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Activation of Gαi3 and interacting protein, TNFAIP8, inhibitis TNFα-induced death and promotes transformation in mouse fibroblast

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Activation of Gαi3 and interacting protein, TNFAIP8, inhibits TNFα-induced death and promotes transformation in mouse fibroblast

Ben Laliberté

A thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the degree of Master of Science in Neuroscience

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Abstract:

Stimulation of dopamine D2S receptor introduced in Balb/c-3T3 cells induces Gαi3-dependent transformation. Gαi3 interacts with DED2-containing protein, TNFAIP8, in yeast mating and in FLAG-TNFAIP8 transfected Balb/c-3T3 cells. TNFAIP8 inhibits caspase-8 activity and is elevated in certain cancers as well as in metastatic, radiation resistant, chemo-resistant and angiogenic tumours. This study looks at Gαi3 activation of TNFAIP8 leading to the inhibition of TNFα-induced cell death in Balb/c-3T3 cells coexpressing D2S and either TNFAIP8 over-expression or TNFAIP8 antisense-knockdown with assays for foci formation, cell death and executioner caspase activation. The data showed D2S increases basal foci formation; this is blocked in TNFAIP8 antisense cells. D2S activation further increases foci formation; also completely blocked in TNFAIP8 antisense cells. D2S activation reduces cell death except in TNFAIP8 antisense cells. D2S activation reduces caspase-active cells. These results show that D2S mediated inhibition of caspase activity and death resulting in transformation is dependent on TNFAIP8.

Table of contents

List of Figures:

List of Tables

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Chapter 1

General Introduction

This project investigates the interaction between inhibitory Galpha (Gαi/o) proteins and a cytosolic protein, Tumor Necrosis Factor Alpha Inducible Protein 8 (TNFAIP8), which is reported to inhibit the extrinsic death pathway leading to apoptosis. Apoptosis is a form of programmed cell death (PCD) and is essential to development and maintenance of tissue homeostasis. The extrinsic death pathway is activated by death ligands binding to their cognate death receptors. Ligand bound death receptor is internalized by endocytosis where the death inducing signalling complex (DISC) is formed and caspase cascade to apoptosis begins. G-protein coupled receptors (GPCRs) transduce numerous cellular responses from a large variety of ligands across cell membranes by inducing GTP-binding to Gα subunits. This results in the activation of heterotrimeric G-proteins, which interact with their effectors to produce cellular responses. Here we intend to show how the activation of Gαi can inhibit the extrinsic death pathway by interactions with the effector, TNFAIP8.

Programmed Cell Death (PCD)

In the classical view of cell death, there was caspase-dependent apoptosis and necrosis. Necrosis was described as uncontrolled cell death characterized by plasma membrane rupture and cell content leakage resulting in inflammation of surrounding tissues. Apoptosis on the other hand, was highly controlled, resulted in cell rounding, detachment, nuclear condensation and the cell "blebbing" into vesicles that could be engulfed by macrophages thus preventing inflammation of the surrounding tissues. Recently, caspase-independent cell death (type III PCD) and programmed necrosis (type

II PCD) have emerged to expand the field of PCD research (Lorenzo HK and Susin SA, 2007). In this study, I investigate a novel inhibitory mechanism involved in caspase-dependent apoptosis (type I PCD), and therefore, the discussion shall focus on this form of PCD.

Caspase-Dependent Apoptosis

Caspase-dependent apoptosis is an evolutionarily conserved process that was first studied at the genetic and molecular level in C. *elegans* (Dive C et al., 1992). In mammals, two distinct caspase-dependent pathways leading to apoptosis have been identified. The intrinsic death pathway relies on the mitochondrial dysfunction for effecting cell death. The extrinsic death pathway relies on death receptors on plasma membrane, which transduce extracellular death signals. Both pathways involve adaptor proteins, caspases and Bcl-2 family proteins. In addition to these three classes of proteins, Bcl-2 inhibitors (BH3-only proteins) have been identified in C. *elegans* and subsequently in mammals (McDonald 3rd ER and El-Deiry WS, 2005). In mammals, new modulators of the death pathways continue to be discovered. Dysfunctions of the death pathways have been attributed to human diseases including cancer and neuro-degenerative diseases.

Note: A third, p53-induced nuclear pathway, activated by genotoxic stress has emerged, involves adaptor proteins, PIDD and RAIDD and activates CARD-containing initiator caspase-2 (Bao Q and Shi Y, 2007). This pathway is reported to be upstream of mitochondrial dysfunction. Since there are no reports of it being directly involved in TNFα signalling, it shall not be discussed further here.

Intrinsic Death Pathway

DNA damage and growth factor withdrawal are typical stimuli to initiate the intrinsic death pathway. Under these stress conditions, the permeabilization of the mitochondrial membrane is typically regulated by bcl-2 family proteins, with contributions also possible from caspase-2 (Gogvadze V et al., 2006). In the event of mitochondrial dysfunction, cytochrome c as well as other proapoptotic proteins are released to the cytoplasm. In the cytoplasm, cytochrome c associates with Apaf-1, an adaptor protein, and this induces the recruitment of ATP. Upon binding ATP, a conformational change occurs within Apaf-1 exposing the caspase activation and recruitment domain (CARD). Procaspase-9 also has a CARD and it is through these domains that Apaf-1 recruits the initiator caspase. Via CARD-CARD interactions, procaspase-9 associates with Apaf-1 (McDonald 3rd ER and El-Deiry WS, 2005). Three models have been proposed for how this leads to caspase-9 activation but the exact mechanism remains undetermined (Bao Q and Shi Y, 2007). Several procaspase-9, Apaf-1, cytochrome c and ATP complexes join to form the holoenzyme or apoptosome. Procaspase-9 is not cleaved and remains associated with the apoptosome while active (Bao Q and Shi Y, 2007). The apoptosome propagates apoptosis signalling by cleaving and activating executioner caspases (i.e. caspase-3). The reliance of this pathway on mitochondrial dysfunction distinguishes it from the extrinsic death pathway.

Extrinsic Death Pathway

In the extrinsic death pathway, homotrimeric death ligands bind the homotrimeric death receptors, which eventually leads to clathrin-dependent receptor internalization and conformational changes exposing the receptors death domain (DD) to binding of FADD through its DD (Schutze S et al., 2008). FADD also has a death effector domain (DED), as does caspase-8/10. Interactions of these domains allows for the formation of the death inducing signalling complex (DISC), which facilitates the activation by dimerization. Autocatalytic cleavage of initiator caspase (caspase-8/10), stabilizes of the dimer (Boatright KM et al., 2003)(Donepudi M et al., 2003). The initiator caspase then activates the executioner caspase (caspase-3), which commits the cell to death. The ability of the extrinsic death pathway to induce cell death is tissue dependent and sometimes requires mitochondrial amplification, which can be achieved by caspase-8 cleavage of Bid (McDonald 3rd ER and El-Deiry WS, 2005). Other mechanisms for crosstalk between these pathways have been proposed.

Caspases

In the apoptosis pathways, caspases are proteolytic enzymes with multiple targets including: 1) other **caspases,** which amplifies death signalling; 2) **structural targets** related to cytoskeletal elements such as plectin, which induce cell membrane blebbing and cell shrinkage; and 3) **nuclear targets** and DNA repair targets such as PARP that lead to irreparable DNA cleavage and fragmentation. Caspases are named for their cysteine residue in the active site and their substrate specificity for cleavage following aspartic acid residues (McDonald 3rd ER and El-Deiry WS, 2005). Caspases are

expressed in all tissues with higher levels of initiator caspase found in lymphoid and myeloid derived cell lines (Genomics Institute of the Novartis Research Foundation - SymAtlas, Online: http://symatlas.gnf.org/SymAtlas/).

There are two important classes of caspases in mammalian systems with generally distinct functions. The initiator caspases are activated by external (extrinsic) or internal (intrinsic) signals via adaptor proteins. These initiators then activate by cleavage the executioner class of caspases, which then effect cytoskeletal changes and DNA fragmentation necessary for apoptosis. Some exceptions to this do exist, such as initiator caspases targeting plectin, a cytoskeletal protein target (McDonald 3rd ER and El-Deiry WS, 2005). Also, executioner caspases may activate other caspases in a similar fashion to initiator caspases thus amplifying the effect. In general, adaptor proteins activate initiator caspases and these activate executioner caspases.

Structurally, caspases are translated in a precursor form with a regulatory prodomain, a large and small subunit (Fig 1). In executioner caspases, the prodomain is cleaved off by initiator caspases, as are the small and large subunits cleaved from one another. A heterotetramer of the small and large subunits form the holoenzyme (McDonald 3rd ER and El-Deiry WS, 2005).

For initiator caspases, caspase-2 and caspase-8, dimerization is necessary for their activation. This is accomplished by proximity effect induced by activated adaptor proteins RAIDD and FADD in the PIDDosome and DISC respectively (Bao Q and Shi Y, 2007). In the case of Procaspase-8, it is recruited to the death ligand/receptor-FADD complex forming the death-inducing signalling complex (DISC). The DISC is sufficient to induce the caspase activation cascade. Procaspase-8 while in association with the

DISC becomes dimerized due to close proximity. Dimerization is sufficient for caspase-8 activation. Procaspase-8 is then cleaved by autocatalytic cleavage producing dimerized caspase-8. Cleavage stabilizes the catalytically active dimer (Boatright KM et al., 2003) (Donepudi M et al., 2003).

However, the activation of procaspase-9 is not fully understood. While dimerization of procaspase-9 increases activity, it is not equivalent to the activity induced by procaspase-9 association with the apoptosome. Consequently, there are presently three models for caspase-9 activation involving simple dimerization, monomeric procaspase-9 association to the apoptosome and multi-homodimeric association to the apoptosome. The prodomain is not cleaved from caspase-9 and it remains associated with apoptosome while active (Bao Q and Shi Y, 2007).

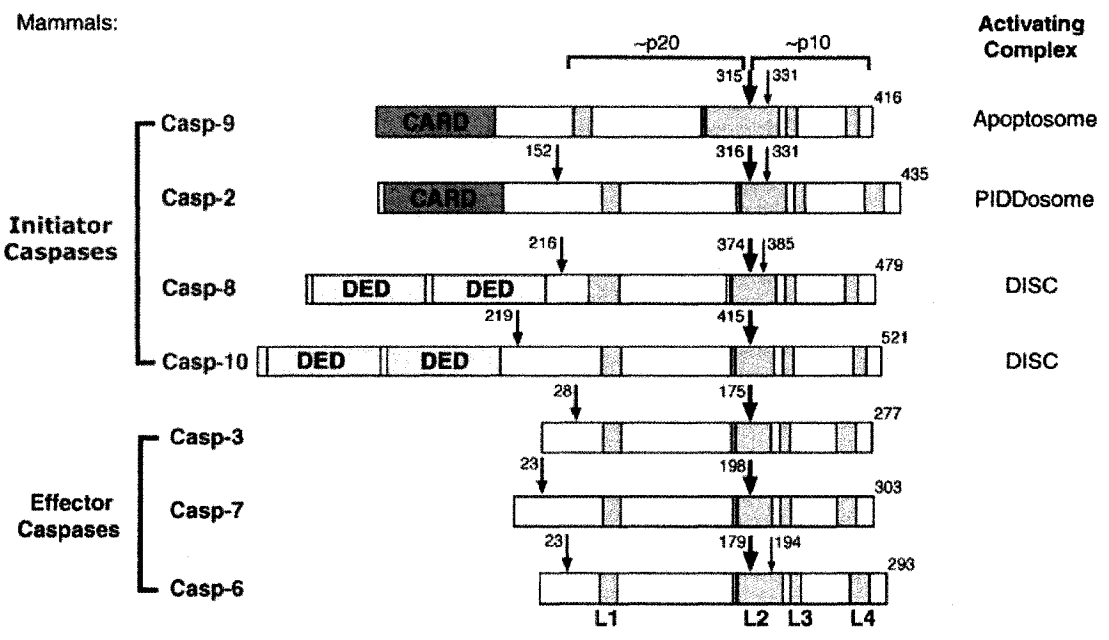


Figure 1. Apoptotic caspases. Six initiator caspases and four effector caspases are drawn to scale. The position of the first intrachain cleavage (between the large and small subunits) is highlighted by a large arrow whereas additional cleavages are represented by medium and small arrows. The prodomains in initiator caspases invariably contain homotypic interaction motifs, such as the CARD and the DED.33 The four surface loops, labeled L1 through L4, form the active site of a caspase.7 The catalytic cysteine residue is shown as a red line at the beginning of loop L2. The p20 and p10 subunits together form a caspase monomer (Reprinted with permission from Macmillan Publishers Ltd: Cell Death Differ. 14(1):56-65 Bao Q and Shi Y, Copyright 2007).

