THE ROLE OF TIPE2 IN THE REGULATION OF INFLAMMATION AND TUMORIGENESIS

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Dedication

To my parents and brother, who helped me through this process, always offering support and words of encouragement.

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To all those people and more, my sincerest thanks.

ABSTRACT
THE ROLE OF TIPE2 IN THE REGULATION OF INFLAMMATION AND TUMORIGENESIS

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TIPE2 is a recently discovered regulator of immunity and inflammation. Here we describe a new function of TIPE2 in the regulation of Ras signaling and Tumorigenesis. By using various stimuli and inhibitors in T Cells and macrophages we discovered that TIPE2 is regulated at both the message and protein level by inflammatory stimuli. TIPE2 mRNA is regulated in the short to intermediate term by an NF-κB induced micro RNA, and TIPE2 is also ubiquitylated and degraded, possibly by SCF-βTRCP. Mechanistically TIPE2 interacts with and inhibits the Ras-interacting domain of the RalGDS family of Ras effectors, leading to a loss of downstream Ral and AKT activity. TIPE2 deficiency led to increased activation of Ral and AKT, resulting in resistance to cell death, increased migration, and dysregulated exocyst complex formation. Overexpression of TIPE2 conversely induced cell death, affected actin polymerization, and reduced exocyst complex assembly. TIPE2 was able to dramatically slow the growth of Ras-induced tumors in mice, and the tumors were required to silence TIPE2 before they were licensed to grow. TIPE2 additionally negatively regulates effectors of the mTOR pathway, including S6K and 4EBP1, possibly via an interaction with, and destabilization of the mTORC2 complex. Crucially TIPE2 expression is either completely lost or heavily down-regulated by human hepatocellular carcinoma. Thus, via its simultaneous role as

a regulator of inflammation and cancer, TIPE2 provides a mechanistic link between these two disease states, and may be a potential drug target for both inflammatory and neoplastic disease.

Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Publications

*Gus-Brautbar, Y., ***Johnson, D**., Zhang, L., Sun, H., Wang, P., Zhang, S., Zhang, L., and Chen, Y.H. (2012). The anti-inflammatory TIPE2 is an inhibitor of the oncogenic Ras. Molecular Cell *45*, 610–618.

*These authors contributed equally to this work

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Ruan, Q., Wang, T., Kameswaran, V., Wei, Q., **Johnson, D.S**., Matschinsky, F., Shi, W., and Chen, Y.H. (2011). The microRNA-21-PDCD4 axis prevents type 1 diabetes by blocking pancreatic beta cell death. Proceedings of the National Academy of Sciences of the United States of America *108*, 12030–12035.

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Sun, H., Zhuang, G., Chai, L., Wang, Z., **Johnson, D**., Ma, Y., and Chen, Y.H. (2012). TIPE2 Controls Innate Immunity to RNA by Targeting the Phosphatidylinositol 3-Kinase-Rac Pathway. Journal of Immunology (Baltimore, Md. : 1950).

Wang, Z., Fayngerts, S., Wang, P., Sun, H., **Johnson, D.S**., Ruan, Q., Guo, W., and Chen, Y.H. (2012). TIPE2 protein serves as a negative regulator of phagocytosis and oxidative burst during infection. Proceedings of the National Academy of Sciences of the United States of America *109*, 15413–15418.

Chapter 1
Introduction

"Inflammation" is a diverse and nuanced process composed of thousands of different reactions and molecules. This process is tightly controlled in order to ensure that the body responds appropriately to a nearly infinite number of infectious agents and dangerous conditions while still maintaining proper bodily homeostasis. If this response is too weak, the body is overtaken by infection and dies, if the response too potent, the weapons employed by the immune system can permanently damage, or, in some cases, destroy the host. The immune system has many tools and methods that it uses to modulate and tweak its response in order to create an outcome with just enough duration and intensity to end the external threat while dealing minimal damage to the host. These tools range from soluble molecules that enter into the peripheral circulation (cytokines, chemokines, etc.) to pro- and anti- inflammatory molecules expressed on the surface of both immune and non-immune cells (TNFαR, TGFβR, CTLA4 etc.) to various intracellular signaling molecules that can control every process from secretion to metabolism to survival. With a careful balance maintained between these myriad inputs, we arrive at a condition that we refer to as "health". Loss of proper regulation of inflammation results in inflammatory disease. Inflammatory diseases afflict tens of millions of people every year (CDC). While crude treatments such as glucocorticoids and non-steroid-anti-inflammatory-drugs (NSAIDs) and more sophisticated treatments (soluble TNFα receptor, anti TNFα monoclonal antibody) exist to treat such diseases, the etiology of many such diseases is unknown (Hanauer, Lakatos). Central to discovering treatments to these debilitating diseases is developing a better

understanding of immuno-modulatory mechanisms that become dysregulated during these disorders. While many of these regulatory mechanisms (PD-1, CTLA4, SOCS-1) have been identified, the failure to control so many diseases of excess inflammation indicates that there is much more to learn.

One family of molecules that has remained fairly thinly recognized for their role as regulators of inflammation are small G proteins, specifically those of the Ras family.

What Are Small GTPases?

Small GTPases are molecular switches that are capable of being in an "on" state in which they are bound to **G**uanosine-**T**ri**p**hosphate (GTP), or an "off" state in which they are bound to **G**uanosine-**D**i**p**hosphate (GDP) (Figure 1.1). Their binding to either of these nucleotides causes changes in the conformation of the GTPase, which enables them to interact with and activate/deactivate different effector molecules. Small GTPases have an intrinsic (albeit very weak) ability to hydrolyze GTP to GDP, hence their possession of an internal "timer" which will set off eventually. More typically these switches are activated by **G**uanine-Nucleotide-**E**xchange-**F**actors (GEFs) which "exchange" GTP into a binding pocket previously occupied by GDP, hence turning "on" the GTPase. **G**TPase-**A**ctivating-**P**roteins (GAPs) activate the intrinsic GTPase activity of these proteins (speeding up the timer), and convert bound GTP into GDP and turn "off" the switch. It is the interplay between the GTPases, their GEFs and GAPs that coordinate the functioning and modulation of many diverse biological processes.

The Ras Superfamily

Ras is a GTPase that was initially found to be mutated in a wide variety of cancers. Over time many similar GTPases were discovered to have a related 3D structure to Ras, and the family now stands at over 150 total members. This large superfamily can be further divided into at least six subfamilies; Ras, Rho, Ran, Rab, Rheb, and ARF. While each of these families of small GTPases has been extensively studied in the context of carcinogenesis, we are only beginning to scratch the surface of how these related families of proteins are able to control non-oncogenic processes such as immunity and inflammation. There is tremendous overlap in the functions performed by each of these groups of GTPases, but the primary functions for each subfamily are summarized in Figure 1.2. The Ras subfamily, the first and most diverse of the subfamilies, can play a vital role in the regulation of immunity and inflammation.

Ras

Ras is the founding and prototypical member of the small GTPase superfamily. It regulates multiple cellular processes including cell survival, growth, and differentiation. Upon stimulation by a growth factor or other extracellular stimuli, a receptor tyrosine kinase (RTK) activates a Ras GEF such as Son of Sevenless (SOS), which activates Ras by loading it with GTP allowing it to bind its downstream effectors (Figure 1.3). Following activation, Ras triggers three primary effector arms, the Raf/Mek/Erk pathway, the PI3K pathway, and the

RalGDS signaling pathway. The RalGDS pathway activates Ral A and Ral B, also members of the Ras GTPase family (to be discussed below).

ERK

When bound to Raf, Ras initiates the Raf/Mek/ERK MAPK signaling cascade. In this signaling cascade, each upstream molecule acts as a kinase to phosphorylate and activate a subsequent downstream molecule, with an eventual result being the modulation of transcription via the phosphorylation of a variety of transcription factors. ERK plays a crucial role in cell proliferation and survival. It is activated in lymphocytes downstream of TCR and BCR stimulation (Donahue 2007). ERK activation can inhibit Fas-mediated apoptosis in T cells (Holmstro 2000). The production of the vital cytokine IL-2 by T cells is dependent upon ERK activation (Tsukamoto 1999, Koike 2003), as is the regulation of glucose metabolism following TCR stimulation (Marko 2010). Diacylglycerol, an important second messenger in the activation of Ras and ERK signaling, plays crucial roles in T cell responses and Fcγ receptor-mediated phagocytosis (Riese 2011, Shin 2012, Botelho 2009). Finally, ERK signaling is also important in TLR-mediated chemokine production in dendritic cells (Mitchell 2010).

PI3K

Class I PI3K enzymes are composed of a regulatory (p85) and catalyitc (p110) subunit. The catalytic subunit has a Ras binding domain and can be activated by GTP-bound Ras. Upon activation, PI3K converts PIP2 to PIP3, which can serve as a docking motif for proteins containing PH domains. Within the immune system, PI3K plays crucial roles in the activation and functioning of all immune

cells. PI3K regulates cytokine responsiveness and functions both in effector and regulatory T cells, and mice with PI3K deficient T cells have several immune defects (Sasaki 2000). The downstream PI3K target mTOR determines whether T cells become activated or undergo anergy (Zheng 2007). PI3K is also crucial for proper functioning of B cells (Shin 2012, Heidt 2008), NK cells (Ackerman 2011), dendritic cells, mast cells (Wai 2008), macrophages, and neutrophils (Sasaki 2000).

Rap

The Rap proteins share approximately 50% sequence homology to Ras, and have an identical amino acid sequence in their effector loops. While there are multiple Rap proteins, Rap1a and Rap1b are the best understood. It was initially hypothesized that Rap1 functioned as a direct inhibitor of Ras by competition for substrate binding. This idea has since been revised as it has become clear that Rap1 has its own set of effector proteins and exists in completely different signaling pathways than does Ras. Rap1 has been implicated in the activation of integrin-mediated adhesion (Cirillo 1991, Tsukamoto 2004), establishment of polarity (Shimonaka 2003, Schwamborn 2004), cell proliferation, and the control of cell-cell interactions (Gerard 2007). The innate immune system has a requirement for Rap1 in both macrophages (Katagiri 2000) and dendritic cells (Caron 2000). In T cells, Rap1 is crucial for chemokine-induced polarization (Gerard 2007, Katagiri 2004), and in B cells it regulates cell spreading and adhesion (Mcleod 2004). Rap1 is a potent activator of LFA1 (Katagiri 2000),

and at least 3 different second messengers can activate Rap: i.e., cAMP, calcium, and diacylglycerol.

Rap1a

Due to having 90% sequence homology, Rap1a and Rap1b were originally assumed to serve redundant roles in the body. However experiments with knockout and transgenic mice have increased our understanding of Rap1 function, as well as the specific and separate functions carried out by Rap1a and 1b. As mentioned above Rap1a was initially thought to exist to antagonize Ras signaling. This initial hypothesis was supported by the observation that large quantities of activated Rap1a are found in anergic T cells, and that activation of CD28 by antibody binding inhibits the induction of Rap1a by TCR stimulation. Additionally in Jurkat T cells, active Rap1a expression inhibits activation of ERK and induction of IL-2 gene expression (Boussiotis 1997). The assumption from these results was that suppression of Rap1 expression is required for a maximal T cell response. Nevertheless the theory that Rap1a inhibits lymphocyte activation has been called into question by the generation of transgenic mice expressing constitutively active Rap1a. Rap1a transgenic mice expressing active Rap1a do not have deficiencies with either Ras signaling or T cell activation (Sebzda 2002). Rap1a deficient T Cells were not anergic, but surprisingly had an enhanced TCR response. Additionally, activation of Rap1a caused T cells to bind more strongly to firbronectin, and induced strong activation of β1 and β2 integrins, a process that generally requires antigen receptor binding to induce. In contrast to the transgenic mice, Rap1a null mice had lymphocytes

that were defective in adhesion to fibronectin and ICAM coated plates. T Cells from Rap 1a null mice had impaired polarization following CD3 stimulation, but these mice had otherwise healthy lymphocyte function (Duchniewicz 2006). Macrophages from Rap1a knockouts had increased haptotaxis but reduced chemotaxis, and had an increase in FcR-mediated phagocytosis. Neutrophils from these mice produce reduced amounts of superoxide in response to fMLP stimulation, which is likely due to the fact that Rap1a interacts with the p22 subunit of NADPH oxidase in neutrophils (Li 2007).

Rap1b

Since Rap1b is highly expressed in B cells, Rap1b null mice were created to understand the role of Rap1b in B cells (Chu 2008). Rap1b knockout mice have reduced T-dependent, but normal T-independent humoral responses. B cells from these mice have a reduction in migration in response to chemokines, and have reduced homing to lymph nodes (Chrzanowska-Wodnicka 2008) Lung endothelial cells from these mice show delayed healing in a wound-healing assay (Chrzanowska-Wodnicka 2008). These mice had fewer pre-B cells in the bone marrow, although splenic B cell proliferation was not affected. Additionally, mice have been generated that lack the Rap1 Gap SPA1 and loss of this enzyme (resulting in increased Rap1 activity) in hematopoietic and peripheral T cells resulted in antigen-induced T cell anergy. Rap1b is also the primary isoform expressed in NK cells, and may play a role in cytokine and chemokine production in these cells (Awasthi 2010). Thus, *in vivo,* Rap1 is crucial for proper functioning and development of T and B lymphocytes.

Ral

The Ral (Ras-like) family of small GTPases is composed of two isoforms, Ral A and Ral B, which share 85% protein sequence identity between them and approximately 50% homology with Ras. Ral proteins have six known GEFs, four of which are of the RalGDS family (RalGDS, RGL1, RGL2, RGL3) and are primarily activated by upstream Ras signaling. Ral A and Ral B are highly pleiotropic and impact many diverse signaling pathways within the cell. The Ral proteins have many downstream binding partners that they interact with in order to modulate cellular conditions and respond to extracellular signals. The primary effectors are Ralbp1, the exocyst complex, and phospholipase D. In addition the RalGDS family itself has the capacity to act as a scaffold to activate the Ser/Thr Kinase AKT and promote cell survival and proliferation (Hao 2008).

RalBP1

Also known as RLIP76 and RIP (Ral interacting protein), RalBP1 is a non-ABC multi-functional membrane transport protein that is responsible for the majority of glutathione electrophile conjugate export in mammalian cells. This transporter is responsible for a large amount of chemotherapeutic drug removal from cancerous cells, and provides protection from a variety of forms of oxidative damage or radiation-induced stress. RalBP1 has been linked to migration, endocytosis of multiple receptor ligand pairs including TGFβ, and has GAP activity towards some RHO family GTPases. Inhibition of RalBP1 with an

antibody directed towards an extracellular region of the molecule induced apoptosis in target cells. Also autoantibodies to the C terminal region of RalBP1 are associated with several immune mediated diseases such as Behçet disease, SLE, and carotid atherosclerosis (Margutti 2008). Ralbp1 is crucial for the proper expression of the transcription factor hsf-1, which is necessary for the expression of many heat shock proteins, which are essential for cell survival under many infectious conditions.

Exocyst

The exocyst complex is a tethering complex that exists to tether intracellular vesicles to the plasma membrane prior to vesicular fusion during polarized exocytosis. It is an octameric complex that exists in two parts, a three-member complex, which exists on the vesicle being trafficked, and a five-member complex that exists on the plasma membrane. Ral binds to and induces fusion between members of each of these complexes, Exo84 on the vesicular complex, and sec5 on the membrane complex. The exocyst complex plays a central role in polarized exocytosis. Additionally the exocyst has been shown to play a role in ciliogenesis, cytokinesis, wound healing, and cell migration (Rosse 2006). Moreover one study has shown that the exocyst complex is needed for appropriate NK cell degranulation and proper NK cell cytotoxicity (Sanchez 2011). Recently it has been reported that RalB and Exo84 can assemble to autophagosomes and initiate autophagy (Bodemann 2011). RalB is also capable of inducing an interaction between sec5 and the non-canonical IKK TBK1, resulting in the activation of TBK1 and the downstream viral defense and survival

pathways it controls (Chien 2006). A systems biology approach to assessing pieces of the drosophila phagosome identified components of the exocyst complex (Stuart 2007) as being involved in phagosome function. Being crucial for a variety of key processes involving cell polarization, the exocyst complex and its multiple components will likely be recognized to play an even more vital role within the immune system in the future.

PLD

Phospholipase D (PLD) is an enzyme that hydrolyses phosphatidylcholine into phosphatidic acid. PLD plays an important role in the internalization and recycling of receptors. Both RalA and RalB can interact with PLD, though it is the RalA interaction with phospholipase D positively regulates FcγR mediated phagocytosis (Corrotte 2010). PLD, via phosphatidic acid production, can activate the central kinase mTOR, and this activation can be modulated by RalA (Xu 2011).

TIPE2

A correlation between inflammation and cancer has been known to exist for some time, but the mechanistic basis for this has not been clear. With strong connections to neoplastic disease, as well as the regulation of inflammation and the immune system, it is logical to consider that the Ras family and its effectors and regulators could compose part of the mechanistic connection between these two disease states. Work presented here will demonstrate that TIPE2 provides one such mechanistic connection. TIPE2 is a member of the TNFAIP8 family of proteins.

TNFAIP8 Family

The Tumor Necrosis Factor Alpha Induced Protein Eight (TNFAIP8) family of proteins, is a group of four proteins (TNFAIP8, TIPE1, TIPE2, and TIPE3) that have to date been very thinly studied. TNFAIP8 is a roughly 20KD cytosolic protein that sensitizes cells to glucocorticoid induced cell death (Woodward 2010) but suppresses TNF-α mediated apoptosis (Laliberte 2010). TNFAIP8 has been correlated with cancer progression and poor cancer prognosis in certain types of cancer (Romanuik 2009). Very little is currently known about TIPE1 and TIPE3.

TIPE2

Tumor necrosis factor alpha induced protein 8 like-2 or TNFAIP8L2 or TIPE2 is a recently discovered member of the *TNFAIP8* family of proteins. It was discovered by comparing the gene expression profiles of spinal cord tissue from healthy mice with that of mice with induced experimental autoimmune encephalomyelitis or EAE, a mouse model for multiple sclerosis. The inflamed EAE spinal cord tissue expressed large amounts of TIPE2, piquing interest in the gene and further study which eventually resulted in the creation of a TIPE2 knockout mouse via germ line gene targeting (Sun 2008). The loss of TIPE2 proved to be an extremely debilitating mutation for these mice. TIPE2 deficient mice develop normally but are prone to many inflammatory diseases which are characterized by heightened inflammatory cytokine production, sensitivity to

septic shock, and premature death. These mice had extraordinarily high levels of circulating cytokines, and had a larger number of both lymphocytes and monocytes, resulting in splenomegaly (despite the mice themselves being significantly smaller than their wild type counterparts). TIPE2 is down-regulated in peripheral blood mononuclear cells in patients suffering from lupus (Li, 2009) and hepatitis (Xi, 2011), and is upregulated in kidneys of diabetic rats (Zhang 2010). Additionally a recent paper has indicated that TIPE2 may provide a protective function against ischemia/reperfusion injury following a stroke (Zhang 2012).

TIPE2 is expressed constitutively and at high levels in all immune cells and shows inducible expression in fibroblast cell lines (Sun 2008). The loss of TIPE2 correlates with increased levels of IkB degradation and increased nuclear localization of NF-κB family members. Likewise overexpression of TIPE2 reduces the level of NF-κB activity in response to TNFα treatment**.** Knockdown of TIPE2 resulted in increased phospho- JNK, phospho- p38, and c-Fos signaling. TIPE2 can be endogenously pulled down with caspase 8 in macrophage cell lines. Caspase 8 has been previously shown to activate NF-kB through a BCL10 / MALT1 complex (Su 2005). Knockdown of TIPE2 in EL4 T Cells results in resistance to FasL induced cell death, while TIPE2 knockout T Cells are resistant to activation induced cell death. TIPE2 knockout T cells develop normally but produce more tetramer positive CD8+ T cells in response to LCMV infection and produce more cytokines following CD3/CD28 stimulation. TIPE2 knockout macrophages produce more cytokines when

exposed to TLR ligands. TIPE2 has recently been shown to bind to and inhibit the small GTPase Rac1. By inhibiting Rac1 TIPE2 inhibits phagocytosis in macrophages (Wang 2012). Due to its regulation of such central and important immunological pathways, TIPE2 plays a role in regulating both the innate and the adaptive arms of the immune system

The TIPE2 protein has a very unique conformation, with no significant homology to anything previously crystalized and catalogued. It is composed solely of six alpha helices, which are arranged into a bowl-like shape, with the inside faces of the alpha helices (inside of the bowl) possessing very hydrophobic residues, while the outer faces of the alpha helices (outside of the bowl) possess highly charged residues (Zhang 2009). While TIPE2 was thought to have a DED domain due to interacting with Caspase 8 as well as due to high homology with DED domains, the crystallization of TIPE2 has shown that it does not in fact possess a DED domain, but instead has an odd DED “mirror image” domain. Due to its potent effects on the immune system and inflammatory state, its preferential expression in lymphoid and myeloid cell types, and its unique and never before seen 3D structure, further knowledge of TIPE2 function and regulation could be crucial towards creating future treatments for a wide variety of diseases.

Hypothesis and Specific Aims

Understanding the mechanisms involved in generating an immune response is a crucial component of creating new treatments for nearly every disease known to man, as well as understanding how environmental factors can affect health, and even how to make better and more efficacious vaccines. TIPE2 appears to be a crucial piece of this puzzle, and will be of importance in unraveling the mysteries of the immune system, and how it communicates and orchestrates cellular actions in conjunction with the rest of the body. TIPE2 itself, a modified TIPE2, or an ability to choose when and where TIPE2 is expressed may all become viable treatments for any number of diseases at some point in the future. Thus the specific aims were to understand both the regulation of TIPE2, as well as the molecular mechanism(s) of its action in regulating cellular processes.

The work described in Chapter 2 begins to narrow down the way in which TIPE2 is regulated within the immune system. Both T Cells and macrophages respond to stimulation by heavily reducing TIPE2 message levels. This reduction in TIPE2 expression was both dependent upon NF-KB activity as well as transcription not being blocked, drawing the conclusion that TIPE2 is regulated by an NF-KB induced micro RNA in response to inflammatory signal reception by both T lymphocytes as well as macrophages. This work has been further investigated and preliminary evidence indicates that mir21 is the crucial microRNA regulating TIPE2. In addition we provide evidence that TIPE2 is ubiquitylated and degraded in response to these same TLR signals, and provide

indications that SCF-βTRCP may be the E3 ligase responsible for TIPE2 ubiquitylation and degradation.

In Chapter 3 the mechanistic underpinnings of TIPE2 began to be investigated. TIPE2 interacts with and inhibits the activity of RGL1 and RalGDS, both GEFS for the Ral family of small GTPase. These GEFs are activated by the binding of Ras, which TIPE2 interrupts, and we went on to exhaustively demonstrate that a multitude of downstream Ral effector pathways were impaired by the presence of TIPE2. RGL1 and RalGDS also possess certain non GEF functions, such as providing scaffolding activity for PDK1 and AKT, allowing PDK1 to phosphorylate and activate AKT. This function was also inhibited by TIPE2. We then went on to test the role of TIPE2 in tumor formation, and found that TIPE2 delayed the appearance of tumors, and had to be silenced (via an unknown mechanism) by the tumors in order for them to grow. We also went on to demonstrate that TIPE2 is heavily downregulated in the hepatocytes of human hepatocellular carcinoma patients. This data indicates that TIPE2 may play a role in tumor suppression as well as in control of the immune system, or, possibly play a role in anti-tumor immunity.

The data presented in Chapter 4 continues to investigate the molecular mechanism of TIPE2s multitude of diverse yet potent phenotypes. The data demonstrates that TIPE2 is capable of heavily interfering with, and reducing, signaling through the mTOR pathway. This is likely occurring through a specific and highly acute reduction of Rictor by TIPE2. It is possible that this is occurring through a binding and sequestration of GBL, an interaction which is

demonstrated in this chapter. This data indicates that TIPE2 is affecting the nutrient sensing pathway within the immune system, and is therefore likely affecting a plethora of functions in both monocytes and lymphocytes via metabolic regulation. This data additionally raises the question of whether or not TIPE2 could be one of the key linkages between diabetes/obesity and inflammation, since it provides a direct connection between inflammation and the mTOR nutrient sensing pathway.

In Chapter 5 the effect of TIPE2 on negatively regulating both the RGL1 and mTOR is investigated by looking into the role TIPE2 plays in inducing cell death. Here we see that overexpression of TIPE2 induces cell death, while coexpressing RGL, mTOR, or parts of the mTOR pathway rescue from death. We also discovered that TIPE2 is likely activated by p70S6K, since the presence of S6K increases TIPE2 induced cell death. Finally we see that TIPE2 alongside GBL and RGL creates a maximal amount of cell death, likely hinting at the role of TIPE2 in providing a linkage between these two pathways.

Overall, the findings presented in this doctoral work will help to better understand the functioning of TIPE2, and how it intersects with various signaling pathways within the cell. This knowledge may serve to provide valuable treatments and therapeutics for an extraordinarily wide array of diseases, from chronic inflammatory disease to diabetes to obesity to cancer. Each of these diseases carries the common thread of being associated with a dysregulation of inflammation. Likewise, TIPE2 has now been established to not only have connections to inflammation, but to each of these diseases. With hope this

information will be able to help solve the problems currently posed by each of these diseases… and more.

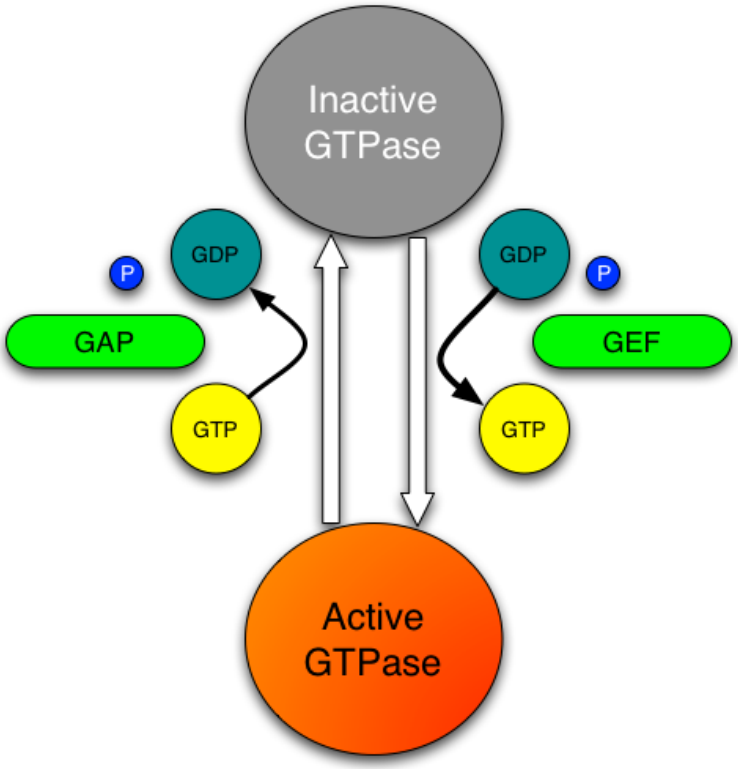


Figure 1.1 Overview of GTPase signaling. Small GTPase is “off” when bound to GDP; a GEF then removes GDP and allows GTP to bind to the GTPase, turning it “on”. All GTPases can eventually hydrolyze GTP to GDP and turn themselves off, though GAPs rapidly accelerate this process.

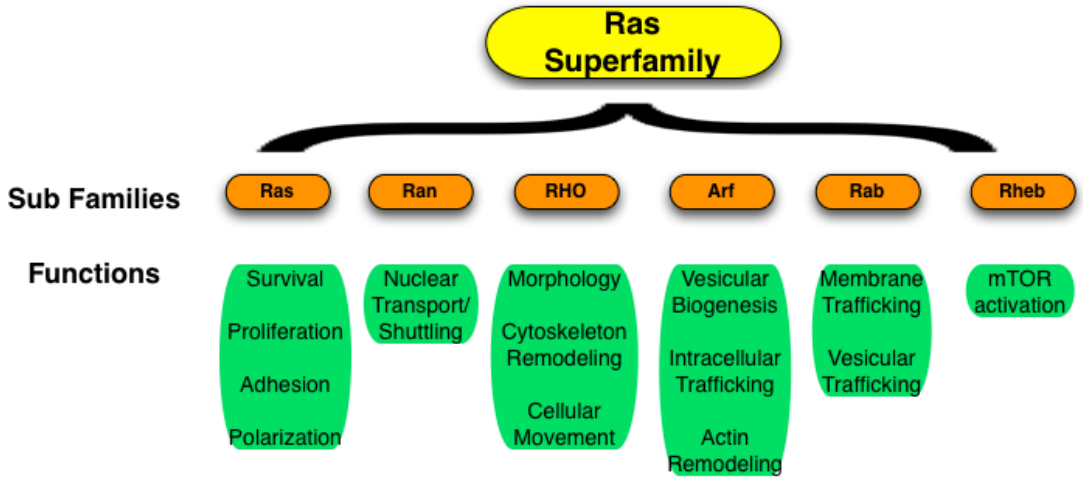


Figure 1.2: The Ras Superfamily. The Ras superfamily is broken down into 6 families, each listed with their primary functions.

