly from work done using small interfering ribonucleic acid (siRNA)-mediated Tradd knockdown in RAW264.7 cells, which showed that signaling in response to IFNγ stimulation increased in the absence of TRADD (Wesemann and Benveniste, 2003). However, our own investigation does not support a negative regulatory role for TRADD in IFNγ-induced responses. In fact, we sometimes observed a slight reduction in STAT1-α phosphorylation in
Tradd-deficient MEFs, BMDMs and T cells. TRADD’s role in IFNγ signaling thus remains an unresolved controversy. Studies of pathogen infection in vivo might be helpful to fully characterize the role of TRADD in IFNγ signaling. It will also be interesting to determine whether TRADD is involved in cytokine signaling mediated by other STATs.

2.2.3 TRADD in TLR signaling

The most exciting result of our study is the uncovering of an important role for TRADD in TLR signaling. We found that TRADD participates in the TLR4 signaling complex formed in LPS-stimulated cells. Our biochemical analyses indicate that this involvement of TRADD occurs very far upstream in the signaling pathway and takes the form of a direct association between TRADD and TLR4-TIR and/or an indirect association between TRADD and RIP1 in the TRIF-dependent pathway. The latter proposition is supported by our studies of the effects of TRADD deficiency on the TLR3 pathway because signaling downstream of TLR3 relies solely on TRIF. Upon TLR3 stimulation by poly(I:C), we noted a significant decrease in cytokine production by Tradd-deficient cells compared to controls. Detailed analyses of TLR3 signaling, as well as assessments of TLR4 signaling in Tradd/Myd88 double knockout mice, will be crucial for the delineation of the precise involvement of TRADD in the TRIF pathway. Nevertheless, our study is seminal in that it has revealed a new role for TRADD in TLR signaling that is independent of its functions in the TNFR1 pathway. A recent paper by Michallet et al. (Michallet et al., 2008) has revealed TRADD’s participation in the RIG-I helicase antiviral pathway. This finding, in combination with our current observations, suggests that TRADD may be a critical player in the host antiviral and antibacterial response. The identification of TRADD as a player in antiviral
immunity may contribute to the development of new therapeutic strategies against viral infections.
CHAPTER 3

TRADD CONTRIBUTES TO TUMOR SUPPRESSION BY 
REGULATING ULF-MEDIATED p19Arf UBIQUITINATION

A version of this chapter is submitted to Nature 2011. (Chio et al., 2011)
3.1 Introduction

Cancer development is a multistep process characterized by uncontrolled cell proliferation, impaired apoptosis, loss of differentiation, immortalization and neovascularization (Hanahan and Weinberg, 2011). Mutations in both oncogenes and tumor suppressor genes are found in end-stage tumors, implying their causal role in cancer development. About 30% of human tumors carry an activating mutation in one of three related oncogenes, Harvey-Rat sarcoma (H-Ras), Kirsten (K)-Ras, or Neuroblastoma (N)-Ras, that generates a constitutively active Ras protein (Bos, 1989). Because Ras is a nodal signaling molecule, aberrant Ras activation dysregulates multiple signaling pathways, leading to profoundly altered proliferation, apoptosis, differentiation, and senescence (Downward, 2003). In some primary cells, activated Ras induces the accumulation of key tumor suppressor proteins, such as p53, p16\(^{\text{Ink4a}}\) and alternative reading frame (ARF, known as p14\(^{\text{ARF}}\) in human and p19\(^{\text{Arf}}\) in mouse), and triggers cellular senescence (Serrano et al., 1997). This oncogene-induced senescence can be bypassed by inactivating Ink4a/Arf function, suggesting that Ras-induced accumulation of tumor-suppressive proteins may have evolved as a mechanism of cancer inhibition. Indeed, mutation of Ras, Raf (Rapidly accelerated fibrosarcoma) or Pten (Phosphatase and tensin homolog) has been shown to result in oncogene (or tumor suppressor gene)-induced senescence in both human cancers and mouse tumor models (Braig et al., 2005; Collado et al., 2005; Lazzerini Denchi et al., 2005; Michaloglou et al., 2005; Serrano et al., 1997; Sun et al., 2007).

Mutations of Ras are prominent in the dimethylbenzanthracene (DMBA)/12-O-tetradecanoylphorbol 13-acetate (TPA)-induced model of mouse skin carcinogenesis, which is perhaps the best-characterized \textit{in vivo} model of epithelial neoplasia. This model has been instrumental in establishing the concepts of tumor initiation, promotion and progression.
Mice are first treated with the carcinogen DMBA, followed by multiple applications of the tumor promoter TPA. This two-stage protocol induces benign skin tumors, nearly all of which exhibit an A-T mutation in codon 61 of $H$-Ras (Balmain et al., 1984; Quintanilla et al., 1986). This mutant $H$-Ras allele frequently becomes amplified upon disease progression and generates a constitutively active H-Ras protein. In conjunction with H-Ras activation, loss of $p19^{ARF}$ enhances the number and growth rate of tumors developing in DMBA/TPA-treated mice, and accelerates the progression to malignancy (Kelly-Spratt et al., 2004).

ARF was originally identified as an alternative transcript of the INK4a-ARF locus on human chromosome 9p21.1 (Ozenne et al., 2010). Disruption of this locus, which encodes two tumor suppressor proteins, $p16^{INK4a}$ and $p14^{ARF}$, by deletions or point mutations, is a common event in human cancer, second only to the loss of p53 (Ruas and Peters, 1998). Whereas $p16^{INK4a}$ antagonizes the activities of cyclin D-dependent kinases, ARF’s tumor-suppressive activity is largely attributed to its ability to regulate p53 in response to aberrant growth or oncogenic stresses such as H-Ras activation (Palmero et al., 1998; Sherr, 2000). ARF stabilizes p53 protein by binding to and neutralizing two ubiquitin ligases, murine double minute (Mdm)-2 and ARF-binding protein (Arfbp)-1/Mcl-1 ubiquitin ligase E3 (Mule), which promote p53 protein degradation (Chen et al., 2005; Pomerantz et al., 1998). This ARF-mediated accumulation of p53 in response to oncogene activation is a major means of combating cellular transformation. In addition, ARF displays p53-independent tumor-suppressive activities such as attenuation of ribosomal RNA processing (Itahana et al., 2003; Sugimoto et al., 2003), sumoylation of its binding partners (Tago et al., 2005) and promotion of autophagy (Abida and Gu, 2008).
Although ARF’s transcriptional regulation has been well studied, relatively little is known about the post-translational regulation and/or stabilization of the ARF protein. Both the mouse and human ARF proteins are relatively stable, with estimated half-lives ranging from 1-8 hrs, depending on the cell type (Kuo et al., 2004; Ozenne et al., 2010). N-terminal ubiquitination followed by proteasomal degradation has been implicated in ARF turnover (Bertwistle et al., 2004; Kuo et al., 2004; Pollice et al., 2008), and ubiquitin ligase for ARF (ULF), (also known as thyroid receptor interacting protein 12 (TRIP12)), a protein previously identified as a binding partner of the thyroid hormone receptor, was recently identified as an E3 ubiquitin ligase for ARF (Chen et al., 2010).

In the past decade, research on the intersection between inflammation and cancer pathogenesis has produced abundant and compelling evidence that immune cells (largely of the innate immune system) can promote neoplastic progression (Colotta et al., 2009; Coussens and Werb, 2002). In this context, TNF, a major inflammatory cytokine, has been shown to play a crucial, cell-non-autonomous role in driving tumor growth in various models of cancer (Balkwill, 2002; Kulbe et al., 2007; Moore et al., 1999; Popivanova et al., 2008; Scott et al., 2003; Suganuma et al., 1999). The best studied of these models is DMBA/TPA-induced carcinogenesis in mouse skin, in which mice deficient of Tnf or Tnfr1 have been shown to be highly resistant to skin tumor formation (Arnott et al., 2004; Moore et al., 1999; Suganuma et al., 1999).

As described in the previous chapter, TRADD is a central adaptor in the TNFR1 signaling complex, mediating cell death signals through the recruitment of FADD, and pro-inflammatory signals through the activation of NF-κB, JNK and MAPK (Aggarwal, 2003). In addition, we have also shown that TRADD is required for signaling downstream of TLR3
and TLR4, which are key players of the innate immune response. Due to its role as an adaptor in the above signaling pathways, TRADD is generally considered to be a cytoplasmic protein, but there is some evidence that TRADD may also have a function in the nucleus (Morgan et al., 2002; Wesemann et al., 2004). The physiological relevance of nuclear TRADD is still uncertain.

Given TRADD’s role in proinflammatory TNFR1 signaling, as well as in the TLR3 and TLR4 signaling cascades of innate immunity, it would be reasonable to postulate that TRADD functions would generally support tumorigenesis. However, genomic studies have established that the human TRADD gene is located within chromosome 16q22.1 (Scheuerpflug et al., 2001), a region in which loss of heterozygosity (LOH) is observed at high frequency in human liver, breast, prostate and Wilms tumors (Filippova et al., 1998; Hainsworth et al., 1991; Kihana et al., 1996; Kim et al., 2009; Knuutila et al., 1999; Sakai et al., 1992; Wang et al., 2000a). Such findings, along with the ability of TRADD to trigger apoptosis in vitro, argue for a potential tumor-suppressive role for TRADD. To clarify TRADD’s function in tumorigenesis, we have applied the DMBA/TPA-induced skin carcinogenesis model to Tradd-deficient mice and report that TRADD does indeed have a previously unknown tumor-suppressive role that is independent of TNFR1 signaling and independent of apoptosis. In vitro, primary cells lacking TRADD were less susceptible to H-Ras-induced senescence and showed reduced accumulation of p19Arf protein. Our data suggest that TRADD shuttles dynamically from the cytoplasm into the nucleus to modulate the interaction between p19Arf and its E3 ubiquitin ligase ULF, thereby promoting p19Arf protein stability and thus tumor suppression. These results reveal a novel tumor-suppressive
role for nuclear TRADD and constitute an important contribution to the rapidly expanding field of p19Arf post-translational regulation.
3.2 Materials and Methods

3.2.1 Mice

Tradd<sup>−/−</sup> (C57BL/6, F8-9 generation), Tnf<sup>−/−</sup> (Pasparakis et al., 1996b), and Tnfr1<sup>−/−</sup> (Rothe et al., 1994) mice have been described previously. Mice were maintained at the Ontario Cancer Institute Animal Facility in compliance with the regulations of the Animal Ethics and Animal Care Committees at the Princess Margaret Hospital. These bodies approved all experiments.