Inhibitors of Caspases

Inhibitors of caspases have been identified in mammalian cells as well as in Drosophila. The inhibitor of apoptosis (IAP) class of proteins block dimerization of caspases-3,-7 and -9 and in some cases induce ubiquination of caspases-3, and -7. IAPs were initially of interest because some are elevated in certain forms of cancer and are believed to contribute to resistance to cell death (Srinivasula SM and Ashwell JD, 2008). However, since their discovery, numerous other functions have been assigned to IAPs including mitotic chromosome segregation, cellular morphogenesis, copper homeostasis, NF-κB activation, and MAP kinase signaling (Srinivasula SM and Ashwell JD, 2008).

There are eight proteins in this family found in humans that exhibit some or all of these functions including Survivin, XIAP, ILP-2, ML-IAP, c-IAP1, c-IAP2, NAIP and BRUCE (Srinivasula SM and Ashwell JD, 2008). The IAPs contain baculovirus IAP repeats (BIR) domains and linkers that provide specificity for caspase inhibition. In some cases, they also contain a RING domain that provides E3 ubiquitin ligase function. This ligase function allows for autoubiquination in response to glucocorticoids or etoposide and favours apoptosis. XIAP and c-IAP2 ubiquinate caspases leading to their proteosomal degradation, and thus inhibiting apoptosis. c-IAP1 and c-IAP2 bind to the TNFR2 receptor complex and have been shown to inhibit caspase-3 and -7. No IAP has been shown to inhibit DED-containing caspases (McDonald 3rd ER and El-Deiry WS, 2005).

Some inhibitors of caspase-8 have also been identified. The first to be identified have viral sources and serve to enhance host survival in response to an attacking immune

system. These viral caspase-8 inhibitors include CrmA, vICA and 14.7K adenovirus protein (McDonald 3rd ER and El-Deiry WS, 2005). CrmA interacts directly with caspase and serine protease classes at their catalytic sites (Swanson R et al., 2007). vICA constitutively associates with the prodomain of procaspase-8 and interferes with assembly to FADD (Goldmacher VS, 2005). On the other hand, it was recently reported that 14.7K inhibits TNFR1 internalization and subsequent DISC formation thus not interacting directly with caspase-8 (Schneider-Brachert W et al., 2006). In addition to these viral inhibitors are the DED-containing viral FLICE-inhibitory proteins (v-FLIPs). Based on crystal structure and protein mutation studies, the tandem death effector domains (DED1 and DED2) of theses proteins compete with FADD and caspase-8 DED domains thus inhibiting DISC formation and caspase-8 activation (Li F-Y et al., 2006).

Following the discovery of viral FLIP proteins a family of cellular FLIP proteins (c-FLIP) were identified. Splice variants of the c-FLIP gene produce c-$FLIP_L$, c-$FLIP_S$ (not found in mice) and c-$FLIP_R$. c-$FLIP_S$ is well established as an antiapoptotic protein (Thome M and Tschopp J, 2001). However, at physiological levels in some tissues, c-$FLIP_L$ has a proapoptotic function by forming a heterodimer with caspase-8 and requiring its enzymatically inactive C-terminal (Micheau O et al., 2002) (Chang DW et al., 2002). When it is over-expressed or amplified in cancer, it has an antiapoptotic function (Chang DW et al., 2002)(Sharp DA et al., 2005)(Safa AR et al., 2008). PEA-15 is another DED-containing protein that inhibits death signalling by disrupting FADD and caspase-8 interactions or regulates ERK/MAPK signalling depending on its phosphorylation state (Hao et al., 2001)(Renganathan H et al., 2005)

In addition to FLIPs and PEA-15, a new DED2-containing inhibitor of caspase-8 activity has been identified as Tumor Necrosis Factor Alpha Inducible Protein 8 (TNFAIP8) and it is this protein that is the focus of my investigation (Kumar D et al. 2000) (You Z et al., 2001). A detailed introduction of TNFAIP8 is provided below. It should also be mentioned, that a DED2-containing TNFAIP8 family member, TIPE2, has been identified as proapoptotic regulator of immune homeostasis in myeloid and lymphoid cell lines (Sun H et al., 2008).

Intrinsic Death Pathway: Bcl-2 Family Proteins

The mechanisms involved in transducing cellular stress to mitochondrial dysfunction and permeabilization are not completely understood. However, it is apparent that bcl-2 family proteins play a key role. The bcl-2 family proteins consist of 3 groups of proteins based on structure having either antiapoptotic or proapoptotic functions. The antiapoptotic bcl-2 proteins have 3 to 4 bcl-2 homology (BH) domains and consist of Bcl-2, Bcl-X_L, BCL-w, A1, Mcl-1, Bcl-B and Boo. One proapoptotic group is referred to as the BH3-only group and consists of Bid, Bad, Bik, Bim, Hrk, Blk, Bmf, Noxa, Puma and Bcl-G_S. The other proapoptotic group, referred to as Bcl-2-like (Bax), has two to three BH domains and consists of Bax, Bak, Bok, Bcl-G_L and Bcl-X_S (McDonald 3rd ER and El-Deiry WS, 2005).

The antiapoptotic bcl-2 proteins localize at the inner membrane of the mitochondria and inhibit multi-BH domain, proapoptotic proteins (Bak and Bax) that would destabilize the outer membrane. Until recently, Bax/Bak-induced mitochondrial fragmentation was associated with cytochrome c release but a new study has shown that

these are coincidental and that other bcl-2 family proteins may be involved (Sheridan C et al., 2008). Sequestered and inactive BH-3-only proteins are released upon cellular stress and target antiapoptotic bcl-2 family members thus releasing multi-BH proteins to multimerize and coordinate with tBid to destabilize the outer membrane (McDonald 3rd ER and El-Deiry WS, 2005). Bcl-2 family proteins are of general interest to the study of apoptosis but in particular Bid conversion to tBid is mediated by active caspase-8 and Asp-protease cathepsin D (CTSD) in a TNFR1-dependent manner making it directly relevant to this project (Schutze S et al., 2008).

Mitochondrial Apoptosis: Modulators

Upon mitochondrial dysfunction, cytochrome c is released from the inner membrane of the mitochondria, activating the adaptor protein Apaf-1 as the initial step in the formation of the apoptosome. In addition to cytochrome c, mitochondria release smac/DIABLO and Omi/HtrA2 from the intermembrane space of the mitochondria. Both smac/DIABLO and Omi/HtrA2 have IAP binding motifs (IBM). Interactions of these proteins with IAPs (XIAP, IAPc) blocks IAP inhibition of caspase activity thus providing a proapoptotic effect (McDonald 3rd ER and El-Deiry WS, 2005). Omi/HtrA2 has serine protease activity that is essential in maintaining mitochondrial homeostasis (Fulda S and Debatin K-M, 2006). In addition to unblocking caspase activity upon mitochondrial release, Omi/HtrA2 migrates to the nucleus and cleaves p53 family member, p73, which is a proapoptotic transcription factor (Marabese M et al., 2008). Given the proapoptotic character of these proteins, they have become the recent focus for cancer therapy development.

The mitochondria also release AIF, which is known to promote DNA cleavage through mechanisms that have not been completely determined. Upon mitochondrial membrane permeabilization, AIF is released to the cytoplasm and is translocated to the nucleus. AIF has been shown to interact with peptidyl-polyl *cis-trans* isomerase to induce the breakdown of DNA in mammalian cells (Cande et al., 2004). In neurons, AIF release and caspase-independent glutamate-induced death is Bid-dependent (Landshamer S et al., 2008). The mitochondria also releases endonucleas G which translocates to the nucleus to induce DNA fragmentation. The combined effect of these mitochondrial proteins is to promote apoptosis through the p53-inducible nuclear death pathway (Fulda S and Debatin K-M, 2006).

Death Ligands and Death Receptors

The concept of a tumor necrosis factor was first formulated in the 1800's based on observations of acute bacterial infections causing tumor shrinkage in patients and in culture (McDonald 3rd ER and El-Deiry WS, 2005). Since then, molecular cloning has lead to the identification of the cytokines forming the TNF superfamily of protein ligands comprising at least 19 members (Shen H-M and Shazib P, 2006). This superfamily of TNF ligands evolved primarily to regulate immune homeostasis (McDonald 3rd ER and El-Deiry WS, 2005). TNFα, FasL and TRAIL are members of this superfamily known to induce cell death through some of their cognate receptors. Death ligands are active in homotrimeric form and have homology only in the regions necessary for trimerization while non-conserved residues determine receptor specificity (McDonald 3rd ER and El-Deiry WS, 2005).

Death receptors (DR) are a subgroup of the tumor necrosis factor receptor (TNFR) superfamily consisting of 23 members (Schutze S et al., 2008). DRs exist in a trimeric form in the plasma membrane prior to binding their ligands. The DRs are distinguished from other TNFR superfamily receptors by their intracellular death domains (DD) (McDonald 3rd ER and El-Deiry WS, 2005). TNFα interacts with TNFR1 and TNFR2 but only TNFR1 is capable of mediating cell death due to its DD. TNFR superfamily receptors that do not induce death are often called decoy receptors (DcR) and can be considered antiapoptotic because they sequester the death ligand (McDonald 3rd ER and El-Deiry WS, 2005). In addition to sequestering death ligands some DcRs can also mediate other intracellular signals, such as TNFR2 activation of c-AIP1.

FasL is known to bind to the death receptor Fas and the soluble decoy receptor, DcR3, while TRAIL induces death through DR4 and DR5 and has decoys DcR1, DcR2 and osteoprotegerin (OPG) (McDonald 3rd ER and El-Deiry WS, 2005). Both FasL- and TRAIL-mediated death signals are typically less complex than TNFα-mediated signals because their receptors act primarly to activate the death machinery through caspase-8. While TNFα binding TNFR1 will eventually form a DISC and activate caspase-8, it starts by activating multiple signalling pathways that regulate cell proliferation and inflammatory response. These pathways can have either a pro- or anti-apoptotic effect depending on cell line and conditions (Schutze S et al., 2008).