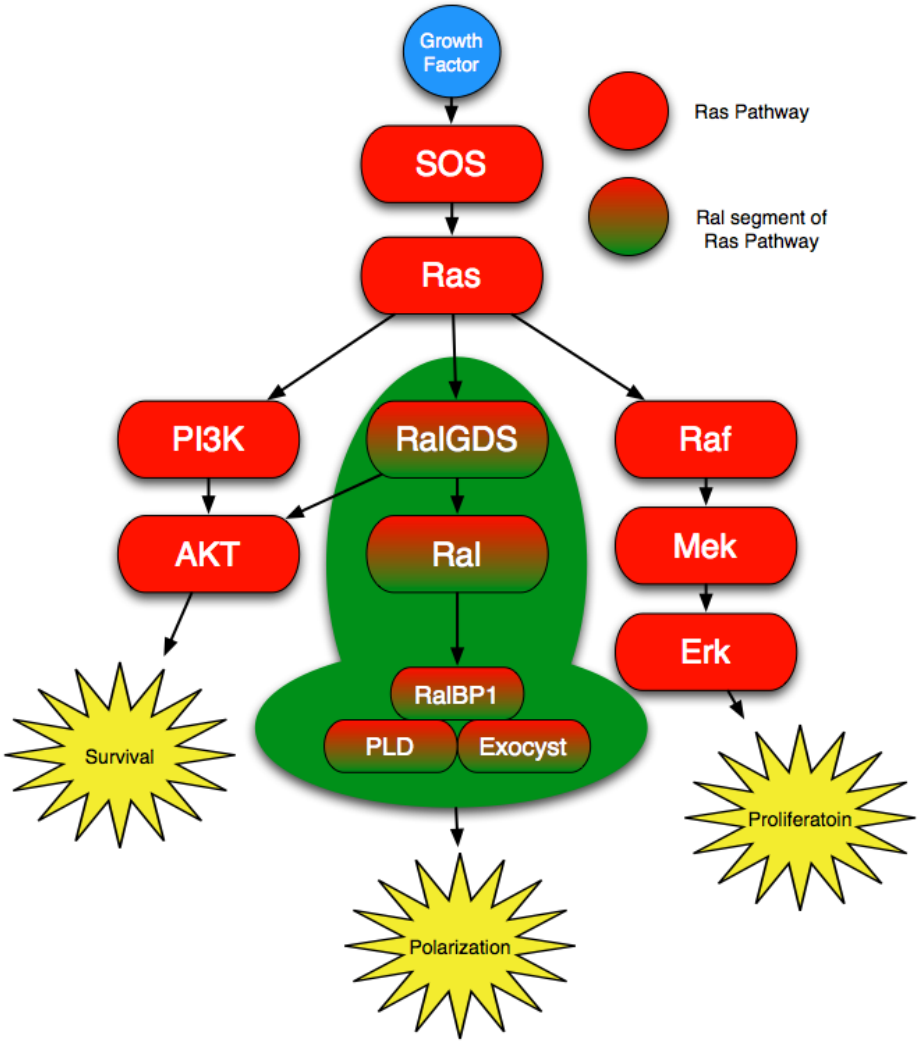


Figure 1.3. Overview of Ras Signaling. Ras signaling pathway is highlighted in Red. The Ral signaling segment of the Ras pathway is additionally marked in Green.

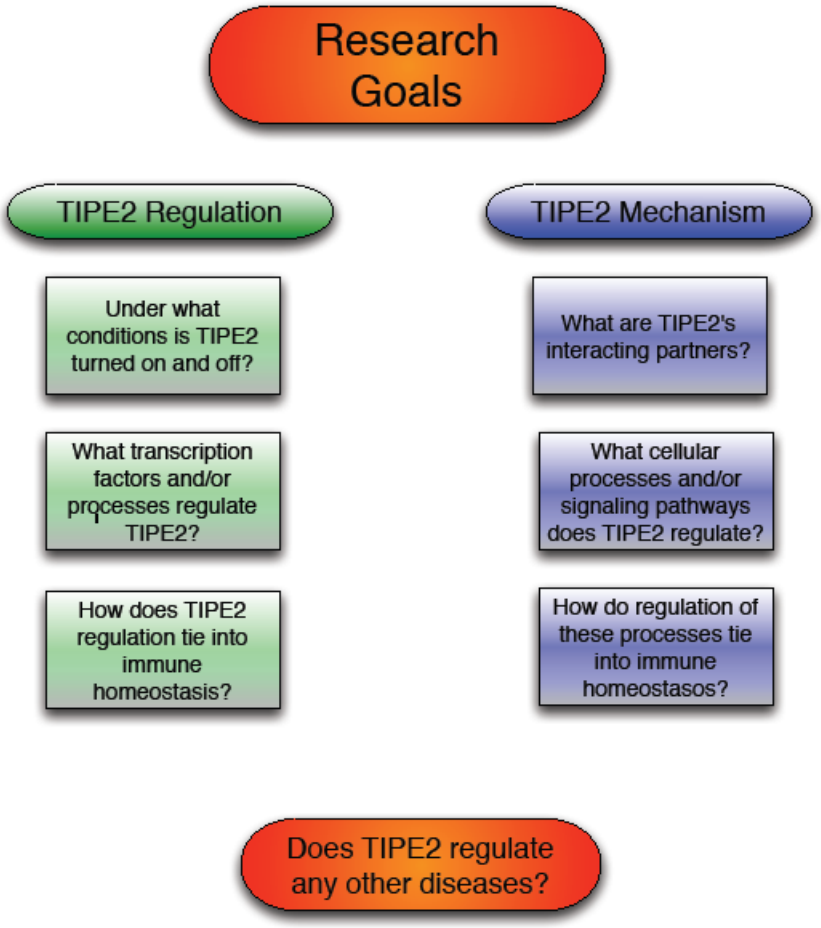


Figure 1.4 Outline of Research Goals

Chapter 2

TIPE2 Regulation

by NF-KB

Work from this chapter is from:

Gus-Brautbar, Y., Johnson, D., Zhang, L., Sun,H., Wang, P., Zhang, S., Zhang, L., and Chen, Y.H. (2012). The anti-inflammatory TIPE2 is an inhibitor of the oncogenic Ras. Molecular Cell *45*, 610–618.

Wang, Z., Fayngerts, S., Wang, P., Sun, H., Johnson, D.S., Ruan, Q., Guo, W., and Chen, Y.H. (2012). TIPE2 protein serves as a negative regulator of phagocytosis and oxidative burst during infection. Proceedings of the National Academy of Sciences of the United States of America *109*, 15413–15418.

Abstract

TIPE2 is a known regulator of inflammation, yet little is known about how TIPE2 responds to inflammatory signals. Here we investigated the effect of stimulating macrophages with various TLR ligands on TIPE2 expression. TIPE2 is rapidly and specifically downregulated by all TLR ligands tested. The same downregulation occurred in T cells stimulated with PMA and Ionomycin. This downregulation was further assessed with an inhibitor analysis and was found to be dependent upon both active transcription, and upon active NF-KB activity. Neither translation nor JNK activity appear to play a role in the downregulation of TIPE2. These data suggest that an NF-KB induced miRNA may be responsible for downregulating TIPE2 upon the presence of inflammatory signals. We additionally show that TIPE2 is ubiquitylated for its degradation, and that TIPE2 interacts with the BTRCP E3 Ligase, which may be the ligase that regulates the TIPE2 protein levels.

Introduction

The first step towards understanding a new molecule is to deduce how it is regulated. By understanding the "how" and "why" of a new molecule's regulation, we can begin to gain insight into both its function, and understand the magnitude of its importance. While it has been established that TIPE2 is a regulator of inflammation via work with TIPE2 -/- knockout mice and tissues as well as *in vitro* work, how TIPE2 was connected to inflammation had remained a mystery.

In the cell there are generally two primary forms of regulation; nucleic acid regulation and protein regulation. Nucleic acid regulation occurs when the transcription of DNA into mRNA, or the mRNA itself is altered (Figure 2.1). This form of regulation typically occurs via one of two mechanisms; direct action of transcription factors on genomic DNA, or action of microRNAs towards target mRNA sequences. Transcription factors are proteins which enter into the nucleus of a cell and bind to a specific DNA sequence. The transcription factor then either recruits cellular machinery to begin transcription of the target gene into mRNA, or it recruits cellular machinery to shut down transcription of the target gene into mRNA. Which effect occurs depends upon the particular transcription factor(s), DNA sequences, and cellular conditions involved. Micro RNAs (abbreviated miRNA) are small regulatory RNAs that are encoded in the genomes of eukaryotic cells. miRNAs will bind to one or more specific target mRNAs, typically on the 3‘ UTR, and are able to affect mRNA function via marking them for destruction or in negatively regulating the translation of the target mRNA.

Protein regulation occurs when cellular conditions or signaling events dictate a change be made to an already existing protein (Figure 2.2). Certain changes, such as lipid modifications like palmitoylation, farnesylation, or geranylgeranylation usually assist a protein in anchoring to the plasma membrane or another vesicular structure; this anchoring both alters localization of the modified protein and drastically extends its half-life. Other modifications such as poly ubiquitylation on certain lysine residues, marks the protein for destruction by the proteasome – the protein garbage disposal unit of the cell. While ubiquitylation occurs at a steady state rate to destroy old proteins within the cell, anything that increases the rate of ubiquitylation will decrease the half-life of the target substantially. Phosphorylation is another common method of regulating proteins. Phosphorylation typically activates whichever protein is phosphorylated by altering its conformation, or by altering which other proteins it interacts with. However, depending upon the cellular conditions, and the specific phosphorylation target, phosphorylation can have many other diverse effects.

Nuclear Factor Kappa B (NF-κB) is a term for a well-known family of transcription factors (for review see Pasparakis 2009 and Perkins 2012). These transcription factors exist as dimers of five possible subunits; p65, cREL, p50, p52, RelB. NF-KB has been heavily studied in the field of immunology, and most inflammatory signals activate NF-KB activity. Thus, NF-KB provides one possible method by which inflammatory signals are capable of signaling to the cell that inflammatory regulators, such as TIPE2, need to be turned on or turned off.

Materials & Methods

Cell Lines and Animals

Raw 264.7 and EL4 cells were purchased from ATCC and grown in DMEM supplemented with 10% FBS, penicillin and streptomycin. C57BL/6J (B6) mice that carry a *Tipe2* gene null mutation were generated by backcrossing *Tipe2*$^{-/-}$ 129 mice (Sun et al., 2008) to B6 mice for 12 generations.

Bone marrow deried macrophages were isolated from mouse femurs and were cultured for seven days in DMEM supplemented with 10% FCS, 1% Penicillin/streptomycin, 1% glutamine, and 30% L-929 cell culture supernatant. Following culturing cells were washed with cold PBS and rested overnight in complete DMEM before being lysed for testing. Bone marrow derived macrophages were >95% cd11b+ and F4/80+ as determined by flow cytometry.

Inhibitors:

Bay-11-7082 in solution was purchased from emdmillipore (part# 196870-10MG). and was used at a concentration of 100 uM.

Actinomycin D was purchased from Sigma (part# A1410) and was used at a concentration of 1 ug/mL.

Cycloheximide was purchased from Sigma (part# 01810-5g) and was used at a concentration of 5 ug/ml.

Treatment:

TLR agonists were purchased from invivogen part# tlrl-kit1mw and were used per manufacturer's instructions for maximum stimulation.

Quantitative RT-PCR

Total RNA was extracted with RNEasy (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription was performed with oligo dT primers. Real-time PCR was carried out in an Applied Biosystems 7500 system with Power SYBR Green PCR Master Mix (Applied Biosystems) and with specific quantitect primers (Qiagen, Valencia CA) for mouse GAPDH and TIPE2. Relative levels of gene expression were determined by normalizing TIPE2 levels to the endogenous control gene GAPDH. Relative TIPE2 levels were set to a value of 1 at time point zero, subsequent time points compare relative TIPE2 levels to this initial level.

Results

TLR Treatment

In order to assess how TIPE2 was affected by inflammatory signals, RAW 264.7 macrophages were treated with LPS over an eight hour time course (Figure 2.4A), and the level of TIPE2 mRNA was measured at various time points throughout the time course via real time PCR. TIPE2 expression was reduced to approximately 35% of initial levels by 2.5 – 3 hours. Signal levels returned to normal levels within 8 hours. To test whether other TLRs were involved in this signaling process TLR3, TLR7, and TLR9 were tested by stimulating cells with polyI:C, flagellin, and cpg respectively. Each of these TLR ligands showed a similar effect to that of LPS. To ensure that this phenomenon was not limited only to RAW cells, bone marrow derived macrophages were subjected to similar treatments, and EL4 T Cells were subjected to treatment with PMA and Ionomycin (Figure 2.4B). PMA/Ionomycin of EL4 T cells showed a similar pattern of TIPE2 reduction by 2.5-3 hours, and a slow return to normal levels by 8 hours (Figure 2.4B). Bone marrow derived macrophages showed a similar pattern of reduced expression, but expression stayed reduced out to at least 24 hours. Protein levels of bone marrow derived macrophages were also checked, to ensure that changes in message levels correlated with changes in protein levels (Figure 2.5).

Inhibitor Analysis

To test whether TLR signaling was inducing production of a protein that was destroying TIPE2 mRNA or preventing its transcription, cells were pretreated with cycloheximide to inhibit translation before TLR agonist treatment (Figure 2.6). Pre-treatment with cylclohexamide had no effect on the TLR mediated reduction of TIPE2 message levels. Since preventing protein production did not stop the reduction in TIPE2 message levels, we next pre-treated cells with actinomycin D in order to prevent transcription (Figure 2.6). This treatment prevented the TLR mediated reduction in TIPE2 message levels, implying that fresh transcription was required for the reduction in TIPE2 expression. Finally in order to identify the transcription factors responsible for inducing the transcriptional event that was reducing TIPE2 expression, both Sp100125 and Bay 11-7082 were used to pretreat cells (Figure 2.7). These compounds inhibit JNK and NF-KB respectively. While JNK inhibition had no effect on TIPE2 expression, inhibition of NF-KB by Bay 11-7082 prevented the TLR driven reduction in TIPE2 expression.

Protein Regulation

In order to assess the effect of regulation on the TIPE2 protein, we performed a pulse chase assay in which Raw 264.7 cells were treated with cycloheximide in order to stop fresh translation, followed by either LPS or vehicle administration. LPS treated Raw cells showed a significantly reduced TIPE2 protein half-life compared to vehicle treated controls (Figure 2.8B). In order to identify the likely method of regulation a ubiquitin ligase assay was performed (Figure 2.8A), which shows that TIPE2 is able to be ubiquitylated and thus may

be marked for degradation by the proteasome. Data discussed in Chapter 3 (figure 3.11c) indicates that inhibition of the proteasome can restore repressed TIPE2 protein expression, further lending support to the idea that TIPE2 is able to be regulated by the proteasome.

Discussion

Understanding the cellular events that regulate TIPE2 expression is important to gain insight into its function as a modulator of inflammation. Expression of TIPE2 is heavily downregulated by treatment with poly I:C, LPS, Flagellin, and imidazoquinoline; agonists for tlrs 3,4,5, and 7 respectively. This group of TLR ligands signal via both MyD88 and TRAM/TRIF signaling, indicating that that multiple intracellular signaling complexes have been activated, leading to downstream activation of the NF-KB, AP-1, and IRF transcription factor complexes (for review see O'Neill 2007). This effect is phenocopied by treatment of T Cells with PMA/Ionomcyin, which activates many of the same downstream transcription factors, therefore activation of one or more of these pathways is responsible for the loss of TIPE2 message and therefore the loss of TIPE2 protein.

In order to narrow down the molecule(s) responsible for this regulation, RAW 264.7 macrophages and EL4 T Cells were pre-treated with either SP600125, an inhibitor of JNKs 1,2, and 3, or Bay 11-7082, an inhibitor of IKBa, which effectively inhibits NF-KB activation. Following pre-treatment with inhibitor cells were either activated with TLR ligands (RAW 264.7 cells) or PMA/Ionomycin (EL4 T Cells) in order to assess the importance of the targeted pathways on TIPE2 message destruction. While SP600125 pre-treatment had no effect on TIPE2 message levels, pre-treatment with Bay 11-7082 completely prevented the TLR and PMA/Ionomycin mediated reduction in TIPE2 levels, indicating that NF-KB activity was required for the cellular activation ligands to transmit the signal that negatively regulate TIPE2 mRNA levels.

Having identified NF-KB as the relevant transcription factor involved, it was now necessary to identify how NF-KB was regulating TIPE2 message levels. In order to identify the method of TIPE2 down-regulation, both RAW 264.7 macrophages and EL4 T Cells were pre-treated with inhibitors of translation (cycloheximide), or transcription (actinomycin D) before stimulating them with either TLR ligands or PMA and Ionomycin. Cycloheximide had no effect on the TLR or PMA/Ionomycin mediated reduction in TIPE2 mRNA levels, indicating that fresh translation of some RNA editing enzyme or transcriptional repressor was not responsible for the loss of TIPE2 message. Actinomycin D however completely ablated the activation induced reduction in TIPE2 message levels, oddly indicating that fresh transcription was required to reduce TIPE2 mRNA levels.

Since the down-regulation of TIPE2 has a dependence upon fresh transcription, but not fresh translation, and a dependence upon NF-KB activity, we conclude that during periods of acute inflammation TIPE2 is down-regulated by an NF-KB targeted miRNA. Preliminary evidence (Chen Lab unpublished data) indicates that the miRNA of interest is mir21.

Interestingly when primary cells were used instead of cell lines, the loss of TIPE2 was not reversed even after 48 hours (Chen Lab unpublished data). This implies that *in vivo* TIPE2 expression likely remains reduced for a fairly significant amount of time following an infectious challenge, in order to allow the immune system to have time to mount a full and complete defense. We have additionally identified that TIPE2 is degraded at the protein level in response to LPS stimulation, and that this degradation is likely due to ubiquitylation and subsequent degradation by the proteasome. Additional work will be required in order to deduce which E3 ligase is involved in this process.

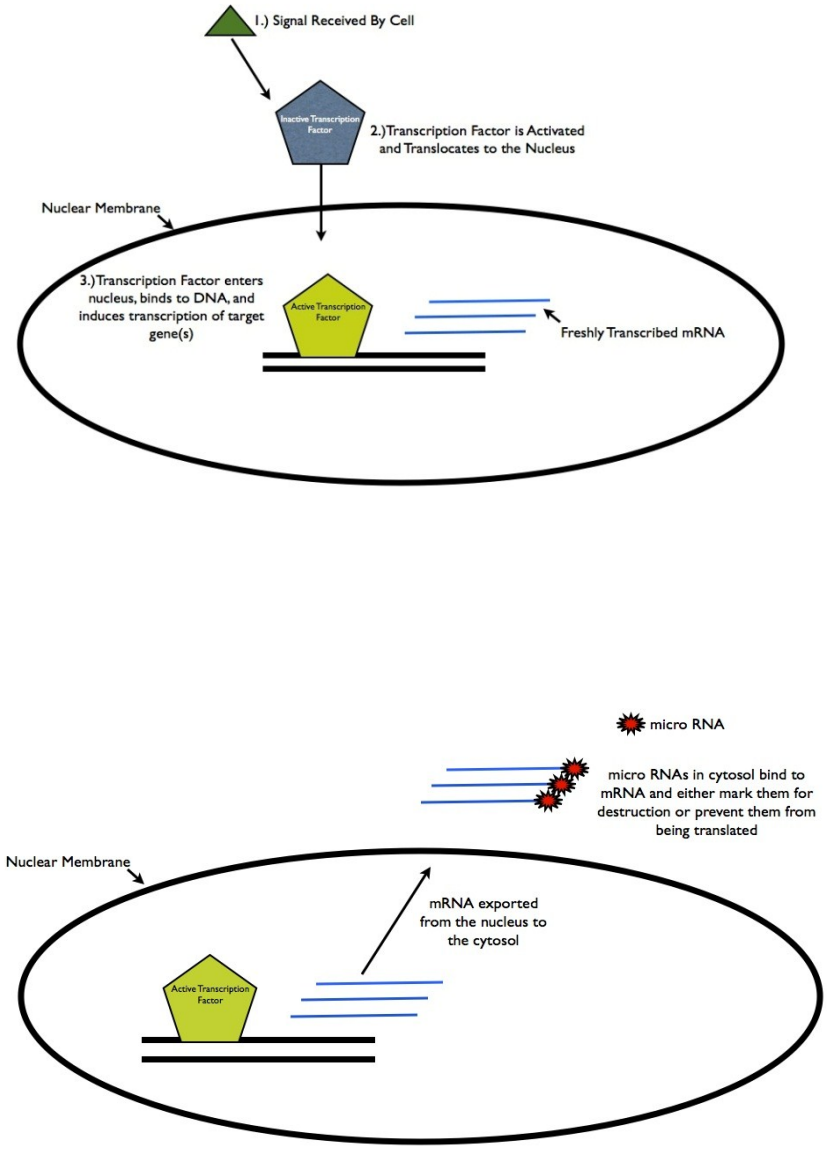


Figure 2.1 Mechanism of Nucleic Acid Regulation. Nucleic acids are regulated via two key mechanisms. The first involves a transcription factor entering the nucleus and binding directly to genomic DNA. The transcription factor then acts as a platform which

either recruits other molecules which either activate or inhibit transcription. The second mode of regulation is via microRNAs (miRNAs). miRNAs are small strands of RNA, averaging about 22 nucleotides in length. They bind to complementary messenger RNAs (mRNA) and typically either cause the mRNA to be destroyed, or interfere with its ability to be translated.

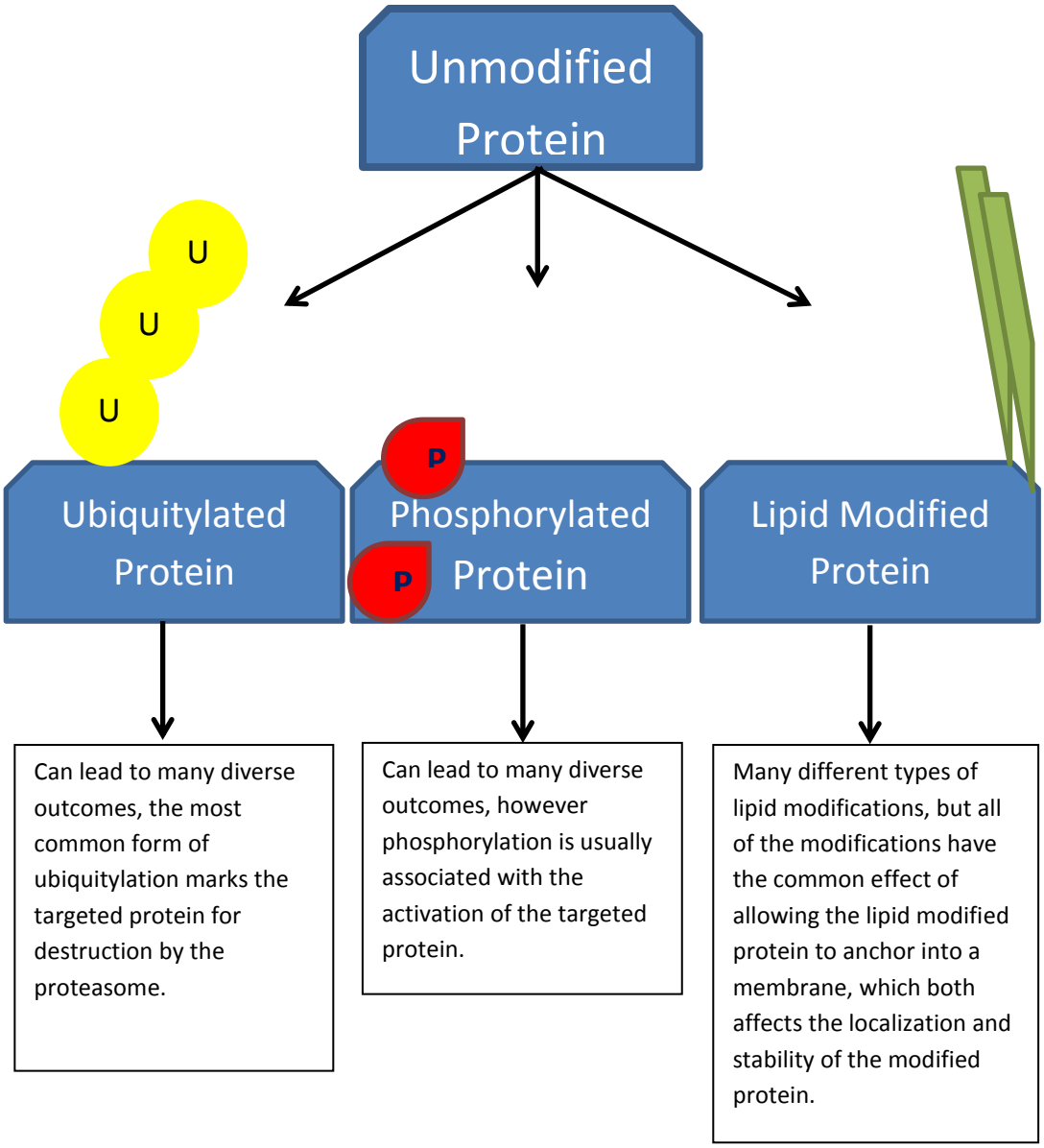


Figure 2.2 Mechanism of Protein Regulation. Some of the common modifications which regulate protein function and stability: Ubiquitylation typically marks proteins for destruction by the proteasome. Phosphorylation typically switches proteins into an active conformation. Lipid modifications typically contribute to protein stability by allowing them to associate with membranes or vesicular structures.

Figure 2.2

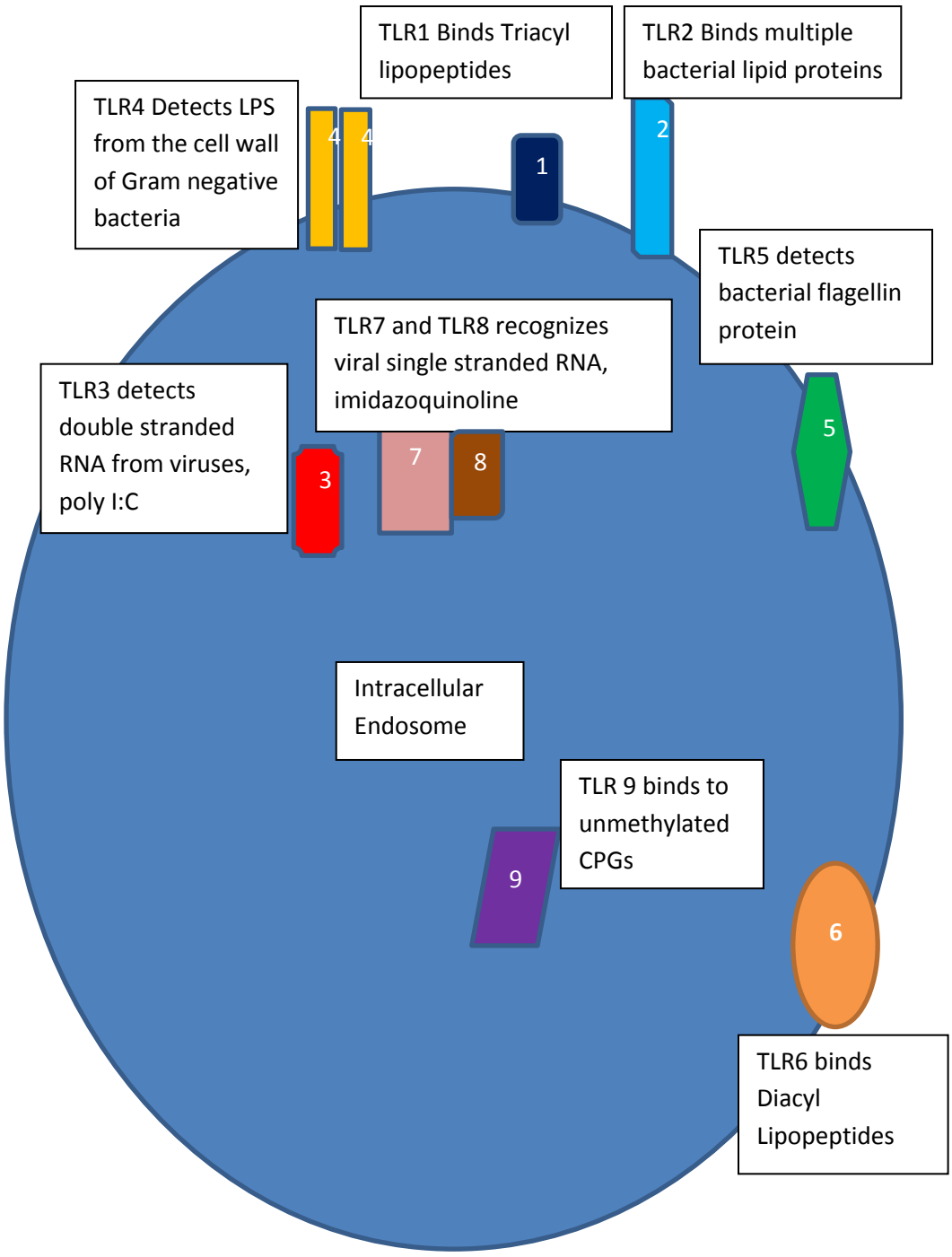


Figure 2.3 Overview of TLRs on Macrophages. TLRs (toll like receptors) are pattern recognition receptors which bind to various microbial products and alert the cell to the presence of a microbe in or around the cell.