3.2.2 Cell culture

Murine embryonic fibroblasts (MEFs) were isolated as described in chapter 2 of this dissertation and cultured in DMEM with 10% FCS. The 293T, HeLa and B16F10 cell lines were obtained from ATCC and maintained in DMEM with 10% FCS. HCT116 cells obtained from ATCC were maintained in McCoy’s 5A medium with 10% FCS.

3.2.3 Plasmids

Vectors expressing murine TRADD-FLAG, nuclear TRADD-FLAG, TRADD-Myc, N-terminal TRADD-Myc, C-terminal TRADD-Myc, cytoplasmic TRADD-Myc, p19<sup>Arf</sup>-FLAG, ULF-V5 and ULF-FLAG were constructed by cloning TRADD, p19<sup>Arf</sup>, or ULF cDNA into pBABEPuro or pcDNA3.1 (Invitrogen). Oligonucleotides for shRNA-mediated targeting of human TRADD were cloned into pSUPER.retro according to a published protocol (Brummelkamp et al., 2002). siRNA targeting human ULF was custom-designed by Dharmacon using the published sequence (Chen et al., 2010). The human ULF construct (Chen et al., 2010) was the kind gift of Dr. Wei Gu (New York, NY, U.S.A.). The TRADD-
DN construct was the kind gift of Dr. Arnd Kieser (München, Germany). Sequences of shRNAs and siRNAs are as follows:

Oligonucleotides for shRNA-mediated targeting of human TRADD were:
5’ gatcccgaggatgcgtgcgaaatttttaagagaaatttcgcagcgcatcctctctttta3’ and
5’ agcttaaaaaggagatgctgcgaaattttctcttgaaaaatttcgcagcgcatcctccggg3’.

The sequence of the siRNA targeting human ULF was 5’-gguagugacuccacccauuu-3’.

3.2.4 Virus-based gene transduction

Retrovirus preparations were made in phoenix packaging cells transfected with 10 µg of the pBABE plasmid encoding the gene of interest. Culture supernatant was harvested 48 hrs after transfection and filtered through a 0.45 µm filter. Virus-containing medium was used immediately or snap frozen for storage. For MEF infections, virus-containing medium was added to cells in the presence of 5 µg/ml protamine sulphate. MEFs were then spun at 2500rpm for 45 min. Lentivirus preparations were made in a manner similar to retrovirus preparations, using a three-plasmid packaging system in 293JD cells. All transduced primary MEFs and cell lines were rested for 24 hrs post-infection before culture in 2 µg/ml puromycin for 2 days or 1 week, respectively.

3.2.5 Immunoblot analysis

MEFs, HCT116 cells or B16F10 cells that were infected with retrovirus or lentivirus were lysed in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris HCl pH8.0) containing protease inhibitor tablets (Roche) and phosphatase inhibitor tablets (Roche). Lysates (10-20 µg) were separated into nuclear and cytoplasmic membrane
fractions using a nuclear/cytosol protein extraction kit (BioVision) according to the manufacturer’s instructions. Fractions were resolved by standard SDS-PAGE and immunoblotting. Primary antibodies recognizing the following proteins were used: β-actin, anti-FLAG M2, anti-NPM1/B23 (Sigma); p53 FL-393, p21\textsuperscript{WAF1} C-19, p16 M-156, HA probe, TRADD, USF2 (Santa Cruz); p19\textsuperscript{Arf}, vinculin, V5 tag (Abcam); ULF/TRIP12, MULE/ARFBP1 (Bethyl Laboratories); phospho-Erk, Erk, pAKT, Myc-tag (Cell Signaling); p53 CM5 (Novocastra); Ras (BD Pharmingen); and p14ARF NB200-111 (Novus Biologicals). Anti-FLAG antibody (clone M2) conjugated to agarose beads was from Sigma.

3.2.6 Immunoprecipitation (IP)

Cells were washed, harvested and lysed in IP buffer (50 mM Tris HCl pH 7.4, 100 mM NaCl, 5 mM EDTA, 0.4% NP40, 5 mM NaF, 10% glycerol, with complete proteinase inhibitor cocktail). Lysates (1 mg, unless stated otherwise) were precleared with rabbit IgG and protein A/G beads (Santa Cruz Biotechnology) for 1 hr at 4°C. Precleared supernatants were immunoprecipitated with anti-TRADD (Santa Cruz), anti-ULF (Bethyl laboratories) or anti-Myc antibodies (Santa Cruz) or with beads conjugated to anti-FLAG antibody (Sigma) for 3 hrs or overnight at 4 °C. The beads plus the antibodies and associated proteins were washed 5 times in IP buffer and resuspended in SDS-PAGE sample buffer. Samples were separated by SDS-PAGE and protein components of receptor complexes were determined by immunoblot analysis.

3.2.7 Senescence

Analysis of senescence in cell culture was performed as described previously (Serrano et al.,
1997). Briefly, cells were washed once with PBS (pH 7.2), fixed with 0.5% glutaraldehyde in PBS, and washed in PBS (pH 7.2) supplemented with 1 mM MgCl₂. Cells were stained in X-gal solution (1 mg/ml X-gal (Sigma), 0.12 mM K₃Fe[CN]₆, 0.12 mM K₄Fe[CN]₆, 1 mM MgCl₂ in PBS at pH 6.0) overnight at 37 °C. To quantify SA-β-gal⁺ cells, at least 200 cells were counted in random fields in each of three triplicate wells per sample.

3.2.8 Histology
Tumors and skin were excised and apportioned. For H&E staining, TUNEL staining, or immunohistochemical staining to detect p19ARF, Ki-67, F4/80 or CD3, portions were fixed in formalin for 48 hrs at room temperature (RT) followed by immersion in 70% ethanol and storage at 4°C. For CD31 or SA-β-gal staining, portions were mounted in O.C.T. compound (Sakura Finetek) and stored at -80°C. H&E staining was performed using a standard method. SA-β-gal assays were performed using a published protocol (Sun et al., 2007).

3.2.9 Tumorigenesis in allografted and xenografted mice
For xenografts, HCT116 cells (1x10⁶), transduced with shTRADD or control vector as described above, were suspended in 100 µl DMEM and injected subcutaneously (s.c.) into the left or right flanks, respectively, of 6-week-old female NIH athymic nude mice. For allografts, B16F10 cells (1x10⁶) were injected s.c. into the right flanks of 6-week-old Tradd+/+ and Tradd−/− mice. Tumor growth was analyzed at 2 weeks (B16F10) or 4 weeks (HCT116) post-injection.
3.2.10 DMBA/TPA skin carcinogenesis

The dorsal skin of 3-week-old $Tradd^{+/+}$, $Tradd^{-/-}$, $Tnfr1^{-/-}$, $Tnf^{-/-}$ and $Tradd^{-/-}Tnf^{-/-}$ mice was treated once with 50 µl 0.5% DMBA in acetone followed by treatment with 6.25 µg TPA in 100 µl acetone twice a week starting at 14 days post-DMBA. For single stage carcinogenesis, mice were treated with 50 µl 0.5% DMBA only on postnatal day 1–5 and monitored weekly. For TPA-induced epidermal hyperplasia, mice were treated with 6.25 µg TPA in 100 µl according to a published protocol (Oskarsson et al., 2006).

3.2.11 In vivo ubiquitination

In vivo ubiquitination was assayed as described previously (Chen et al., 2010) with some modifications. Calcium phosphate-transfected 293T cells were harvested at 24 hrs post-transfection and disrupted in lysis buffer (50 mM Tris HCl pH7.4, 100 mM NaCl, 5 mM EDTA, 0.4% NP40, 5 mM NaF, 10% glycerol) plus protease and phosphatase inhibitors. Lysates (1 mg) were precleared with rabbit IgG and protein A/G beads (Santa Cruz Biotechnology) for 1 hr at 4°C. Precleared supernatants were immunoprecipitated with rabbit anti-p19Arf antibody ab-4 (Labvision) or beads conjugated to anti-FLAG antibody (clone M2; Sigma). Immune complexes were recovered, washed three times in lysis buffer, and boiled in SDS-PAGE sample buffer for 5 min. Denatured immune complexes were resolved by SDS-PAGE. Proteins were detected by immunoblotting as described above.

3.2.12 Real-time quantitative reverse transcription (qRT)-PCR

Total RNA was prepared using the RNeasy kit (Qiagen) and treated with RNase-free DNase (Promega). Total RNA (1 µg) was used for cDNA synthesis using iScript according to the
manufacturer's instructions (BioRad). Real-time qRT-PCR using SYBR green dye was carried out on an ABI 7700 (Applied Biosystems) according to the manufacturer's instructions. Samples were assayed in triplicate and results were normalized to 18S rRNA or GAPDH. Relative expression was calculated using the ΔΔCT method. Primers used for real-time qRT-PCR analysis are as follows:

<table>
<thead>
<tr>
<th>Table 3.1 Realtime qRT-PCR primers</th>
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<tbody>
<tr>
<td>p19Arf Forward primer</td>
</tr>
<tr>
<td>p19Arf Reverse primer</td>
</tr>
<tr>
<td>p16Ink4a Forward primer</td>
</tr>
<tr>
<td>p16Ink4a Reverse primer</td>
</tr>
<tr>
<td>p21Cip/Waf1 Forward primer</td>
</tr>
<tr>
<td>p21Cip/Waf1 Reverse primer</td>
</tr>
<tr>
<td>Tp53 Forward primer</td>
</tr>
<tr>
<td>Tp53 Reverse primer</td>
</tr>
<tr>
<td>mTradd Forward primer</td>
</tr>
<tr>
<td>mTradd Reverse primer</td>
</tr>
<tr>
<td>hTRADD Forward primer</td>
</tr>
<tr>
<td>hTRADD Reverse primer</td>
</tr>
<tr>
<td>hULF Forward primer</td>
</tr>
<tr>
<td>hULF Reversed primer</td>
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</tbody>
</table>
3.3 Results