In vivo, inflammation, injury and environmental stresses cause hematopoeitic cell types to produce and release TNFα, which mediates responses including cell proliferation, differentiation, apoptosis, and necrosis. TNFα signalling dysfunction can lead to septic shock, arthritis, irritable bowel disorder and cachexia (McDonald 3rd ER

and El-Deiry WS, 2005). Since FasL primarily mediates cell death, dysfunction leads to lymphoproliferation diseases. Consequently, systemic application of these ligands to treat illnesses such as cancer has adverse effects such as inflammatory response and liver failure (McDonald 3rd ER and El-Deiry WS, 2005). TRAIL on the other hand does not produce system adverse effects and can kill some tumour cells. This is due to the relatively high expression of TRAIL death receptors in relation to decoy receptors in some cancer tissues. Consequently, the TRAIL-mediated death pathway is currently an active area in cancer research (McDonald 3rd ER and El-Deiry WS, 2005). In this study, TRAIL failed to induce caspase-3 activation and apoptosis in transfected Balb/c-3T3 cell lines (data not shown). This might be due to a high expression level of decoy receptors in relation to death receptor or just too little DR4 and/or DR5 to mediate the death response.

TNFR1 Complexes

TNFR1 is constitutively expressed in most cell types and is responsible for mediating most of the cellular responses attributed to TNFα. Upon binding TNFα, TNFR1 sheds its silencer of death domain (SODD) and recruits TNFR1-associated death domain protein (TRADD). Binding to TRADD are two complexes: 1) plasma membrane bound complex I and 2) the cytoplasmic complex II (Shen H-M and Shazib P, 2006). The plasma membrane complex I consists of TNFR1, TRADD, the receptor interacting protein (RIP), and TNF receptor-associated factor 2 (TRAF2) which mediates the activation of NF-κB. The cytoplasmic complex II consists of TNFR1, TRADD, FAS-associated death domain protein (FADD), and caspase 8, which signals to apoptosis (Fig.4)(Micheau O and Tschopp J, 2003) (Schutze S et al., 2008). Most TNFα cellular

responses including cell growth, development, oncogenesis, inflammation, stress-induced signaling, and cell death are mediated through these complexes (Shen H-M and Shazib P, 2006).

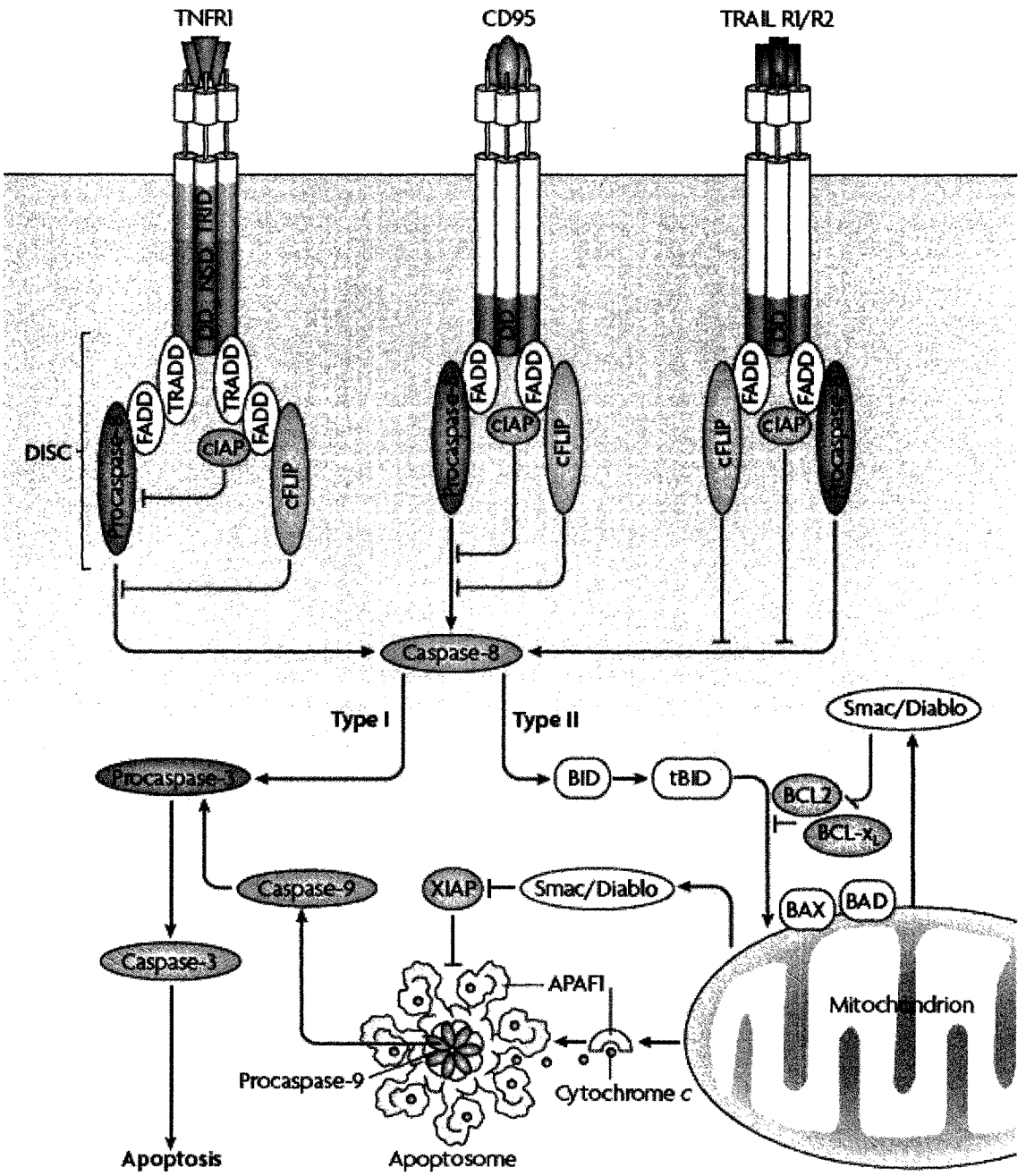


Figure 2. Death receptor ligands, receptors and their downstream effectors. In type 1 cells, death receptor induced caspase-8 activation is sufficient to induce apoptosis. In type 2 cells, Bid-dependent mitochondrial amplification is required for cell death. (Reprinted with permission from Macmillan Publishers Ltd: Molecular Cell Biology. 9(8):655-62 Schutze et al., Copyright 2008).

Ligand-bound TNFR1 internalizes via clathrin-coated pits (CCP) to form the cytoplasmic complex II (Fig. 3 and 4). RIP1 is degraded by ubiquitin E3 ligases, caspase-associated ring protein-2 (CARP2) and A20, terminating NF-κB activation, and is replaced by FADD and caspase-8, which activates the caspase-3 signalling cascade. The TNF-receptosome fuses with *trans*-Golgi vesicles and caspase-8 activates pro-acid sphingomyelinase (pro-aSMase) and pre-pro-CTSD. This structure is associated with endolysosomal aSMase activation and ceramide production, which is proposed to activate CTSD and Bid cleavage (Fig.4) (Schutze S et al., 2008).

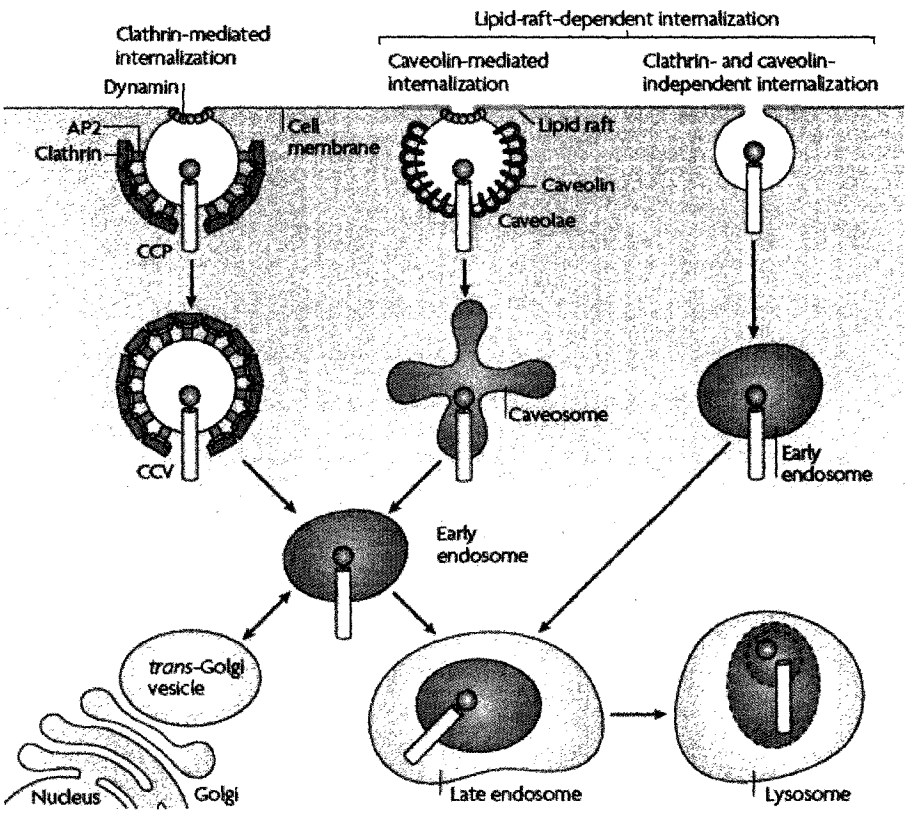


Figure 3. Receptor Endocytosis: "Receptors and ligands can be internalized from the cell surface by various routes. The first step in clathrin-mediated endocytosis involves the selective recruitment of transmembrane receptors and their bound ligands into specialized membrane microdomains (known as clathrin-coated pits, CPP). Several adaptor-protein complexes participate in this process, which is initiated by the binding of adaptor protein complex-2 (AP2) to the plasma membrane through its lipid binding domains. Interaction of AP2 with the GTPase dynamin results in a ring formation around the neck of budding vesicles that leads to membrane fission and generation of free clathrin-coated vesicles (CCVs). After uncoating during intracellular

trafficking, CCVs fuse with early endosomes and with *trans*-Golgi vesicles, from which the ligand-receptor complexes are sorted to various intracellular compartments, such as late endosomes (also known as multivesicular bodies) and lysosomes. Receptor complexes that are destined for degradation fuse with lysosomes and are degraded." Two clathrin-independent routes are also shown. (Reprinted with permission from Macmillan Publishers Ltd: Molecular Cell Biology. 9(8): 655-62 Schutze et al., Copyright 2008).

As mentioned, TNFα can stimulate a number of responses including cell death. In the cases where cell death has been shown, the mechanisms underlying cell death have not always coincided from study to study. In type 1 cells, the activation of caspase cascade by TNFR1 cytoplasmic complex II is sufficient to induce apoptosis. In type 2 cells, mitochondrial amplification via caspase-8 cleavage of Bid to tBid is necessary to induce cell death (Fig. 2 and 4). In these cells, tBid contributes to permeation of the mitochondrial membrane, which amplifies the signal to apoptosis (Schutze S et al., 2008).

Another important factor regulated by TNFR1 and influencing apoptosis and necrosis is the production of reactive oxygen species (ROS). TNFR1 can induce the accumulation of ROS by activation of the JNK pathway. It has also been shown that ROS accumulation contributes to sustained activation of JNK, which completes a feed-forward loop (Shen H-M and Shazib P, 2006). On the other hand, NF-κB activation has been shown to inhibit ROS accumulation while at the same time NF-κB activation is inhibited by high ROS levels (Shen H-M and Shazib P, 2006). Therefore, both JNK and NF-κB pathways can be activated by TNFR1 and both pathways can negatively regulate the other. What determines the fate of a cell might be duration of activation of these pathways (Shen H-M and Shazib P, 2006).