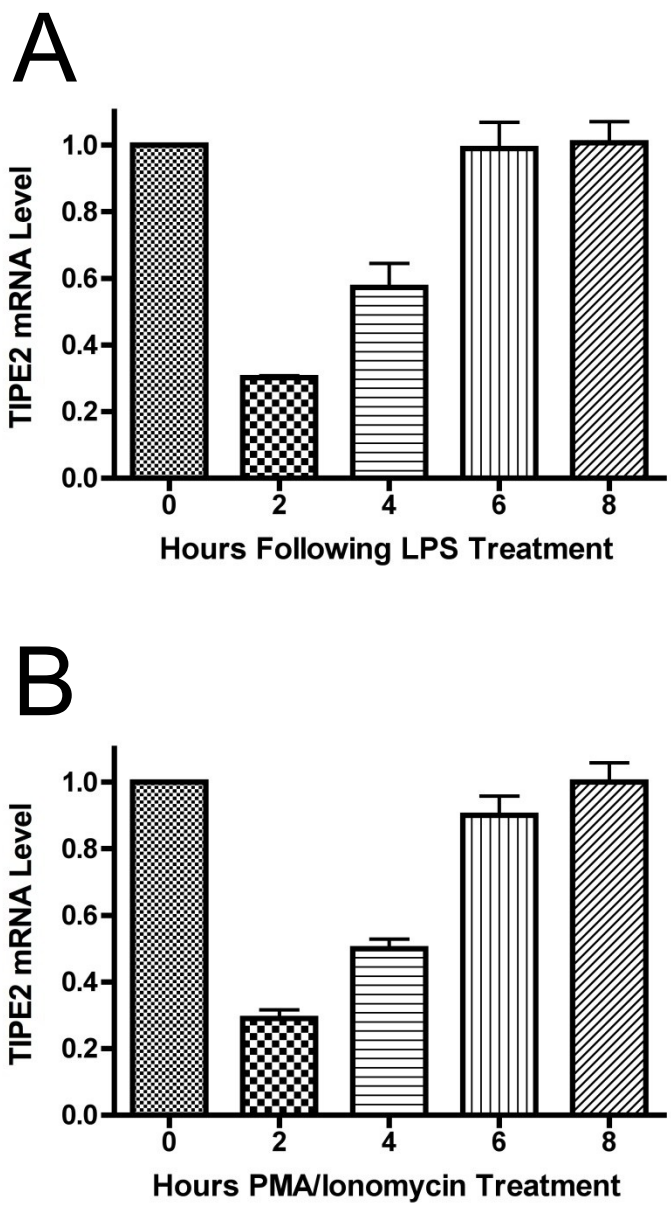


Figure 2.4 Reduction in TIPE2 expression Following Activation with LPS or PMA/Ionomycin. A.) Raw 264.7 macrophages were treated with 100 ng/ml LPS for 8 hours and Cells were lysed and RNA extracted at various timepoints along the time course, and levels of TIPE2 mRNA were measured via qPCR. B.) EL4 T Cells were treated with PMA/Ionomycin for 8 hours and cells were lysed and RNA extracted at various timepoints along the time course, and levels of TIPE2 mRNA were measured via qPCR.

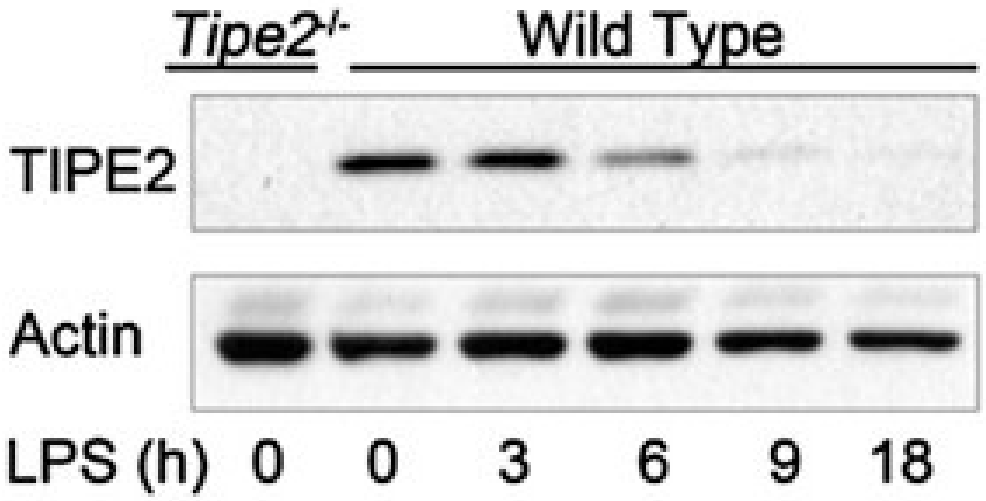


Figure 2.5 TIPE2 Protein level in primary cells following LPS Stimulation. Bone marrow derived macrophages were treated with 100 ng/ml of LPS and were analyzed by Western Blot.

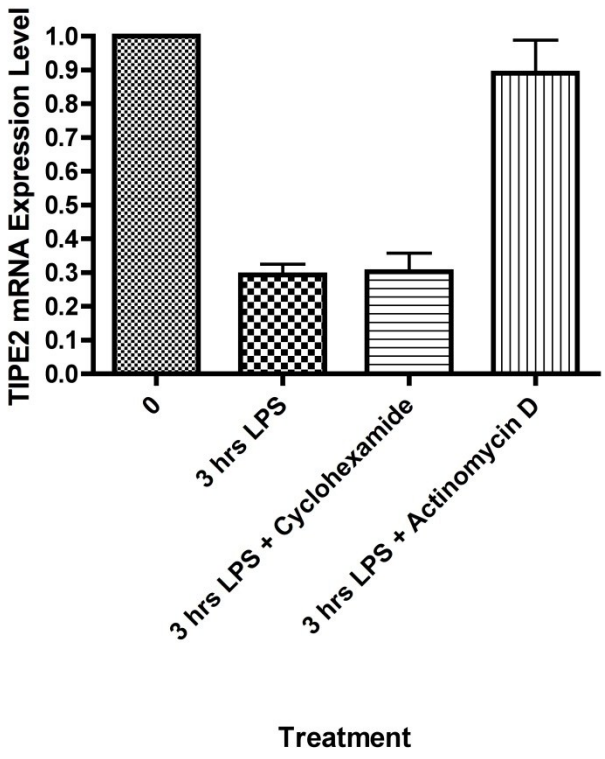


Figure 2.6 Cell Process Inhibitor Treatments of Stimulated Cells. Raw 264.7 macrophages were treated with 100 ng/ml LPS for 3 hours, and were treated with cycloheximide to inhibit fresh protein translation, or were treated with Actinomycin D in order to inhibit fresh transcription.

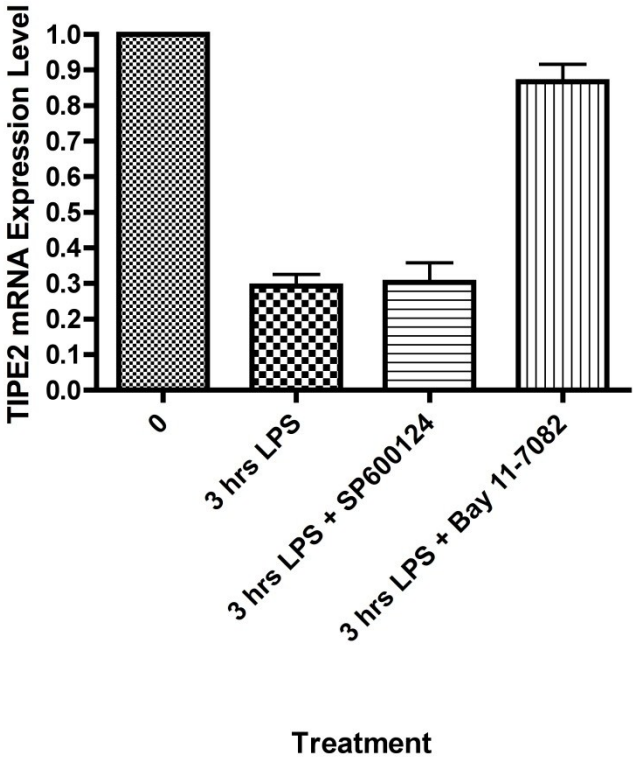


Figure 2.7 Cell pathway inhibitor treatments of stimulated cells. Raw 264.7 macrophages were treated with sp600125 in order to inhibit JNK Signaling, or were treated with Bay 11-7082 to inhibit NF-KB signaling.

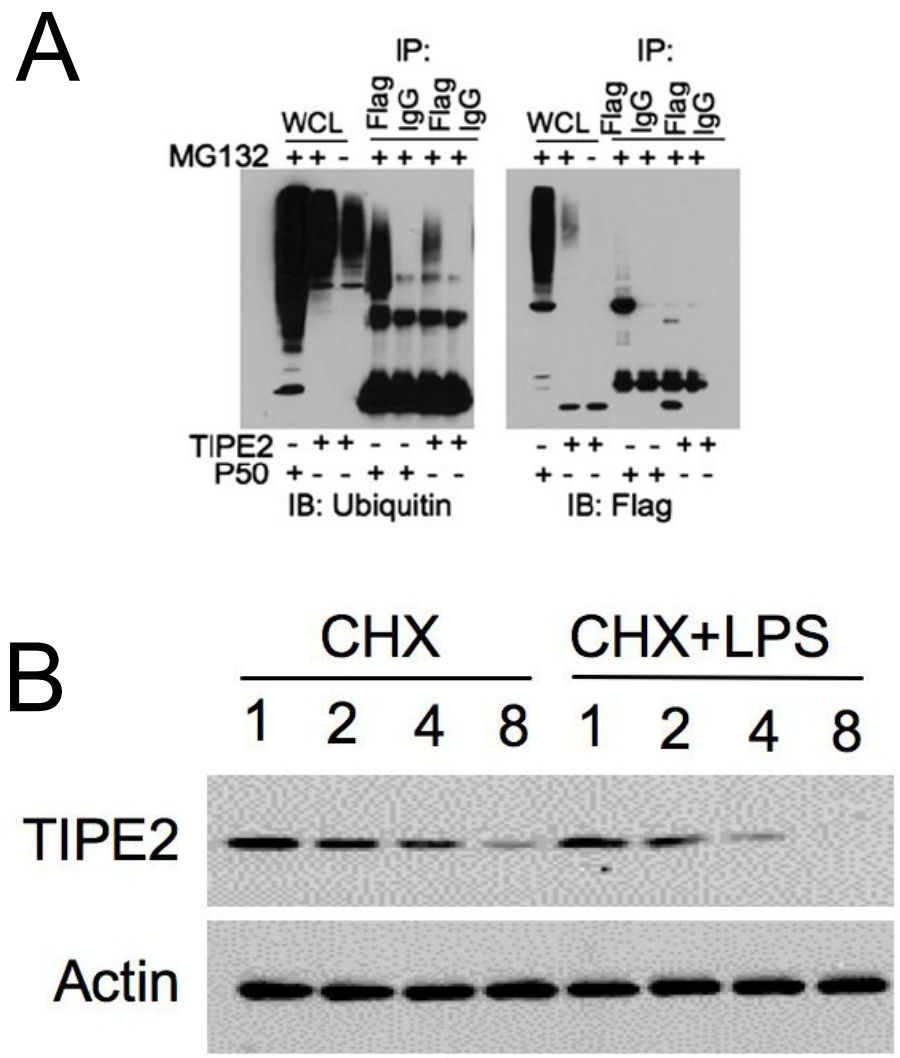


Figure 2.8 TIPE2 is Ubiquitylated and Regulated at the Protein Level. A.) Ubiquitination of TIPE2. 293T cells were transfected with either TIPE2-Flag or p50-Flag as a positive control. These cells were treated with MG132 (10 um), the lysates were immunoprecipitated with an anti flag antibody, and ubiquitination status was examined as described in Carmody et al., 2007). B.) TIPE2 protein half-life is reduced by LPS treatment. Raw 264.7 cells were treated with LPS (100 ng/ml) and cycloheximide (100 um) for the indicated times. Cells were lysed and endogenous TIPE2 protein levels were examined using anti-TIPE2 antibody.

Chapter 3

TIPE2 Binds to and Inhibits RGL1

Work from this chapter is from: *Gus-Brautbar, Y., *Johnson, D., Zhang, L., Sun, H., Wang, P., Zhang, S., Zhang, L., and Chen, Y.H. (2012). The anti-inflammatory TIPE2 is an inhibitor of the oncogenic Ras. Molecular Cell *45*, 610–618.

***These Authors Contributed Equally To This Work**

Abstract

TIPE2 has been established as a regulator of immunity and inflammation, yet the mechanism(s) by which this occurs have remained elusive. Here we report that TIPE2 interacts with the RalGDS family of GEFs, and by doing so inhibits their activity. This inhibition affects both their scaffolding activity for AKT and PDK1, as well as their GEF activity towards the Ral small GTPases. Inhibition of the Ral proteins leads to a plethora of cellular effects, including inhibition of the exocyst complex, inhibition of NF-KB, as well as impacts cell survival, wound healing, migration, and anchorage independent growth. Expression of TIPE2 in tumor cells delays tumor onset, and tumor onset can only proceed following the silencing of the TIPE2 protein expressed in these cells. Finally TIPE2 is highly downregulated in human hepatocellular carcinoma, indicating that TIPE2 may be a future therapeutic target or provide a diagnostic indicator for human cancer.

Introduction

Ras is a major regulator of cell survival, proliferation, migration, and transformation. Alongside phosphatidylinositol (PI) 3 kinase and Raf1, Ral guanine nucleotide dissociation stimulator (RalGDS) family makes up the third arm of Ras effectors. The RalGDS family members are Guanine nucleotide Exchange Factors (GEFs) for the small GTPases RalA and RalB, switching GDP-bound inactive to GTP-bound active form of Ral (Ferro and Trabalzini, 2010; Spaargaren and Bischoff, 1994). A subset of the RalGEFs, including RalGDS, RGL and RGL2/Rlf, are direct effectors for activated Ras, which binds their C-termini and enhances their GEF activity towards Ral (White et al., 1996; Wolthuis et al., 1996). The RalGEF pathway plays a prominent role in mediating Ras-induced oncogenic transformation in humans. RalGDS deficiency suppresses Ras-mediated tumor formation (Gonzalez-Garcia et al., 2005). In rodent fibroblasts, the RalGEF effector pathway cooperates with the MAPK pathway to promote transformation and metastasis (Ward et al., 2001; White et al., 1996). In humans, the activation of this pathway is essential for transformation in a variety of cell types (Hamad et al., 2002; Rangarajan et al., 2004).

The RalGDS transforming ability is mediated by the active RalA and RalB. Active Rals regulate various biological processes including cell proliferation, motility, endo- and exocytosis, and cellular architecture (Feig, 2003). RalA and RalB have distinct and sometimes conflicting roles, during oncogenic transformation despite their high sequence identity (over 80%). RalA, but not

RalB, is critical for RalGDS-mediated transformation, and RalA is more potent than RalB in promoting anchorage-independent growth and targeted delivery of proteins to basolateral membrane in epithelial cells. On the other hand, RalB is more effective than RalA in promoting cell migration and activation of TBK1, and in suppressing apoptosis and promoting metastasis (Chien et al., 2006; Chien and White, 2003; Lim et al., 2005; Lim et al., 2006). Constitutively active forms of RalA and RalB can transform human cell lines (Lim et al., 2005). Both RalA and RalB are activated in human malignancies such as bladder, pancreas and colon cancers, and they collaborate to promote and maintain oncogenic transformation (Lim et al., 2006; Martin et al., 2011; Smith et al., 2007).

Recent studies suggest that the Ral effects on motility, secretion and cell proliferation are largely mediated through the regulation of the exocyst complex. This octameric complex regulates targeting and tethering of secretory vesicles to specific plasma membrane domains, such as the leading edge of migrating cells (Rosse et al., 2006; Spiczka and Yeaman, 2008). Two exocyst subunits, Sec5 and Exo84, are bona fide Ral effectors (Moskalenko et al., 2002), each of which belongs to a different sub-complex. One sub-complex contains Exo84 and Sec10, and is localized on the plasma membrane, whereas the other contains Sec 5, 6, 8 subunits, and is located on secretory vesicles (He and Guo, 2009). Active Ral promotes assembly of these two sub-complexes through dual subunit interaction, leading to vesicle tethering to the plasma membrane (Jin et al., 2005; Moskalenko et al., 2003). As the exocyst complex is being better understood, its involvement in carcinogenesis has been brought to the forefront. Exocyst subunit

interaction with active Ral is required for tumorigenesis of colorectal carcinoma, progression of skin cancer, motility, anchorage-dependence, and survival of transformed cells (Chien et al., 2006; Martin et al., 2011; Sowalsky et al., 2011).

An important function of the RalGDS family is to promote cell survival. This may be mediated through both Ral GTPases (Chien and White, 2003) and non-canonical activation of AKT (Hao et al., 2008). Canonical AKT activation requires the generation of PIP3 by PI3K at the membrane. The PH domain-containing proteins AKT and PDK1 bind these phoshoinositides, allowing AKT to be phosphorylated by PDK1 (T308) and mTOR (S473). By contrast, in the non-canonical AKT activation pathway, RalGDS acts as a scaffold for PDK1 and enhances its kinase activity, resulting in increased phosphorylation of AKT. Active AKT phosphorylates a large number of substrates thereby protecting cells from death (Sale and Sale, 2008). RalGDS-mediated AKT activation is responsible for the proliferative effect of RalGDS in NIH3T3 cells (Hao et al., 2008). In vivo, RalGDS regulates tumor growth by providing survival signals to tumor cells, and consequently, in RalGDS-/- mice, apoptosis of carcinogen-induced papillomas is enhanced (Gonzalez-Garcia et al., 2005). Thus, the regulation of the RalGEF effector pathway is key to Ras-mediated transformation. Here, we describe an unexpected and previously unknown connection between TIPE2 and the RalGDS family, and demonstrate its relevance to cell survival, motility, and Ras-induced oncogenesis.

Materials and Methods

Animals and human subjects

C57BL/6J (B6) mice that carry a *Tipe2* gene null mutation were generated by backcrossing *Tipe2*$^{-/-}$ 129 mice (Sun et al., 2008) to B6 mice for 12 generations. Male nude mice (nu/nu) were purchased from Jackson Laboratories. Mice were housed in the University of Pennsylvania Animal Care Facilities under pathogen-free conditions. All animal procedures used were pre-approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

A total of 116 hepatocellular carcinoma specimens and 111 normal adjacent hepatic tissue specimens were obtained from 116 patients aged between 30 and 82 years who underwent operations at the Qilu Hospital of Shandong University from January 2005 to October 2006. The pathological diagnosis was made according to the current World Health Organization (WHO) criteria for hepatocellular carcinoma. None of the patients studied had received radiotherapy, chemotherapy, or adjuvant immunotherapy prior to surgery in order to eliminate their effects on gene expression. All human procedures used were pre-approved by the Institutional Review Board of the Shandong University.

Immunohistochemistry

Paraffin sections (4μm) were stained with rabbit anti-TIPE2 antibody (IgG) overnight at 4°C. Secondary staining was performed with HRP-conjugated anti-rabbit IgG using a MaxVision^{TM} Kit and a DAB Peroxidase Substrate kit (Maixin

Co., Fuzhou, China). The sections were counterstained with hematoxylin. Unrelated rabbit IgG was used as a control for the primary antibody. All slides were independently analyzed by two pathologists in a blinded manner, and scored based on both staining intensity and the percentage of positive cells as follows. Staining intensity: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. The percentage of positive cells: 0, <1%; 1, 1-33%; 2, 34-66%; 3, 67-100%. The two scores for each slide were then combined to produce a final grade of TIPE2 expression: 0, total score = 0; 1+, total score = 1 to 2; 2+, total score = 3 to 4; 3+, total score = 5 to 6. When there were discrepancies between the two pathologists, the average score was used.

Cell lines and plasmids

The 293T, Raw 264.7, NIH 3T3, and Ras V12 NIH 3T3 cells were grown in DMEM supplemented with 10% FBS, penicillin and streptomycin. To generate stable cell lines, NIH 3T3 or Ras NIH3T3 cell lines (gift from Dr. Rotem Karni, Hebrew University of Jerusalem) were infected with pBABE-puro retroviral vector expressing TIPE2-Flag. Culture medium was replaced 24 hrs. after infection, and after an additional 24 hrs., infected cells were selected with puromycin (1-1.5 μg/ml) for 3 days. Expression of TIPE2-Flag was verified by Western blotting. pRK5 and TIPE2-Flag-pRK5 were described previously (Sun et al., 2008). TIPE2 Δ105-132 was generated from TIPE2 cDNA by PCR and cloned in-frame with a C-terminal Flag tag into vector pRK5. pcDNA3-HA-AKT AAA was a gift from Dr. Morris Birnbaum (University of Pennsylvania). Active AKT (pCDNA3-AKT T308D, S473D), myr-PDK1 (pWZL Myr Flag PDK1), Active RalA (pBABE-RalAV23),

Active RalB (pBABE-RalBQ72L) were purchased from Addgene. GFP-WT-RalA and GFP-WT-RalB plasmids were a gift from Dr. Wei Guo (University of Pennsylvania). Murine RGL cDNA (cDNA clone MGC:18430, IMAGE:4241244, RGL-1 complete CDS) was obtained from ATCC. Full length RGL (amino acids 1-768) was generated from the cDNA clone by PCR, and cloned in-frame with a C-terminal myc tag into vector pRK5 using BamHI-XhoI sites. ΔN RGL (amino acids 300-768), Δ RID RGL (amino acids 86-496), and RID RGL (amino acids 599-768) were generated from the cDNA clone by PCR, and cloned in-frame with a C-terminal myc tag into vector pRK5 using BamHI-XhoI sites. TIPE2-Flag-pBABE was generated by cloning PCR-amplified TIPE2-Flag fragment into vector pBABE using BamHI/EcoRI sites.

Ral activity assay

The 293T cells, 2 x10^6 per 10-cm plate, were cultured for 24 hrs., and then transfected with 10 μg/plate pRK5 or TIPE2-Flag-pRK5 plasmid. Cells were lysed at different time points after transfection with RAB buffer (Millipore) supplemented with protease inhibitor cocktail tablet (Complete, Roche) and 1mM PMSF. Protein concentration was determined by Bradford assay. 0.5 or 1 mg of lysate was mixed with GST-RalBP1 agarose beads (Millipore) for 30 minutes at 4 ^{0}C. After washing, protein on beads and in total cell lysates was subjected to Western blot to determine the level of active RalA. The levels of active RalA in *Tipe2*$^{-/-}$ or wild type macrophages were determined in the same manner.

Yeast-two-hybrid screen

Yeast-two-hybrid screen for TIPE2-interacting proteins was carried out by ProteinLinks Inc. (Pacadena, California, USA). A mouse splenic cDNA library ($1.7x10^7$ cDNA clones) was used. 38 positive clones were initially found, and 24 of them were verified in a second screen. Of these, two clones encoded a murine RGL (ref NM_016846.3, Mus Musculus Ral guanine nucleotide dissociation stimulator-like 1) fragment, corresponding to amino acids 484-768.

Mass spectrometry

The 293 cells were transiently transfected with TIPE2-Flag or pRK5, and in the mean time, Raw264.7 macrophages were plated in 15-cm plates. 24 hrs. later, cells were lysed in CellLyticM buffer (Sigma) supplemented with protease inhibitor cocktail. Lysates were cleared by centrifugation and protein concentration was determined by Bradford assay. TIPE2-Flag or control lyastes were immunoprecipitated using the anti-Flag-M2 affinity gel (Sigma), overnight at 4 ^{0}C. After three washes with CellLyticM buffer, pre-cleared Raw264.7 lysate was added and incubated overnight, at 4 ^{0}C. Both TIPE2-Flag and pRK5 control were then eluted using 3X Flag peptide (Sigma) and the eluates were separated on 10% SDS-PAGE gel. The gel was either stained with coomassie or silver, and bands specific to the TIPE2-Flag lane were excised and sequenced.

F-actin determination

The total F-actin content in Raw 264.7 cells transiently transfected with TIPE2 or pRK5, or in *Tipe2*$^{-/-}$ and wild type splenocytes, was measured as follows. After cells were treated with LPS (200 ng/ml, Sigma), the reaction was

stopped by addition of formaldehyde (to a final concentration of 3.7%) for 15 min at room temperature. The fixed cells were permeabilized with ice-cold solubilizing buffer (10 mM imidazole (pH 7.2), 40 mM KCl, 10 mM EGTA, 1% Triton X-100, 1 mM PMSF, 1 mM $MgCl_2$, and protease inhibitor mixture tablet). F-actin was then stained with NBD-phallacidin (Molecular Probes) for 2 hrs. at room temperature. After two washes with PBS, F-actin-bound NBD-phallacidin was extracted with methanol. Extracts were centrifuged and relative fluorescence was measured using a fluorescent plate reader (Synergy 2, Biotek, USA) with excitation and emission wavelengths set at 465 nm and 535 nm, respectively.

Wound healing assay

Tipe2$^{-/-}$ and wild type macrophages were grown to confluence in 10-cm plates. Monolayers were wounded using a micropipette tip, and visualized using a phase-contrast microscope. Images were acquired at various time points, and the number of cells in the wounded area was counted using the ImageJ software.

Immunoprecipitation

Cells were lysed with CellLyticM buffer (Sigma) supplemented with protease inhibitor (complete, Roche) and phosphatase inhibitor (PhosStop, Roche) cocktail tablets. The lysates were cleared by centrifugation for 15 min, and protein concentration was determined by Bradford assay. 40 μl of 50% protein G-sepharose beads was incubated for 1 hour at 4 ^{0}C, with one of the following antibodies: myc (1:1000, cell signaling), Flag (2 μg, Sigma), TIPE2 (1:500, Novus biological), and Sec5 (2 μg, ProteinTech) antibodies, or IgG

isotype controls (BD biosciences). The beads were incubated with 1 mg of total protein from each lysate overnight at 4 ^{0}C, washed four times with CellLyticM buffer, and boiled for 5 minutes in 40 μl 2X SDS sample buffer. After SDS-PAGE and transfer, the membranes were probed with antibodies against Flag (Flag-M2-HRP, 1:1000, Sigma), Myc-HRP (1:1000, Cell signaling), Exo84 (Lifespan Biosciences), Sec6 (1:1000 Assay Designs), TBK1 (1:1000, Cell Signaling), PDK1 (1:1000, Cell Signaling), and RGL (1:500, abnova).

Immunoblotting

Cells were lysed in SDS and total protein concentration determined. 30 μg protein was loaded to each lane, and separated by SDS-PAGE. After transferring to a nitrocellulose membrane, it was blocked with 5% milk in TBST and probed with the following primary antibodies, overnight at 4 ^{0}C: Phospho–AKT (Serine 473, 1:1000, Cell signaling), total AKT (1:1000, Cell Signaling), Actin (1:1000, Sigma), RalA (1:5000, BD Transduction Laboratories), Ras (1:500, abcam), Phospho-IRF3 (1:1000, Cell Signaling), total IRF3 (1:1000, Cell Signaling) antibodies. Detection was done using enhanced chemiluminescence of HRP-conjugated secondary antibodies (anti-mouse or anti-rabbit IgG, 1:1000, GE healthcare).

Quantitative RT-PCR

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription was performed with oligo dT primers. Real-time PCR was carried out in an Applied Biosystems 7500

system with Power SYBR Green PCR Master Mix (Applied Biosystems). Relative levels of gene expression were determined with *GAPDH* as the control.

Anchorage-independent growth

Cells were cultured in duplicates in soft agar plates at 37 ^{0}C and 5% CO_2. After 10–14 days, colonies from ten different fields in each plate were counted, and the average number of colonies per field was calculated. The colonies were photographed using a GelDock camera.

Statistical analyses

Student's *t*-test was used to evaluate the statistical significance of differences in cell death, migration, and F-actin content. Mann-Whitney *U* test was used to evaluate tumor onset, and Wilcoxon signed-rank test was used to evaluate TIPE2 protein expression in human hepatic tissues.

Results

TIPE2 prevents Ras from binding the Ras-interacting domain of RGL

The mechanisms of TIPE2 function are not clear. To address this issue, we searched for binding partners of TIPE2 using a two-pronged approach. Firstly, we conducted a yeast two-hybrid screen of a mouse splenic cDNA library using TIPE2 as the bait, and secondly, we performed a large-scale coimmunoprecipitation of TIPE2-binding proteins followed by mass spectrometry. Among several clones isolated in the yeast-two hybrid screen, two were found to encode the C-terminus region of the RGL. Consistent with this finding, mass spectrometry results showed that TIPE2 pulled down with proteins of the RGL-Ral pathway. Together, these results suggested a role for TIPE2 in Ras-mediated signaling. While other clones from the yeast-two hybrid screen encoded proteins for Cytip, ORC1, and BRD2, these interactions were not supported by co IP / tandem mass spectrometry data, and also were not able to be endogenously immunocprecipated with TIPE2.

To establish whether endogenous TIPE2 interacts with RGL in mammalian cells, we immunoprecipitated TIPE2 from the murine macrophage cell line, Raw 264.7. We found that endogenous RGL co-precipitated with endogenous TIPE2 (Figure 3.1), as did two other RalGEF family members, RalGDS and RGL2. To map the region within RGL that is responsible for TIPE2 interaction, we cloned the murine full-length RGL (amino acids 1-768) or truncated RGL (Figure 3.2B), in frame with a myc tag and co-transfected the full-length or truncated RGL constructs into cells together with the TIPE2-Flag

plasmid. TIPE2 pulled down with full-length RGL and the C-terminus of RGL. However, TIPE2 did not pull down with a truncated RGL that lacked the C-terminal region (Figure 3.2A). These data indicate that TIPE2 binds to the C-terminus of RGL, which contains the Ras Interacting Domain (RID) (Murai et al., 1997).