3.3.1 *Tradd* deficiency promotes DMBA-induced tumor formation independently of TNF signaling

TRADD is a perplexing case in the context of tumorigenesis. This adaptor protein is a central mediator of responses triggered by the tumor-promoting inflammatory cytokine TNF (Chen et al., 2008), but is encoded by a gene within a chromosomal region exhibiting frequent LOH in human cancers. We sought to clarify the role of TRADD in tumorigenesis through the use of *Tradd*-deficient murine models. On average, *Tradd*+/– mice exhibited a shorter lifespan than wildtype (*Tradd*+/+) mice (552 vs. 790 days) (Figure 3.1A). Two of 35 *Tradd*–/– mice developed spontaneous tumors within the first 18 months of life (Figure 3.1B), whereas no tumors were seen in 30 age-matched *Tradd*+/+ littermates over the same period. To examine tumor development in the context of TNF signaling, we treated wildtype, *Tradd*–/–, *Tradd*+/+*Tnfr1*–/–, *Tradd*+/+*Tnf*–/– and *Tradd*–/–*Tnf*–/– mice with DMBA/TPA to induce skin tumors. As expected, both *Tradd*+/+*Tnfr1*–/– and *Tradd*+/+*Tnf*–/– mice were highly resistant to DMBA/TPA and exhibited prolonged tumor latency compared to the wildtype controls (Figure 3.2A). However, *Tradd*–/– mice developed tumors much faster than the wildtype, starting as early as 7 weeks post-DMBA/TPA. Similarly, although more resistant than the wildtype, *Tradd*–/–*Tnf*–/– mice developed tumors faster than *Tradd*+/+*Tnf*–/– mice (Figure 3.2A). *Tradd*–/– and *Tradd*–/–*Tnf*–/– mice also accumulated much greater numbers of tumors (Figure 3.2B) of larger size (Figure 3.2C, D) than their control counterparts. These data show that TRADD has a tumor-suppressive role that is independent of TNF.

The skin tumors in DMBA/TPA-treated *Tradd*+/+ and *Tradd*–/– mice were histologically similar (Figure 3.2E), with comparable skin tumor subtype distribution (Table 3.2). No squamous cell carcinomas occurred in either group. Immunohistological analyses
(Figure 3.2F) revealed slightly decreased CD31 staining in $Tradd^{+/}$ tumors, ruling out enhanced angiogenesis as an explanation for the increased tumor growth. TUNEL staining was very low in tumors of both genotypes, as was the level of CD3$^+$ T cell infiltration. However, Ki-67 staining was modestly enhanced and senescence-associated beta galactosidase (SA-β-gal) staining was reduced in $Tradd^{+/}$ tumors. Importantly, $Tradd^{-/-}$ tumors also showed a consistent decrease in p19$^{Arf}$ positivity but not in p16$^{Ink4a}$ positivity. These results indicate that TRADD’s tumor-suppressive effect is not related to immunosurveillance, apoptosis or angiogenesis, but rather to effects on p19$^{Arf}$. 
Figure 3.1 *Tradd*⁻/⁻ mice exhibit a shorter lifespan and a modest increase in spontaneous tumorigenesis.
Figure 3.1  *Tradd*−/− mice exhibit a shorter lifespan and a modest increase in spontaneous tumorigenesis.

(A) The survival of *Tradd*+/+ (n=30) and *Tradd*−/− (n=35) mice was monitored for 1000 days. Results shown are percent survival. P values were determined using the log rank test.

(B) Images of 2 spontaneous tumor cases (white arrows indicate tumors) found in *Tradd*−/− mice within the first 18 months of life.
Figure 3.2 Tradd deficiency promotes skin tumorigenesis following DMBA/TPA treatment.
Figure 3.2 *Tradd* deficiency promotes skin tumorigenesis following DMBA/TPA treatment.
Figure 3.2 *Tradd* deficiency promotes skin tumorigenesis following DMBA/TPA treatment.

(A) Tumor-free survival of wildtype (n=17), *Tradd*<sup>−/−</sup> (n=17), *Tradd*<sup>+/−</sup>*Tnfr1<sup>−/−</sup> (n=17), *Tradd*<sup>+/+</sup>*Tnf<sup>−/−</sup> (n=9) and *Tradd*<sup>−/−</sup>*Tnf<sup>−/−</sup> (n=9) mice treated with DMBA/TPA. P values were determined using the log-rank test.

(B) Mean count of tumors of all sizes in the mice in (A). P values, unpaired Student’s *t*-test.

(C) Mean count per mouse of tumors of the indicated sizes in the indicated groups of mice from (A).

(D) Gross appearance of tumors (indicated by white arrows) developing in DMBA/TPA-treated mice of the indicated genotypes.

(E) Representative H&E staining of tumor samples from the *Tradd*<sup>+/+</sup> and *Tradd*<sup>−/−</sup> mice in (D).

(F) Immunohistological staining to detect the indicated markers in representative tumor samples from the *Tradd*<sup>+/+</sup> and *Tradd*<sup>−/−</sup> mice in (D).
### Table 3.2 Distribution of skin tumor subtypes in DMBA/TPA-treated \textit{Tradd}^{+/+} and \textit{Tradd}^{-/-} mice.

<table>
<thead>
<tr>
<th></th>
<th>Papilloma</th>
<th>Keratoacanthoma</th>
<th>Sebaceous Adenoma</th>
<th>Follicular Keratosis</th>
<th>Total tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Tradd}^{+/+} (n=17)</td>
<td>23 (67.6%)</td>
<td>3 (8.8%)</td>
<td>8 (23.5%)</td>
<td>0 (0%)</td>
<td>34</td>
</tr>
<tr>
<td>\textit{Tradd}^{-/-} (n=17)</td>
<td>32 (59.3%)</td>
<td>7 (12.9%)</td>
<td>14 (25.9%)</td>
<td>1 (1.9%)</td>
<td>54</td>
</tr>
</tbody>
</table>
3.3.2 \textit{Tradd} deficiency compromises cellular responses to DMBA but not to TPA

As the promoting agent in the DMBA/TPA model, TPA induces robust proliferation that leads to epidermal hyperplasia within days (Kennard et al., 1995). To determine whether the increased tumor formation in \textit{Tradd}\textsuperscript{+/−} mice was due to an enhanced proliferative response to TPA, we treated the dorsal skin of 4-week-old \textit{Tradd}\textsuperscript{+/+} and \textit{Tradd}\textsuperscript{−/−} mice with TPA alone. Equivalent epidermal thickening and proliferation as detected by Ki-67 staining were observed at 4 days post-treatment (Figure 3.3A), excluding a difference in proliferative responses to TPA. We then asked whether \textit{Tradd} deficiency could overcome the need for TPA in the skin carcinogenesis model; i.e. whether tumors could be generated in these mutants by DMBA alone. We treated neonatal \textit{Tradd}\textsuperscript{+/+} and \textit{Tradd}\textsuperscript{−/−} mice once with DMBA and monitored tumor formation over 12 months. Only 23% (3/13) \textit{Tradd}\textsuperscript{+/+} mice developed small lesions (1-2 mm in diameter) on the skin surface, as expected, whereas 67% (8/12) \textit{Tradd}\textsuperscript{−/−} mice developed tumors (Figure 3.3B, C). Two of these tumors were greater than 10 mm in diameter (Figure 3.3D). These data suggest that a lack of TRADD renders cells more vulnerable to oncogenic stress.
Figure 3.3 *Tradd* deficiency compromises the cellular response to DMBA but not to TPA.
Figure 3.3 *Tradd* deficiency compromises the cellular response to DMBA but not to TPA.

(A) Comparable proliferation. Left panels: H&E staining of untreated (no TPA) or treated (4 days TPA) areas of dorsal skin from the same individual mice of the indicated genotypes. Thickness of epidermis (Epi) is indicated. Right panels: Immunohistochemical analysis of cell proliferation in the skin samples in the left panels as detected by Ki-67 staining.

(B) Tumor-free survival of *Tradd*<sup>+/+</sup> (n=13) and *Tradd*<sup>−/−</sup> (n=12) littermates treated with DMBA alone. P value, log-rank test.

(C) Mean count of tumors of all sizes in the DMBA-treated *Tradd*<sup>+/+</sup> and *Tradd*<sup>−/−</sup> mice in (B). P value, unpaired Student’s t-test.

(D) Gross appearance of representative tumors (white arrows) from the DMBA-treated mice in (B).
3.3.3 The tumor suppressive effect of TRADD is cell intrinsic

To determine if TRADD’s tumor-suppressive function was cell-autonomous or mediated through effects on the tumor stroma, we transplanted B16F10 murine melanoma cells into Tradd\(^{+/+}\) or Tradd\(^{−/−}\) hosts and monitored tumorigenesis. No differences were observed in tumor formation (Figure 3.4A), indicating that Tradd is not required in the tumor stroma, at least in this context. We also performed shRNA-mediated TRADD knockdown in HCT116 cells, a human cancer cell line harbouring constitutively active K-Ras (Wang et al., 2004), and injected these cells subcutaneously as a xenograft into athymic nude mice. Tumor growth was significantly enhanced by TRADD depletion (Figure 3.4B, C), confirming that the TNF-independent tumor-suppressive role of TRADD is cell-autonomous.
Figure 3.4 The TNF–independent role of TRADD in tumor suppression is cell-autonomous.
Figure 3.4 The TNF–independent role of TRADD in tumor suppression is cell-autonomous.

(A) Murine melanoma B16F10 cells were transplanted into $Tradd^{+/+}$ (n=6) and $Tradd^{-/-}$ (n=6) mice and weights of tumors were determined at 10 days post-injection. Horizontal lines, median values. NS, p=0.88 (unpaired Student’s t-test).

(B) HCT116 cells transfected with control shRNA vector (shVector), or shRNA against TRADD (shTRADD), were injected subcutaneously into the right or left flanks of athymic nude mice, respectively (n=11/group). Weights of tumors developing in the xenografted mice were determined at 2 weeks post-injection. Horizontal lines, median values. P value, unpaired Student’s t-test.