Some studies have shown TNFα-induced caspase-3 activation mediated by caspase-8 or caspase-9 or both pathways (Yamashita K et al., 1999)(Kulik G et al., 2001)(McDonnell MA et al., 2003). In a study of TNFα induced cell death in fibroblasts,

the importance of caspase-8 activation has been clearly demonstrated *in vitro* and *in vivo* for human and mouse cells respectively (Alikhani M et al., 2004). The *in vitro* portion of the Alikhani study used 20 ng/ml TNFα applied for 6 hours in 0.5% fetal bovine serum (FBS) and also resulted in the significantly elevated expression of proapoptotic proteins including TNFα, TNFR1, FasL, Fas, TRAIL, caspase-2,-3,-6,-7,-8, TRADD,CRADD and FADD (Alikhani M et al., 2004).

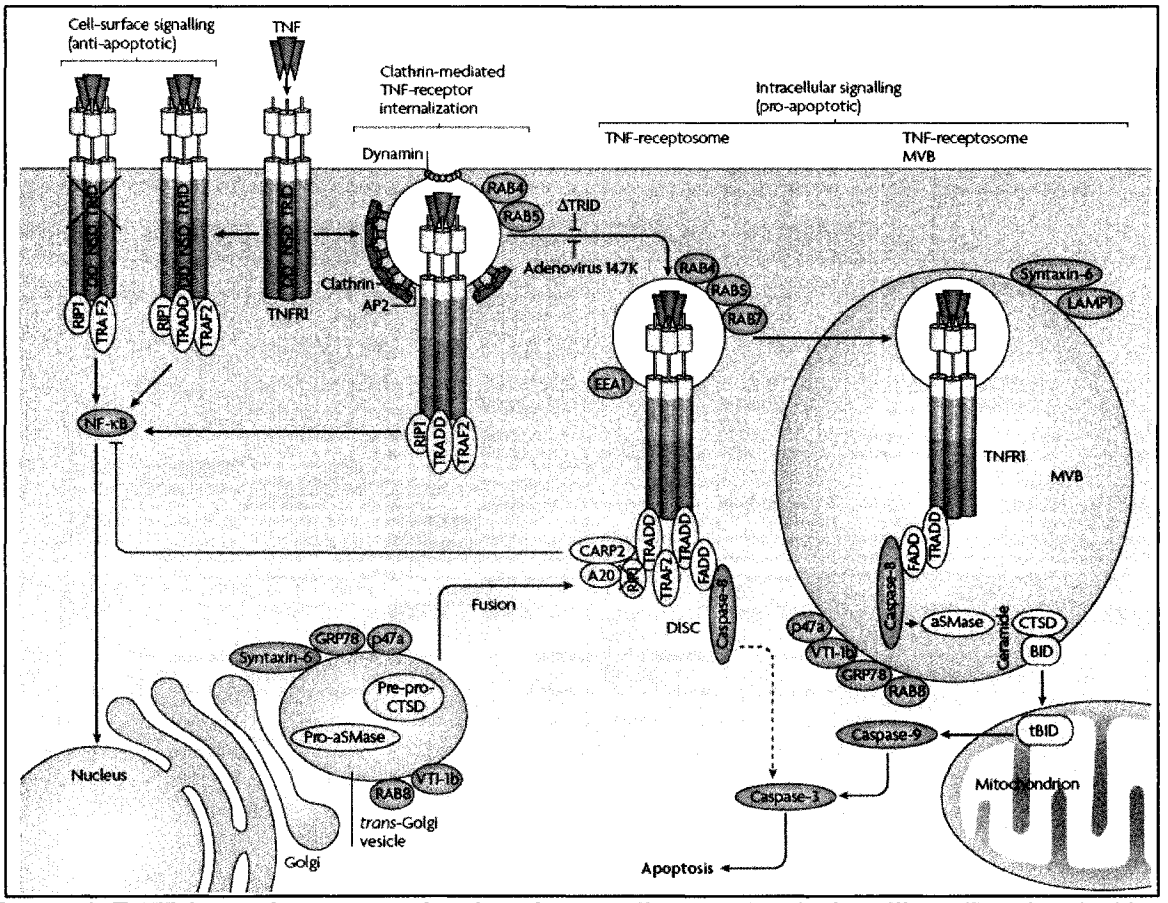


Figure 4. TNFR1 membrane complex 1 and cytosolic complex 2 signalling (Reprinted with permission from Macmillan Publishers Ltd: Molecular Cell Biology. 9(8):655-62 Schutze et al., Copyright 2008).

In my study, experimental conditions were optimized for TNFα-induced cell death in mouse Balb/c-3T3 fibroblasts and involved serum deprivation for periods ranging from 6 to 23 hours in 0.5% FBS and TNFα treatments coupled with

cycloheximide. Serum deprivation contributes to cell death by potentiating the intrinsic death pathway, which is known to augment the response of the extrinsic death pathway in some cells. Cycloheximide blocks protein translation and therefore can block TNFα-induced NF-κB-activated gene expression. Another report indicates that TNFα-induced cell death requires blockade of NF-κB and the release of mitochondrial apoptotic factors mediated by a JNK- and Bid-dependent pathways (Deng Y et al., 2003). Cycloheximide has also been shown to induce TNFR1 internalization via p38 MAPK and ERK activation (Ogura H et al., 2008). In any case, the combination of translation blockade and/or the potentiation of the mitochondrial pathway by serum deprivation produce strong TNFα-induced cell death response in fibroblast cells and have been used in other studies (Alikhani M et al., 2004) (Guillermet-Guibert J et al., 2007). Cycloheximide treatment also quickly lowers the levels c-FLIP proteins, modulators of caspase-8 activation, which are known to cycle quickly through translation and degradation processes (Ueffing N et al., 2008). Future work with this cell line should also look at FasL-mediated cell death since cycloheximide treatment is not typically required for this death pathway.

Anoikis: When Cell Homelessness Leads to Death

Most cells require adhesion to the extracellular matrix (ECM) to grow normally and avoid a form of apoptosis called *anoikis* (*greek:* homelessness) in an integrin-dependent manner (Chiarugi P and Giannoni E, 2008). *Anoikis* is mediated by both the intrinsic and extrinsic death pathway via integrin engagement of the ECM. Integrin-ECM interactions activate several pro-survival routes including ERK, JNK and Akt which stimulate fos, jun and NF-κB transcription factors and, inhibit pro-apoptotic proteins.

Loss of proper cell-ECM contact can result in upregulation of Fas receptor and FasL expression (Chiarugi P and Giannoni E, 2008). *Anoikis* is an important form apoptosis in this study because it may be involved in an evaluation of TNFAIP8 with respect to anchorage-independent growth in soft agar foci formation assay.

G-protein coupled receptors (GPCR)

GPCRs comprise a large family of transmembrane proteins that sense a molecular signal (ligand) outside the cell and transduce this signal across the membrane to internal signalling pathways resulting in a cellular response. They have 7 transmembrane domains and are found ubiquitously throughout eukaryotes. The transmembrane domains are alpha helices and are linked by 3 extra-cellular loops and 3 intracellular loops. The N-terminal tail is extracellular while the C-terminal is intracellular (Bohm SK et al., 1997). Ligands include light-sensitive compounds, odors, pheromones, hormones, and neurotransmitters, and vary in size from small molecules to peptides to large proteins (Bohm SK et al., 1997), which specifically bind to motifs on the extracellular loops and transmembrane domains inducing conformational changes through the alpha-helices and intracellular loops. These conformational changes can either induce activity (agonist) of G-proteins or inhibit activity (inverse agonist). Some ligands may also block the effects of agonists and inverse agonists and these are considered antagonist. Some ligands may interact with many types of receptors however with varying affinity and this is what defines receptor specificity. The strength of the signals transduced to different signalling pathways can vary by ligand. While G-protein coupled receptors are in involved in many diseases, they are also the targets of approximately half of all commercial pharmaceuticals (Filmore D, 2004).

Dopaminergic System

In this study, the dopamine D2S receptor is introduced into mouse fibroblast cells to provide a receptor that can specifically activate Gαi/o proteins for the purpose of

modulating the death receptor pathway. Consequently, I will provide a brief review of the dopaminergic system as a whole with some emphasis on D2S signalling and regulation in the context of mouse fibroblasts.

Dopamine was first synthesized in the 1910 and was assumed to be an intermediary in the production of norepinephrine. Later in the 1950's, it was recognized as an important neurotransmitter (Marsden CA, 2006). Subsequent studies have shown that dysfunction of the dopaminergic system is a major contributor to neurological diseases such as Parkinson's disease, schizophrenia, attention deficit hyperactivity disorder, addiction and some forms of depression. The study of dopamine receptors and their ligands has become an important research area for the development of treatments for these diseases (Marsden CA, 2006).

Dopamine (DA or 4-(2-aminoethyl)benzene-1,2-diol) is produced from cytosolic tyrosine in the neurons of the ventral tegmental area (VTA), substantia nigra and the hypothalamus. *Tyrosine hydroxylase* converts l-tyrosine to l-dopa and l-dopa is converted to dopamine by *dopa decarboxylase*. Cytosolic dopamine is stored in vesicles by VMAT and these vesicles are delivered to the synapses for eventual excitation-driven release.

Dopamine neurons account for less than 1% percent of the central nervous system but mediate important behaviour. They are organized into 4 major pathways:

1) **Tuberoinfundibulary pathway** run from the periventricular nuclei of the hypothalamus to the median eminence and is secreted into the portal blood stream to reach the pituitary gland, providing inhibitory control of prolactin mediated by D2 receptors.

2) **Mesolimbic pathway** runs from the VTA to the limbic areas (accumbens, ventral striatum and amygdala) and is associated with reward and schizophrenia. This pathway may be involved in the positive symptoms of schizophrenia.

3) **Mesocortical pathway** runs from the VTA to the cortex (medial, prefrontal, cingulate and entorhinal cortex) and is also associated with reward and schizophrenia. This pathway may be involved in the negative symptoms of schizophrenia.

4) **Nigrostriatal pathway** runs from the substantia nigra to the caudate putamen (dorsal striatum) and is associated with Parkinson's disease. This pathway is involved in the control and initiation of movement (Marsden CA, 2006).

Dopamine Receptor Structure

Dopamine receptors are members of the 7 transmembrane (7 TM) receptor family also called G-protein coupled receptors (GPCR). There is considerable homology between dopamine receptors with greater homology found between members in the same subfamily, either D1-like or D2-like (Missale C et al. 1998). The structural components sharing the greatest homology are found in the transmembrane domain segments and the intracellular loops regions closest to the TMs, which have been shown to couple to G-proteins.