Active Ras binds the RID of RalGEFs and activates their GEF activity (Murai et al., 1997; Urano et al., 1996). Our finding that TIPE2 binds the RID of RGL (Figure 3.2A) suggests that in the presence of TIPE2, Ras would be unable to bind RGL. We examined the presence of Ras in complex with RGL in 293T cells transiently expressing full-length RGL and increasing amounts of TIPE2. TIPE2 inhibited endogenous Ras from forming a complex with RGL, in a dose-dependent manner (Figure 3.3). This indicates that TIPE2 can exclude active Ras from binding to RGL.

TIPE2 inhibits RGL-induced activation of Ral

We then asked whether the outcome of TIPE2 binding to RGL could be inhibition of RGL GEF activity towards its substrate Ral. In 293T cells transiently overexpressing TIPE2, we detected more than 60 percent decrease in Ral GTP level compared to the control (Figure 3.4A). Similar results were obtained in Raw 264.7 macrophages (data not shown). Active Ras levels were not affected by overexpression of TIPE2. TIPE2 protein and mRNA levels were downregulated in Raw 264.7 cells treated with LPS (Figure 2.4), and Ral activity was elevated as a result of the treatment (Figure 3.4C) Moreover, TIPE2-deficient bone marrow-derived macrophages showed a three-fold increase in active Ral level

over wild type control cells (Figure 3.4B). These results indicate that TIPE2 serves as an inhibitor of RGL activity by blocking active Ras binding to RGL.

The RGL-PDK1 complex is induced by growth stimuli, and activated Ras plays an important role in its formation. PDK1 relieves the intra-molecular inhibition of the catalytic domain of RalGEFs by binding to its N-terminus, and RalGEFs enhance PDK1 kinase activity preferentially towards AKT (Hao et al., 2008)(Tian et al., 2002). Ras binding to RGL seems to be a necessary but insufficient step in promoting RGL-PDK1 interaction, since a Ras mutant that preferentially binds RalGEFs could not activate AKT under serum starvation conditions (data not shown)(Tian et al., 2002). Expression of TIPE2 significantly reduced PDK1 binding to RGL (Figure 3.5), implying that the disruption of Ras binding to RGL may prevent RGL-PDK1 complex formation.

TIPE2 inhibits cell motility and exocyst complex assembly.

A hallmark of Ral function is its regulation of cell motility. Ral activation promotes cellular protrusions and is essential for directional movement of cells (Rosse et al., 2006; Sugihara et al., 2002). Active Ral mediates chemotaxis in lymphocytes, plays a critical role in tumor metastasis, and contributes to cytokinesis progression (Oxford et al., 2005). Ral mediates these effects by regulating both actin dynamics and exocyst complex assembly. Actin polymerization is essential for maintaining cell shape, internalization processes (endocytosis and phagocytosis), and cell motility. We therefore examined the effects of TIPE2 expression on actin polymerization. Polymerization of F-actin can be induced by LPS stimulation in monocytes (Kong and Ge, 2008).

Expression of TIPE2 in Raw 264.7 cells resulted in a significant decrease in the total level of F-actin (Figure 3.6A). The F-actin polymerization rate in TIPE2-transfected cells was also reduced. Conversely, in *Tipe2*$^{-/-}$ splenocytes, the rate of F-actin polymerization was significantly enhanced compared to wild type cells (Figure 3.6B). These results suggest that TIPE2 can impact both the rate of actin polymerization and the total levels of F-actin in immune cells.

We next examined the exocyst subunit levels in *Tipe2*$^{-/-}$ splenocytes and bone marrow-derived macrophages, and detected a significant increase in exocyst subunits Sec 5, 6, 8 and 84 (Figure 3.7A). These results are consistent with previous reports showing decreased absolute amounts of Sec5 and Sec6 in RalA- or RalB-depleted rat kidney cells (Rosse et al., 2006). Next, we examined whether TIPE2 impacts the formation of the exocyst complex, by measuring its assembly from its two sub-complexes in wild type (WT) and *Tipe2*$^{-/-}$ cells. While there was no difference in Sec5/Sec6 sub-complex assembly between *Tipe2*$^{-/-}$ and WT cells, the association between Sec5 and Exo84 increased by about 3-fold in *Tipe2*$^{-/-}$ cells (Figure 3.7A). Therefore, the Ral-regulated step of exocyst assembly is defective in *Tipe2*$^{-/-}$ cells. Consistent with this observation, TIPE2 overexpression resulted in destabilization of the exocyst complex. In 293T cells expressing TIPE2, the assembly of the sub-complex Sec5/Sec6 was unchanged, while the assembly of Exo84 and Sec5 was markedly decreased (Figure 3.7B).

Ral depletion blocks exocyst complex formation at the leading edge of migrating cells, and inhibits cell migration (Rosse et al., 2006; Spiczka and Yeaman, 2008). We tested directional cell migration of *Tipe2*$^{-/-}$ macrophages in a

wound-healing assay (Figure 3.8A). The “wound” was created in confluent *Tipe2*$^{-/-}$ and wild type cultures (time zero), and migration of cells into the gap was monitored after 3 and 6 hrs. Wild type macrophages started moving into the wound after 3 hrs., and by 6 hrs. the wound was still visible. However, *Tipe2*$^{-/-}$ macrophages moved into the wound faster, and completely closed the gap by 6 hrs. Quantification of the number of cells that moved into the gap showed that the rate of *Tipe2*$^{-/-}$ cell migration was 3-fold higher than that of the wild type (Figure 3.8B). Moreover, *Tipe2*$^{-/-}$ macrophages that had moved into the wound were elongated, and had increased number of cellular extensions, generally assuming a “migratory” form. In contrast, wild type cells looked round, with smaller number of extensions. Consistent with this finding, in vivo migration of TIPE2 knockout leukocytes into skin air-pouches injected with the chemokine KC (keratinocyte chemoattractant) was significantly enhanced as compared to wild type controls (Sun 2012). The enhanced migratory phenotype of *Tipe2*$^{-/-}$ cells could be mediated by irregularities of both actin dynamics and exocyst complex assembly. These abnormalities may partly explain the increased inflammation in *Tipe2*$^{-/-}$ mice.

Recently, it has been established that active RalB induces Sec5 dimerization and subsequent activation of TBK1 (Chien et al., 2006). This pathway results in AKT activation, protects cancer cells from apoptosis, and is required for mounting host defense responses. However, as described above, the inhibition of Sec5-TBK1 interaction by TIPE2 does not appear to be a main contributor to TIPE2-induced cell death. RalB and Sec5 are required for TLR3-

induced IRF3-dependent interferon-b production. We observed reduced interaction between Sec5 and TBK1 in TIPE2-overexpressing cells (Figure 3.7C), and a reduction in phosphorylated IRF3. These results suggest that the Ral/Sec5/TBK1 pathway is inhibited by TIPE2. It was shown previously that TIPE2-deficient cells exhibit increased NF-kB activity. Therefore, TIPE2 may regulate the NF-kB pathway through the Ral/Sec5/TBK1 axis.

TIPE2 inhibits tumorigenesis *in vivo*.

Activating mutations of Ras are found in ~30% of all malignant tumors. The best-characterized Ras effector pathways are the Raf-MAPK and PI3K pathways, and their importance in Ras-mediated oncogenesis has been extensively studied. However, a growing body of evidence supports an important role for the RalGDS family in Ras-induced growth and transformation of human cells. To determine the potential roles of TIPE2 in tumorigenesis, the Ras-transformed NIH 3T3 fibroblasts (Ras G12V) were used to stably express Flag-tagged TIPE2. Expression of TIPE2 significantly reduced the growth of Ras 3T3 cells (Figure 3.9A). The effect was the most dramatic under low serum conditions, where cells were more dependent on Ras for survival. Consistent with these results, overexpression of TIPE2 in Ras 3T3 cells reduced colony formation in soft agar (Figure 3.9B). Expression of TIPE2 in NIH 3T3 alone did not result in colony formation. To test the effect of TIPE2 on tumor formation in vivo, Ras 3T3 cell line stably expressing TIPE2 was injected into nude mice. TIPE2 significantly delayed tumor onset in two independent experiments, in comparison to control injections (Figure 3.10). NIH 3T3 or NIH 3T3 stably

expressing TIPE2 did not give rise to tumors. TIPE2 tumors, once formed, could grow to the same weight as control, suggesting that TIPE2 tumors did not have a growth disadvantage as compared to Ras 3T3 tumors. In addition, while Ral activity was inhibited in the pre-injected cell lines, it was restored in isolated tumor cells. This paradox could be explained if somehow the tumors in mice had lost expression of TIPE2. Indeed, upon examining the tumors 14 and 22 days after tumor cell inoculation, we could not detect any TIPE2 protein by immunoblotting (Figure 3.11A). However, by quantitative RT-PCR, we could clearly show that TIPE2 tumors expressed similar amounts of TIPE2 transcript compared to TIPE2-expressing Ras 3T3 cells before injection (Figure 3.11B). Therefore, it appears that TIPE2 downregulation in the tumor occurred at the protein level. The half-life of TIPE2 protein is rather short, around 4 hrs. (Figure 2.8B), and the TIPE2 protein is heavily ubiquitylated in cells (Figure 2.8A). These findings indicate that TIPE2 protein is likely regulated by ubiquitylation and proteasomal degradation. Indeed, the reduced TIPE2 level in tumor cells could be restored to that of pre-injected cells after treatment with the proteasome inhibitor MG132 (Figure 3.11C). This indicates that TIPE2 degradation is enhanced in the tumor cells. Therefore, cells that formed tumors were those that had TIPE2 protein actively suppressed. These cells were likely present in the pre-injected pool but were outnumbered by those that did express TIPE2. However, once injected into the animal, cells that suppressed TIPE2 protein had a significant survival advantage and were therefore positively selected. Although TIPE2 tumors might have originated from fewer cells, as the delay in tumor onset

suggests, they eventually reached the same size as the control tumors. Since TIPE2 -/- mice do not develop spontaneous tumors, it is likely that loss of TIPE2 is a necessary, though not sufficient mutation for cancer progression. Treatment of TIPE2 -/- mice with various carcinogens will help to better understand the relationship between TIPE2 and cancer in the future. This unexpected result suggests that mechanisms responsible for TIPE2 elimination may also result in acquisition of a growth advantage over Ras3T3 control cells. These results point to a role for TIPE2 as a novel tumor suppressor involved in carcinogenesis.

TIPE2 is markedly down-regulated in human hepatocellular carcinoma.

It was recently published that TIPE2 plays an important role in HBV-induced hepatitis (Xi et al., 2011). Chronic HBV infection is a major cause of HCC and is prevalent among a large world population. Interestingly, RalGEF plays a more prominent role in transforming human cells than murine cells (Hamad et al., 2002). To test the possibility that TIPE2 regulates carcinogenesis in humans, we examined the level of TIPE2 expression in the livers of 116 patients suffering from hepatocellular carcinoma. We found that TIPE2 was expressed in normal hepatocytes adjacent to carcinoma cells. Remarkably, ~20% of carcinoma expressed little or no TIPE2 and the rest expressed significantly lower levels as compared to adjacent hepatocytes (Figure 3.12 A-B). TIPE2 re-expression in three cultured human HCC cell lines (HepG2, BEL7402, and SMMC-7721) significantly reduced their growth and viability as measured by flow cytometry and MTT [(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (unpublished data). Consistent with the murine tumor data,

down-regulation of TIPE2 occurred at the protein but not at mRNA level, because RT-PCR revealed no significant difference in TIPE2 mRNA between hepatocellular carcinoma and its adjacent tissues (unpublished data). Thus, development of human hepatocellular carcinoma is associated with the down-regulation of TIPE2 protein.

In summary, we have discovered a novel mode of Ras regulation that is carried out by TIPE2, a recently described anti-inflammatory protein containing a novel fold. This mode of regulation is essential for maintaining an organism's immune homeostasis, because its defect leads to severe inflammation and cancer progression. This finding provides a novel molecular bridge between inflammation and cancer, a connection widely recognized, but poorly understood (Karin and Greten, 2005). Thus, inflammation may cause cancer by inhibiting the expression of the tumor suppressor TIPE2 (Figure 3.13), in addition to activating the oncogenic NF-kB (Karin and Greten, 2005). Due to its diverse effects on cell survival and motility, the Ras inhibitor TIPE2 represents an attractive new drug target for neoplastic and inflammatory diseases.

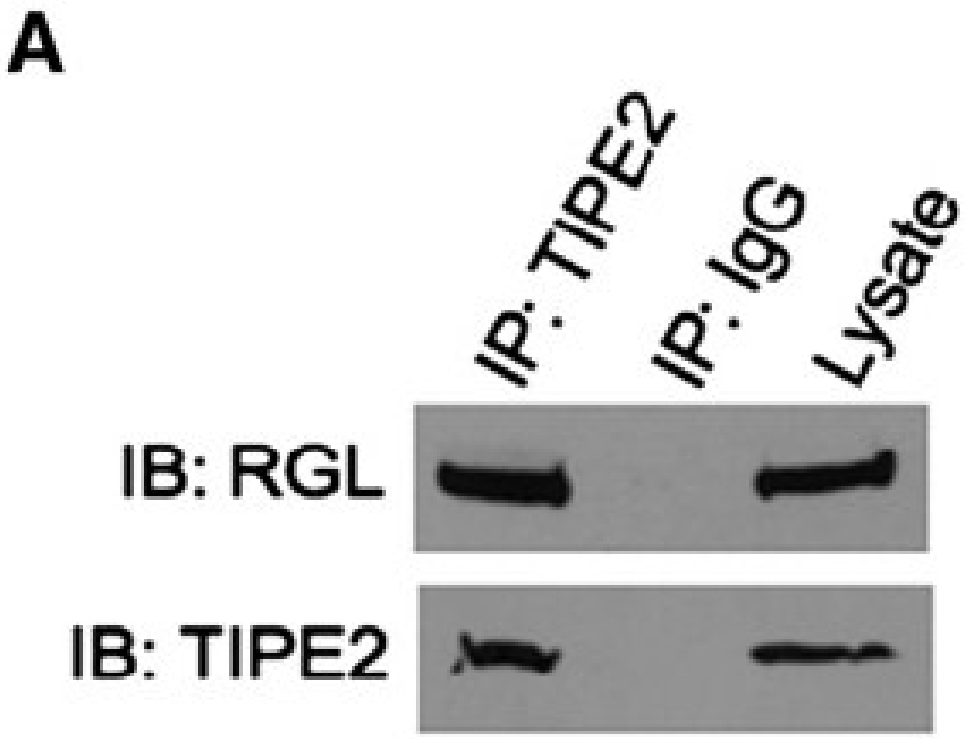


Figure 3.1 TIPE2 and RGL1 Co Immunoprecipitation. Raw 264.7 cells were lysed and immunoprecipitated with anti TIPE2 antibody (Novus Bio). IPs were then analyzed by SDS-PAGE and western blot.

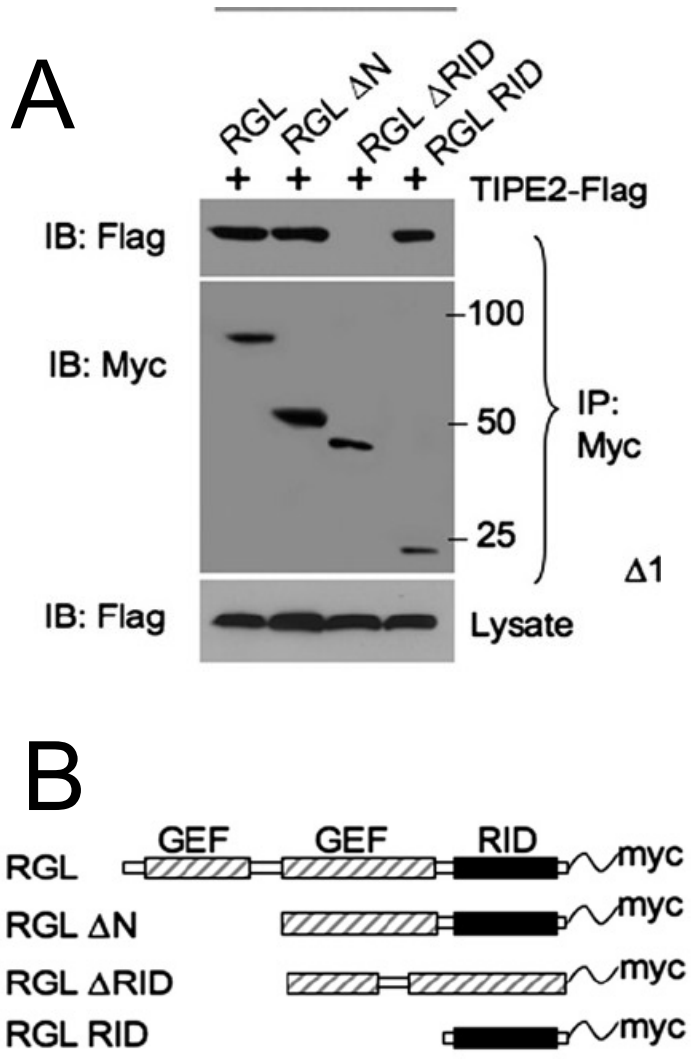


Figure 3.2 TIPE2 interacts with the "Ras interaction domain" of RGL. A series of RGL truncations were created deleting various important domains of the molecule. These mutants were then coexpressed alongside flag tagged TIPE2 and co-immunoprecipitations were performed. The only mutant that TIPE2 could not coIP with was the mutant lacking the RID of RGL.

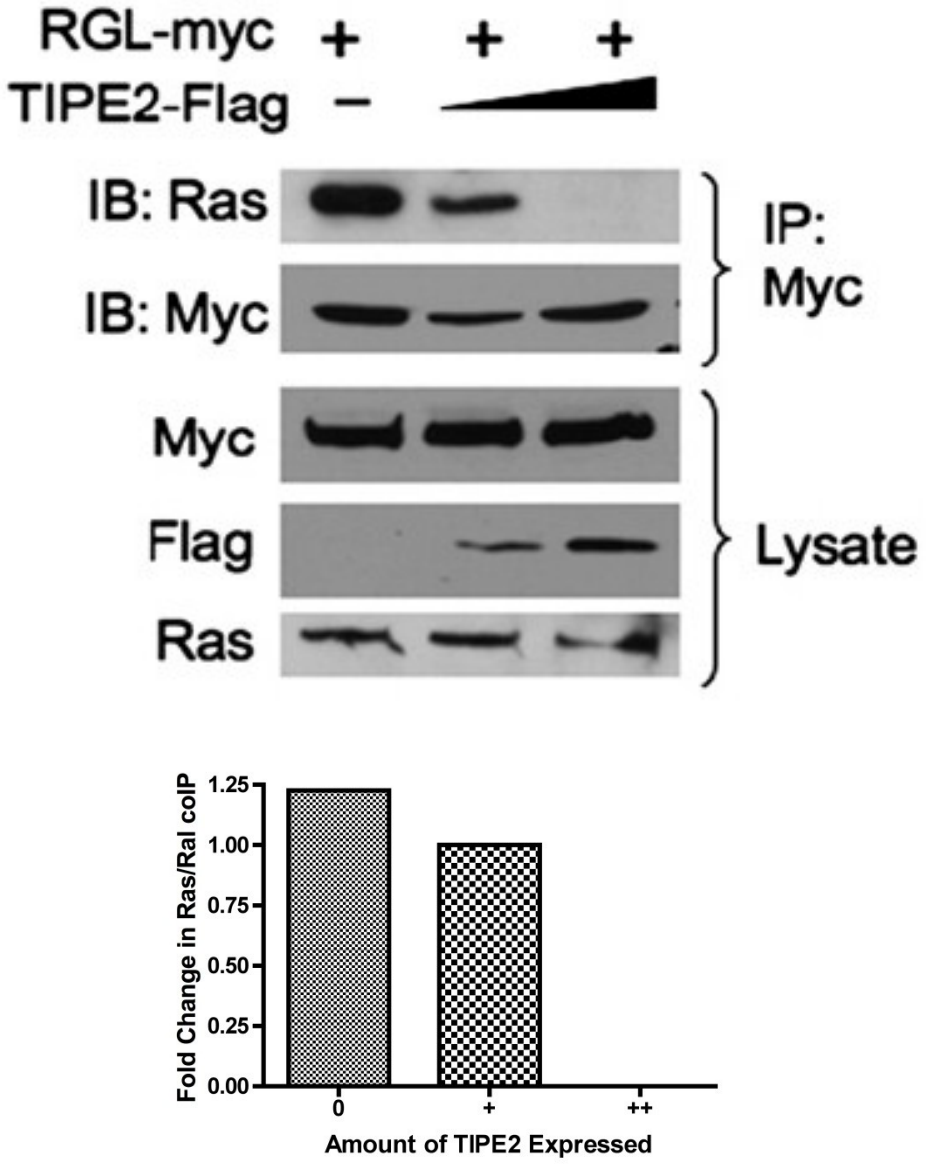


Figure 3.3 TIPE2 Displaces Ras from RGL. Myc tagged RGL1 was co expressed with increasing amounts of flag tagged TIPE2. RGL1 was then IP'd using an anti myc antibody and the amount of Ras bound to RGL was measured via western blot. Increasing amounts of TIPE2 lead to decreased Ras/RGL1 interaction

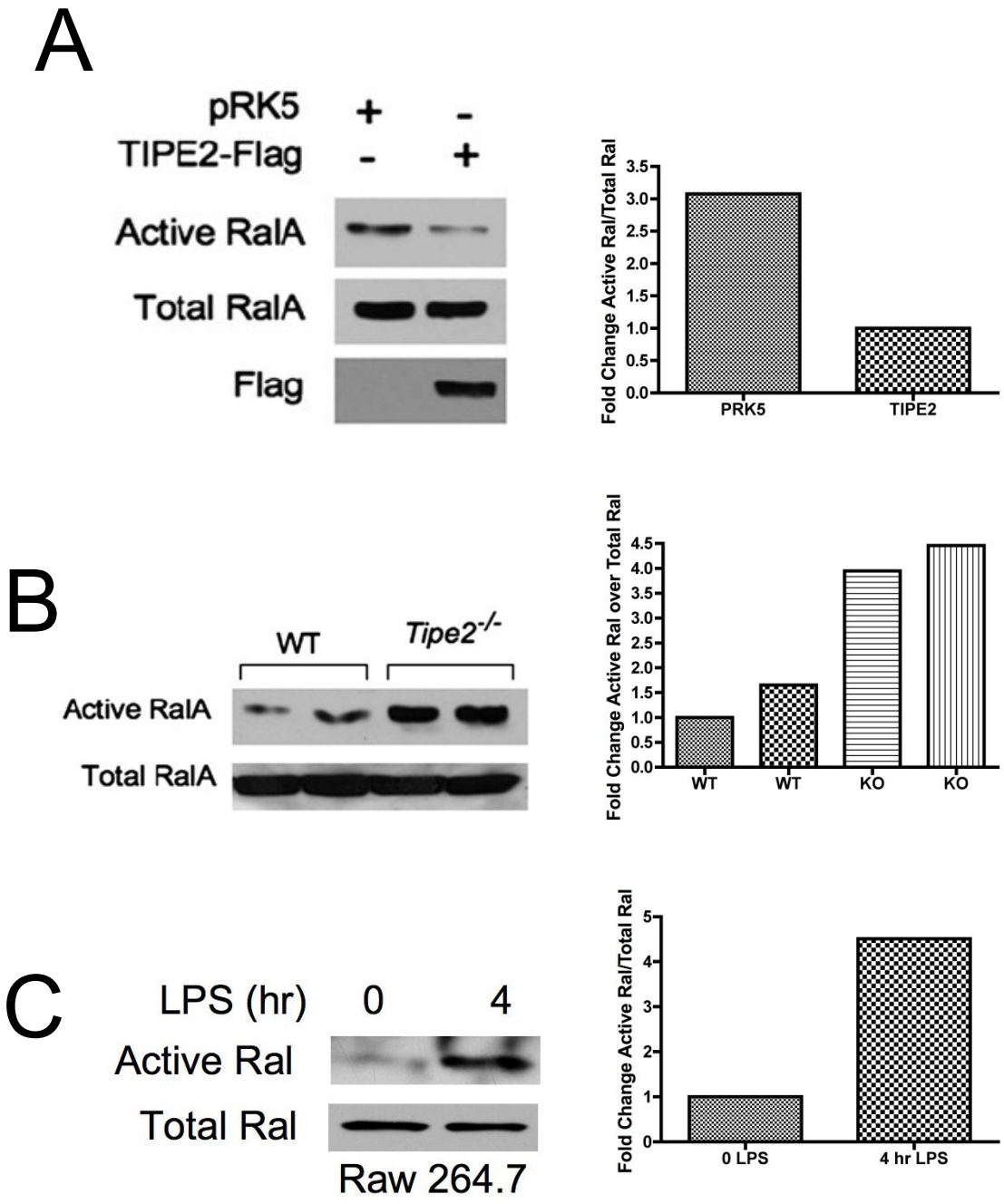


Figure 3.4 TIPE2 Regulates Ral Activity. A.) 293T cells were transfected with Flag tagged TIPE2, and GTP bound Ral was measured. TIPE2 Reduces the amount of GTP bound Ral. B.) TIPE2 -/- bone marrow derived macrophages were lysed and active Ral levels were compared to those of w.t. bone marrow derived macrophages. TIPE2 -/-

macrophages possess more GTP bound RalA than their wild type counterparts. C.) RAW 264.7 macrophages were stimulated with LPS and active Ral levels were assayed via a Ral activity assay and western blot.

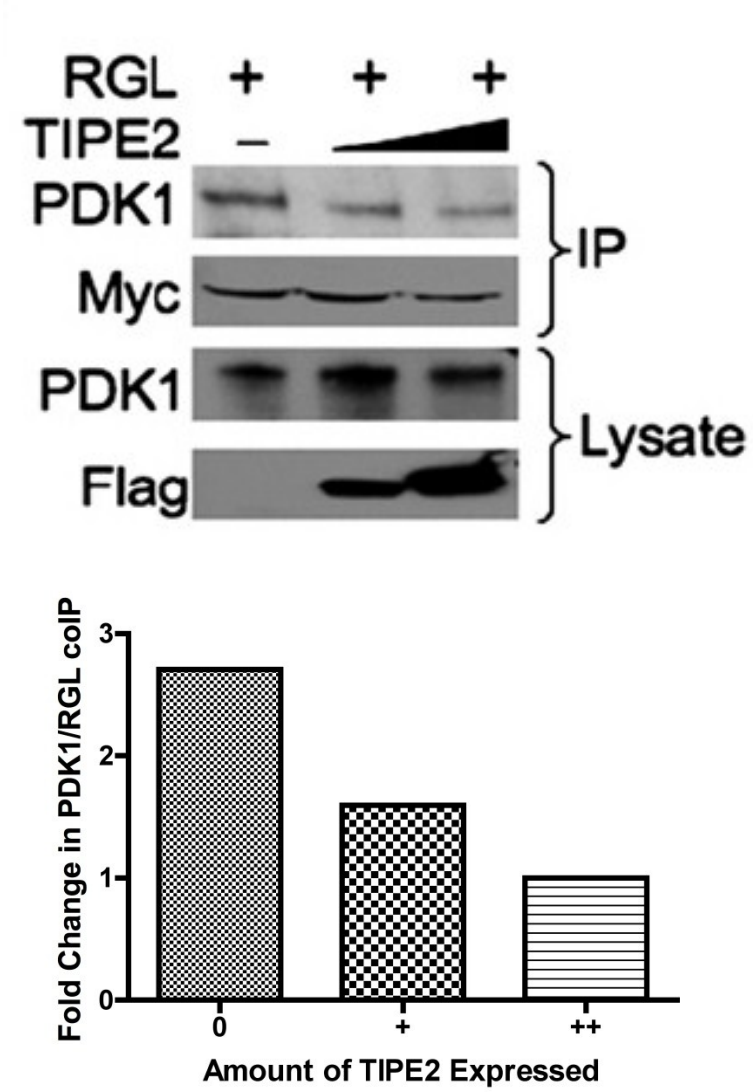


Figure 3.5 TIPE2 Displaces PDK1 From RGL1. Myc tagged RGL1 was co expressed with increasing amounts of flag tagged TIPE2. RGL1 was then IP'd using an anti myc antibody and the amount of PDK1 bound to RGL was measured via western blot. Increasing amounts of TIPE2 lead to decreased PDK1/RGL1 interaction.