(C) Left: Images of representative xenografted mice from (B). Right: Immunoblot validation of TRADD knockdown in HCT116 cells by shTRADD. Vinculin, loading control.
3.3.4 *Tradd* deficiency impairs cellular responses to H-RasV12 expression

More than 90% of DMBA/TPA-induced tumors carry amplified copies of activated *H-Ras* mutations, and H-Ras hyperactivation can trigger oncogene-induced senescence that serves as a barrier against cellular transformation (Collado and Serrano, 2010). Such senescence has previously been shown to block tumor growth in the DMBA/TPA model (Sun et al., 2007). Because our data showed that DMBA/TPA-induced tumor formation was enhanced in *Tradd*−/− mice, we postulated that TRADD’s tumor-suppressive function might contribute to or regulate components of the senescence barrier. To assess this possibility, we examined the role of TRADD during H-Ras-induced senescence in primary murine embryonic fibroblasts (MEFs). First, upon transduction with constitutively active H-RasV12, *Tradd*−/− MEFs showed less growth arrest than wildtype MEFs, in that the mutant cells maintained higher levels of bromodeoxyuridine (BrdU) incorporation (Figure 3.5A). Secondly, transduced *Tradd*−/− MEFs showed reduced senescence, as indicated by senescence-associated-beta-galactosidase (SA-β-gal) positivity, compared to transduced wildtype MEFs (Figure 3.5B). No such alterations were observed in similarly treated *Tnfr1*−/− cells (Figure 3.5B). *Tradd*−/− cells were not readily transformed by H-RasV12, as determined by focus formation assays (Figure 3.5C). Interestingly, when *Tradd* deficiency was combined with *Tp53* heterozygosity, focus formation at 3 weeks post-H-RasV12 transduction was increased compared to *Tradd*+/+*Tp53*+/− cells (Figure 3.5D), as was colony formation in soft agar at 2 weeks post-H-RasV12 transduction (Figure 3.5E). These results imply that *Tradd*−/− MEFs must sustain additional genetic lesions to undergo complete transformation.
Figure 3.5 *Tradd* deficiency attenuates H-RasV12-induced growth arrest and senescence.
Figure 3.5 Tradd deficiency attenuates H-RasV12-induced growth arrest and senescence in MEFs.
Figure 3.5 *Tradd* deficiency attenuates H-RasV12-induced growth arrest and senescence in MEFs.

(A) BrdU incorporation. *Tradd*+/+ and *Tradd*–/– primary MEFs at passage 2 were transduced with retrovirus expressing H-RasV12 or empty vector (pBABE). Left panel: BrdU incorporation was assessed on the indicated days by flow cytometry. Cells were stained with APC-conjugated anti-BrdU antibody (BD Biosciences) to detect BrdU incorporation (vertical axis) and with 7-AAD to detect total DNA content (horizontal axis). Right panel: Quantitation of BrdU incorporation. Results are the mean percentage of BrdU+ cells ± SD of triplicate samples per genotype. P values, unpaired Student’s *t*-test.

(B) SA-β-gal staining. wildtype, *Tradd*–/– and *Tnfr1*–/– MEFs were transduced with H-RasV12 or pBABE and SA-β-gal staining was assessed 8 days later. Left panel: Representative cell culture images. Right panel: Quantitation. Results are the mean percentage of SA-β-gal+ cells ± SD in triplicate cultures. P values, unpaired Student’s *t*-test.

(C, D, E) *Tradd* deficiency alone is insufficient for transformation. (C) *Tradd*+/+ and *Tradd*–/– MEFs transduced with pBABE or H-RasV12 were seeded at 1x10^5 cells/10 cm dish. Plates were fixed 4 weeks after seeding and stained with 0.5% crystal violet to detect focus formation. (D) *Tradd*+/+p53+/– and *Tradd*–/–p53+/– MEFs transduced with pBABE or H-RasV12 were seeded as for (C). Plates were fixed 3 weeks after seeding and stained as for (C). (E) *Tradd*+/+p53+/– and *Tradd*–/–p53+/– MEFs transduced with pBABE or H-RasV12 (5x10^3 cells) were suspended in medium containing 0.3% soft agar and seeded onto 0.6% soft agar in 12-well plates. Plates were fixed 2 weeks after seeding and stained with 0.002% crystal violet to detect colony formation in soft agar (top). Bottom, quantitation. Results are the mean ± SD of triplicates. P values, unpaired Student’s *t*-test.
3.3.5 *Tradd* deficiency modulates p19<sup>Arf</sup> at the protein level

Wildtype MEFs engineered to express constitutively active oncogenic Ras undergo cell cycle arrest marked by upregulation of p16<sup>Ink4a</sup>, p19<sup>Arf</sup> and p53 (Serrano et al., 1997). Consequently, when either p19<sup>Arf</sup> or Tp53 is absent, MEFs can be transformed by oncogenic H-Ras without the need for other immortalizing oncogenes (Kamijo et al., 1997; Serrano et al., 1997). When we infected *Tradd<sup>+/−</sup>* and *Tradd<sup>/−</sup>* MEFs with H-RasV12-expressing retrovirus and performed immunoblot analyses, both the TRADD and p19<sup>Arf</sup> proteins were induced in the transduced wildtype cells (Figure 3.6A). Interestingly, induction of p19<sup>Arf</sup> protein was impaired in transduced *Tradd<sup>/−</sup>* MEFs compared to either transduced wildtype cells (Figure 3.6A) or transduced *Tnfr1<sup>/−</sup>* MEFs (Figure 3.6B). The accumulation of p53 protein in transduced *Tradd<sup>/−</sup>* cells was also decreased compared to controls (Figure 3.6A), consistent with negative regulation of Mdm2-mediated p53 degradation by p19<sup>Arf</sup> (Pomerantz et al., 1998). However, p21<sup>Waf1/Cip1</sup> accumulation occurred normally in the absence of *Tradd* (Figure 3.6B), implying an intact p53-independent means of p21 induction (Aliouat-Denis et al., 2005; Macleod et al., 1995). In addition, the induction of p16<sup>Ink4a</sup>, a senescence-associated protein that is more important in humans than in mice (Collado and Serrano, 2010), was not compromised by *Tradd* deficiency (Figure 3.36A). Similar results were obtained for transduced *Tradd<sup>+/−</sup>*Tp53<sup>+/−</sup> MEFs. (Figure 3.6C). Lastly, the mitogen-activated kinases (Erk1/2) and phosphoinositide 3-kinase (PI3K) signaling cascades appeared intact in the absence of *Tradd* (Figure 3.6A).

At the transcriptional level, real-time qRT-PCR analysis of transduced *Tradd<sup>+/+</sup>* MEFs showed that *Tradd* mRNA was induced 2-fold upon H-RasV12 transduction, in line with our immunoblot data (Figure 3.6Di). However, in contrast to our protein results, there was no significant difference in *p19<sup>Arf</sup>* mRNA levels between transduced *Tradd<sup>+/+</sup>* and
Tradd<sup>−/−</sup> MEFs (Figure 3.6Dii), nor any differences in transcript levels of p16<sup>Ink4a</sup>, p21<sup>Waf1/Cip1</sup>, Tp53, or IL-6, a TNF-inducible cytokine driving oncogene-induced senescence (Coppe et al., 2008; Kuilman et al., 2008) (Figure 3.6Diii-vi). These results suggest that Tradd deficiency impairs H-RasV12-induced senescence and increases susceptibility to tumorigenesis by decreasing p19<sup>Arf</sup> at the protein level.
Figure 3.6 Tradd deficiency reduces p19Arf protein accumulation induced by H-RasV12 activation.
Figure 3.6 *Tradd* deficiency reduces p19\(^{Arf}\) accumulation at the protein level.
Figure 3.6 *Tradd* deficiency reduces p19^{Arf} accumulation at the protein level.

(A, B, C) Lysates (10 µg) of (A) *Tradd^{+/+}* and *Tradd^{−/−}* MEFs, (B) *Tradd^{+/+} Tnfr1^{−/−}* and *Tradd^{−/−}* MEFs, and (C) *Tradd^{+/+} Tp53^{+/−}* and *Tradd^{−/−} Tp53^{+/−}* MEFs that were transduced with H-RasV12 or pBABE were immunoblotted to detect the indicated proteins on the indicated days post-transduction. Tubulin, loading control.

(D) qRT-PCR measuring levels of the indicated mRNAs in *Tradd^{+/+}* and *Tradd^{−/−}* MEFs on the indicated days after transduction with H-RasV12 or pBABE. Results are the mean ± SD of triplicates.

For (A-D), data are representative of at least three independent experiments.
3.3.6 Nuclear Tradd regulates p19\textsuperscript{Arf} ubiquitination

The stability of the p19\textsuperscript{Arf} protein is controlled by the ubiquitin-proteasome pathway (Chen et al., 2010; Kuo et al., 2004). To investigate whether TRADD regulates p19\textsuperscript{Arf} protein by influencing this pathway, we incubated \textit{Tradd}\textsuperscript{+/+} and \textit{Tradd}\textsuperscript{−/−} MEFs with the 26S proteasome inhibitor MG132, followed by treatment with the translational inhibitor cycloheximide (CHX) over a time course of 36 hrs. We found that the half-life of p19\textsuperscript{Arf} protein was clearly shorter in the absence of TRADD (Figure 3.7A). We then used an \textit{in vivo} ubiquitination assay (Chen et al., 2010) to show that TRADD overexpression in cells significantly reduced ubiquitinated p19\textsuperscript{Arf} (Ub-p19\textsuperscript{Arf}) levels in a dose-dependent manner (Figure 3.7B, C).

Because p19\textsuperscript{Arf} is localized within the nucleus, we postulated that TRADD had to be able to function within the nucleus to regulate p19\textsuperscript{Arf} protein stability. The TRADD amino acid (aa) sequence does contain both a putative nuclear localization signal (NLS) and a putative nuclear export signal (NES) (Figure 3.8A), which might allow TRADD to shuttle dynamically between the cytoplasm and the nucleus. To investigate the dynamics of TRADD localization, we expressed C-terminal GFP-tagged TRADD in HeLa cells that were then treated with leptomycin B (LMB), a drug that blocks exportin-1-mediated nuclear export of proteins (Kudo et al., 1999). Upon inhibition of nuclear export, TRADD clearly accumulated within the nucleus (Figure 3.8B), consistent with a previous report (Morgan et al., 2002). These data support our hypothesis that TRADD shuttles constantly between the cytoplasm and the nucleus.