The extracellular N-terminal tail is of similar length in all dopamine receptor types and has a variable number of glycosylation sites (Missale C et al. 1998). There is a large degree of variability in glycosylation possible for a given receptor. For instance, deglycosylated D2 receptor has a MW of 44kDa, yet recombinant D2 expressed in

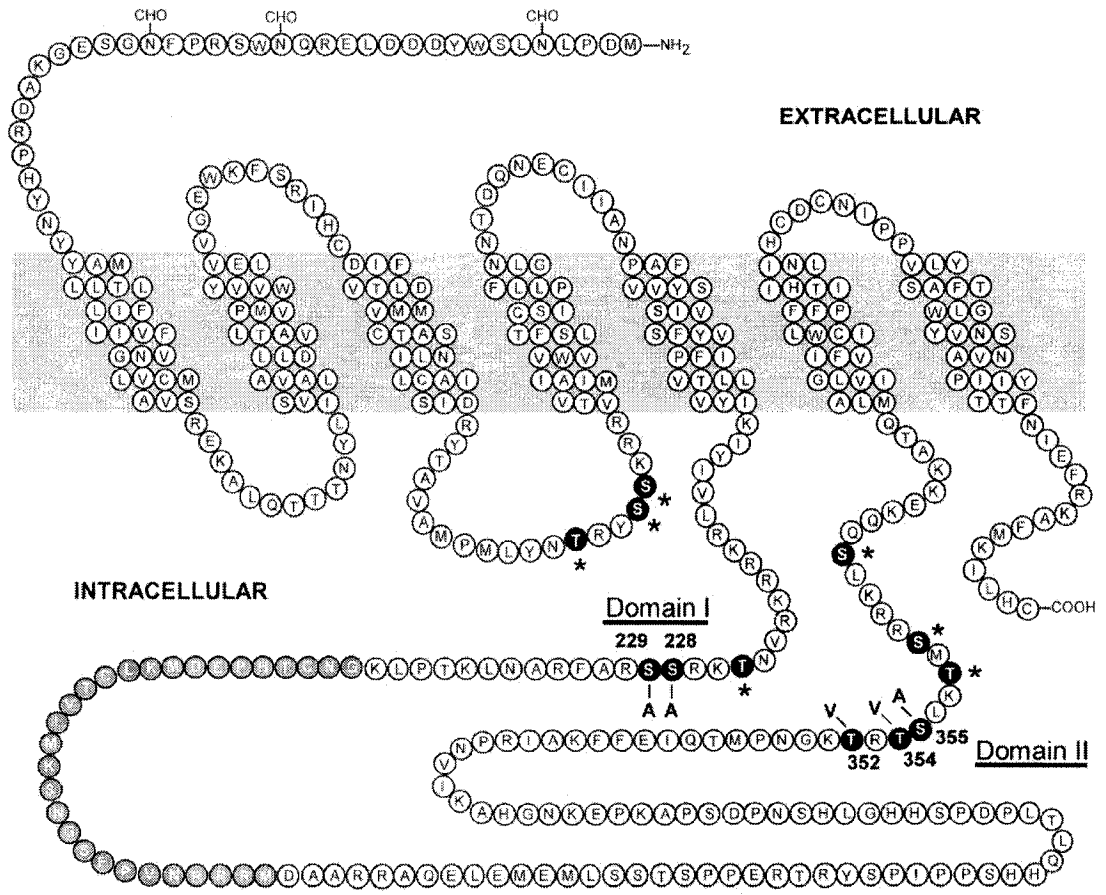


Figure 5. D2 receptor sequence and signalling: Grey residues represent those absent in the D2S isoform. Black residues represent putative PKC phosphorylation sites. (Reprinted with permission from American Society for Biochemistry and Molecular Biology: J Biol Chem. 279(47):49533-41 Namkung Y and Sibley DR, Copyright 2004).

various cell lines or endogenous D2 isolated from brain tissues may have MW ranging from 75kDa to 140kDa (Neve KA et al., 1997).

The C-terminal tail of D1 receptors is approximately seven times longer than the D2 C-terminal tail. The D1 receptor also has a short third cytoplasmic loop compared to the D2 receptor. These structural differences are typical of GPCRs that bind Gαs (D1-like) versus Gαi/o (D2-like) proteins (Missale C et al. 1998).

The intracellular loops and C-terminal tail of dopamine receptors are rich in serine and threonine residues. Some of these residues become available to G-protein receptor kinases upon agonist binding while others are located in second-messenger kinase consensus sites (such as PKA or PKC) and can also be phosphorylated. Phosphorylation of these sites has been shown to regulate dopamine signalling (Missale C et al. 1998) (Fig 5) (Claing A et al., 2002).

Splice variants of the D2 receptor gene produce two forms of the receptor called D2 short isoform (D2S) and D2 long isoform (D2L). Both isoforms share similar structure and pharmacology. The primary difference between these D2 receptors is the omission of 29 residues in the third intracellular loop of D2S (Fig 5).

D2-like Receptor Expression in the Brain

D2 receptors are found primarily in the caudate/putamen, nucleus accumbens, olfactory tubercle and at low levels in the cerebral cortex. D3 receptors are expressed primarily in the nucleus accumbens, olfactory tubercle, islands of Calleja and cerebral cortex (low). While D4 is predominately found at low levels in the frontal cortex, midbrain, amygdala, hippocampus, hypothalamus, medulla and retina (Marsden CA,

2006). Although the expression profile of these D2-like receptors varies in the brain it should also be noted that cells in some tissues might express more than one type of dopamine receptor. For instance D1 and D2 receptors are coexpressed in some tissues of the brain and are responsible for a heterooligomer-Gq/11-dependent calcium signal (Rashid AJ et al., 2007).

D2 Pharmacology of Drugs in this Study

The pharmacology of most agonist and inverse agonists does not vary greatly between members of the D2-like family and there is practically no difference between D2S and D2L. In D2 receptors, dopamine binds the receptor with a Ki of approximately 600 nM. In this study, the agonist apomorphine and the inverse agonist spiperone are used. Apomorphine was chosen for its high affinity for D2 (Ki ~ 50 nM) and because it is more stable than dopamine in media. Spiperone was chosen for its extremely high affinity for D2 receptors (Ki < 0.5 nM) (Missale C et al. 1998) (PDSP Ki database, on line: http://pdsp.med.unc.edu/kidb.php).

D2S and D2L

In previous studies, conflicting differences between D2S and D2L coupling to G-proteins and the strength of their signals to downstream effectors have been reported in heterologous expression systems (Missale C et al. 1998). Physiologically, D2S has been shown to act primarily as the presynaptic autoreceptor regulating dopamine and D2L is important in postsynaptic signal transmission (Usiello A, et al., 2000)(Wang Y et al., 2000). In a recent study looking at the two isoforms in terms of their abilities to act as

autoreceptors, enhanced green fluorescent protein (EGFP)-tagged D2S and D2L transfected in mesencephalic neurons showed no differences in terms of subcellular localization, regulation of cell excitability, regulation of neurotransmitter release or regulation of intracellular calcium (Jomphe C et al., 2006). In another recent study of D2S and D2L signalling, the differential inhibitory regulation of ERK1/2 has been reported in transfected GH4 pituitary cells and is attributed D2L's PKC pseudosubstrate site in the third intracellular (Van-Ham II et al., 2007). The D2S receptor, lacking the PKC pseudosubstrate site, has been shown to have higher susceptibility to homologous desensitization by the PLC-PKC pathway where both receptors can activate PLC β and calcium mobilization in mouse fibroblasts (Morris SJ et al., 2007). From these results, it is apparent that D2S and D2L share many common features however, there is evidence supporting differential signalling and receptor regulation in some systems.

Dopaminergic Signal Transduction

A. Adenylyl Cyclase: It has been shown in neuronal tissues as well as in heterologous expression systems that D1 and D5 receptors can stimulate cAMP accumulation via AC. This was initially believed to be mediated by D1 and D5 coupling to Gαs proteins but it has also been shown $G\alpha_{olf}$ can couple to these receptors and stimulate AC activity (Missale C et al. 1998).

D2, D3 and D4 have been shown to negatively couple to AC-mediated cAMP accumulation (specifically through AC-5 and in some cases AC-6) in most tissues including CNS and pituitary via Gαi/o proteins. However, D3 has a very weak coupling to AC inhibition such that it is not apparent in some cell lines (Missale C et al. 1998)

(Neve KA et al., 2004). While Gαi/o subunits negatively couple to AC, D2-like receptors also activate Gβγ subunits, which have been shown to have a stimulatory effect on AC-2 and -4 (Neve KA et al., 2004). This may explain some of the tissue specific instances where D2-like activation does not inhibit the production of cAMP. In transfected mouse fibroblasts, D2S activation strongly couples to inhibition of cAMP accumulation via Gαi2 and Gαi3 proteins (Ghahremani M et al. 2000).

B. Calcium Channels: D1-like receptor-mediated calcium mobilization is complex and cell line dependent. In neuronal cells, agonist activation of L-type channels generally increases calcium influx while activation of N and P/Q-type channels decrease influx. These effects can be modulated by PKA, PKC, DARPP-32, and PP-1 interactions as well as by D1-mediated decreases in K^+ currents. Whether D1 activation results in a net influx of calcium or not is determined by tissue specific interactions of these proteins and will not be discussed in detail here (Neve KA et al., 2004). In some non-neuronal transfected cell lines, like Ltk^- fibroblast cells, calcium mobilization is mediated by PLC activation and phosphatidylinositol (PI) hydrolysis but is not apparent in chinese hamster ovary or baby hamster kidney cells (Missale C et al. 1998).

In neurons, D2-like receptor-mediated activation of pertussis toxin-sensitive G-proteins results in the inhibition of calcium currents via L-, N- and P/Q-type calcium channels. The activation of Gβγ subunits can also decrease the activity of N-type channels by direct interaction in neostriatal large aspiny interneurons and it can decrease the activity of L-type channels via a PLC-$[Ca^{+2}]_i$-calcineurin(PP2B) dependent pathway in striatal medium spiny neurons (Neve KA et al., 2004). D2-like-mediated decreases in calcium conductances in neurons may serve to inhibit neurotransmitter release. In

transfected mouse fibroblast, D2-activation induces an increase in calcium mobilization due to the activation of PLC β via Gβγ subunits (Ghahremani M et al., 2000).

C. Mitogen-Activated Protein Kinase (MAPK): MAPK activation is known to mediate cellular functions such cell proliferation (including DNA synthesis and mitogenesis), differentiation and survival by transmitting extracellular signals to the nucleus. D1-like receptors are reported to activate MAPK including ERK, p38 MAPK and c-jun amino-terminal kinase (JNK). Studies have shown that JNK and p38 can be activated via a cAMP-PKA dependent pathway. However a PKA-independent pathway to ERK activation involving Rap GTPase has also been proposed (Neve KA et al., 2004).

D2-like receptors have also been shown to activate MAPK in some cell-lines. Several mechanisms involving Gβγ have been proposed for this activation based on cell type. In non-neuronal cells, D2 transactivation of RTK (platelet-derived growth factor receptor) has produced ERK activation. The activation of platelet-derived growth factor receptor can induce activation of ERK by two pathways: RTK-Ras-MEK-ERK and RTK-PI3K-Atk. In neostriatal neurons Gβγ-PLCβ-$[Ca^{+2}]$i-PP2B pathway may also contribute to the activation of ERK as well as CREB (Neve KA et al., 2004). In D2S or D2L transfected GH4 pituitary cells, D2S activation inhibited thyrotropin-releasing hormone-induced ERK phosphorylation, while D2L failed to do this suggesting a difference in ERK regulation by these two isoforms (Van-Ham II et al., 2007). In mouse fibroblast, D2S-mediated ERK (MAPK p42/44) activation is proposed to occur not only through Gβγ activation but also by Gαi2 activation. Similarly, increased DNA synthesis was also mediated by these two G-protein subunits (Ghahremani M et al., 2000).