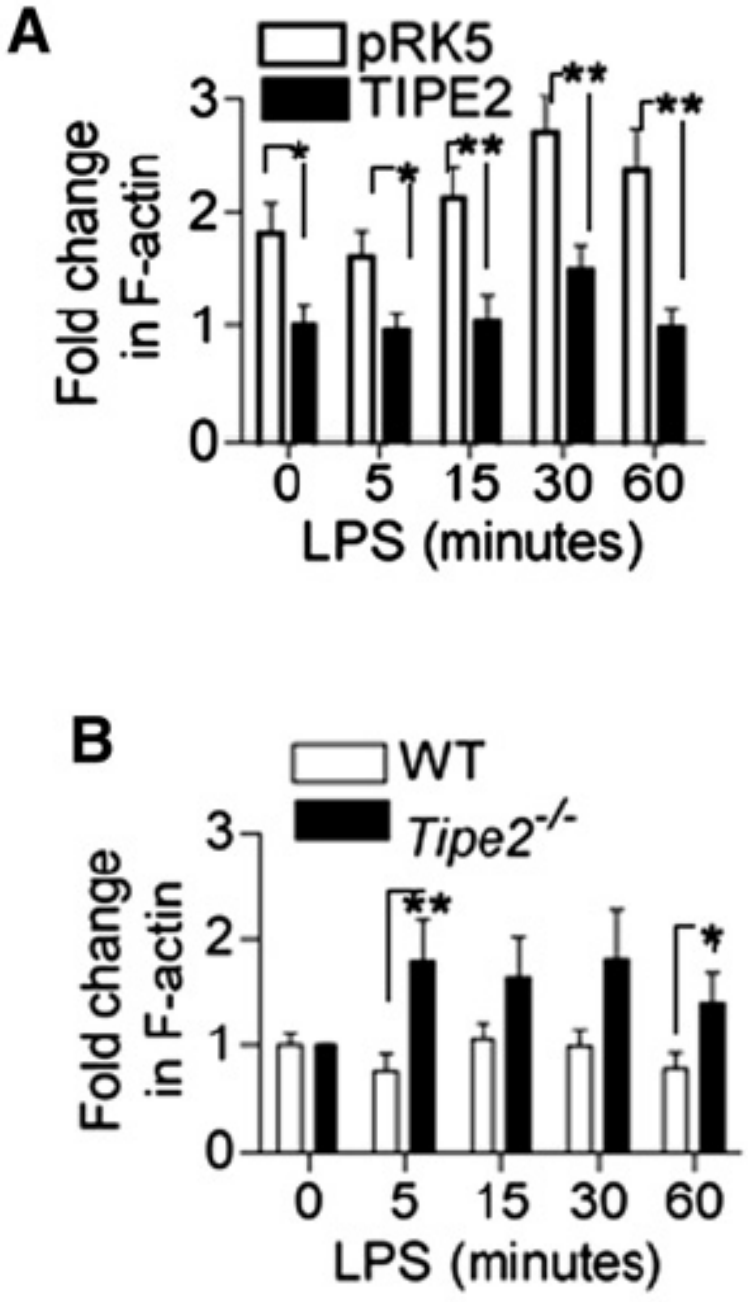


Figure 3.6 Effect of TIPE2 on Actin Polymerization. A.) Raw 264.7 macrophages transfected with either flag tagged TIPE2 or empty plasmid (pRK5) following LPS stimulation (200ng/ml) for the indicated times. F (Filamentous) actin levels were determined as described in methods on pages 47-48. The value of the TIPE2 group at

time point zero was set to a value of 1. Results are means +/- SEM and were pooled from three independent experiments (n=12). *p<0.05; ** p<0.01. B.) F actin levels were measured in splenocytes from TIPE2 -/- animals or their wild type counterparts at the indicated timepoints following 200 ng/ml LPS stimulation as described in methods on pages 47-48. The value at time point zero was set to a value of 1. Results are means +/- SEM and were pooled from three independent experiments (n=6). * p<0.05; ** p<0.02.

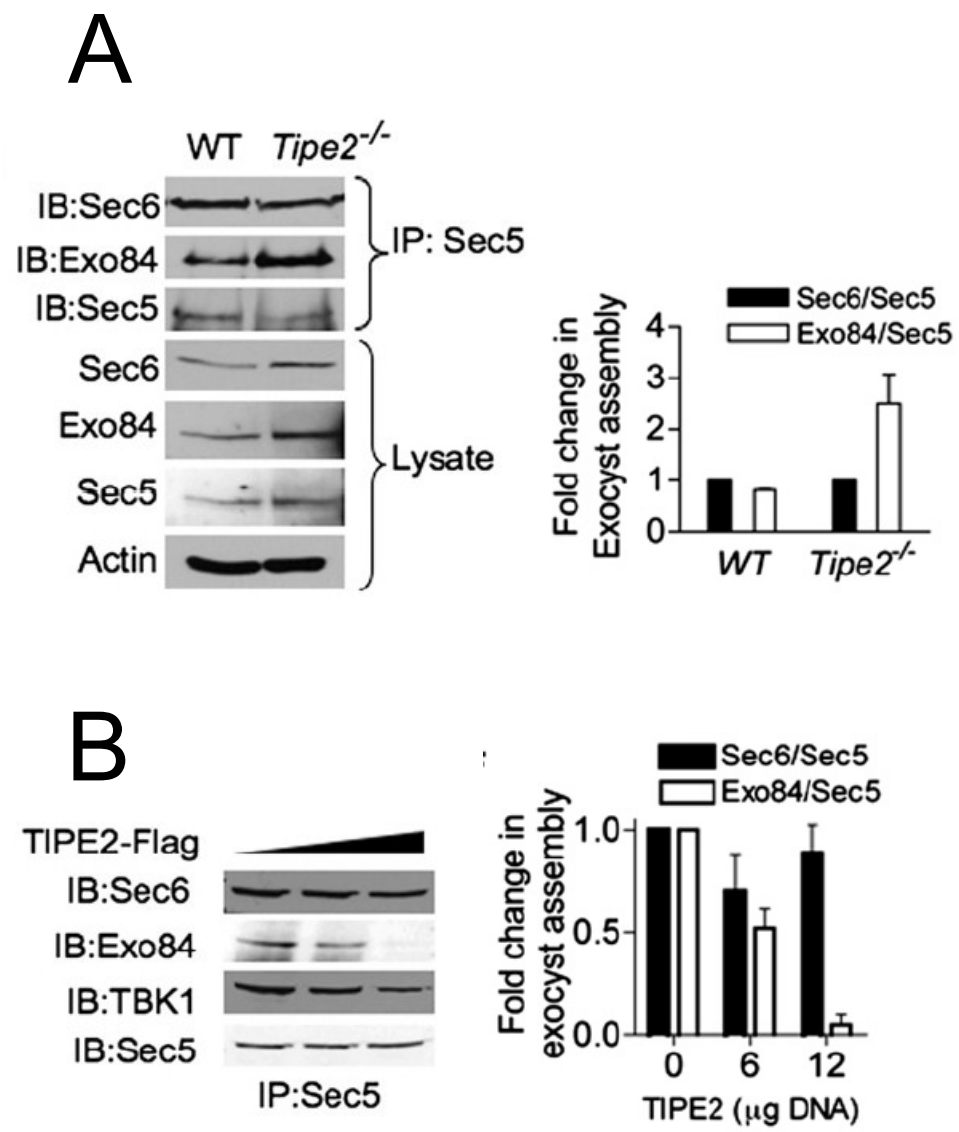


Figure 3.7 TIPE2 induces a dysregulation of the Exocyst Complex. A.) Co immunoprecipitations were carried out for Sec5 and Exo84 and Sec6 in both knockout and wild type bone marrow derived macrophages. Loss of TIPE2 increased the strength of the Sec5/Exo84 interaction. B.) Flag tagged TIPE2 was transfected in increasing amounts in 293T cells and Sec5 was co immunoprecipitated with Sec6 and Exo84. Increasing amounts of TIPE reduce the Sec5/Exo84 interaction (Ral regulated step) but have no effect on the Sec5/Sec6 interaction.

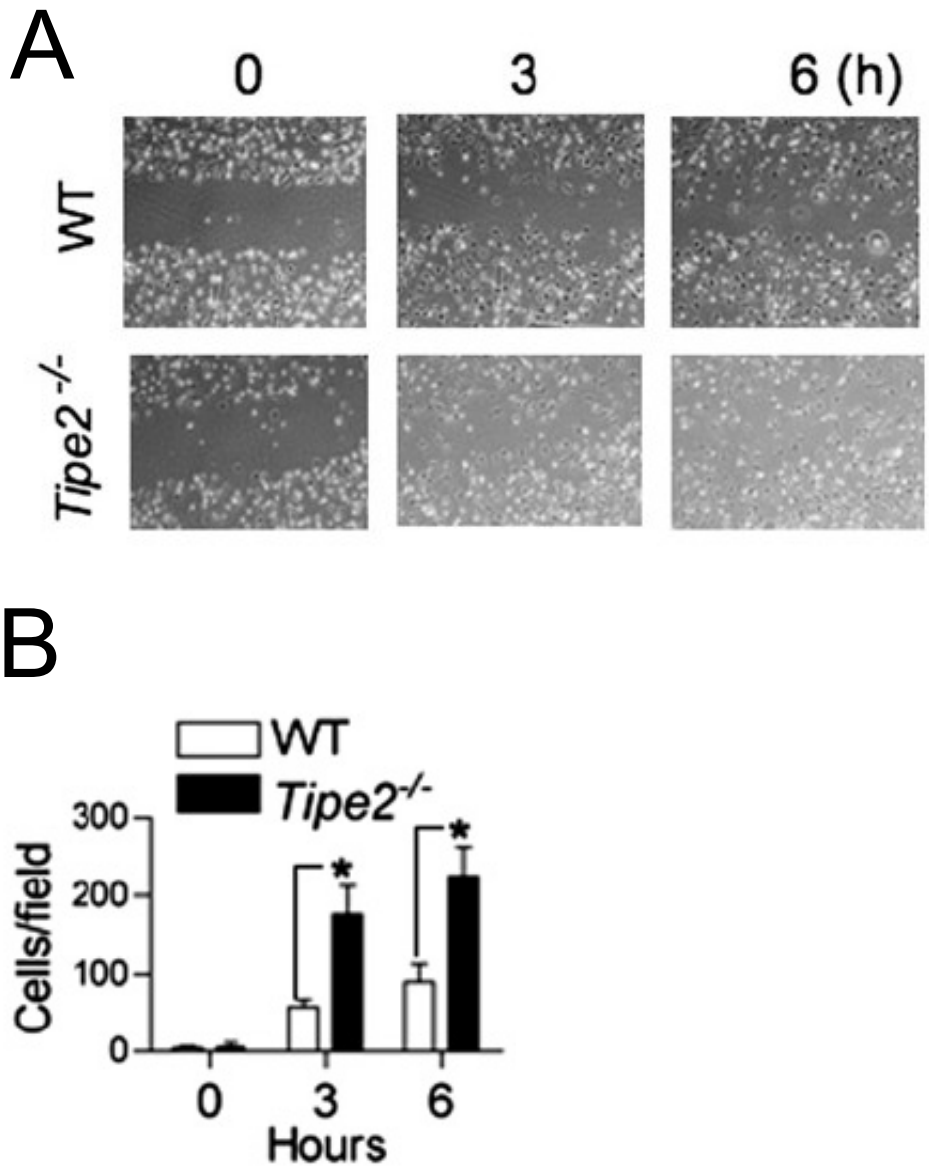


Figure 3.8 Loss of TIPE2 Increases Macrophage Movement. TIPE2 -/- and wt bone marrow derived macrophages were grown on polystyrene dishes. A "wound" was created in the monolayer with a sterile pipette tip and the resulting movement of the macrophages into the wound was followed by microscopy.

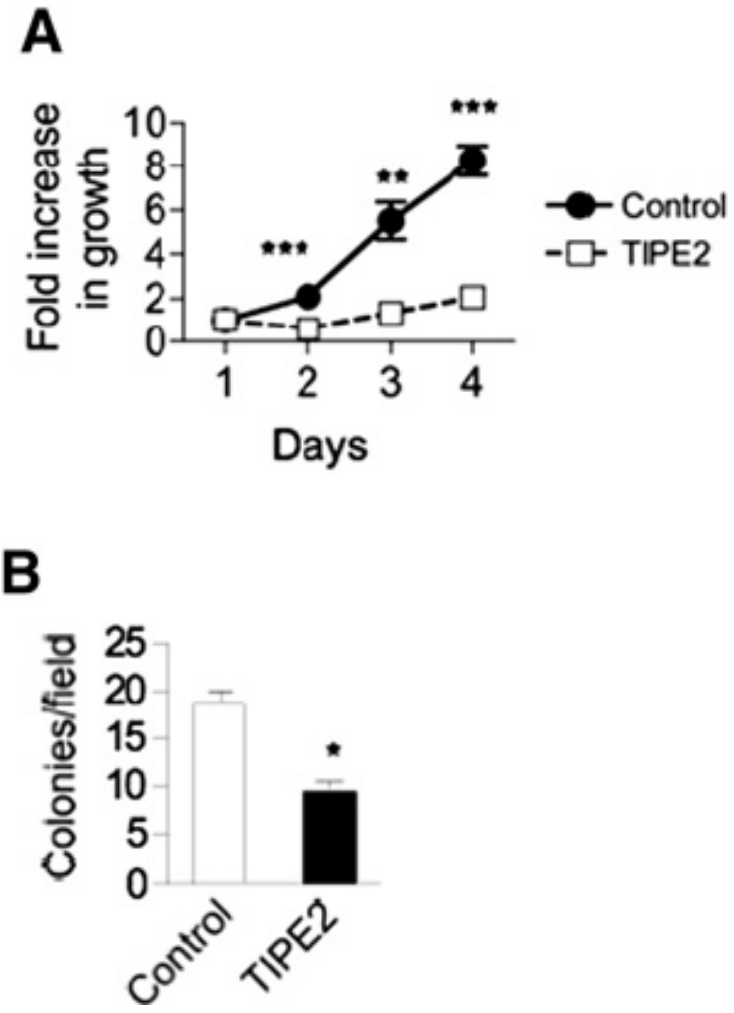


Figure 3.9 TIPE2 Affects Growth and Colony Formation in Soft Agar. A.) TIPE2 was stably expressed via viral transduction into NIH-3T3 cells and cell growth was monitored. TIPE2 significantly reduced cell growth. B.) NIH-3T3 cells were transformed with TIPE2 and ability to form colonies in soft agar was compared with control NIH-3T3 cells. TIPE2 significantly reduced colony formation in soft agar.

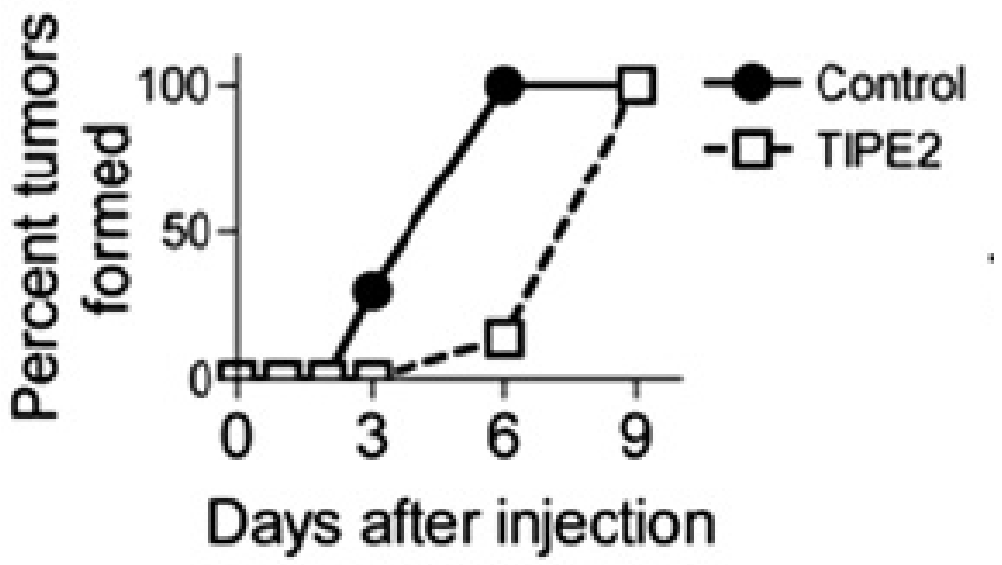


Figure 3.10 TIPE2 delays tumorigenesis in nude mice. Ras-transformed NIH 3T3 cells that did or did not express TIPE2 were injected subcutaneously into the rear flanks of nude mice (2 x10^6 cells/injection, n = 3), and tumor formation was monitored daily. All sites injected with Ras-transformed NIH 3T3 cells eventually developed tumors, whereas control NIH 3T3 or NIH 3T3-TIPE2 cell lines did not give rise to tumors during the course of this study. Data shown are percent of tumors formed and are representative of two independent experiments. The differences between the two groups are statistically significant (p = 0.014).

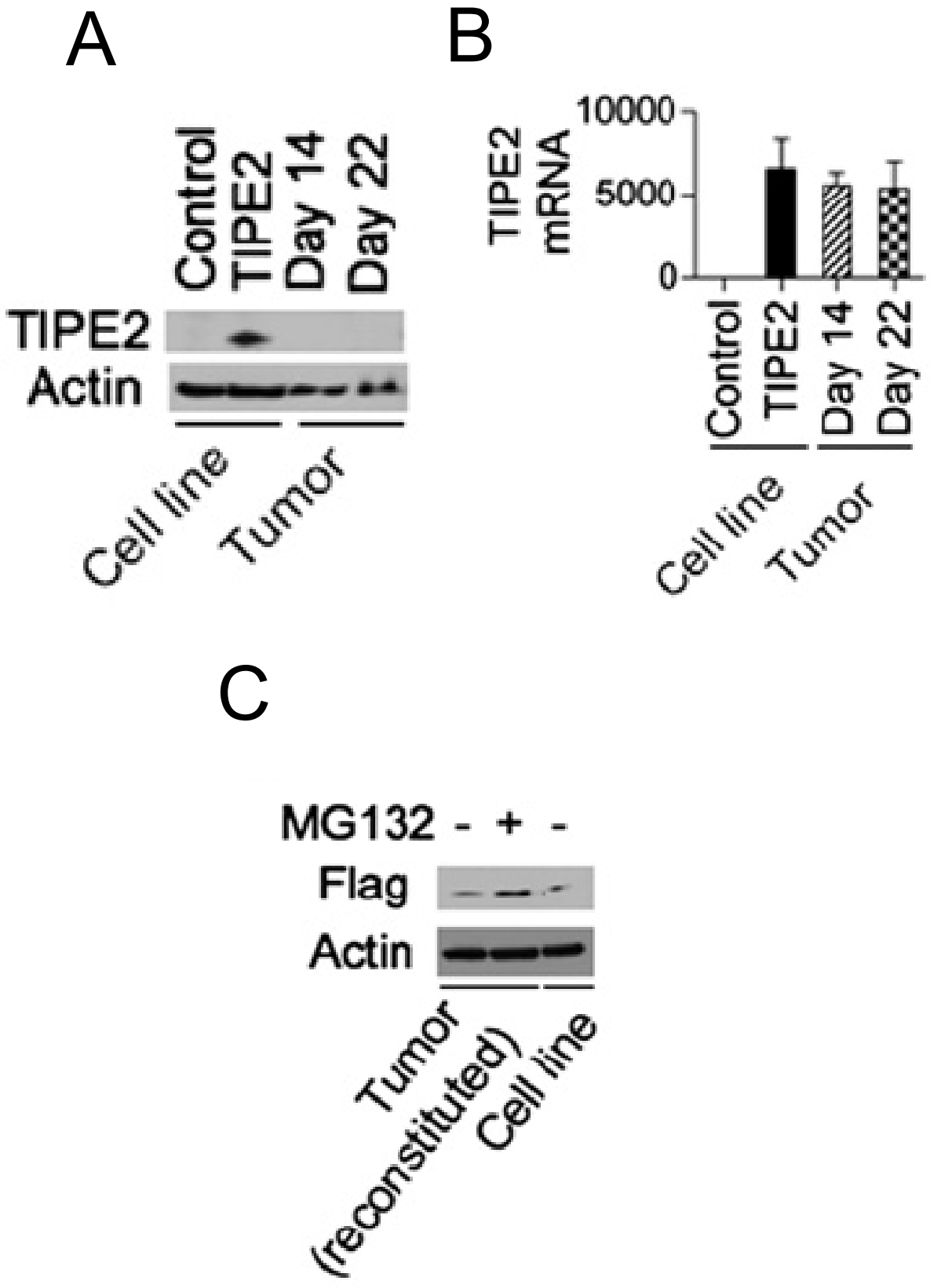
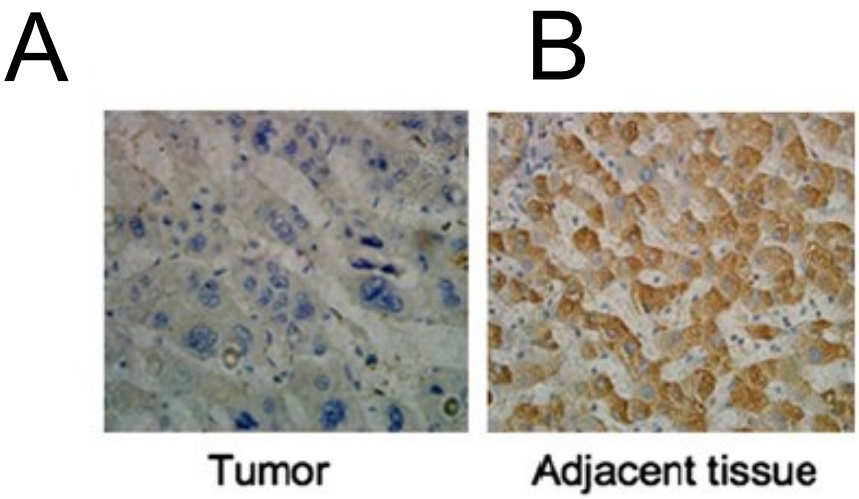

A
Control
TIPE2
Day 14
Day 22
TIPE2
Actin
Cell line
Tumor
B
TIPE2 mRNA
10000
5000
0
Control
TIPE2
Day 14
Day 22
Cell line
Tumor
C
MG132 - + -
Flag
Actin
Tumor (reconstituted)
Cell line

Figure 3.11 Tumors Silence TIPE2 in order to grow. A.) TIPE2 protein levels were measured in Ras transformed NIH-3T3 cells expressing TIPE2 before injection into Nude mice, and then at 14 and 22 days following tumor onset. TIPE2 was not detectable in tumors. B.) TIPE2 message levels were checked in pre injection cells as well as tumors, and TIPE2 RNA levels were not changed between samples. C.) Reconstituted tumors were treated with MG132 in order to inhibit the proteasome to check the effect on TIPE2 levels. Inhibition of the proteasome allowed TIPE2 levels to rise even beyond those in the pre injection cell lines.



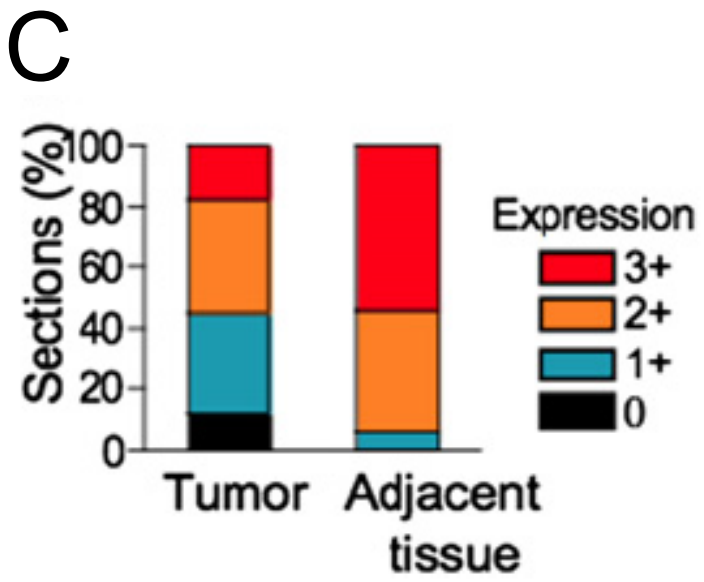


Figure 3.12 Amount of TIPE2 expression in human hepatocellular carcinoma in. A.) Tumor tissue and B.) control hepatic tissue adjacent to the tumor from the same patient was determined by immunohistochemistry as described in materials&methods beginning on page 43. TIP2 positive cells are stained in brown, magnification x400. C.) Quantificatino of the TIPE2 signal from 116 patients was performed as described in methods beginning on page 43. The differences between the two groups are statistically significant ($p<.001$).

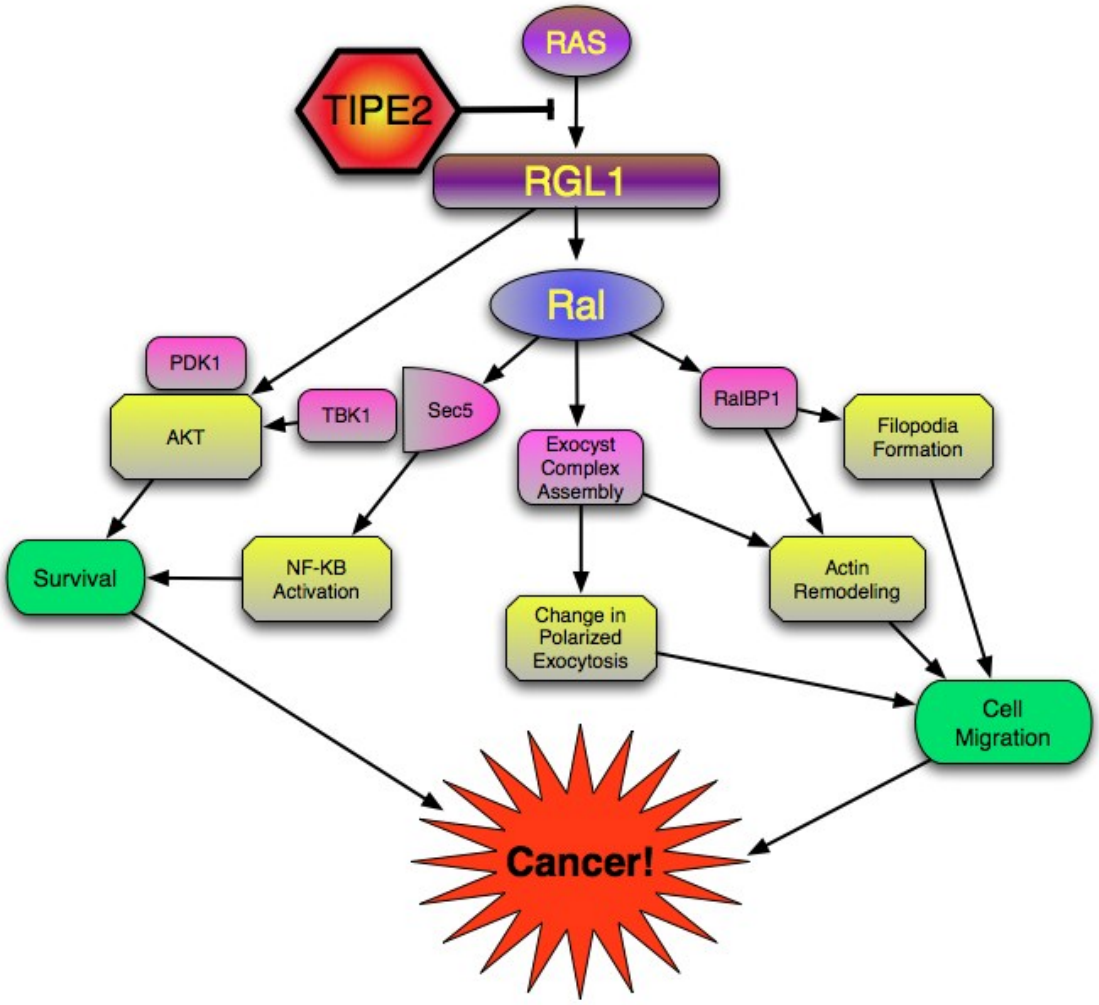


Figure 3.13 Putative TIPE2 and RGL1 Mechanism.

Chapter 4

TIPE2 and mTOR Inhibition

The work in this chapter is unpublished data, and is intended to be published in the near future.

Abstract

mTOR is a serine/threonine kinase that plays a central role in responding to nutrient and environmental cues. mTOR interprets these cues, and dictates to the cell whether anabolic or catabolic programming should be initiated, as well as a host of other important cellular changes. TIPE2 negatively regulates the mTOR pathway, resulting in a marked change in primary mTOR effectors. Overexpression of TIPE2 induces a marked down-regulation of the Rictor component of mTORC2, while TIPE2 -/- cells show an increased amount of Rictor. TIPE2 interacts with the GBL common subunit of both the mTORC1 and mTORC2 complexes, and this interaction may be responsible for the loss of Rictor induced by TIPE2, which in turn likely explains the marked reduction in downstream mTOR signaling.