To evaluate whether nuclear TRADD regulates p19\textsuperscript{Arf} ubiquitination, we generated a mutated TRADD protein (Nuc-TRADD) in which the 5 leucine residues within the TRADD NES were mutated to alanines (Figure 3.8C), causing this mutant protein to localize
exclusively in the nucleus (Figure 3.8D). *In vitro*, Nuc-TRADD was even more potent than wildtype TRADD in blocking p19\textsuperscript{Arf} ubiquitination (Figure 3.9A). In contrast, a mutant TRADD protein that was membrane-anchored (Figure 3.8C) and thus cytoplasm-specific (Cyt-TRADD) (Figure 3.8D) had no such effect (Figure 3.9B). Similarly, TRADD-NT, a mutant TRADD protein that contained only the N-terminal aa1-192 and lacked the NLS and DD (Figure 3.8C), was exclusively cytoplasmic (Figure 8D) and did not diminish Ub-p19\textsuperscript{Arf} (Figure 3.9C). However, TRADD-CT, a mutant protein that contained the C-terminal aa193-310 encoding the NLS and DD but not the NES (Figure 3.8C), was restricted to the nucleus (Figure 3.8D) and reduced Ub-p19\textsuperscript{Arf} (Figure 3.9C). Lastly, TRADD-DN, a dominant negative mutant of TRADD lacking an intact DD due to mutations in aa 296-299 (Park and Baichwal, 1996b), actually enhanced Ub-p19\textsuperscript{Arf} (Figure 3.9C, D). A similar result was achieved by siRNA-mediated *TRADD* knockdown in 293T cells (Figure 3.9D). These data indicate that nuclear TRADD regulates p19\textsuperscript{Arf} ubiquitination, and that TRADD’s C-terminus is important for this process.
Figure 3.7 TRADD regulates p19\textsuperscript{Arf} protein stability.
Figure 3.7 TRADD regulates p19Arf protein stability.

(A) Tradd+/+ and Tradd−/− MEFs pre-treated with MG132 were treated with 10 µM CHX for the indicated times and lysates (10 µg) were immunoblotted to detect the indicated proteins. Vinculin, loading control.

(B) 293T cells were transfected with the indicated tagged expression vectors and treated for 6 hrs with 10µM MG132. Lysates were immunoprecipitated with anti-FLAG beads to recover FLAG-tagged p19Arf complexes and immunoblotted to detect poly- and mono-ubiquitinated p19Arf. WCL, whole cell lysate (10 µg) used as the input.

(C) An increasing dose of TRADD decreased the ubiquitination of p19Arf.

For (A-C), data are representative of at least three independent experiments.
Figure 3.8 TRADD shuttles between the nucleus and the cytoplasm.
Figure 3.8 TRADD shuttles between the nucleus and cytoplasm.

(A) Schematic diagram showing TRADD domains. NES, nuclear export sequence in the leucine-rich region [amino acids (aa) 147-163]. NLS, nuclear localization signal in a highly basic bipartite region of the death domain (DD) (aa 229-242).

(B) Dynamics of TRADD localization. HeLa cells were transfected with green fluorescent protein (GFP)-tagged TRADD. DAPI was used to visualize nuclei. Transfected HeLa cells were treated with DMSO (vehicle) or the nuclear export inhibitor leptomycin B (LMB) for 6 hrs. TRADD accumulated in the nucleus of all GFP\(^+\) cells.

(C) Schematic representation of TRADD constructs used in ubiquitination experiments. WT TRADD was tagged with either FLAG or Myc. Nuc-TRADD, nuclear-specific TRADD mutant. (*) denotes the mutation of 5 leucine residues (aa 147, 152, 155, 159, 162) to alanines, abrogating NES function. Cyt-TRADD, cytoplasmic-specific TRADD mutant. PIP2 denotes the PIP2-binding domain. TRADD-NT, C-terminal truncation mutant (aa1-192); TRADD-CT, N-terminal truncation mutant (aa193-310). TRADD-DN, dominant negative mutant (Park and Baichwal, 1996b). (**) denotes the mutation of residues 296-299 to alanine.

(D) HeLa cells were transfected with the indicated TRADD expression vectors. At 24 hrs post-transfection, cells were fixed in 4% paraformaldehyde and then incubated with anti-FLAG or anti-Myc antibody. Primary antibodies were visualized with Alexa Fluor 488 (Invitrogen)-labeled secondary antibody. Cells were examined by confocal microscopy.
Figure 3.9 Nuclear TRADD regulates $p19^{Arf}$ ubiquitination.
Figure 3.9 Nuclear TRADD regulates p19\textsuperscript{Arf} ubiquitination.

(A-C) 293T cells were transfected with the indicated tagged expression vectors and treated for 6 hrs with 10 µM MG132. Lysates were immunoprecipitated with anti-FLAG beads or anti-p19\textsuperscript{Arf} antibody to recover p19\textsuperscript{Arf} complexes and immunoblotted with anti-HA to detect poly- and mono-ubiquitinated p19\textsuperscript{Arf}. WCL, whole cell lysate (10 µg) used as the input.


(D) 293T cells were transfected with the indicated vectors, or siRNA against TRADD (si\textit{TRADD}), and treated with MG132 as in (A). Lysates were immunoprecipitated with anti-FLAG and immunoblotted with anti-HA.

For (A-D), data are representative of at least three independent experiments.
3.3.7 Nuclear TRADD interacts with ULF E3 ligase and prevents its interaction with ARF

ULF was recently identified as an E3 ubiquitin ligase for ARF (Chen et al., 2010). We first verified that both mouse and human ULF could induce p19\(^{\text{Arf}}\) ubiquitination in the absence of MG132 (Figure 3.10A). We then determined that the ability of TRADD to reduce Ub-p19\(^{\text{Arf}}\) was comparable to that of ULF siRNA treatment (Figure 3.10B, C). Conversely, ULF overexpression enhanced Ub-p19\(^{\text{Arf}}\), but this enhancement was ablated in the presence of TRADD overexpression (Figure 3.10D). To investigate how TRADD regulates ULF’s E3 ligase activity toward p19\(^{\text{Arf}}\), we examined p19\(^{\text{Arf}}\)-ULF interaction. We found that p19\(^{\text{Arf}}\) binding to endogenous ULF was diminished in the presence of either siULF or TRADD (Figure 3.10C). Likewise, the interaction between p19\(^{\text{Arf}}\) and exogenous ULF was reduced by TRADD (Figure 3.10D). Overexpression of TRADD dampened p19\(^{\text{Arf}}\)-ULF interaction in a dose-dependent manner (Figure 3.10E), and p19\(^{\text{Arf}}\)-ULF binding was markedly enhanced in the presence of TRADD-DN or siRNA targeting TRADD (Figure 3.10F). Thus, TRADD regulates p19\(^{\text{Arf}}\) ubiquitination through regulating the interaction between p19\(^{\text{Arf}}\) and its E3 ligase, ULF.

Immunoprecipitation (IP) of ULF immunocomplexes identified TRADD as a binding partner of ULF in overexpression settings (Figure 3.11A), which was confirmed by a reciprocal experiment showing that both ectopically expressed TRADD and Nuc-TRADD interact specifically with endogenous ULF, but not with several other known p19\(^{\text{Arf}}\) binding partners, including MULE (ARFBP1) and NPM1/B23 (Bertwistle et al., 2004; Chen et al., 2005) (Figure 3.11B). The interaction between ULF and TRADD can also be detected at the endogenous level in HCT116 cells with stable TRADD expression (Figure 3.11C) as well as in primary MEFs (Figure 3.11D). This interaction is dependent on the C-terminal sequences
(comprising mainly the death domain) of TRADD and is abolished in the dominant negative TRADD mutant, which contains mutations in the death domain (Figure 3.11E, F). Thus, the interaction between TRADD and ULF may serve to prevent the binding of ULF to p19^{Arf}, thereby reducing p19^{Arf} ubiquitination.
Figure 3.10 TRADD disrupts the interaction between ULF and p19Arf and impairs p19Arf ubiquitination.
Figure 3.10 TRADD disrupts the interaction between ULF and p19\(^{Arf}\) and impairs p19\(^{Arf}\) ubiquitination.
Figure 3.10 TRADD disrupts the interaction between ULF and p19^Arf and impairs p19^Arf ubiquitination.

(A) 293T cells were transfected with the indicated tagged expression vectors. hULF, human ULF; mULF, mouse ULF. Lysates were immunoprecipitated with anti-FLAG beads and immunoblotted with anti-HA to detect ubiquitination of p19^Arf.

(B) ULF antibody validation. 293T cells were transfected with 150 pmol control scramble siRNA (siScramble) or siRNA targeting ULF (siULF). At 24 hrs post-transfection, lysates were split into two portions that were separated independently 4-12% Bis-Tris gel or a 6% Tris-Glycine gel. Top panel: The band representing endogenous ULF is clearly reduced in the presence of siULF. The doublet bands observed for ULF in these gradient gels suggest that post-translational modification of this protein occurs. Bottom panel: ULF mRNA in the cells in the top panel was quantitated by qRT-PCR to confirm the efficient knockdown of ULF in siULF-transfected cells.

(C) 293T cells were transfected with siRNA targeting ULF (siULF) using lipofectamine 2000, and transfected 24 hrs later with the indicated tagged expression vectors using calcium phosphate precipitation. Cells were treated with MG132 at 24 hrs after the last transfection. Lysates were immunoprecipitated with anti-FLAG beads and immunoblotted with anti-HA antibody.

(D) 293T cells were transfected with the indicated vectors and then treated with MG132. The indicated proteins were detected by IP and immunoblotting as for (C).

(E) 293T cells were transfected with FLAG-tagged p19^Arf (6 µg) and increasing doses of Myc-tagged TRADD (1 µg, 3 µg or 6 µg). Lysates were immunoprecipitated with anti-FLAG beads and immunoblotted to detect endogenous ULF (left panel). Right panel, input.
(F) 293T cells were transfected with 150 pmol siTRADD followed 24 hrs later by transfection with the indicated tagged expression vectors. Lysates were harvested 24 hrs later, immunoprecipitated with anti-FLAG beads, and immunoblotted to detect endogenous ULF (left panel). Right panel, input.