Other Relevant Dopamine Signals

A. Ceramide Formation: D2S stimulation with apomorphine (EC_{50} = 10nM) induced increased ceramide and diacylglycerol formation maximally after 1h and up to 6h in transfected Balb/c-3T3 cells via Gβγ-PLCβ pathway and was blocked by PLC inhibitors (Liu G et al., 2003). In Balb/c-3T3 cells, C2-ceramide (ceramide analog) leads to ERK1/2 activation and cell proliferation (Liu G et al., 2003). In other cell lines, ceramide can induce apoptosis by stress activated protein kinases p38 or JNK, however neither of these showed ceramide-induced activation in Balb/c-3T3 (Liu G et al., 2003). Recently, acute D2 stimulation (30 min) has been shown to induce apoptosis in snail saliva gland cystic cells by ceramide-dependent induction of mitochondrial release of cytochrome c activating caspase-3 (Pirger Z et al., 2008). However, in my work with Balb/c-3T3 cells I have not observed caspase-3 activation given 3 hours of apomorphine or apomorphine+cycloheximide treatment (data not shown).

B. D2 interaction with PAR-4: Prostate apoptosis response 4 (PAR-4) constitutively interacts with D2 receptor via the calmodulin binding motif in the third cytoplasmic loop where it competes with Ca^{+2}-activated calmodulin (Park SK et al., 2005). The Park study focused on the anti-inhibitory effect on D2 signalling mediated by PAR-4 competition with calmodulin. However, PAR-4 is known to be a pro-apoptotic factor induced by apoptotic stimuli (Park SK et al., 2005). A recent study has shown PAR-4 siRNA-silencing contributing to decreased glutamate-induced caspase-3 activity in human mesenchymal cells (Lu C et al., 2008). PAR-4 over-expression coupled with apoptotic stimuli increases caspase-3 activity and apoptosis (Lee J-W et al., 2007)(Lee T-

J et al., 2008). At this time, it remains an open question whether D2-mediated pathways can modulate PAR-4's pro-apoptotic activity. As stated previously, D2-activation in Balb/c-3T3 did not correlate with increased caspase-3 activity and therefore, whatever role D2 plays in PAR-4 induction, it should not affect this study.

C. D2-sGαi2-mediated Caspase-3 Activity: A splice variant of Gαi2 (sGαi2) is reported to be a mediator of dopamine-D2-induced cell death by activation of ERK1/2 and p38 (Lopez-Aranda MF et al., 2008). Intracellular D2 receptors bind sGαi2 but release sGαi2 upon dopamine treatment followed by receptor translocation to the membrane. A study by Lopez-Aranda shows that these cytosolic free-sGαi2 subunits induce caspase-3 activation by ERK1/2 and p38 activation in baby hamster kidney cells. Whether this mechanism involves ceramide or not is not discussed. The mechanism appears to be dependent on a transient imbalance of free-sGαi2 compared D2-sGαi2 complex. Therefore, this pathway to cell death is not likely to occur in cells over-expressing D2S but not sGαi2 such as those used in this study.

D. D2S/D2L-NF-kappaB Activation: A study in NG108-15 cells stably expressing D2S and D2L showed that these receptors activate NF-κB via different mechanisms (Takeuchi Y et al., 2004). The Takeuchi study showed that quinpirole stimulated D2L-NG108-15 cells couples more strongly to NF-κB activation and that this coupling is mediated partially by calcineurin (PP2B) activity and partially by ERK activation. The activation of NF-κB in D2S-NG108-15 was weaker and apparently mediated by ERK activation (Takeuchi Y et al., 2004). NF-κB is a transcription factor that enhances the expression of many pro- and anti-apoptotic genes where activation produces a cell and condition specific response towards death or survival. It is not known

if D2 stimulation in Balb/c-3T3 cells activates NF-κB or what affect this might have on cell survival or transformation. However, given the use of translation inhibitor cycloheximide and the short duration of most experiments in this study, this effect (if it exists in this system) should be minimal for all but the soft agar foci formation assay.

Regulation of Dopamine Signalling

Regulation of chemical neurotransmission is complex and can occur at many levels in and outside of the cells involved. For instance synthesis, degradation, storage, delivery to synapse, release and reuptake of neurotransmitter are important components in the regulation of neurotransmitter signalling. While these processes are important for *in vivo* signalling, they are not relevant to this study since neurotransmitter is applied to tissue culture in media and therefore they will not be discussed here. Of greater significance to this study are the regulation of dopamine receptors and their G-proteins.

D2 receptor regulation

GPCRs can be desensitized to repeated and prolonged exposure to agonists resulting increased refractoriness to mediate intracellular signals. This process of desensitization is invoked by receptor phosphorylation by kinases followed by binding to intracellular proteins that inhibit signalling and/or mediate receptor internalization. Once internalized, the receptor can be targeted for degradation or reintegration in the plasma membrane resulting in resensitization (Bohm SK et al. 1997)(Claing A et al., 2002).

There are two types of desensitization referred to as homologous and heterologous desensitization. In homologous desensitization, the active agonist-bound GPCR interacts

with G-protein receptor kinases (GRK) leading to phosphorylation and stabilized β-arrestin-receptor interaction. There are 7 GRK family members with differing distribution and localization depending on cell type. Second-messenger kinases (PKA and PKC) can also contribute to receptor phosphorylation. In heterologous desensitization, non agonist-specific interactions with other GPCR types results in the activation of second-messenger kinases that phosphorylate and desensitize the receptor (Bohm SK et al. 1997). In this study, a single receptor specific agonist (apomorphine) is used and thus homologous desensitization is most likely to occur as a result of experimental treatments while constitutive activity of endogenous receptors may also contribute to desensitization.

Phosphorylation of GPCRs occurs at the intracellular loops and cytoplasmic C-terminal tails. Kinases target these intracellular peptide sequences based on conformational changes in activated receptor structure for GRKs or at specific kinase consensus sites for second messenger kinases. When its agonist activates the dopamine receptor, GRK proteins can then interact with the activated-receptor and phosphorylate specific sites. Once the receptor has been phosphorylated, β-arrestin can associate with the agonist-bound receptor. A recent study has shown that β-arrestin dissociates from the receptor quickly after agonist dissociation despite being phosphorylated (Kelly E et al., 2008). In addition to GRK phosphorylation, active Gα and Gβγ can induce the production of second messengers cAMP, diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) thus activating PKA and PKC. Both second-messenger kinases can contribute to phosphorylation of either active or inactive receptor (Claing A et al., 2002).

Mechanisms for GRK regulation have been proposed. Down regulation of GRK function has been shown via GRK-interactor (GIT) family proteins (Claing A et al.,

2002). Second-messenger kinases have been shown to increase GRK2 interactions with some GPCRs while PKA activation decreases GRK2 interaction with mGLUR1a (Kelly E et al., 2008). Therefore, the effect of second-messenger kinases appears to be receptor and/or context specific.

The association of β-arrestin to the receptor is believed to be an important step in one type of receptor internalization. Studies have shown high affinity binding between β-arrestin-1 or -2 and clathrin and these proteins co-localize with active receptor at the plasma membrane. β-arrestin interacts with adaptor protein 2 (AP-2), which also binds clathrin. Mutant studies of β-arrestin and AP-2 have highlighted their importance in clathrin-coated vesicle internalization (Figure 3). In addition to these proteins, dynamin is essential for vesicle budding and is activated by phosphorylation by non-receptor tyrosine-kinase (cSrc), which is recruited to the membrane by β-arrestin. Dynamin can interact with AP-2 complex and clathrin (Claing A et al., 2002). The regulation of β-arrestin 1 has been proposed to occur by phosphorylation. Cytosolic β-arrestin is primarily phosphorylated at Ser412 but the membrane-associated form is not. The phosphorylated form of β-arrestin 1 is unable to bind clathrin and induce receptor internalization. Other arrestin- and clathrin-independent internalization processes have been proposed but less is known about these mechanisms and they will not be discussed here (Claing A et al., 2002).

Desensitization of D2-like receptors has been investigated in a number of studies. COS-7 cells co-transfected with D2 receptors and GRK2 are susceptible to homologous desensitization by dynamin-dependent internalization (Iwata K et al, 1999). Similar results were achieved for D2S and D2L in COS-7 and Hek 293 cells with co-expression

of GRK2 or GRK5 with evidence of clathrin-coated vesicle internalization (Ito K et al., 1999). Interestingly, these cells showed little or no D2 internalization without over-expression of GRK proteins (Iwata K et al, 1999) (Ito K et al., 1999). Hetero-oligomeric D1-D2 complexes showed GRK2 and GRK3 dependent desensitization when either D1 or D2 receptors were activated (So CH et al., 2007). A recent study in mouse fibroblasts shows PKC-dependent pathway that affects D2S and D2L receptors differently. The existence of a PKC pseudosubstrate site in the third intracellular loop of the D2L receptor makes it resistant to PKC-induced desensitization in comparison to D2S (Morris SJ et al., 2007). Earlier literature showed discrepancies concerning agonist-induced desensitization with some studies showing D2 sequestration and desensitization while others showed D2 receptor up regulation at the plasma membrane in response to agonist treatment (Callier S et al., 2003). The exact mechanism by which D2S receptor might become desensitized in Balb/c-3T3 cells is not entirely clear from the existing data. Whether any of these mechanisms contribute to the results presented in this study remains to be determined.

G-protein regulation by RGS proteins

In addition to the regulation of G-protein coupled receptors, the regulation of active G-proteins is also an important determinant of signal transduction. As mentioned, the conformational changes induced in GPCRs by their agonists leads to the activation of G-proteins by Gα exchanging GDP for GTP. Once activated, GTP-bound Gα and decoupled Gβγ produce intracellular signals via high affinity interactions with downstream effectors (Siderovski DP et al., 2005). The activity of the G-proteins is

partially determined by the duration of GTP binding and is terminated by hydrolysis of GTP to GDP by Gα's inherent GTPase activity. GDP-bound Gα has low affinity for effectors but high affinity for Gβγ, which effectively terminates the active signalling phase (Siderovski DP et al., 2005). A class of proteins called regulators of G-protein signalling (RGS) first discovered in 1996 dramatically increase the GTPase catalytic activity (also called GTPase-accelerating proteins or GAP) of Gα subunits thus providing the ability to down regulate their signals. RGS proteins may also down regulate Gα-GTP signals by competition with downstream effectors (Siderovski DP et al., 2005).

There are over three-dozen human RGS proteins categorized in several subfamilies with differing cellular distributions and Gα subunit selectivity based on their RGS-box domains. Important effects on signalling specificity and strength are modulated by domains outside the RGS-box. For instance, the GoLoco motif of RGS12 and AGS3 provide these proteins with guanine-nucleotide dissociation inhibitor (GDI) function on GDP-bound Gαi thus inhibiting the formation of Gαβγ and consequently the formation of high affinity receptor-Gαβγ complex. Some RGS proteins such as RGS12 permit coupling to distinct signalling pathways via its RhoA guanine-nucleotide exchange factor (RhoGEF) binding motif. In addition to these functional domains, many RGS proteins have domains, such as PDZ in RGS12, designed to target the protein to specific locations thus providing enhanced selectivity (Siderovski DP et al., 2005).