Introduction

mTOR (mechanistic Target Of Rapamycin, also referred to as the mammalian Target Of Rapamycin) is a serine/threonine kinase that plays the predominant role in interpreting environmental signals to control cell growth and protein synthesis, cell survival, cell proliferation, and cell motility (Zoncu 2011). This kinase was originally identified as the target of the immunosuppressive macrolide rapamycin, and his since been recognized as an extraordinarily important molecule in regulating proper cell function. Due to its central function in regulating so many different processes mTOR dysregulation has been linked to numerous disease states, including obesity (Um 2004), cancer (Aoki 2001, Shah 2001, Podsypanina 2001), type 2 diabetes, neurodegeneration (Chong 2012), as well as others.

mTOR exerts its numerous biological functions via two protein complexes named mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is composed of six protein subunits; mTOR, GBL, DEPTOR, Raptor, PRAS40, and the Tti/Tel2 complex. mTORC2 contains seven subunits; mTOR, GBL, DEPTOR, Rictor, Sin1, Protor, and the Ti1/Tel2 complex.

mTORC1

mTORC1 functions as a cellular computer whose inputs are cellular environmental conditions, and whose outputs are either growth and fresh protein synthesis, or autophagy to conserve energy and materials (Zoncu 2001). mTORC1 is capable of sensing the energy status of the cell via AMPK activity

(Reviewed in Hardie 2007), the relative amount of amino acids present via detecting the branched chain amino acid leucine (Sancak 2008,), the relative amounts of lipids via binding phosphatidic acid (Fang 2001, Yoon 2011), it can sense the presence of growth factors, the relative abundance of oxygen, as well as cellular stress. When mTORC1 has received signals acknowledging the presence of oxygen, growth factors, energy, raw materials in the forms of lipids and amino acids, as well as no negative stress signals, it will signal down to its downstream effectors p70S6 Kinase (S6K) and eIF4E binding protein 1 (4EBP1) to begin protein synthesis (Haghinghat 1995, Hara 1997, Holz 2005, Max Ma 2008,). It will also inhibit the lipid inhibitor lipin 1 (Peterson 2011) as well as signal to inhibit autophagy (Nobukini 2005). S6K is a kinase which performs multiple functions to increase protein synthesis. Its primary target is the S6 ribosomal protein, which it phosphorylates to induce protein synthesis at the ribosome. S6K also increases translational initiation and elongation (Max Ma 2008), and can induce biogenesis of mRNA (Max Ma 2008) as well as biogenesis of ribosomes (Mayer 2004, Jastrzebski 2007). 4EBP1 is an inhibitor of the cap binding protein eIF4E (Pause 1994). eIF4E is a crucial part of the eIF4F cap binding complex which is necessary in order to efficiently undergo efficient cap dependent translation. mTORC1 can phosphorylate 4EBP1 which marks it for degradation (Schalm 2003). Once 4EBP1 has been degraded eIF4E is free to initiate cap dependent translation. Since protein synthesis is crucial for the growth of a cell, mTORC1 is a master regulator of cellular growth.

mTORC2

mTORC2 responds to growth factors, but not to nutrients in the way that mTORC1 does. mTORC2 is the primary kinase that activates the AGC kinase AKT (Sarbassov 2005), as well as the kinases SGK1 and PKC (Garcia 2008, Facchinetti 2008). AKT is an extremely important survival signaling molecule which promotes survival and reduces apoptois via multiple downstream effectors such as the FoxO family of proteins (reviewed in Zhang 2011), which in turn can affect the apoptosis modulators FasL and Bim, as well as the cell cycle activators Cyclin D1 and D2. mTORC2 also plays a role in the organization of the actin cytoskeleton (Sarbassov 2004).

mTOR in the Immune System

Due to the central position occupied by mTOR in regulating survival, proliferation, and growth, it is no surprise that mTOR has been shown to have an essential role within both the adaptive and innate immune system. Within the adaptive immune system mTOR alters cell surface receptors that control T cell trafficking (Finlay 2010). Inhibition of mTOR in activated CD8+ T cells increases expression of CCR7 and CD62L (Sinclair 2008), increasing the trafficking of these cells to secondary lymphoid organs (Sinclair 2008). Additionally there are multiple threads of evidence indicating that mTOR modulates CD8+ memory T Cell differentiation, inhibition of mTOR during the expansion phase of T Cell response results in an increased number of memory CD8+ T cells (Rao 2010, Li

2011). Within CD4+ T cells mTOR plays crucial roles in the appropriate differentiation into different CD4+ subsets. Loss of mTOR results in a loss of differentiation into Th1, Th2, and Th17 subsets (Delgoffe 2009). When loss is restricted to mTORC1 components Th1 and Th17 differentiation is impaired (Delgoffe 2011), while loss of mTORC2 activity resulted in a failure to develop properly differentiate into Th2 subsets (Delgoffe 2011).

mTOR also plays an important role in the proper operation of the innate immune system. Animals expressing hypomorphic mTOR have smaller spleens with fewer monocytes (Zhang 2011). mTOR inhibition decreases generation of human myeloid DCs (va de Laar 2010). Additionally DCs treated with rapamycin have a poor ability to induce allogeneic t cell responses and instead induce Treg differentiation (Haidinger 2010). Moreover in various DC subsets rapamycin treatment can impair type I interferon production (Cao 2008 and Colina 2008.), and can also impair both pro- and anti-inflammatory cytokine production. Due to its negative effect on autophagy, mTOR can reduce DC function by impairing DC autophagy, a process important for proper antigen processing and presentation (Lee 2007). Paradoxically this indicates that depending upon the specific conditions mTOR can either inhibit or enhance DC cell mediated T Cell activation.

Inflammation and mTOR

Both diabetes and obesity are often linked to highly dysregulated mTOR signaling. Additionally “diabesity” as it is sometimes referred to is often

described as a disease of inflammation, as both obesity and diabetes are highly correlated with excess inflammation. Attempting to infer causality between inflammation and diabesity has proven to be difficult, and a better understanding of how inflammatory signaling can intersect with the metabolic signaling involved in diabetes and obesity is needed to understand this issue.

Materials and Methods

Animals

C57BL/6J (B6) mice that carry a *Tipe2* gene null mutation were generated by backcrossing *Tipe2*$^{-/-}$ 129 mice (Sun et al., 2008) to B6 mice for 12 generations. Mice were housed in the University of Pennsylvania Animal Care Facilities under pathogen-free conditions. All animal procedures used were pre-approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Cell lines and plasmids

HEK 293T cells were purchased from ATCC. Cells were grown in DMEM supplemented with 10% FBS, penicillin and streptomycin. mTOR, Rictor, Raptor, S6K1, and S6K2 plasmids were purchased from Addgene. Bone marrow was removed from femur and was cultured for 7 days in DMEM supplemented with 10% FBS, penicillin and streptomycin and mCSF.

Immunoprecipitation

Cells were lysed with CellLyticM buffer (Sigma) supplemented with protease inhibitor (complete, Roche) and phosphatase inhibitor (PhosStop, Roche) cocktail tablets. The lysates were cleared by centrifugation for 15 min, pre-cleared with 100 ul beads for 30 minutes, and protein concentration was determined by Bradford assay. Antibody was added to 300 ug protein lysate and rotated for 2 hours at 4C followed by addition of beads and rotation for an additional 2 hours at 4C. Lysates were spun down, resuspended in Laemmli

loading dye, and 30 ul of IP run through 4%-12% miniTGX gradient gels, purchased from Biorad. After SDS-PAGE and transfer, the membranes were probed with various antibodies.

Immunoblotting

Cells were lysed in SDS and total protein concentration determined. 30 μg protein was loaded to each lane, and separated by SDS-PAGE. After transferring to a nitrocellulose membrane, it was blocked with 5% milk in TBST and probed with the following primary antibody.

Results and Discussion

TIPE2 has been reported to regulate the immune system, inflammation, and survival, but the mechanisms by which it performs these functions are not clear. While attempting to identify survival pathways regulated by TIPE2, we discovered that AKT phosphorylation is heavily dysregulated in bone marrow derived macrophages isolated from TIPE2 knockout mice (Figure 4.2b). Loss of TIPE2 leads to a marked increase in the amount of AKT phosphorylated at S473, indicating increased survival signaling in these cells. This correlates with a prior report of TIPE2 knockout cells being resistant to activation-induced-cell-death (Sun 2008). Since loss of TIPE2 led to increased AKT phosphorylation, it raised the question of whether additional TIPE2 could reduce AKT phosphorylation. To test this effect the converse experiment was carried out and TIPE2 was overexpressed in order to see if overexpression could inhibit AKT phosphorylation at S473. This overexpression did lead to a significant reduction in phosphorylation of AKT (Figure 5.5b).

mTOR signals from two distinct complexes, mTORC1 and mTORC2 (Figure 4.1). While AKT is phosphorylated and activated by the mTORC2 complex, 4EBP1, S6K, and S6 are all downstream of the separate mTORC1 complex. Since AKT is a downstream effector of the mTORC2 complex, and TIPE2 had such a potent effect on its signaling status, we next sought to determine whether effectors downstream of mTORC1 were dysregulated by TIPE2. Overexpression of TIPE2 led to large reductions in phosphorylated 4EBP1, p70S6K, and S6 (Figure 4.2 A,C), indicating that TIPE2 negatively

regulates mTORC1 as well as mTORC2. When co-expressed with its binding partner RGL, the TIPE2 mediated reduction in mTOR signaling is reduced (Figure 4.2c), suggesting that TIPE2 has a separate and new binding partner via which it is regulating mTOR signaling, and that overexpression of RGL is able to compete away some of the TIPE2 from this binding partner.

Since it seemed likely that TIPE2 was regulating the mTORC1 and mTORC2 complexes via a new interacting partner, a series of co-immunoprecipitations were performed between TIPE2 and members of the two complexes. Since the two subunits that are common to each complex are mTOR and GBL, the effort was first focused on these two members. While TIPE2 will coIP with mTOR itself, the interaction is quite weak and sporadic, implying that the interaction is either transient or indirect. When co-immunoprecipitated with GBL however, TIPE2 showed a robust interaction, much stronger than with mTOR (Figure 4.3). Since GBL binds directly to mTOR, it is likely that any co-immunoprecipitation seen between TIPE2 and mTOR was indirect and due to an interaction between TIPE2 and GBL, not an interaction with mTOR itself. Also coIPs were carried out between TIPE2 and the Raptor and Rictor subunits of mTORC1 and mTORC2 respectively, and no interaction was seen.

Next we sought to identify how an interaction between TIPE2 and GBL could lead to such a marked reduction in mTOR downstream signaling. We hypothesized that TIPE2 was binding and sequestering GBL, preventing it from forming into functional mTORC1 and mTORC2 complexes, and since loss of

GBL has been previously shown to lead to a loss of Rictor (Guertin 2006), the effect of TIPE2 expression on Rictor was tested. Overexpression of TIPE2 led to a severe reduction in Rictor levels present in cells, but not of either GBL or of Raptor, implying that the reduction in protein was specific to just the Rictor component of the mTORC2 complex (Figure 4.4b). Since overexpression of TIPE2 leads to increased cell death (Chapter 5), it was necessary to ensure that the loss of Rictor expression was a specific function of TIPE2, and not a non-specific cell-death related effect. To test this, cells overexpressing Rictor with and without TIPE2 were treated with etoposide to induce apoptosis, and only those cells with TIPE2 present showed a reduction in Rictor protein levels. Additionally TIPE2 knockout bone marrow derived macrophages were compared to their wild type counterparts, and the knockout macrophages had significantly more rictor present (Figure 4.4A).

TIPE2 interacts with the GBL constant subunit of both the mTORC1 and mTORC2 complexes, effectively inhibiting their downstream signaling and leading to a reduction in the amount of Rictor present in the cell. Rictor is the key scaffold which maintains mTORC2 integrity, while Raptor is the key scaffold that maintains mTORC1 integrity. While loss of GBL has been shown to correlate with reduced levels of Rictor, its loss did not affect Raptor levels (Guertin 2006). In line with this a loss of GBL caused mTORC2 signaling to AKT to be affected to a more significant degree than mTORC1 signaling to either p70S6K or 4EBP1. This correlates strongly with what we see from TIPE2 overexpression – a very strong reduction in phosphorylated AKT, a substrate of mTORC2, and a

significant but less intense reduction in phosphorylated levels of p70S6K and 4EBP1, both substrates of mTORC1. It is both possible and likely that the effect of TIPE2 on mTORC1 signaling is in actuality primarily occurring via its inhibition of AKT via reduction in Rictor levels. AKT is capable of inhibiting the TSC1/2 complex, which in turn inhibits the activity of mTORC1. When AKT inhibits the inhibitor, it allows mTORC1 to become active, phosphorylating its downstream targets S6K and 4EBP1 (Vander Haar 2007, Sancak 2007).

The inhibitory effect of TIPE2 on mTOR signaling is particularly relevant when considering TIPE2 through the prism of an inflammatory regulator. Animals expressing hypomorphic amounts of mTOR have smaller spleens with fewer monocytes (Zhang 2011). TIPE2 knockout mice have an opposite phenotype, having larger spleens (Sun 2008), with a larger percentage of monocytes over wild type controls (Wang 2012). This is exactly what we would expect if TIPE2 knockout cells are missing a crucial negative regulator of mTOR signaling and thus mTOR is able to transduce a stronger signal than would normally occur. Rapamycin treatment will inhibit mTOR activity, and treatment of dendritic cells with rapamycin can impair type I interferon production (Cao 2008, Colina 2008). In contrast, TIPE2 knockout dendritic cells produce significantly more type I interferons (Sun 2012). It is distinctly possible that much of the ability for TIPE2 to regulate inflammation is due to its ability to modulate mTOR signaling.

While its role in regulating inflammation and immunity via mTOR is an intriguing possibility, a perhaps more clinically relevant and important avenue to

consider is the role TIPE2 may play in regulating diabetes and obesity. TIPE2 is a regulator of the mTOR nutrient sensing pathway, and is in turn regulated by inflammatory signals (see Chapter 2) which down-regulate TIPE2 expression. Obesity and type II diabetes have a strong correlation between excessive inflammation and dysregulated nutrient signaling. While some molecular pathways that connect these two themes have been discovered, such as the inflammation induced JNK mediated inhibitory phosphorylation of IRS1, the precise connection between inflammation and metabolic syndrome is incompletely understood. While it is primarily expressed within the immune system, TIPE2 is expressed in both liver and skeletal muscle (Zhang 2011), both extremely important insulin responsive tissues, which can become insulin resistant if proper homeostatic regulation is lost. The putative role for TIPE2 as a regulator of metabolic syndrome will be a line of work worth investigating in the future.

In addition to its potential role in connecting inflammation with nutrient signaling, TIPE2 may also play an important role in atherosclerotic plaque formation and the subsequent heart disease caused by said plaque formation. Like diabetes and insulin resistance, atherosclerosis is highly associated with inflammation. One key component of atherosclerotic plaques is deposited foam cells, which are macrophages engorged with cholesterol that become sticky and adhere to the vascular endothelium, constricting blood flow and eventually causing a blockage. A recent report has indicated that inhibition of mTOR during foam cell formation can reduce at least some aspects of foam

cell formation (Yu 2011). Considering that TIPE2 negatively regulates mTOR, and is itself negatively regulated by inflammatory signals, and that TIPE2 is highly expressed in macrophages, it could prove to be worthwhile to investigate the putative role of TIPE2 in foam cell formation.

By providing a new and exciting linkage between inflammation and the primary nutrient and metabolic signaling pathway within the cell, TIPE2 holds a great deal of promise as a potential druggable target to treat plethora of diseases that currently ail millions of people.

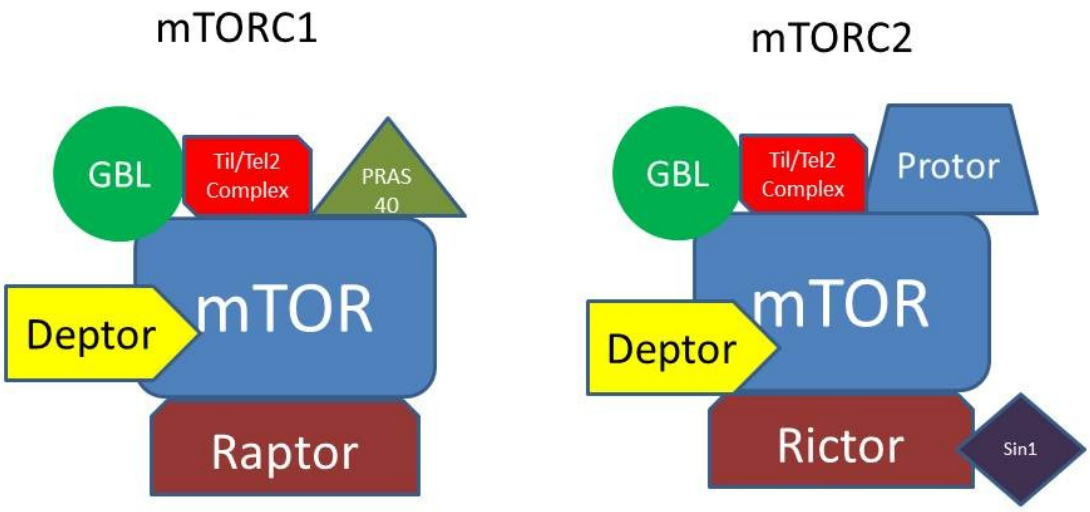
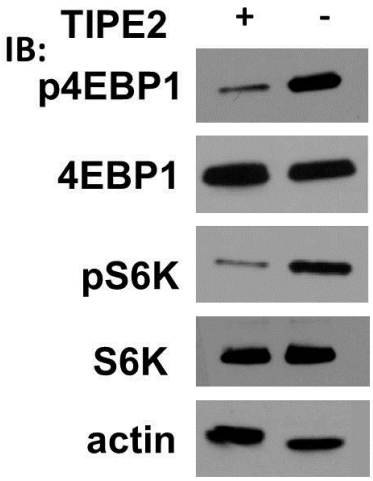
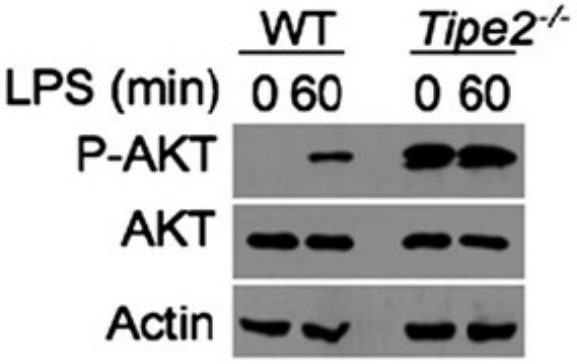


Figure 4.1 Mechanistic diagram of mTORC1 and mTORC2 complexes. GBL and the recently discovered inhibitor DEPTOR are constant members of each complex. Raptor provides the mTORC1 specific scaffolding activity while Rictor provides mTORC2 specific scaffolding activity.

A


TIPE2
+
-
IB:
p4EBP1
4EBP1
pS6K
S6K
actin

B


WT
Tipe2-/-
LPS (min)
0 60
0 60
P-AKT
AKT
Actin

C

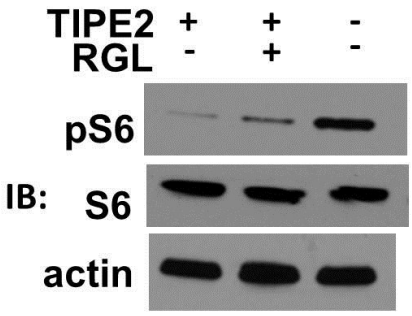

TIPE2
+
+
-
RGL
-
+
-
pS6
IB:
S6
actin

Figure 4.2 mTOR Signaling is negatively regulated by TIPE2. A.) TIPE2 was overexpressed in 293T cells for 24h and the activation status of the downstream the mTOR effectors 4EBP1 and S6K were assayed via western blot. B.) Bone marrow derived macrophages were isolated from WT and TIPE2 KO mice as described in methods. Following culturing BMDM were either treated with vehicle or LPS for 60 minutes, cells were lysed and the activation status of AKT at S473 was assayed via western blot. C.) TIPE2 was overexpressed in 293T cells with or without co-expression of RGL for 24h. The activation state of S6, a target of the mTOR effector S6K was assayed via western blot.

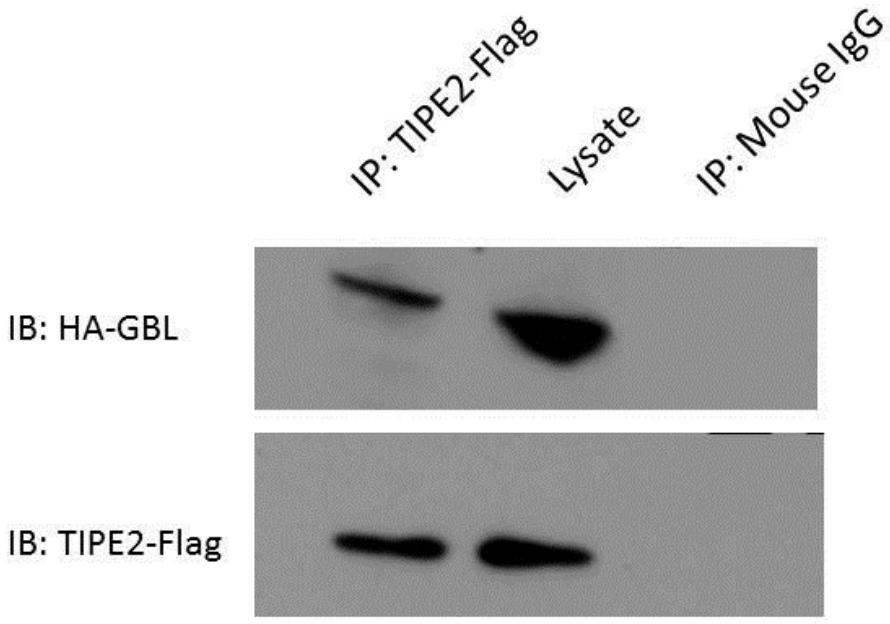
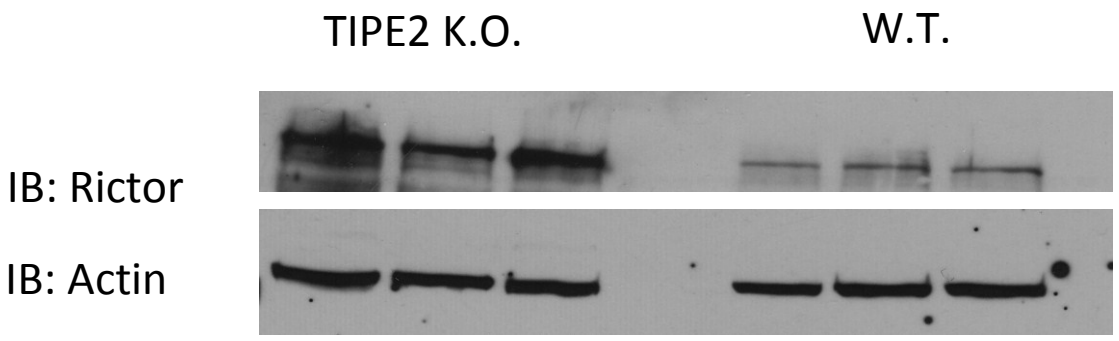


Figure 4.3 TIPE2 Co-Immunoprecipitates with GBL. Flag-tagged TIPE2 and HA-tagged GBL were co-expressed in 293T cells for 24h. Following lysis, lysates were cleared of the insoluble fraction, and were incubated with either anti-flag or anti mouse IgG for 2h at 4^{o}C. After a two hour incubation protein G agarose beads were added to the lysate in order to immunoprecipate all Ig bound protein. Following a 2h incubation with beads protein was eluted and assayed via western blot.

A


TIPE2 K.O.
W.T.
IB: Rictor
IB: Actin

B

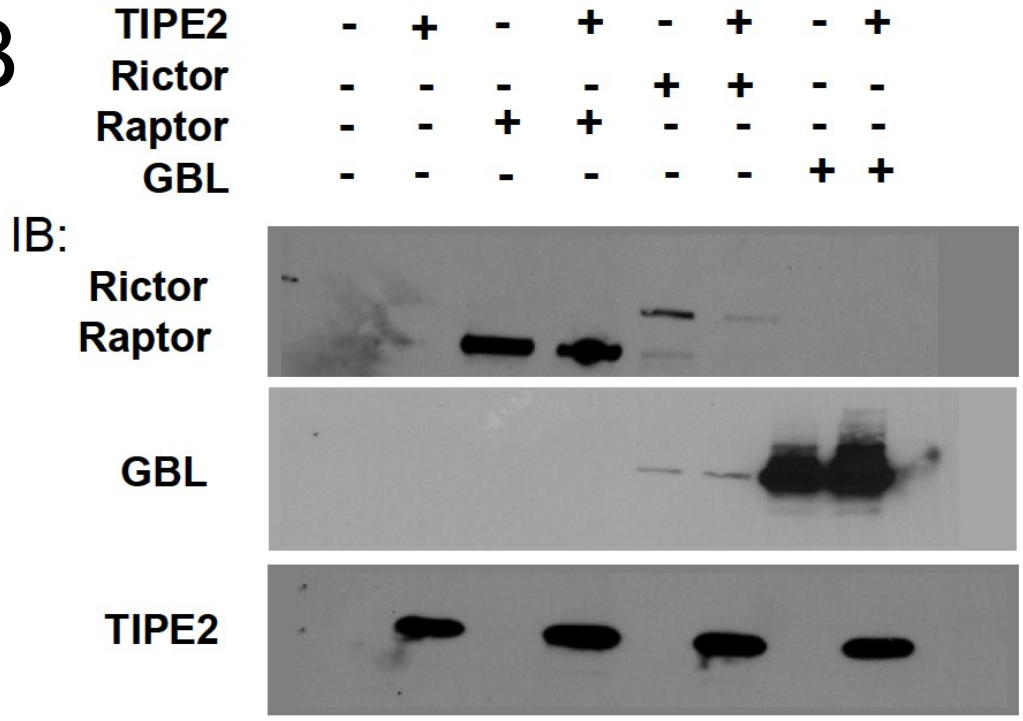

TIPE2 - + - + - + - +
Rictor - - - - + + - -
Raptor - - + + - - - -
GBL - - - - - - + +
IB:
Rictor
Raptor
GBL
TIPE2

Figure 4.4 TIPE2 Regulates Rictor. A.) Bone marrow derived macrophages were isolated from WT and TIPE2 KO mice as described in methods. Following culturing cells were lysed and assayed via western blot to compare Rictor levels. Lysates were pooled from 3 animals. B.) 293T cells were either transfected with TIPE2 alone, or co-transfected with TIPE2 and one of the following plasmids; Raptor, Rictor, and GBL for 24h. Following transfection cells were lysed and lysates were analyzed via western blot to compare the effect of TIPE2 on different members of mTOR complexes.

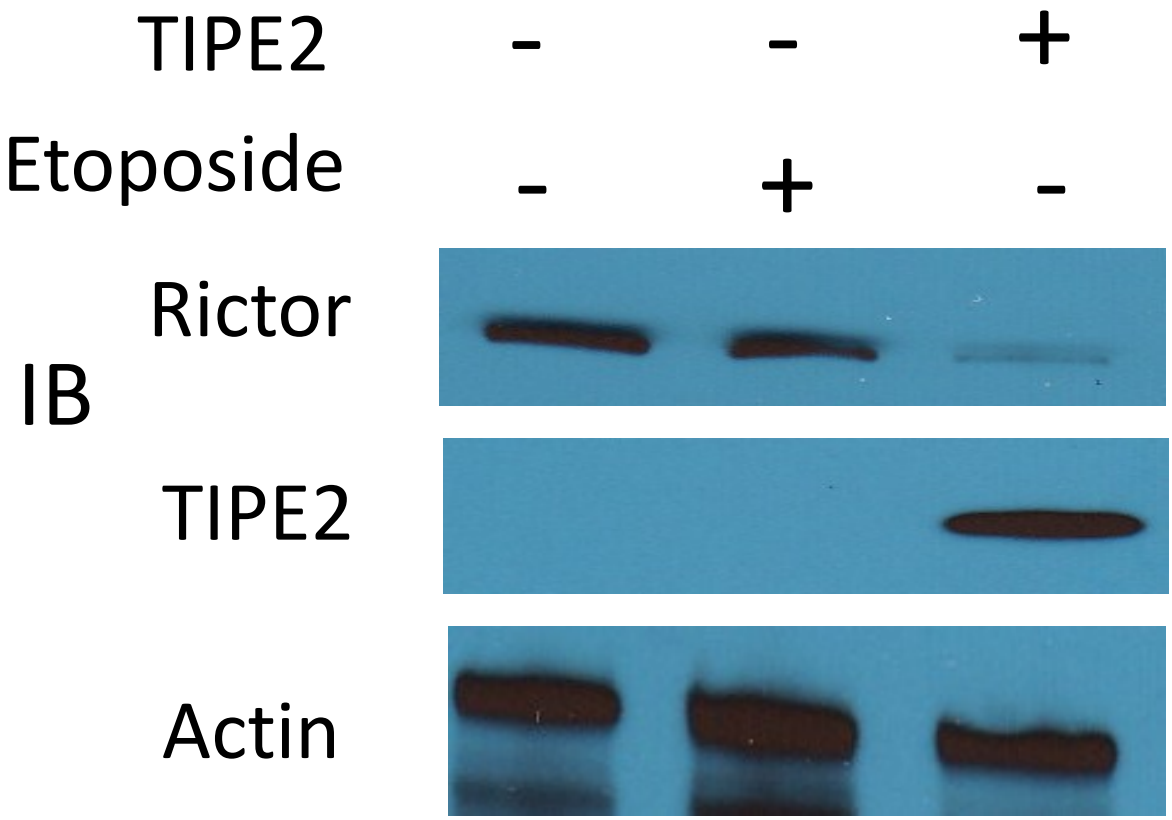


Figure 4.5 TIPE2 specifically affects Rictor levels independently of cell death. 293T cells were either treated with etoposide 10 uM 14h or transfected with TIPE2 24h. Following treatment cells were lysed and lysates were analyzed by western blot for the effect of death on Rictor levels.