For (A-F), data are representative of at least three independent experiments.
Figure 3.11 TRADD-ULF interaction occurs at the endogenous level and is mediated through the C-terminal sequences of TRADD.
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Figure 3.11 TRADD-ULF interaction occurs at the endogenous level and is mediated through the C-terminus of TRADD.

(A) 293T cells were transfected with the indicated vectors. At 24 hrs post-transfection, lysates were immunoprecipitated with anti-FLAG beads to isolate ULF complexes and immunoblotted to detect ULF and TRADD.

(B) 293T cells were transfected with empty vector (pcDNA3) or vectors expressing FLAG-tagged WT TRADD, Nuc-TRADD, or p19Arf. Lysates were immunoprecipitated with anti-FLAG beads and immunoblotted to detect the indicated known binding partners of p19Arf (left panel). Right panel, input.

(C) FLAG-tagged TRADD was stably expressed in HCT116 cell line. Lysates were immunoprecipitated with anti-ULF antibody or anti-FLAG antibody and immunoblotted to detect TRADD and ULF (left and middle panels). Right panel, input.

(D) Primary MEFs were treated for 6 hrs with leptomycin B (10 ng/ml) to induce nuclear accumulation of TRADD. 10 mg of lysates were immunoprecipitated with anti-TRADD antibody and immunoblotted to detect TRADD and ULF (left panel). Right panel, input.

(E) 293T cells were transfected with vectors expressing Myc-tagged WT TRADD, TRADD-NT TRADD-CT, TRADD-DN or FLAG-tagged ULF. Lysates were immunoprecipitated with anti-Myc antibody and immunoblotted to detect ULF (left panel). Right panel, input.

(F) 293T cells were transfected with vectors expressing Myc-tagged WT TRADD, TRADD-NT TRADD-CT, TRADD-DN or FLAG-tagged ULF. Lysates were immunoprecipitated with anti-FLAG beads and immunoblotted with an anti-Myc antibody to detect various TRADD constructs (left panel). Right panel, input.

For (A-F), data are representative of at least three independent experiments.
3.3.8 Stabilization of p19\textsuperscript{Arf} mediated by nuclear TRADD is required for H-RasV12-induced senescence

To investigate whether TRADD’s regulation of p19\textsuperscript{Arf}-ULF interaction was relevant during H-RasV12-mediated cellular senescence, we analyzed the intracellular distribution of TRADD in transduced MEFs. H-RasV12 expression resulted in a 2-fold increase in both cytoplasmic and nuclear TRADD, as determined by nuclear/cytoplasmic fractionation (Figure 3.12A, B, Ci). However, the overall ratio of nuclear to cytoplasmic TRADD was unaltered by H-Ras activation (Figure 3.12Cii). These data imply that the cellular distribution of TRADD does not change upon H-Ras activation, and that a 2-fold increase in nuclear TRADD triggered by oncogenic stress is sufficient to attenuate p19\textsuperscript{Arf}-ULF interaction and promote p19\textsuperscript{Arf} protein stabilization. In support of this hypothesis, while H-RasV12-transduced \textit{Tradd}\textsuperscript{−/−} MEFs reconstituted with wildtype TRADD showed resumption of p19\textsuperscript{Arf} accumulation (Figure 3.12D) and SA-β-gal activity (Figure 3.12E), transduced cells reconstituted with Cyt-TRADD did not (Figure 3.12D, E). Even without H-Ras activation, overexpression of wildtype TRADD increased the basal level of p19\textsuperscript{Arf} protein (Figure 3.12D). With respect to Nuc-TRADD, we have yet to achieve a satisfactory level of reconstitution, suggesting that excessive levels of nuclear TRADD may be toxic to cells.

In summary, results presented in this chapter suggest that TRADD regulates intracellular levels of p19\textsuperscript{Arf} protein by regulating its interaction with ULF, its E3 ubiquitin ligase. In the absence of TRADD, p19\textsuperscript{Arf}-ULF interaction is sustained such that p19\textsuperscript{Arf} protein degradation is increased. As a result, both \textit{Tradd}\textsuperscript{−/−} MEFs transduced with H-RasV12, and mice treated with DMBA/TPA, show reduced p19\textsuperscript{Arf} protein accumulation. Thus, TRADD demonstrates tumor-suppressive functions that are mediated by its post-translational regulation of p19\textsuperscript{Arf} (Figure 3.13).
Figure 3.12 Stabilization of p19<sup>Arf</sup> by nuclear TRADD is required for Ras-induced senescence.
Figure 3.12 Stabilization of p19ARF by nuclear TRADD is required for H-RasV12-induced senescence.
Figure 3.12 Stabilization of p19Arf by nuclear TRADD is required for H-RasV12-induced senescence.

(A) Lysates of $Tradd^{+/+}$ and $Tradd^{-/-}$ H-RasV12-transduced MEFs were subjected to nuclear and cytoplasmic fractionation. Fractions (20 µg) were immunoblotted to detect the indicated proteins on the indicated days post-transduction. Usf2, nuclear marker; tubulin, cytoplasmic marker.

(B) Lysates of pBABE- or H-RasV12-transduced wildtype MEFs were subjected to nuclear and cytoplasmic fractionation. Fractions (20 µg) were immunoblotted to detect the indicated proteins on the indicated days post-transduction. Usf2, nuclear marker; Tubulin, cytoplasmic marker.

(C) Quantitation of relative TRADD induction in the cytoplasm vs. nucleus. (i) TRADD protein levels in the nuclear and cytoplasmic fractions in (A) were normalized to Usf2 or Tubulin, as appropriate. Results are the mean relative fold increase ± SD of three experiments. (ii) The nuclear/cytoplasmic (N/C) TRADD ratio was determined by comparing nuclear and cytoplasmic TRADD band intensities on the same blot.

(D, E) $Tradd^{+/+}$ or $Tradd^{-/-}$ MEFs were reconstituted with empty retroviral vector (pBABE) or retroviral vector expressing WT TRADD or Cyt-TRADD, as indicated. Cells were rested 24 hrs without selection and then transduced with empty lentiviral vector (pLenti) or lentiviral vector expressing H-RasV12, as indicated. (D) Lysates were harvested 4 days after the last infection and samples (10 µg) were immunoblotted to detect the indicated proteins. (E) (i) Representative SA-β-gal staining. (ii) Quantitation. Results are the mean percentage ± SD of SA-β-gal$^+$ cells. P values, unpaired Student’s $t$-test.

For (A-E), data are representative of at least three independent experiments.
Figure 3.13 Model of TRADD’s tumor-suppressive function in the context of oncogenic Ras.
Figure 3.13 Model of TRADD’s tumor-suppressive function in the context of oncogenic Ras.

TRADD has known roles as an adaptor protein in TNFR1-, TLR3-, and TLR4-triggered intracellular signaling. Data present in this current chapter suggest that TRADD also has a tumor-suppressive role that is independent of these receptors. We propose that, in wildtype cells at steady state (TRADD+), TRADD shuttles continuously between the cytoplasm and the nucleus. Upon constitutive H-Ras activation, the transcription of both p19Arf and TRADD is induced. Levels of TRADD protein rise coordinately in the cytoplasm and the nucleus. Elevated nuclear TRADD disrupts the interaction between nuclear p19Arf and its E3 ubiquitin ligase ULF, resulting in reduced p19Arf ubiquitination and thus enhanced p19Arf stability. In TRADD-deficient cells (TRADD−), ULF unrestrictedly drives the degradation of p19Arf protein, reducing levels of this key tumor suppressor. Our results indicate that the accumulation of p19Arf promoted by TRADD function contributes to oncogene-induced senescence, and thus helps to create a barrier against tumorigenesis.
3.4 Discussion

In this chapter of the dissertation, we used genetically modified mice to investigate \textit{in vivo} the potential tumor-suppressive properties of TRADD. We have demonstrated that deletion of \textit{Tradd}, regardless of \textit{Tnf} status, enhances tumor growth in the DMBA/TPA skin tumor model. Our data are thus the first \textit{in vivo} evidence that the TRADD adaptor protein has tumor-suppressive properties distinct from its long established involvement in TNFR1 signaling and inflammatory responses. These results shed light on a new avenue of TRADD function in cancer signaling pathways, namely the post-translational regulation of p19\textsuperscript{Arf}.

3.4.1 A cell intrinsic tumor suppressive role of TRADD independent of TNFR and other immune signaling pathways

Our data definitively show that TRADD’s tumor-suppressive properties are independent of TNFR1 signaling, but we did not assess whether these effects were related to TRADD’s known involvement in signaling mediated by TLR3 or TLR4. In contrast to \textit{Tradd} deficiency, however, mice lacking components of TLR signaling pathways exhibit reduced skin tumor formation upon DMBA/TPA treatment (Mittal et al., 2010; Swann et al., 2008). Likewise, TRADD participates in death receptor-3 (DR3) signaling, which is prominent mainly during T cell activation triggered when the TL1a ligand engages DR3 (Migone et al., 2002). However, T cell infiltration in \textit{Tradd}\textsuperscript{+/+} and \textit{Tradd}\textsuperscript{−/−} skin tumors was comparable (Figure 3.2F). We therefore believe that the novel tumor-suppressive properties of TRADD are also independent of its roles in TLR and DR signaling.
3.4.2 A novel role of TRADD in p19\textsuperscript{Arf} regulation

H-Ras activation in Tradd-deficient cells resulted in decreased cell cycle arrest but did not lead to cellular transformation, unlike H-Ras activation in cells lacking p19\textsuperscript{Arf} or Tp53 (Kamijo et al., 1997). Tradd\textsuperscript{−/−} MEFs became transformed only upon loss of one Tp53 allele. This observation is consistent with our finding that, although Tradd\textsuperscript{−/−} mice showed heightened growth of skin tumors following DMBA/TPA administration, none was a squamous cell carcinoma, which occur at high frequency in DMBA/TPA-treated p19\textsuperscript{Arf}\textsuperscript{−/−} or Tp53\textsuperscript{−/−} mice (Kelly-Spratt et al., 2004). We therefore believe that TRADD bolsters intracellular p19\textsuperscript{Arf} protein levels, and that additional genetic lesions are thus required before Tradd-deficient cells can fully evade the senescence barrier and become malignant.

p19\textsuperscript{Arf} protein levels are known to be regulated both transcriptionally and post-translationally (Ozenne et al., 2010). c-Myc governs both of these processes because it can induce p19\textsuperscript{Arf} mRNA transcription as well as interact with ULF to modulate p19\textsuperscript{Arf} ubiquitination, thus preventing p19\textsuperscript{Arf} protein degradation (Chen et al., 2010). p19\textsuperscript{Arf} transcription can also be induced by constitutive H-Ras activation, but H-Ras lacks c-Myc’s ability to inhibit ULF (Chen et al., 2010). Our data suggest that TRADD may serve this function for H-Ras, since H-Ras induces TRADD expression. Minimal amounts of nuclear TRADD must be sufficient to carry out this function since we detected no alterations to TRADD’s intracellular distribution upon oncogenic stress. Because endogenous ULF levels are low (Dr. Wei Gu, personal communication), it is plausible that small changes in nuclear TRADD might significantly alter p19\textsuperscript{Arf}-ULF interaction.