RGS proteins have been shown to modulate D2 receptor signalling. The RZ-subfamily RGS19 (GAIP) protein was shown to interact with PDZ domain-containing protein GIPC and GIPC was shown to interact with D2 receptors. In fact, D2 receptor, GAIP and GIPC are co-expressed in several tissues of the brain and form a complex in

transfected cells (Jeanneteau et al., 2004). Jeanneteau et al. showed that GAIP-GIPC-receptor complex could down regulate D2 signalling to cAMP accumulation and arachidonic acid release by reducing GPCR and G-protein coupling (Jeanneteau et al., 2004). Inhibition of G-protein signalling was demonstrated in another study investigating the effect of GoLoco motif peptides on D2S activation of potassium channels (GIRK) in transfected atT20 (mouse pituitary tumour) cells (Webb CK et al., 2005). GoLoco motif peptides did not reduce the initial response to quinpirole but inhibited subsequent stimuli-induced potassium currents suggesting GoLoco motif peptides interfered with Gβγ binding to Gα-GDP subunits and recycling to the receptor (Webb CK et al., 2005). Together, these results show that D2 receptor signalling can be down regulated by RGS protein interactions.

In another study, regulation of RGS protein by external signals has been proposed. TNFα treatment blocks 14-3-3 interactions with RGS7 through the activation of an unknown phosphatase thus enabling RGS7 down regulation of Gαi signalling to GIRK in co-transfected Xenopus oocytes (Benzing T et al., 2002). Clearly the regulation of D2S signalling through Gαi/o proteins is complex, cell line dependent and can possibly be modified by extracellular signals. Understanding the significance of all these mechanisms on the results presented here is beyond the scope of this study but would represent interesting avenues for future work.

Gi/o-protein signalling in Balb/c-3T3 derived cells

In a study by Ghahremani el al., the specific role of D2S-activated Gi/o alpha subunits and the Gβγ subunit were evaluated in Balb/c-3T3 cells. To accomplish this study, mutant Gα subunits were created to be insensitive to Pertussis toxin (PTX), which inactivates Gα subunits by selectively ADP-ribosylating C-terminal cysteine (here mutated to Ser). PTX-insensitive Gαi1/2/3 and Gαo were created and stably transfected in Balb/c-3T3 cells stably co-transfected with D2S receptor. To evaluate the role of Gβγ subunits C-terminus of G-protein receptor kinase (CT-GRK), known to scavenge Gβγ subunits, was also co-transfected in Balb/c-3T3 cells expressing D2S. Using these cells lines, apomorphine (D2 agonist) and PTX treatments, Ghahremani et al. were able to establish the roles of some G-protein subunits in fibroblasts with respect to cAMP production, MAPK p42/p44 activation, calcium mobilization, DNA synthesis and transformation.

In summary, Gαi2 and Gαi3 were identified as key mediators of inhibition of adenylyl cyclase production of cAMP. Gβγ was identified as the mediator of calcium influx, likely through the activation of phospholipase C β2 and β3. Both Gβγ and Gαi2 play an important role in MAPK p42/p44 activation and DNA synthesis (Fig 6A and B). Finally, apomorphine induced foci formation was conserved only in the Gαi3 PTX-insensitive clones and was completely blocked in Gαo PTX-insensitive clones (Fig 7). This indicates that D2S-mediated transformation in Balb/c-3T3 cells is enhanced by Gαi3 signalling. While other D2S-Gi/o pathways are important to cell proliferation they do not directly contribute to transformation (Ghahremani M et al., 2000).

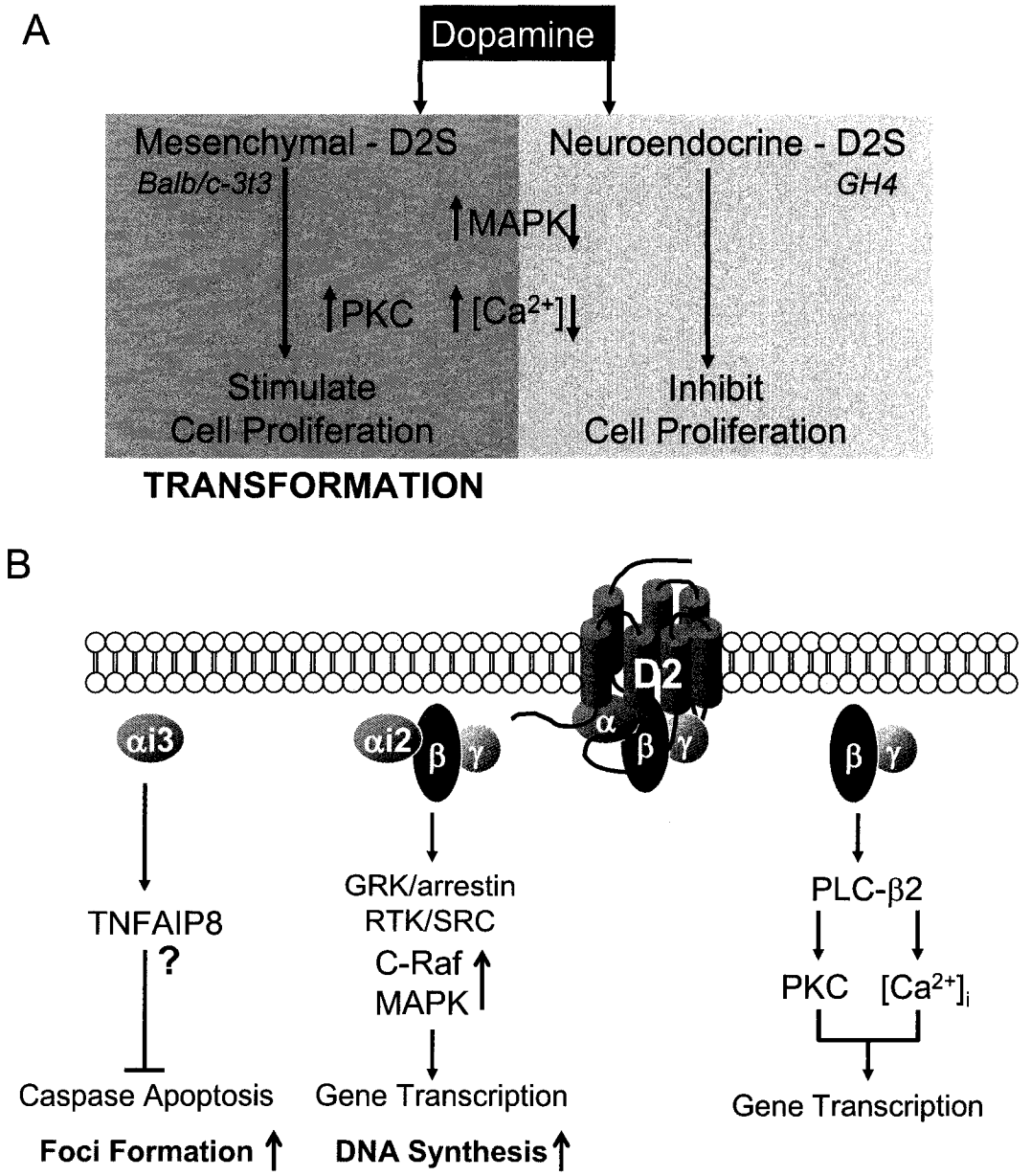


Figure 6. D2S signalling to cell proliferation and transformation. A) Dopamine stimulation of stably transfected D2S receptor induces cell proliferation and transformation in mouse fibroblasts (Balb/c-3T3) but inhibits cell proliferation in pituitary cells (GH4). Downstream effectors of D2S are differentially regulated in these tissues such that MAPK, calcium mobilization and PKC activation are enhanced in fibroblast but inhibited in pituitary cells. B) These effectors are regulated by specific G protein subunits in fibroblasts such that Gβγ and Gαi2 are primarily enhancing cell proliferation processes, while Gai3 is mediating cellular transformation.

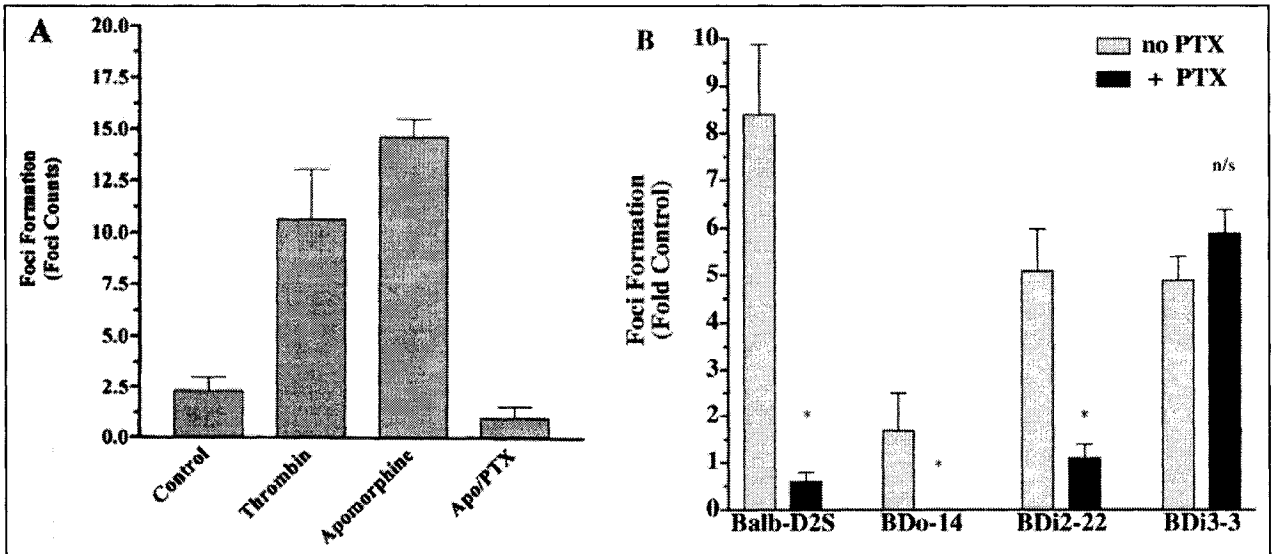


Figure 7. Apomorphine induces focus formation in BALB-D2S cells. "(A) BALB-D2S cells were treated with apomorphine (1 μM), thrombin (1 U/ml), or apomorphine (1 μM) and pertussis toxin (PTX) (1 ng/ml, added every 2 days) (Apo/PTX). The foci were counted as indicated in Materials and Methods. The data represent the results from four independent experiments (n = 4). (B) Apomorphine stimulation of focus formation in BALB-D2S cells expressing PTX-insensitive Gαi/o mutants. The results are presented as fold increase over the basal for each clone. The data are expressed as mean 6 SEM of at least three independent experiments and were analyzed by repeated-measures analysis of variance with Bonferroni multiple comparison posttest (*, P < 0.01, PTX treated compared to no PTX treatment; n/s, not significant). BALB-D2S cells, parent cell line; BDo- 14, BALB-D2S cells expressing Go-PTX; BDi2-22, expressing Gi2-PTX; BDi3-3, expressing Gi3-PTX." (Reprinted with permission from Macmillan Publishers Ltd: Molecular and Cellular Biology. 20(5): 1497-1506 Ghahremani MH et al., Copyright 2000).

Identification of TNFAIP8 as a Gαi3 interacting protein

Given the potentially important role of Gαi3 in fibroblast transformation, identification of interacting proteins and downstream pathways was investigated. A cDNA library from NIH-3T3 cells was screened in yeast two hybrid assays using constitutively active Gαi3 (Gαi3-QL mutant) as bait and a novel interacting protein was identified. This protein, formally named TNFAIP8, (also called SCC-S2, NDED, GG2-1 and MDC-3.13) shares 94% identity with its human homologue, contains a death effector domain (DED2) and is amplified in human carcinomas (Kumar D et al., 2000). The DED domain of TNFAIP8 shares significant sequence homology to DED2 domains found in

caspase 8, FADD and cFLIP, where cFLIP is believed to be a strong anti-apoptotic factor (Fig. 8) (Kumar D et al., 2000).