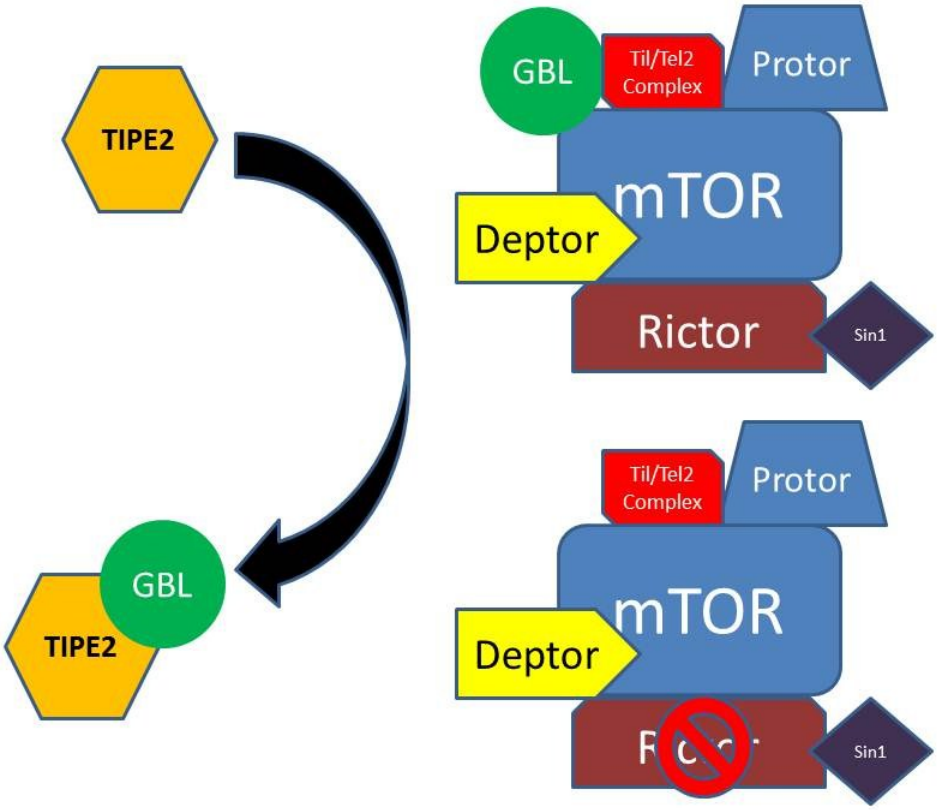


Figure 4.6 Proposed mechanism of TIPE2 Regulation of mTOR Signaling. Upon activation TIPE2 sequesters GBL away from mTOR complexes, resulting in the destabilization of mTORC2 and the subsequent destruction of Rictor and a loss of function in the mTORC2 complex.

Chapter 5

TIPE2 and Cell Death

Work from this chapter is from:

*Gus-Brautbar, *Y., Johnson, D., Zhang, L., Sun,H., Wang, P., Zhang, S., Zhang, L., and Chen, Y.H. (2012). The anti-inflammatory TIPE2 is an inhibitor of the oncogenic Ras. Molecular Cell *45*, 610–618.

*These authors contributed equally to this work

Along with additional unpublished work, intended for publication in the near future.

Abstract

Cell death is a central process crucial to the proper functioning of the immune system; and dysregulation of cell death can result in diseases of both severe inflammation and immunodeficiency. TIPE2 has been previously shown to regulate AICD in CD4+ murine T cells. Additionally a role for TIPE2 in the AKT and mTOR pathways, both crucial for survival has been established. Here the role of TIPE2 in regulating cell death was investigated. Overexpression of TIPE2 is toxic to cells in a dose dependent manner. Coexpression of TIPE2 alongside RGL1, AKT, PDK1, or mTOR, had the effect of reducing TIPE2 mediated cell death. Surprisingly S6K enhanced TIPE2 mediated cell death, indicating that it may play a role in activation of TIPE2. Even more surprisingly when TIPE2 is coexpressed with RGL1 and the GBL subunit of the mTOR pathway a massive amount of cell death is induced, indicating that TIPE2 may play a role in connecting the Ral and mTOR pathways.

Introduction

Cell death is a fundamental biological process that is highly regulated and absolutely crucial for the proper development and functioning of all multi cellular organisms. When the death machinery is not functioning appropriately multiple different disease states can arise, many of which are related to a loss of control within the immune system.

In a typical immunological response to a foreign antigen, the immune system will first recognize the presence of a foreign invader, it will make a decision as to the best way to combat this foreign invader, it will begin to multiply whatever specific pieces of the immune system are needed to combat the invader (T Cells, B Cells, etc.), and following clearance of the foreign antigen the immune system will allow for the orderly destruction of the majority of the newly created immune cells (Figure 5.1). If these immune cells fail to properly die off, they can begin to non-specifically attack any number of host cells within the body, causing a variety of inflammatory disease (Chen 2011).

Within T cells a common function known as activation-induced-cell-death is a well characterized process in which a T cell that is subject to multiple stimulations of its T cell receptor undergoes apoptosis and commits suicide (Ashwell 1987, Smith 1989). This functions as a form of protection since a T cell which is routinely encountering its cognate antigen is far more likely to be attacking a “self” molecule, instead of a foreign invader. The primary way in

which this occurs is via Fas/FasL (discussed in Nagata 1997 and Maher 2002). Upon activation a T cell will upregulate Fas, which is a cell surface protein which, when bound by Fas ligand (FasL) rapidly induces apoptosis within the cell. Additional mechanisms of AICD may include p73, IkBaM, TRAIL, and Bim (Reviewed in Strasser 2009, Kaufmann 2011).

Types of Cell Death

Cell death is a complicated process, and there are multiple types of cell death. The differing types of cell death can be divided into one of three general categories; apoptosis, necrosis, and autophagic cell death.

Apoptosis is a type of programmed cell death in which a suicide program is activated by the cell. Apoptosis can be triggered by several different pathways, but is morphologically characterized by several distinct steps:

1.) Cell Shrinking and rounding.
2.) Condensation of the chromatin.
3.) Fragmentation of the DNA in the nucleus.
4.) Breakdown of the plasma membrane and budding away into “blebs”.
5.) Cellular contents are loaded into and removed by the blebs, to later be phagocytosed and recycled.

In a human billions of cells self-terminate by apoptosis each and every day (Elmore 2007). If cells become defective and unable to properly apoptose, a variety of diseases can result. Some of these include autoimmune and inflammatory diseases if the immune system cannot properly induce apoptosis in

inflammation producing cells (Chen 2011). Numerous forms of cancer can also result from incomplete or ineffective apoptosis (Cotter 2009). If cells apoptose too easily certain types of neurodegenerative disease may result, as well as immunodeficiency.

While apoptosis is a type of pre-programmed cellular suicide that all cells are capable of undergoing, necrosis is an “unplanned” form of cell death in which acute injury or toxicity causes the cell to die catastrophically. The end result of apoptosis is the loading of cellular contents into small blebs of membrane, to be later phagocytosed and recycled, while the end result of necrosis is usually an uncontrolled spillage of cellular contents into the general circulation. This uncontrolled spillage can have varying effects, amongst them it can be highly toxic to adjacent cells, and can also be highly immunogenic. Morphologically necrotic cells oftentimes appear to be swollen and puffy. This is due to a lack of cohesion off their plasma membrane, and they become swollen full of fluid.

Autophagy is a catabolic process whereby a cell will begin to degrade its own internal components in order to generate essential nutrients. This most often occurs in response to starvation or stress within the cell. While a variety of autophagic processes and triggers exist, they all result in the degradation of internal proteins and organelles by the lysosome. While autophagy always occurs at a low basal level, under certain conditions such as starvation or stress it can be upregulated to cause a larger bulk degradation. In many circumstances a large buildup of autophagosomes, as well as heavy upregulation of autophagic

machinery is strongly correlated with cell death (Codogo 2005, Tsujimoto 2005). It is still not entirely clear exactly how autophagy can cause cell death.

TIPE2 has previously been established to regulate AICD in primary murine CD4+ T cells (Sun 2008). Due to the central role of appropriate cell death in regulating the immune system, as well as the central role of TIPE2 in regulating the immune system, the role of TIPE2 in regulating cell death was investigated.

Materials & Methods

Cell Lines and Plasmids

RAW 264.7 and HEK 293T cells were purchased from ATCC. Cells were grown in DMEM supplemented with 10% FBS, penicillin and streptomycin. mTOR, Rictor, Raptor, myr pdk1, AKT, S6K1, and S6K2 plasmids were purchased from Addgene. TIPE2-Flag plasmid was used as previously described (Sun 2008).

Cell Death Quantification

The 293T cells, $0.5x10^6$/dish, were plated in 6-cm dishes and transfected with various plasmids. All transfections were carried out using FugeneHD reagent (Roche) according to the manufacturer's instructions. 24 hrs later, supernatant was collected, and adherent cells were trypsinized and mixed with the supernatant. Cells were centrifuged (1000 rpm, 10 minutes), resuspended in equal volume of media, and stained with trypan blue. Dead and live cells were counted on a hemocytometer. Four fields of the hemocytometer were counted and then averaged for each sample.

Etoposide Treatment

Etoposide was purchased from Sigma (St. Louis, MO) part #E1383-25MG. Cells were treated with 50 uM etoposide for the indicated times.

Immunoblotting

Cells were lysed in Cell Lytic M cell lysis buffer (Sigma St. Louis, MO) and total protein concentration determined via Bradford analysis. 30 μg protein was loaded to each lane, and separated by SDS-PAGE. Protein was transferred to a nitrocellulose membrane, and was blocked with 5% milk in TBST. The membrane was then probed with the following primary antibodies overnight at 4 ^{0}C: Phospho–AKT (Serine 473, 1:1000, Cell signaling), total AKT (1:1000, Cell Signaling), Myc (1:1000 Cell Signaling), Flag (1:1000, Sigma. Detection was performed using enhanced chemiluminescence of HRP-conjugated secondary antibodies (anti-mouse or anti-rabbit IgG, 1:1000, GE healthcare).

Results

An initial observation was made that TIPE2 expression is upregulated in dying cells (Figure 5.2). Since TIPE2 could either be upregulated to assist in inducing cell death, or could be a protective protein upregulated in order to try to save dying cells, this effect was further investigated. In order to determine the role of TIPE2 in regulating cell death, flag tagged TIPE2 was overexpressed at increasing concentration in HEK 293T cells, and cell death was quantified at 24 hours (Figure 5.3). Overexpression of TIPE2 killed cells in a dose dependent manner. In order to identify which pathway(s) TIPE2 was affecting in order to kill cells, TIPE2 was co-transfected alongside multiple constructs expressing genes from both the Ral and mTOR pathways which TIPE2 has previously been linked to (see chapters 3 and 4 of this dissertation for more information on these pathways). Since Ral has been linked to numerous survival and proliferation pathways,TIPE2 was first co-expressed alongside Ral A and Ral B, these protein failed to compensate for TIPE 2 induced death. In order to ensure that both of the Ral Isoforms were active, constitutively active forms of both Ral A and Ral B were also co-expressed alongside TIPE2 (Figure 5.4A). These also failed to provide any rescue from TIPE2 induced death. Because TIPE2 inhibits RGL1, and RGL1 has other functions in addition to acting as a RalGEF, TIPE2 was coexpressed alongside RGL1, which provided a rescue of approximately 60% of the TIPE2 induced death (Figure 5.4B). Since RGL provided a rescue, and because RGL1 can function as a scaffold to bring PDK1 into contact with its target AKT, we next decided to test if active forms of PDK1 and AKT were capable of rescuing from TIPE2 induced cell death (Figure 5.4A). Both myristolated PDK1 (an activating mutation) and a constitutively active AKT were capable of providing a rescue from TIPE2 induced cell death equal to that of

RGL1. To confirm that the scaffolding activity of RGL1 was necessary for rescue from TIPE2 induced cell death a mutant of RGL1 was created in which the N terminus was truncated. The N terminus of RGL1 is required to scaffold for PDK1 and AKT, but is not required for its GEF activity. This N terminal deletion mutant which could not activate AKT was also not able to rescue from TIPE2 induced cell death (Figure 5.4B). Finally, in order to confirm the central importance of dysregulated AKT in TIPE2 induced cell death, a dominant negative construct of AKT was coexpressed alongside TIPE2 (Figure 5.5A). If TIPE2 were killing in an AKT independent fashion, death induced by a dominant negative AKT would be additive with death induced by TIPE2. If TIPE2 were killing via inhibition of AKT, expression of TIPE2 alongside dominant negative AKT should provide no further cell death over dominant negative AKT alone. These conditions were tested and there was no additive effect, indicating that TIPE2's ability to kill cells is via its inhibition of AKT. To further confirm the central role of AKT in regulating TIPE2 induced cell death, the level of AKT phosphorylation was checked in a variety of rescue conditions (Figure 5.5B), further supporting the importance of AKT.

While the activation of AKT by RGL1 is always occurring in cells, the primary method by which AKT is activated is via the MTORC2 complex, which is a complex composed of mTOR, GBL, Rictor, Protor, and Sin1. Since TIPE2 has a negative regulatory effect that occurs intermittently with mTOR, and always with Rictor, we needed to determine if this method of AKT activation was also involved in TIPE2 induced cell death. Overexpression of mTOR alongside TIPE2 rescues cells from death to a similar degree as RGL1, active AKT, and myristolyated PDK1 (Figure 5.4A). Since AKT can lie both upstream and downstream of mTOR, depending on which cellular signaling events are active at

any given time, we decided to test some additional downstream mTOR substrates in order to try to further hone in on which pathways were being dysregulated by TIPE2. p70S6K is a well-known and studied downstream mTOR effector. It is involved in ribosome biogenesis and protein synthesis, and lack of all S6K isoforms leads to marked reduction in viability (Pende 2004). While we hypothesized the co-expression of TIPE2 and S6K would either help to rescue from TIPE2 induced death, or have no effect, S6K and TIPE2 co expression instead increased cellular death (Figure 5.6). This effect was unexpected, so all previous rescue experiments were repeated, and only RGL1 could provide a slight rescue from TIPE2+S6K induced death (Figure 5.7). TIPE2+S6K+mTOR actually led to slightly more death than TIPE2+S6K alone (Figure 5.7).

In order to identify the type of death occurring in cells transfected with TIPE2, lysates were checked for cleaved caspase 3 and supernatant was checked for HMGB1. Neither was readily detectable indicating that cell death was not occurring via a canonical apoptotic or necrotic pathway.

Discussion

TIPE2 induces death in cells, and the mode of killing was investigated via two different approaches. The first approach was to check for common apoptotic (cleaved caspase 3) and necrotic (HMGB1) markers in order to identify what type of cell death TIPE2 was inducing. Neither cleaved caspase 3 nor HMGB1 were readily detectable, indicating that a third type of cell death, or a combination of different types of death are occurring in cells overexpressing TIPE2.

The second approach was to use co-expression analysis in order to identify which signaling pathway(s) were involved in TIPE2 induced cell death. Full Length RGL1 as well as a variety of truncation mutants were co-expressed alongside TIPE2, but only full length RGL1 was capable of rescuing from TIPE2 induced cell death. Since full length RGL1 can function as both a GEF and a scaffold, each function had to be tested in order to see which was responsible for rescuing from TIPE2 induced cellular death. In order to test whether GEF activity was responsible for the rescue an RGL mutant was created lacking the N-terminal region, which functions as an autoinhibitory region of RGL, resulting in an RGL mutant with higher than normal GEF activity. This region is also crucial for RGL scaffolding activity. In addition both wild type and constitutively active forms of Ral A and Ral B were co-expressed alongside TIPE2. Neither of these approaches were able to rescue from TIPE2 induced cell death (Figure 5.4), indicating that despite the well-known pro-survival functions of the Ral proteins, that TIPE2 mediated inhibition of RGL GEF function, and thus downstream Ral A and Ral B activity, were not responsible for the TIPE2 induced cell death. With inhibition of the GEF activity of RGL ruled out as the method by which TIPE2 induces cell death, it was next necessary to determine that RGL1 scaffolding activity was responsible for this function.

We had already established that TIPE2 disrupts the scaffolding activity of RGL (figure 3.5), which brings the kinase PDK1 into close contact with its target AKT, allowing AKT to become phosphorylated and activated. In order to assess whether loss of PDK1/AKT was responsible for TIPE2 causing the death that it does, active forms of AKT and PDK1 were co-expressed alongside TIPE2. Both active AKT and PDK1 rescued from cell death to the same degree as RGL1, indicating that the AKT pathway was likely involved.

In order to further confirm that TIPE2 induced death was occurring through the AKT pathway, TIPE2 was co-expressed alongside dominant negative AKT. If TIPE2 were killing via a means other than AKT, TIPE2 expressed alongside dominant negative AKT would show an additive effect on total cell death. Alternatively, if TIPE2 were killing via inhibition of AKT, then expressing a dominant negative AKT alongside TIPE2 should not induce any additional cell death. The addition of dominant negative AKT to TIPE2 resulted in no additional cell death, confirming that TIPE2 kills via inhibition of the AKT pathway.

mTOR, another activator of AKT can also rescue from TIPE2 induced cell death, further supporting the idea that inhibition of the AKT pathway is how TIPE2 is killing cells. A dosage of TIPE2 corresponding to 1.5ug on the dose response curve in Figure 5.3 was used for nearly all of the signaling assays performed earlier in this work. Taken together, these data indicate that dysregulation of the AKT pathway, at least partially by inhibiting PDK1/AKT scaffolding provided by RGL, is responsible for the TIPE2 death effect on cells.

P70S6K is a kinase that transduces downstream signals to induce anabolism, cell growth, and protein synthesis. When overexpressed in cells, it confers a survival advantage to cells, but paradoxically when co-expressed

alongside TIPE2 it has a synergistic effect in which cell death with TIPE2+S6K is more severe than with TIPE2 alone. Co-expression of mTOR, the upstream activator of S6K, alongside S6K and TIPE2 results in an increase in death over TIPE2+S6K, despite the fact that mTOR co-expression alongside TIPE2 heavily reduces death induced by TIPE2. These data, taken together, indicate that S6K may be functioning as an upstream activator of the TIPE2 death “function”.

GBL and RGL are both proteins that TIPE2 interacts with and neither of which cause any toxicity to cells when expressed on their own or together. When co-expressed alongside TIPE2 however they induce a large amount of cell death. This is despite the fact that RGL can rescue from TIPE2 cell death when co-expressed individually alongside TIPE2. This indicates that TIPE2 may somehow be serving as a bridge between the mTOR and RAL pathways, possibly affecting AKT from two different directions. Additionally both mTOR and Ral can affect autophagy. Ral signaling promotes autophagy while mTOR signaling inhibits autophagy. It is possible that the cellular death seen from TIPE2 is from the interface of these two pathways, confusing the cell with both pro- and anti- autophagic signals. Therefore it is possible that TIPE2 induces some sort of autophagic cell death. More work will need to be completed in order to be certain of this.

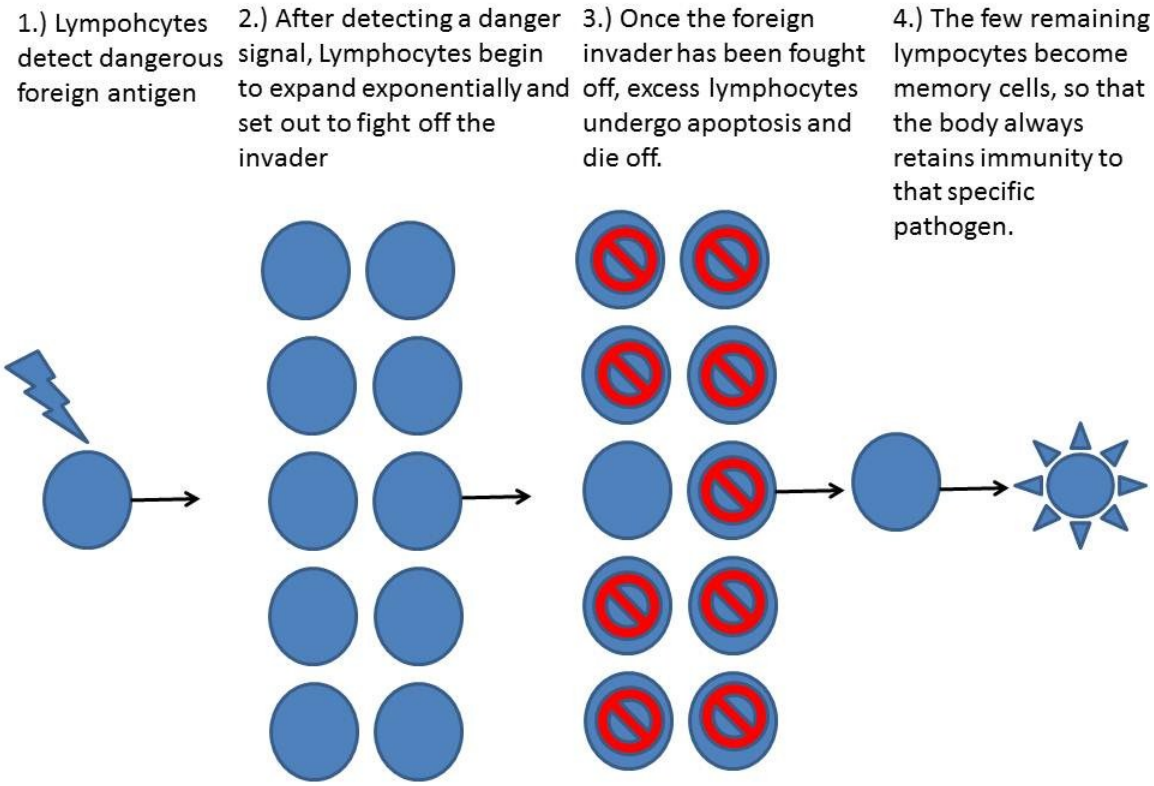


Figure 5.1 Mechanism of how lymphocytes respond to antigenic challenge, divide, and then die off.

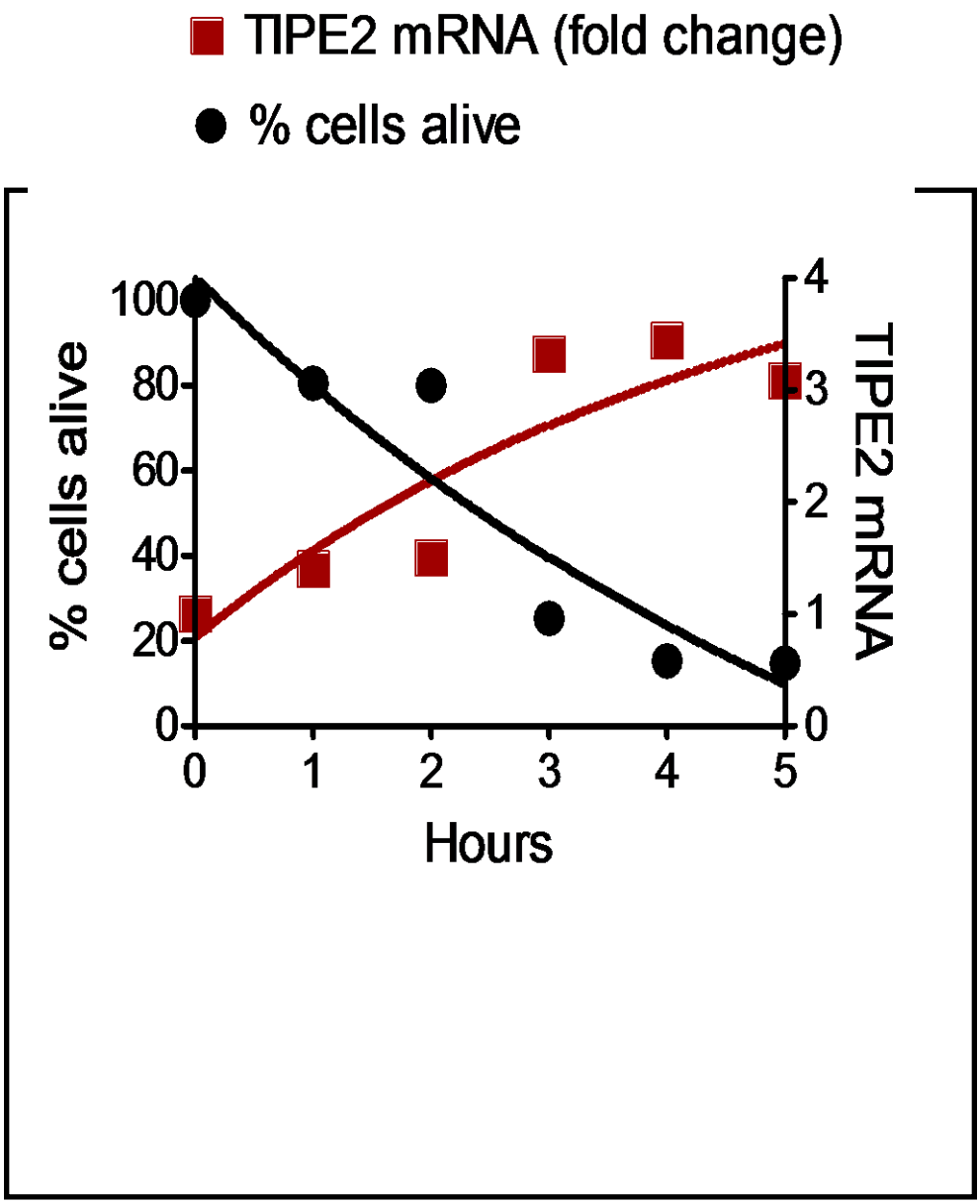


Figure 5.2 Association of TIPE2 Expression with Cell Death. RAW 264.7 macrophage were given a toxic amount of etoposide in order to induce apoptosis (Knethen 1998), and lysates were collected over time and TIPE2 message levels were checked with real time PCR.

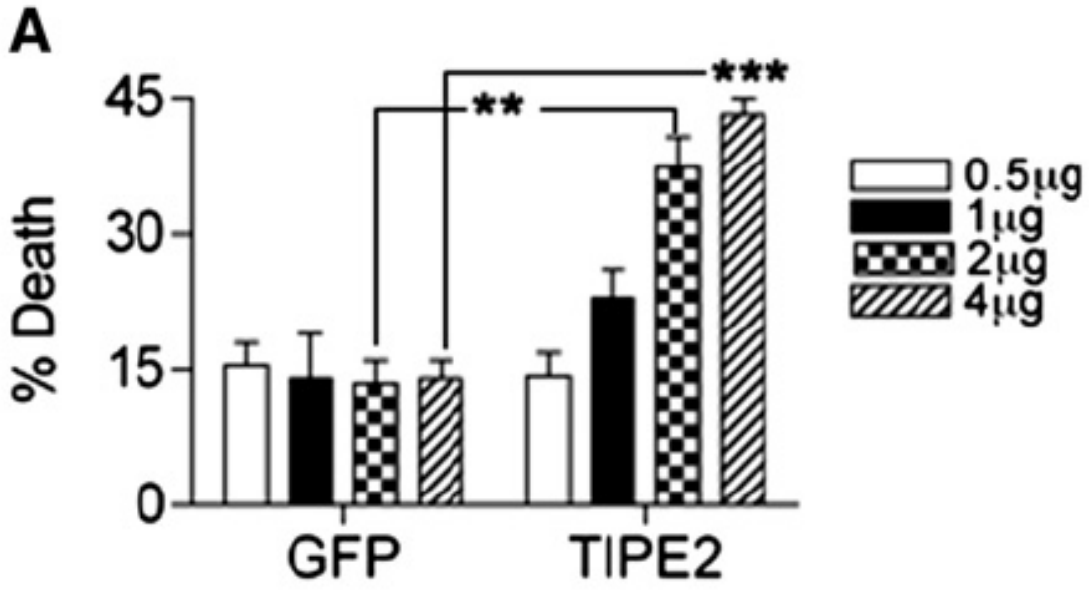
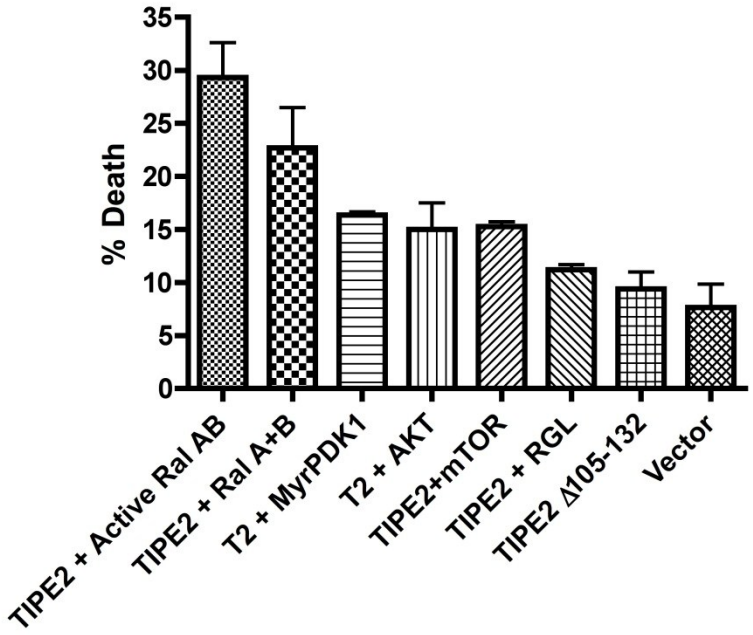


Figure 5.3 Overexpression of TIPE2 induces Cell Death. Increasing amounts of TIPE2 were overexpressed in 293T for 24 hours. Death was assayed at 24 hours via trypan blue staining.