Our analyses of mutant TRADD proteins have revealed the importance of TRADD’s C-terminus in regulating nuclear entry and p19\textsuperscript{Arf} ubiquitination. Given that the C-terminus of TRADD is dominated by the DD, our data suggest that TRADD’s DD exerts two different
regulatory functions through two distinct mechanisms. In combination with data presented in chapter 2 of this dissertation, we propose that, in the cytoplasm, TRADD is a critical mediator of extrinsic cell death and inflammatory signaling pathways, through its recruitment to TNFR1, TLR3 and TLR4 via DD interactions, whereas in the nucleus, TRADD is a sensor that triggers an intrinsic response to oncogenic stress through stabilization of p19^Arf (Figure 3.14). This scheme exemplifies evolutionary elegance in developing a single protein to regulate cell fate in different cellular compartments, and raises the possibility that other DD-containing proteins may exhibit similar functional diversity.
Figure 3.14 The different roles of nuclear and cytoplasmic TRADD.

Upon TNFR1, TLR3 and TLR4 receptor engagement, TRADD is recruited to the receptor complexes, leading to activation of NF-κB and subsequent proinflammatory responses. This is essential for both host anti-bacterial and anti-viral responses. Being able to translocate between the cytoplasm and the nucleus, TRADD also serves a nuclear function, to sequester the E3 ubiquitin ligase ULF competitively away from its substrate, ARF, resulting in ARF stabilization and resistance to tumorigenesis. Ub, ubiquitin.
3.4.3 TRADD is a novel tumor suppressor candidate

The addition of ULF and TRADD to the growing network of molecules regulating p19Arf breaks new ground in the ARF research field. Although our study focused on TRADD’s tumor-suppressive role in the context of H-Ras activation/amplification, we anticipate that TRADD would serve a similar function in other tumor models. As mentioned earlier, the human TRADD gene is located in chromosome 16q22.1 (Scheuerpflug et al., 2001), a region exhibiting frequent LOH in hepatocellular carcinomas and in breast, prostate and Wilms tumors (Filippova et al., 1998; Hainsworth et al., 1991; Kihana et al., 1996; Kim et al., 2009; Sakai et al., 1992; Wang et al., 2000a). Candidate tumor suppressor genes located in this region include CTCF (CCCTC-binding factor) and E-CADHERIN. In breast cancer, E-CADHERIN is believed to function as a tumor suppressor gene but only for the lobular carcinoma histological subgroup. In infiltrating ductal carcinomas, no E-CADHERIN mutations have been identified despite the detection of LOH at 16q22.1 in these malignancies (Berx et al., 1995). This finding implies the existence of at least one other tumor suppressor gene in this locus. Importantly, like Tradd–/– mice, murine models deficient of Ctf or E-cadherin have not been reported to develop spontaneous tumors and require the loss of additional tumor suppressors to develop malignancies (Boussadia et al., 2002; Derksen et al., 2006; Heath et al., 2008). Thus, based on its chromosomal location and our experimental results, we speculate that loss of TRADD may also contribute to cancers exhibiting 16q22 LOH, and that TRADD is therefore a novel candidate tumor suppressor gene. Our hypothesis is reinforced by gene profiling data showing that TRADD mRNA levels are reduced in a variety of human cancers, including acute myeloid leukemia, ovarian and renal tumors (Figure 3.15). In addition, TRADD mRNA levels decrease as prostate cancer progresses (Wang et al., 2009). It has also been shown that in breast cancer, reduced TRADD expression
correlates significantly with a lower probability of relapse free survival (Gyorffy et al., 2010) (Figure 3.16). Finally, missense TRADD mutations have been identified in low frequencies in acute lymphocytic leukemia (Dechant et al., 2008) and in squamous cell carcinoma of the lung (Kan et al., 2010). In view of our data, it would be intriguing to evaluate the relationship between TRADD and ARF mRNA and protein levels in these types of tumors. Although the inactivation of p14ARF in human cancers occurs mainly through gene mutation, homozygous deletion or promoter methylation (Ozenne et al., 2010), in some tumors, p14ARF protein is expressed at very low levels even in the absence of promoter hypermethylation or LOH (Guo et al., 2008). It may be that these latter malignancies have lost TRADD expression or function. If true, TRADD mutation may then be an important new biomarker of disease prognosis.

In conclusion, our findings represent an important conceptual advance in our understanding of ARF regulation, and have provided the first in vivo evidence demonstrating a tumor-suppressive role for TRADD. To delineate the full extent of TRADD’s role in human cancers, future endeavors should include detailed analyses of the status of the TRADD locus in a broad range of human tumors. These studies will no doubt yield valuable insights into potential new therapeutic interventions.
Examination of the Oncomine database revealed the reduction of *TRADD* mRNA in the indicated human tumors.
Figure 3.16 Survival analysis.

Reduced TRADD expression is correlated with poor prognosis in a cohort of 2,472 breast cancer patients.
CHAPTER 4

DISCUSSION
4.1 TRADD in immune signaling cascades

4.1.1 TNFR1 and TLRs: the past and the present

In 1995, TRADD was cloned and identified as an adaptor functioning 'downstream' of TNFR1 (Hsu et al., 1995). This has initiated the 'golden era' of research on the mechanisms involved in TNF receptor superfamily signal transduction. Biochemical studies suggest that the transduction of TNFR1-mediated signals involves four main players, TRADD, TRAF2, RIP1 and FADD, each exerting a different and specific function. As TRAF2 does not efficiently bind TNFR1, even under overexpression conditions, it is generally believed that recruitment of TRAF2 to the receptor complex must be mediated through TRADD. In the case of RIP1, as both TNFR1 and TRADD bear a DD that can associate with the one present in RIP1, it is controversial whether RIP1 recruitment strictly requires TRADD. In any case, the function of both TRAF2 and RIP1, defined by genetic analyses in the mouse (Kelliher et al., 1998; Yeh et al., 1997), is to link the receptor to the activation of MAP kinases and transcription factor NF-κB, which mediate 'downstream' events, including the activation of genes encoding inflammatory molecules. Finally, FADD is required for caspase activation and the induction of apoptosis (Yeh et al., 1998; Zhang et al., 1998).

An apparent gap towards the completion of this simplistic picture of the TNFR1 pathway clearly stems from the lack of definitive genetic data confirming the function of TRADD in this pathway. Moreover, some data based on depletion of TRADD by RNAi have suggested the unexpected possibility that TRADD may not be required for TNFR1-induced apoptosis, thus complicating this scenario (Jin and El-Deiry, 2006). Our description of Tradd-deficient mice has therefore 'cleared the clouds' and provided what is probably the most definitive description of the receptor-proximal events in this signaling pathway. We have demonstrated that TRADD is absolutely required for TNF induced cell death. TRADD
is also indispensable for the recruitment of TRAF2 to TNFR1. While the recruitment of RIP1 to the receptor complex occurs independently of TRADD, its ubiquitination does not occur in the absence of TRADD.

In addition to clarifying the function of TRADD in TNFR1 signaling, Tradd-deficient mice also demonstrate a novel role of TRADD in the TRIF-dependent branch of TLR3 and TLR4 signaling pathways. These results may seem rather unexpected at first glance, given that TNFR1 and TLRs are completely unrelated beyond the fact that both are crucial pathways regulating the immune response. These two receptor families do not share any common features in structure or in signaling mechanisms, with the exception that both employ the use of RIP1 (Meylan et al., 2004b). RIP1 is recruited to TLR-bound TRIF through an interaction between RIP homotypic interaction motifs (Meylan et al., 2004b). After binding to TRIF, the RIP1 DD remains unengaged and should be available to interact with the TRADD DD. It is remarkable that unrelated receptors whose only apparent commonality is their involvement in immune and inflammatory responses have evolved to use TRADD in signal transduction. And in this context, the discovery that an antiviral signaling pathway triggered by the detection of cytoplasmic viral dsRNA by the helicase RIG-I also requires TRADD for 'downstream' signaling is very notable (Michallet et al., 2008). In this pathway, TRADD is recruited to Cardif, a CARD containing adaptor protein, leading to activation of IRF3 and NF-κB, and subsequent production of type I interferons and inflammatory cytokines, respectively. These collectively suppress viral replication and assembly (Michallet et al., 2008). Thus, it appears that TRADD is an important “gatekeeper” of host pathogenic response, such that by manipulating TRADD expression or function, pathogenic microbes are not only able to modulate TNF-induced proinflammatory cytokine
production and TNF-dependent apoptosis, but can also conveniently suppress host antiviral immunity.

4.1.2 Perspectives and future directions

Data presented in chapter 2 of this dissertation demonstrate a role for TRADD in TRIF dependent NF-κB activation, in a manner that is independent of its role in TNFR1 signaling. There remain, however, several open questions. For example, why does TRADD induce potent IRF3 activation only in the RIG-I pathway but not in the TNFR1 and TLR3 pathways described in this study, even though all of these pathways employ TRADD for signal transduction? Conceptually, one could postulate that since TLR3 acts as an initial sensor during a viral infection and RIG-I operates at a later stage of the infection (Slater et al., 2010), it is perhaps logical that the anti-viral role of TRADD is maximized only in the later phase of the infection, by activating both interferon and NF-κB pathways. Biochemically, TNFR1, TLR3 and RIG-I are each situated in different cellular locations. Proximity and access to signaling molecules may contribute to the NF-κB specificity of TRADD downstream of TNFR1 and TLR3. Nevertheless, future molecular and biochemical analyses will be very important to resolve this conundrum.

Another question remaining is, how does TRADD mediate TRIF dependent NF-κB activation? At the moment, we know that the connection between TRADD and TRIF is through RIP1, which is believed to direct activation of NF-κB upon TLR engagement (Meylan et al., 2004b). However, the recruitment of RIP1 to TRIF and hence to the TLR receptor complexes, is independent of its DD and therefore of TRADD (Meylan et al., 2004b), placing TRADD downstream of RIP1. An obvious conjecture from what we have learned about TRADD’s role in TNFR1 signaling is that TRADD may serve to recruit an E3
ubiquitin ligase, possibly TRAF2, to the TLR receptor complex, leading to RIP1 ubiquitination and hence NF-κB activation. Contrary to this idea, however, is a recent study demonstrating that TRAF2 could bind directly to TRIF and mediate ubiquitination of TRIF, but not RIP1, leading to activation of the interferon pathway through IRF3 (Sasai et al., 2010). TRAF6 has been demonstrated to be important in activating MAPK and NF-κB downstream of TLR3 (Kawai and Akira, 2010). It is plausible that TRADD serves to recruit TRAF6 in this context, although interaction between these two proteins has never been reported. Identification of the molecular player(s) connecting TRADD to RIP1 ubiquitination, or to other aspects of TLR activation, will be an important future endeavor. Overall, in depth genetic and biochemical analysis of the role of TRADD in TLR proximal signaling and in complex formation will be imperative to dissect the precise role of TRADD in TLR signaling and in anti-viral immunity in general.
4.2. TRADD in cancer signaling

4.2.1 The ARF tumor suppressor: the past and the present

The identification of a TNFR1-independent role of TRADD in the nucleus regulating p19\textsuperscript{Arf} stability not only broadens our view of the extent of the cellular functions of TRADD, but places it in the enticingly new arena of p19\textsuperscript{Arf} post-translational regulation. The involvement of TRADD’s DD for this function further prompts re-evaluation of the potential functional diversity of other death domain containing proteins.

A variety of oncogenes are able to elicit the activation of ARF, most notably Myc overexpression and oncogenic mutant Ras (Juntila and Evan, 2009). A number of transcriptional modulators have been found to mediate the induction of ARF upon oncogene activation (Kim and Sharpless, 2006). Extensive studies of the transcriptional regulation of ARF have led to the overlooking of the regulatory potential of ARF at the protein level. This is particularly reinforced by the fact that ARF is a “lysine-less” protein, which upon first glance appears incompatible with the general model of proteasome mediated protein degradation, which depends on ubiquitination on lysine residues. In the yeast system, it has been shown that lysine-less proteins can be ubiquitinated at their N-terminus through what is known as N-terminal ubiquitination or the ubiquitin fusion degradation (UFD) pathway (Ciechanover and Ben-Saadon, 2004). The UFD pathway, although not exclusive for lysine-less proteins, is the only available choice for ubiquitin-proteasome degradation in the case of lysine-less proteins. In the same year, Chuck Sherr’s group connected this pathway to ARF post-translational regulation (Kuo et al., 2004). Despite this, the identity of the E3 ubiquitin ligase responsible for the N-terminal ubiquitination of ARF remained elusive. For that matter, the identity of any E3 ubiquitin ligase involved in UFD in mammalian cells was not known.
The identification of ULF as an E3 enzyme of the UFD pathway (Park et al., 2009) and subsequently as the E3 ubiquitin ligase for ARF (Chen et al., 2010) broke new ground for the field and suggested that ULF could act as a novel sensor of oncogenic stress upstream of ARF. Consequently, it became interesting to understand how ULF activity is regulated when normal cells are challenged by oncogenic stress. The addition of TRADD to this evolving picture serves this purpose exactly. In addition to the earlier observation that Myc itself can regulate ARF-ULF interaction upon Myc induced oncogenic stress, our data have revealed that TRADD serves the same function upon Ras induced oncogenic stress. This demonstrates that ULF-mediated ARF degradation is a tightly regulated process, with different molecules involved in fine-tuning ARF ubiquitination upon different types of cellular stresses.

Since unchecked ULF activity will promote ARF degradation and render cells unprotected from oncogenic stress, targeting ULF activity with small drug inhibitors that are structurally similar to TRADD or to the death domain might offer a novel strategy for therapeutic intervention of cancer, thus following the footsteps of agents such as Nutlin3a that targets Mdm2 (Vassilev et al., 2004).

4.2.2 Perspectives and future directions

All tumors are comprised of a neoplastic clonal cell population in the presence of stromal and infiltrating inflammatory cells that provide sustenance and facilitate the metastatic process of the malignant cell (Tlsty, 2001; van Kempen et al., 2003). The tumor stroma may be modulated at several levels by TNF. TNF can promote angiogenesis through various factors including VEGF, basic fibroblast growth factor (bFGF), IL-8, platelet-
activating factor, E-selectin and intercellular adhesion molecule 1 (ICAM-1) (Bussolino et al., 1988; De Cesaris et al., 1999; Leibovich et al., 1987; Yoshida et al., 1997). TNF can also promote further tumor remodelling by stimulating fibroblast and macrophage activity, tumor cell motility and tumor invasion via the induction of matrix metalloproteinases (MMPs) (Battegay et al., 1995; Leber and Balkwill, 1998; Rosen et al., 1991). Given the prominence of TNFR (and TLRs) in tumor promotion, our initial hypothesis and plan of study had been to determine the contribution of TRADD to these signaling cascades in the context of tumor promotion.

In chapter 3 of this dissertation, solid evidence was presented showing that Tradd-deficient mice exhibit heightened skin tumor growth, in direct contrast to reduced tumor growth observed in Tnfr1 and Tnf-null mice. Although these data define clearly a novel, TNF-independent function of TRADD responsible for mediating this effect, this does not preclude a role of TRADD in the aforementioned mechanisms of TNF-mediated tumor promotion through modifications of the stroma. We believe that the physiological outcome of the process of tumorigenesis depends ultimately on the balance between tumor supporting proinflammatory effects in the stroma (through TNF signaling) and intrinsic tumor suppressive pathways (through ARF regulation). In the case of the tumor model we have studied, the tumorigenic effect of downregulating p19Arf expression in the absence of Tradd must have surpassed the lack of stromal support brought about by the loss of TNF-mediated tumor promotion, leading to heightened skin tumor growth as the end result. In this context, we would expect that keratinocyte-specific Tradd knockouts, which maintain intact TNF signaling in the tumor stroma, would exhibit faster and more severe tumor growth than the Tradd total knockouts used in the current study. The use of other tissue specific Tradd
knockouts in the future, particularly in tumor infiltrating immune cells, will be imperative to define the extent of TRADD’s function in the various known tumor-promoting aspects of TNF.

Meanwhile, based on all the data presented in this dissertation, we believe that the role of nuclear TRADD in ARF regulation may actually constitute a novel aspect of TNF-mediated oncogenicity, in addition to its role in modifying the tumor stroma. It is known that activation of TNFR1 results in the recruitment of TRADD in the cytoplasmic receptor complexes. Chronic levels of TNF present in the tumor stroma may result in persistent engagement of TNFR1 on tumor cells, the consequence of which may be sequestration of TRADD away from the nucleus, hence limiting the “nuclear pool” of TRADD that is available to stabilize ARF. Effectively, this will result in the reduction of ARF expression levels, providing additional growth advantages beyond other known effects downstream of TNFR1 (Fig 4.1). Such a model might also be applied to TLR4, which has also been shown to be oncogenic (Lee et al., 2010). This proposed model would put forward a highly dynamic interaction between events happening at the membrane level and those occurring in the nucleus. This may also explain why TRADD loss of function is not a frequent event in human cancers, possibly because the “built-in inflammatory loop” mediated through TNFR1 and TLR4 is sufficient to serve the purpose. If this holds true, anti-inflammatory approaches in cancer treatment should be highly effective, because they can accomplish two goals; by halting tumor promoting inflammatory effects of TNF, and by liberating otherwise TNFR-bound TRADD into the nucleus to stabilize ARF, thereby enhancing endogenous tumor suppressive pathways.
Figure 4.1 Hypothetical model of the connection between inflammatory and tumor suppressive pathways through TRADD.
Figure 4.1 Hypothetical model of the connection between inflammatory and tumor suppressive pathways through TRADD.

NF-κB activation induced by the binding of TNF to its receptor TNFR1 expressed on inflammatory cells initiates regulated expression of cytokine genes, which control cell migration, proliferation and death. In the context of tumorigenesis, chronic inflammation in the tumor microenvironment results in dysregulated overproduction of proinflammatory cytokines by infiltrating as well as tissue cells. This creates a tumor microenvironment that can promote tumor growth, angiogenesis, motility and invasion. We propose that chronic activation of TNFR1 (and possibly TLR4) on the tumor cell may also result in sequestration of TRADD at the membrane level. As a consequence, the nuclear pool of TRADD available for stabilization of the ARF tumor suppressor is diminished. As ARF levels decrease, the tumor cell gains unlimited proliferative potential and thrives. This model exemplifies the efficiency of tumors in their ability to usurp the common features of unrelated signaling pathways to promote tumor progression, in this case, by linking TNF signaling to ARF stabilization through the TRADD protein. Ub, ubiquitin.
4.3 Concluding Remarks

Identified more than 15 years ago, the adaptor protein TRADD was the first component described in the TNFR1 signaling pathway. For this reason, the field has largely focused on the role of TRADD in signaling events involving TNFR superfamily members. The work described in this dissertation has significantly reoriented the field, creating a new picture in which TRADD is no longer a static protein, but one that shuttles dynamically between the nuclear and the cytoplasmic compartments of the cell. In the cytoplasm, TRADD serves as a crucial adaptor protein for several immune signaling pathways and orchestrates major inflammatory responses. In the nucleus, TRADD plays an important role in regulating the protein stability of the ARF tumor suppressor upon oncogenic stress, thereby influencing intrinsic tumor suppressive mechanisms. This new role augments TRADD’s long established functions in inflammatory and immune signaling cascades, and implies that a re-evaluation of the potential functional diversity of other members of the death domain superfamily may be appropriate.
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