A


% Death
35
30
25
20
15
10
5
0
TIPE2 + Active Ral AB
TIPE2 + Ral A+B
T2 + MyrPDK1
T2 + AKT
TIPE2+mTOR
TIPE2 + RGL
TIPE2 Δ105-132
Vector

B

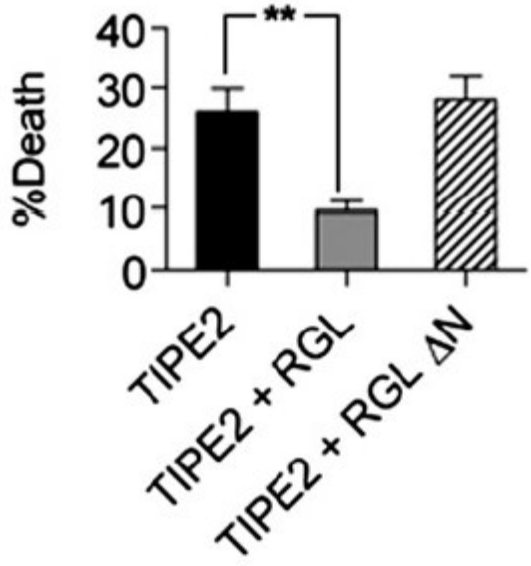
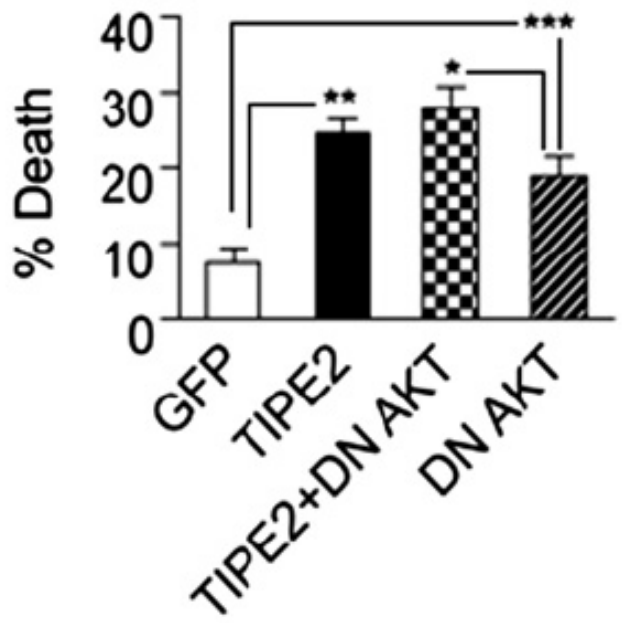

%Death
**
40
30
20
10
0
TIPE2
TIPE2 + RGL
TIPE2 + RGL ΔN

Figure 5.4 Rescue of TIPE2 Induced Death. A.) TIPE2 was transfected alone or with various plasmids from identified affected pathways. After 24h cell death was assayed using trypan blue staining. B.) TIPE2 death is rescued by full length RGL, but is no longer able to be rescued by an N-terminal truncation mutant that lacks the ability to bind to and scaffold for PDK1. C.) Western blot analysis of certain samples from A and B above showing a correlation between AKT phosphorylation and rescue from TIPE2 induced death.

A


% Death
40
30
20
10
0
**
*
***
GFP
TIPE2
TIPE2+DN AKT
DN AKT

B

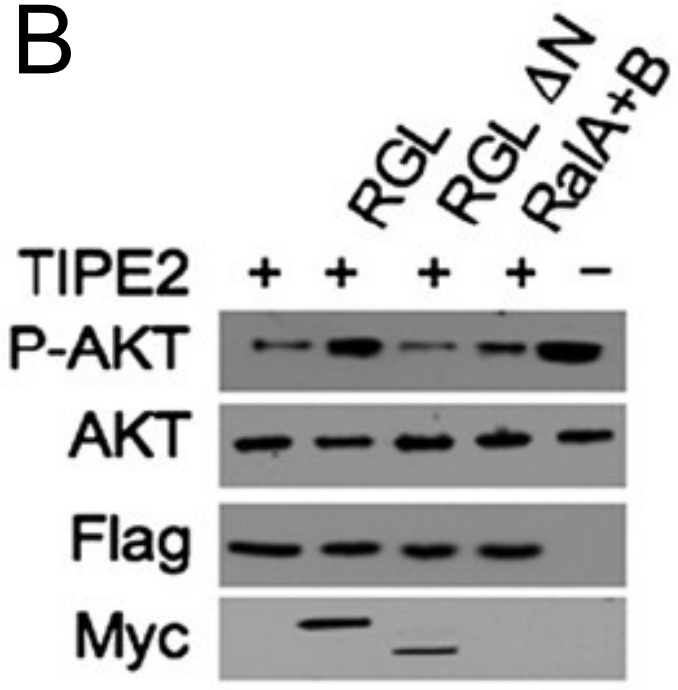
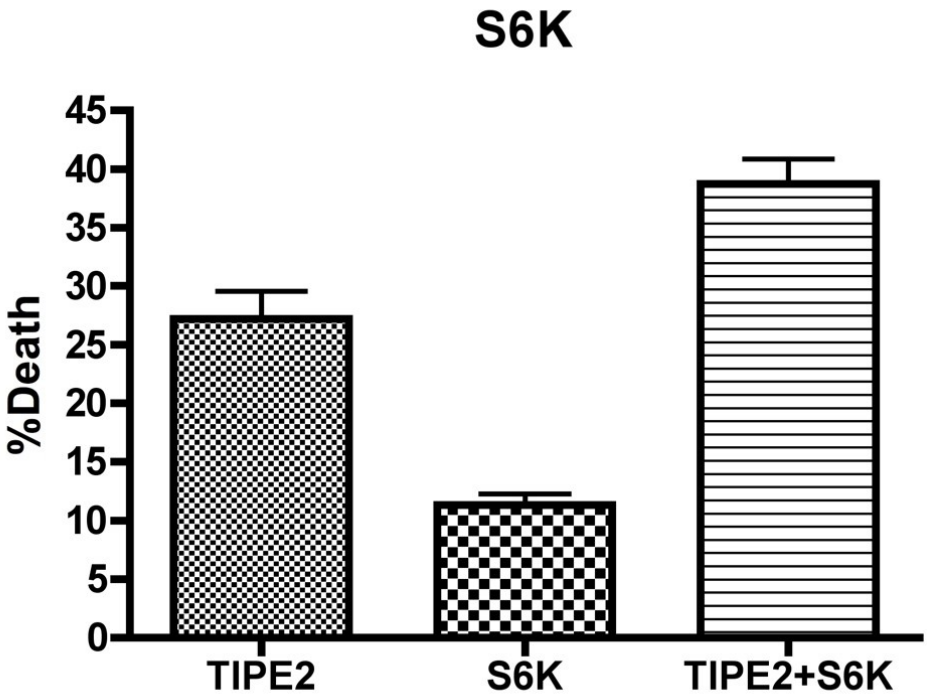

RGL
RGL ΔN
RalA+B
TIPE2 + + + + −
P-AKT
AKT
Flag
Myc

Figure 5.5 TIPE2 affects death by regulating the AKT pathway. A.) TIPE2 was expressed alone or alongside dominant negative AKT and cell death was quantified using Trypan Blue staining. B.) TIPE2 was coexpressed alongside various RGL constructs and levels of phosphorylated AKT (S473) were measured via western blotting.



5.6 S6K and TIPE2 Induce more Cell Death than TIPE2 alone. 293T cells were transfected with either TIPE2, S6K1, or TIPE2+S6K1. 24 hours after transfection cell death was assayed using Trypan blue staining.

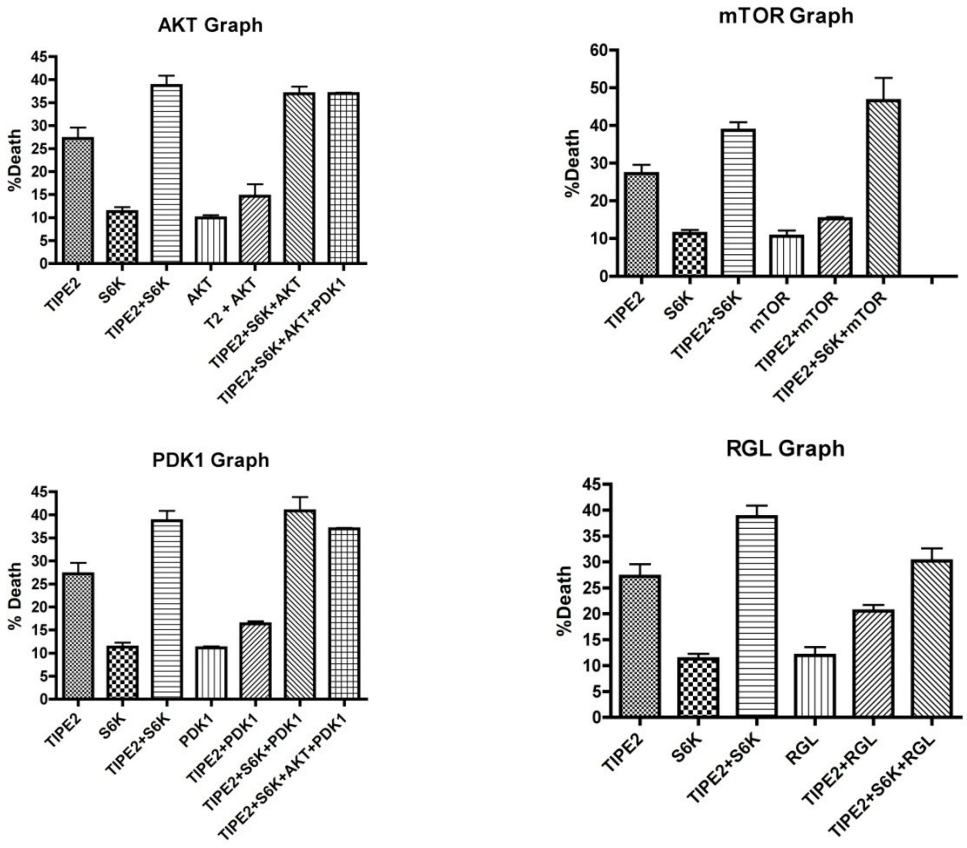


Figure 5.7 S6K and TIPE2 Rescue Attempts. TIPE2 and S6K1 were cotransfected alongside all plasmids which are capable of preventing TIPE2 –only induced cell death. AKT and PDK1 had no effect, while RGL1 still maintained a small rescue effect even in the presence of S6K. mTOR exacerbated the TIPE2+S6K death effect.

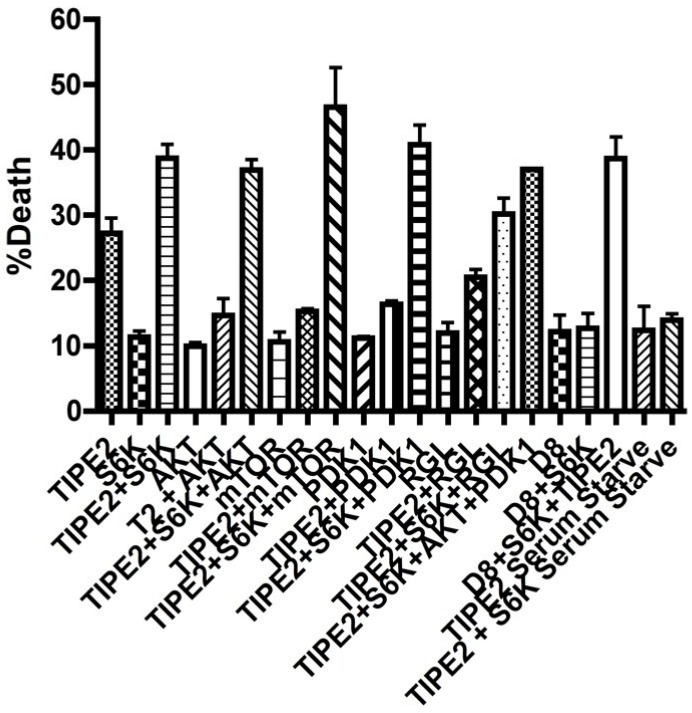
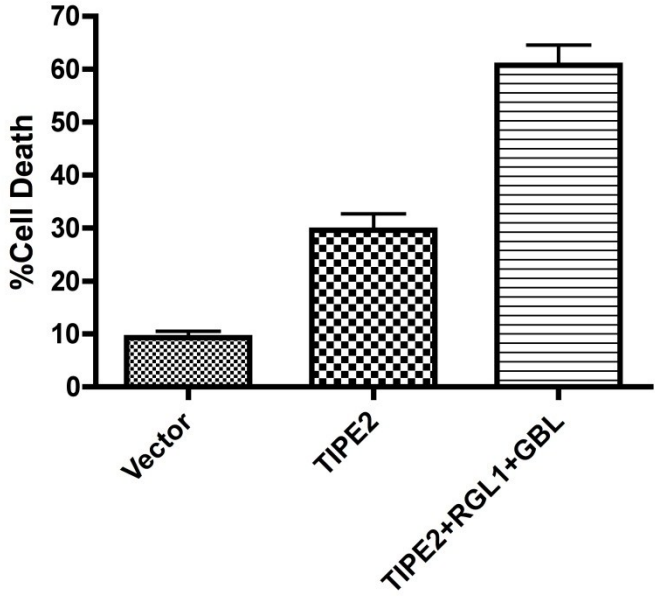


Figure 5.8 Compilation of S6K and TIPE2 Death Effects.



5.9 RGL and GBL synergize to cause cell death with TIPE2. 293T cells were transfected with either vector, TIPE2 alone, or TIPE2 with GBL and RGL1. After 24h transfection time cell death was measured using Trypan blue staining.

Chapter 6

General Discussion and Future Directions

The field of immunity and inflammation has changed tremendously over the past decade, and will continue to change at an ever increasing pace of discovery. TIPE2 plays a central role in regulation of the immune system, and is slowly garnering increased attention for this role.

Understanding how TIPE2 is regulated is crucial towards creating therapies around the immunomodulatory function of TIPE2. Depending upon the pathologic condition it may be desired to either have more or less TIPE2 expressed. For instance as a cancer treatment the expression of TIPE2 could be beneficial to either kill tumors, or to prevent them from metastasizing. In various types of immunodeficiency however, a reduction in TIPE2 level may be warranted, in order to reduce unnecessary death of lymphocytes. In chapter 2 we provided data indicating that TIPE2 is regulated at both the message and protein levels. TIPE2 message is likely regulated by an NF-KB induced microRNA, which further work in the lab has indicated is likely mir21. At the protein level TIPE2 interacts with βTRCP, and this interaction seems to be increased under conditions in which reduced TIPE2 protein is present in the lysate. These data, taken together, indicate that the SCF-βTRCP complex is the likely E3 ligase complex responsible for the destruction of TIPE2 protein.

Since TIPE2 levels drop at both the protein and nucleic acid level in response to detecting inflammation, the role of TIPE2 as a suppressor of inflammatory programming has been supported by this regulation profile. Data from human hepatocellular carcinoma patients from chapter 3 demonstrates that TIPE2 expression in non-immune tissues can minimally correlate, if not play a causative role in hepatocellular carcinoma. This may indicate a potential diagnostic role for TIPE2, using TIPE2 expression as an indicator for carcinogenesis, and possibly metastasis of transformed cells.

With this newfound knowledge of the dual forms of TIPE2 regulation, new treatments can be devised to specifically target TIPE2 levels within various cell types, either promoting or reducing TIPE2 stability. Likewise current drugs can now be reassessed for their effect on TIPE2 expression, possibly allowing for more effective use of said therapies. Hopefully this knowledge will result in a series of viable treatments with which to treat a wide array of different diseases.

Mechanistically TIPE2 appears to modulate the immune system (as well as other cell types and tissues within the body), by regulating key cellular processes involved in metabolism, survival, and polarization. By binding to RGL1 (and other RalGDS family members) TIPE2 is capable of exerting numerous potent effects on the cell, some of which are only in the infant stages of study. By binding RGL1 TIPE2 first and foremost prevents Ras from binding to and activating RGL1, thus inhibiting the activation of the small GTPase RalA and RalB. These GTPases are extremely pleiotropic, and could most aptly be described as regulators of polarization, although the reach of their regulatory events has a hand in nearly every cellular process. RalA is more heavily involved in polarized exocytosis as well as anchorage independent growth, while RalB has been more heavily tied to cell survival. Rapid polarization and recruitment of appropriate membrane proteins and remodeling of the actin cytoskeleton are hallmarks of all cells of the immune system. Without the capacity to modulate these factors the immune system would lack the plasticity required to rapidly adapt to constant onslaught of pathogens it faces. The connection between TIPE2 modulating the immune system via Ral is thus an obvious one, and **future work will be required in order to truly tease apart just how vital the Ral proteins are to the proper functioning of the immune system**. What is truly striking about TIPE2 however, is that it affects polarization

in multiple ways. In addition to inhibiting the activation of the Ral family of small GTPases, TIPE2 also binds to and inhibits the Rac1 small GTPase. Similar to the Ral proteins, Rac1 is a pleiotropic molecule with many downstream effectors, regulating processes as divergent (and important) as cell growth, adhesion, and motility. Rac1 additionally imparts a marked level of regulation upon the actin cytoskeleton, much like Ral A and B. Since the immune system by its very nature is migrating throughout the body and sampling the environment, molecules such as TIPE2 that regulate both the processes of sampling and migration are likely key central regulators for all of these cells.

Beyond its effect on the Ral proteins, the TIPE2 mediated inhibition of RGL1 reduces another important RalGDS family function, the scaffolding activity it performs to link PDK1 to its downstream kinase AKT. Being a central regulator of cell survival, the TIPE2 mediated reduction in AKT activation is a very important signaling event. TIPE2 is also connected to AKT via several other pathways. The TIPE2 binding partner Rac1 can interact with the kinase mTOR, which falls both upstream and downstream of AKT, and thus can both activate AKT and be activated by AKT. TIPE2 itself can weakly IP with mTOR, possibly via using Rac1 as an intermediary. And finally, and most importantly, TIPE2 interacts with GBL, the constant subunit of both mTOR signaling complexes. The mTORC2 complex is the primary upstream kinase of AKT, and a key component of this complex is the scaffold Rictor. TIPE2 markedly affects the level of Rictor present in cells, with overexpression of TIPE2 reducing it, and knockout cells showing increased levels. The most likely reason for this destruction is that TIPE2 sequesters GBL away from mTORC2 complexes, resulting in the destabilization of the complex and the subsequent proteolytic destruction of Rictor. GBL knockout MEFs have been shown to have this precise

phenotype. **The GBL sequestration mediated destruction of Rictor has yet to be demonstrated however, and will need to be completed in the future. An additional possibility is that TIPE2 could bind and sequester away Sin1, another protein that when lost can cause a loss of Rictor. Unpublished data from the Chen lab indicates that TIPE2 can interact with phosphatidic acid (PA). Since mTOR functions as a sensor for this molecule, it is possible that TIPE2 can also intersect with the mTOR pathway by binding to PA and modulating how mTOR can interact with the crucial signaling lipid.** As reported in Chapter 3, the entire mTOR pathway, which is regulated by TIPE2, is extremely crucial for the proper function and response of the immune system. With the affect that mTOR modulation has on appropriate T and B cell differentiation into effector and memory cells, **TIPE2 levels or activation may provide a key target during vaccinations in order to generate more robust memory cell production. To date nothing is known about TIPE2 and B Cell function, and this will have to be further studies in the future**. **It is also important to take note that overexpression of TIPE2 causes a loss of Rictor, and it can also, in certain conditions, cause a loss of mTOR and of RGL. It is thus possible that TIPE2 has some sort of degradatory function, or links certain target proteins to the cellular destruction apparatus. This will require further investigation to deduce how these TIPE2 binding partners or pathway partners are being degraded by the cell.**

With the mechanistic data presented so far it is easy to imagine how and why TIPE2 has such a dramatic effect on the regulation of the immune system. By affecting polarization via Rac and Ral, and by affecting the metabolic state via mTOR regulation, TIPE2 would have a dramatic effect on say, the proper expression of homing receptors and thus proper trafficking of a lymphocyte. Or,

perhaps, the proper recognition and engulfment of a particle, and acidification of a phagolysosome during phagocytosis. (Both of these phenotypes do occur). What is truly interesting though, are the effects of TIPE2 in the non-immune system. While not all cell types express TIPE2, some do, and most express at least one of the TNFAIP8 family members, each of which share high homology with one another. As we see in Chapter 3, TIPE2 is expressed by healthy liver cells, and appears to be silenced by transformed liver cells within the tumor. Since no spontaneous tumors have ever developed in knockout mice, this would imply that (at least in certain tissues) loss of TIPE2 is a necessary but not sufficient step to induce tumor formation. This makes assaying for TIPE2 levels a fantastic early diagnostic indicator for "at risk" cells. Reintroduction of TIPE2 may also be able to destroy already formed tumors. When it comes to cancer however, the effect of TIPE2 would seem to truly be central to metastasis, much more so than tumor formation itself. With TIPE2 regulating pathways involved in survival, metabolism, polarization, motility, and anchorage independent growth, TIPE2 literally regulates **everything** needed for effective metastasis. Thus, TIPE2 as a diagnostic may not only provide information regarding tumor formation, but may also provide extremely crucial diagnosis indicating likely metastasis. And again, an ability to reintroduce TIPE2, even if it were not able to destroy a solid tumor, may prevent that tumor from metastasizing and thus prevent the lion's share of damage and death associated with cancer progression. **More work with human tissues will be required to provide a definitive link between TIPE2 and cancer metastasis.**

It is also important to note that TIPE2 is associated with and regulated by inflammation, and in turn heavily regulates the mTOR pathway. Dysregulation of mTOR signaling as well as inflammation are both highly correlated with type II

diabetes and obesity. **It would be foolish to not investigate the potential role of TIPE2 in functioning as a bridge between inflammation and mTOR, thus possibly providing a new and unknown link between inflammation and nutrient signaling, and possibly providing us with another potential druggable target in the war against metabolic syndrome.**

Also important to note is that when RGL is coexpressed alongside GBL there is no perceived change in any cell viability or health measure. When TIPE2 is cotransfected alongside these two proteins they synergize and kill cells to an extremely high degree, much higher than TIPE2 alone. **Thus, it is possible that TIPE2 function as a bridge between the mTOR and Ral Signaling pathways, quite possibly by nucleating the formation of a new complex. The potential for a complex of this sort has particularly important ramifications on how we interpret autophagy in the future, since mTOR is a very strong negative regulator of autophagy, while Ral is a very strong positive regulator of autophagy. It is possible that TIPE2 is the critical piece that links these two pathways and thus determines whether autophagy should be initiated or not. TIPE2 cell death may thus be some sort of autophagic death due to it confusing the cell's normal autophagy system. This will require further investigation.**

It is rather remarkable that TIPE2 manages to regulate AKT via 3 putative mechanisms and on both of its activating residues. TIPE2 regulates the PDK1 mediated phosphorylation of T308 by inhibiting the scaffolding function of the RalGDS family, TIPE2 potentially affects mTOR itself via Rac1 or via direct binding, and TIPE2 negatively regulates the Rictor subunit of mTORC2, possibly via GBL sequestration. While the importance of AKT cannot be understated, the TIPE2 expression profile when viewed through the prism of cell survival and

metabolic regulation offered by AKT offers an interesting perspective on the role and purpose of TIPE2 within the immune system. To use a monocyte as an example; before activation a monocyte is primarily at rest, migrating through the body and sampling the environment. At this stage in the life of the monocyte TIPE2 is highly expressed, though not toxic, it is only offering a repressive function, preventing the monocyte from becoming highly active, creating a respiratory burst, etc. Upon activation the monocyte rapidly and specifically downregulates TIPE2 expression at both the message and protein level. With TIPE2 no longer present to inhibit metabolism via mTOR, and polarization via Ral and Rac1 the monocyte is free to rapidly migrate, express chemotactic receptors on its surface, and undergo heavily metabolic events like a respiratory burst. After some amount of time – which we have yet to determine experimentally in primary cells – TIPE2 expression is restored. In the now activated monocytes however, TIPE2 is no longer simply repressive, but toxic. This effect is supported by the fact that serum starving TIPE2 overexpressing cells halts any TIPE2 toxicity despite not affecting expression. Additionally TIPE2 only appears to be toxic in active and dividing cells. Cells transfected with TIPE2 that are already dense no longer die en masse, despite equal or greater levels of TIPE2 being expressed in the dense cells. **More work will need to be done to support this theory, such as fully tracking TIPE2 expression levels in primary cells, and using a variety of artificial activation conditions in conjunction with TIPE2 expression in order to fine tune exactly when and under what conditions TIPE2 proves to be toxic to cells, instead of just inhibitory.**

TIPE2 seems to regulate polarization and metabolism in multiple and important ways. These modes of regulation give TIPE2 a role in the regulation of cell survival, and motility. They provide a link between TIPE2 and cancer, and TIPE2 may minimally serve as a diagnostic indicator of transformation and metastasis, if not become a bona fide treatment for these stages of cancer development. TIPE2 regulation of mTOR and Ral have positioned it at a unique crossroads between “go” and “no go” signals for the progression of autophagy. This may result in TIPE2 playing a central role as an autophagic switch (Figure 6.1). Finally TIPE2 may be performing a function similar to AICD, in which it proves toxic to cells that have been activated, thus preventing a buildup of inflammation producing monocytes and lymphocytes following antigenic challenge and clearance. In conclusion we have only begun to scratch the surface of TIPE2 biology and its numerous functions within both the cell and the body. It will prove very interesting to see what we can take from TIPE2 in order to advance medical science and dramatically improve the health of people in the future.

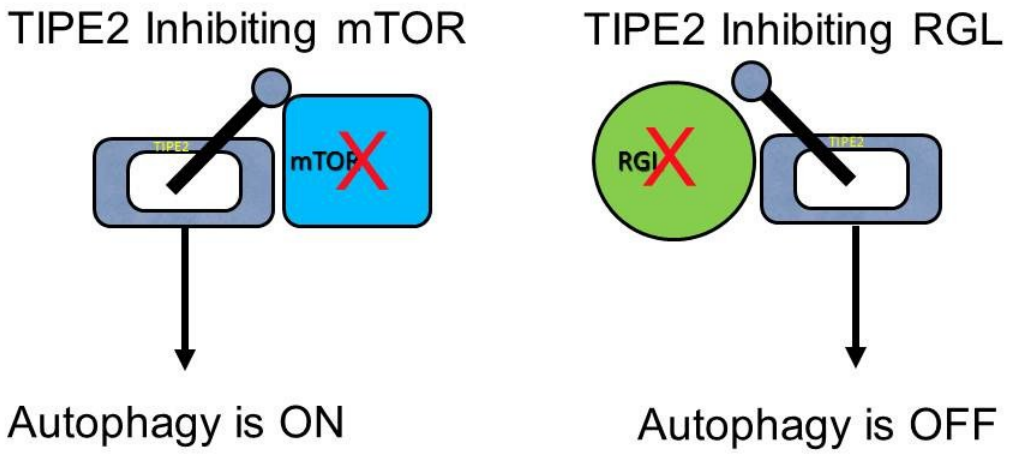


Figure 6.1 TIPE2 may function as an autophagy Switch. Since TIPE2 is capable of inhibiting the anti-autophagy mTOR pathway, and the pro autophagy Ral pathway, and TIPE2 clearly connects these two pathways via strange and incompletely defined phenotypes (chapter 4, chapter 5), it may function as a switch which is either actively inhibiting either mTOR in order to promote autophagy within the cell, or inhibit Ral in order to prevent autophagy within the cell.

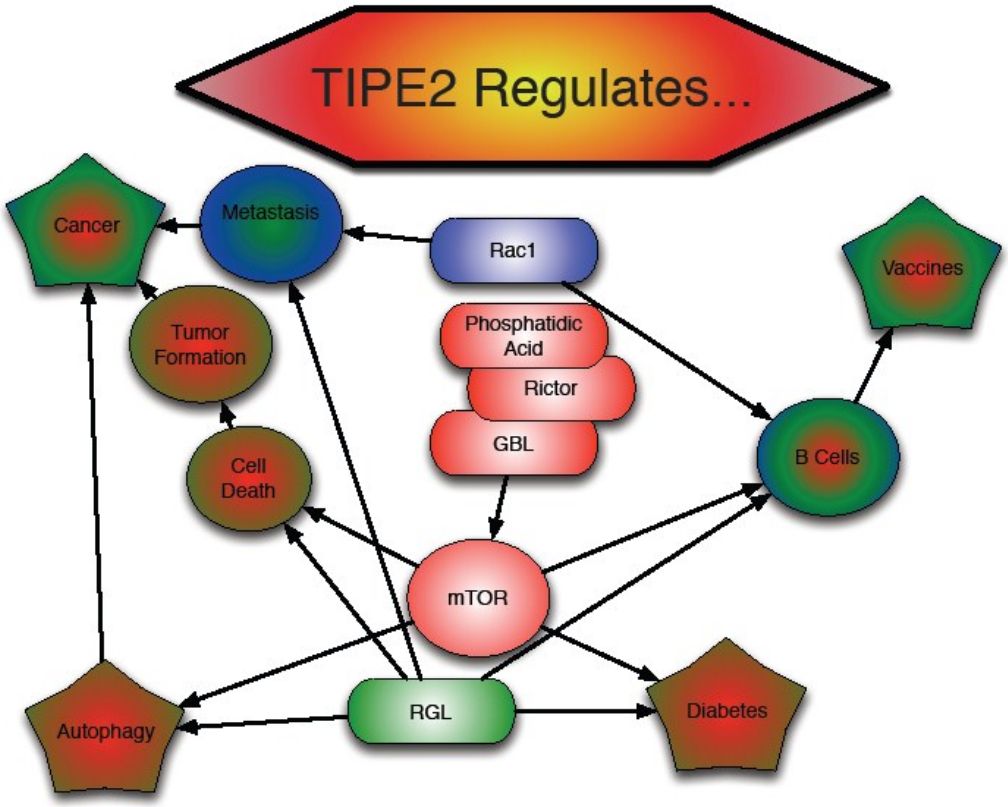


Figure 6.2 Cluster Map of TIPE2 interactions, functions, and potential functions. TIPE2 and RGL functions are highlighted in green, mTOR in red, and Rac1 in blue. Different potential diseases and therapies to investigate are then blurred by color based upon which of these pathways feed into them.

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