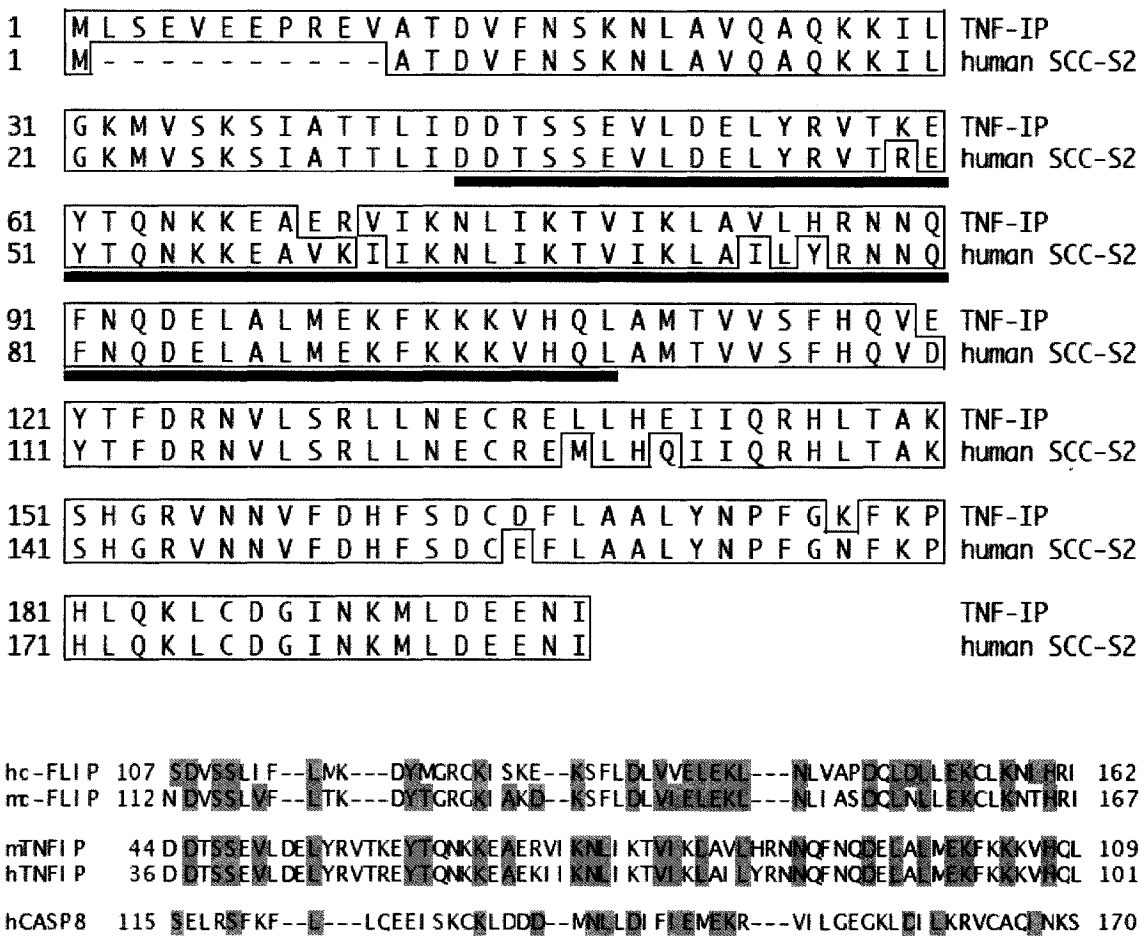


Figure 8. Alignment of human and mouse TNFAIP8 (here called TNF-IP or SCC-S2) as well as DED2 domains [Blue and yellow represent identical and similar residues, respectively, between any of the proteins] (figure credited to Helen Mao). Human and mouse TNFAIP8 share 94% sequence identity. TNFAIP8 contains a putative DED2 domain, which is similar to DED2 found in cFLIP and caspase-8 (Kumar D et al., 2000). Since DED domains are generally recognized as protein-protein interaction domains, it is possible that TNFAIP8 interacts with either or both of these proteins. TIPE2, a TNFAIP8 family member also containing a DED2 domain, has recently been shown to interact with caspase-8 (Sun H et al., 2008).

Additional yeast mating β galactosidase analysis showed strong interactions with constitutively active QL-mutant Gαo and Gαi1,2,3 subunits. Wildtype Gαi3 also showed strong interactions (Fig. 9A). While these results were achieved in yeast, they do suggest that wildtype Gαi3 interacts with TNFAIP8 and that this interaction could be occurring at the receptor prior to activation.

To determine if TNFAIP8 interacted with Balb/c-3T3 endogenous Gi/o proteins, flag-TNFAIP8 was transfected in cells and immunoprecipitated using the flag epitope. Immunoprecipitated proteins were run on SDS-PAGE and anti-Gαi1/2 and anti-Gαi3 were used as probes. Interactions between TNFAIP8 and Gαi3-wildtype and Gαi3-AlF_4 activated proteins were detected (Fig. 9B). In addition, TNFAIP8 interactions with Gαi2-wildtype were detected with weaker interactions for Gαi2-AlF_4. Gαi1 is not expressed in these cells.

Yeast mating β galactosidase assays were performed with several TNFAIP8 constructs to isolate the domains of TNFAIP8 that interact with Gαi3. Constructs included combinations of N-terminal, DED, full-length and truncated C-terminal domains. These experiments revealed that only the full-length TNFAIP8 and DED+C-terminal (here called ΔNT) domains interact with Gαi3. The full-length and truncated forms interact preferentially with wildtype Gαi3 compared to active mutants (Fig 9C). It remains to be seen if the DED domain plus a truncated form of the C-terminal will interact with the G-protein subunit.

Future studies should investigate whether endogenous TNFAIP8 and endogenous Gαi/o proteins interact in cells. However, this will require improved antibodies for TNFAIP8.

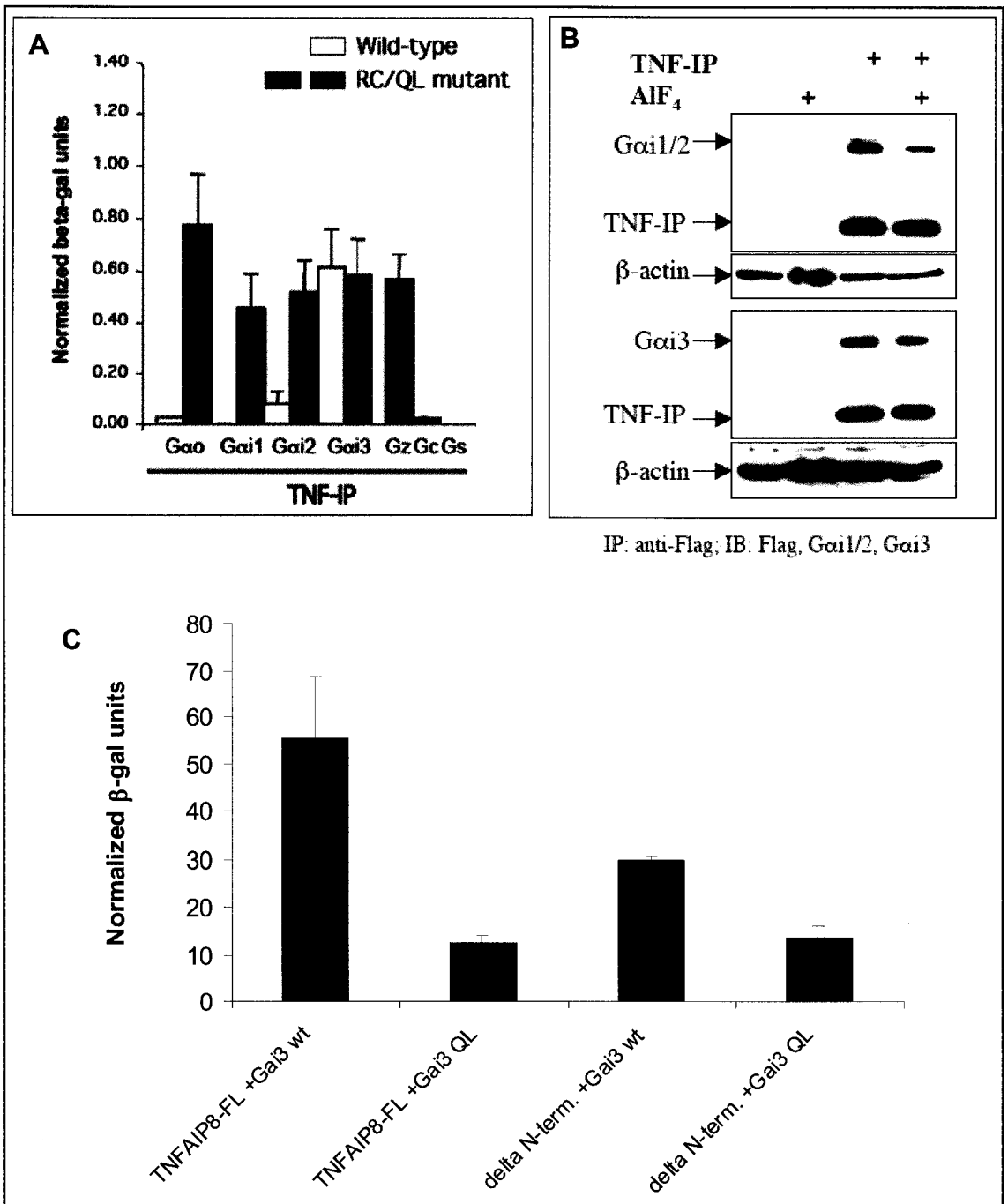


Figure 9. TNFAIP8 and Gi/o interactions in yeast mating and IP. A) Quantitative yeast mating β-galactosidase assay for TNFAIP8 and various Gα proteins showed strong preferential interactions with constitutively active RC/QL-mutant Gαi/o and Gz subunits, but also wildtype Gαi3 (credited to Helen Mao). B) Immunoprecipitation of FLAG-TNFAIP8 with anti-flag in stably transfected Balb/c-3T3 cells showed interactions with endogenous Gαi proteins (credited to Helen Mao). $AlF4^-$ interacts with GDP-bound Gα subunits and mimics the transition state such that GAP proteins bind the complex stably (Ahmadian MR et al., 1997). C) Quantitative yeast mating β-galactosidase assay for full-length TNFAIP8 and truncated delta N-terminal TNFAIP8 and showed strong preferential interactions with constitutively active RC/QL-mutant Gαi3 (credited to Mireille Daigle)

TNFAIP8 and select Gi/o signalling pathways in Balb/c-3T3 derived cells

To determine the effect of TNFAIP8 on previously characterized D2S-Gi/o signalling pathways, Helen Mao and Ariel Wilson in Dr. Paul Albert's laboratory stably transfected Balb/c-3T3 cells co-expressing D2S with either flag-tagged TNFAIP8, ΔNT-TNFAIP8 and antisense-TNFAIP8. This strategy produced a number of cell lines under-expressing and over-expressing TNFAIP8 which could be compared to cells expressing endogenous levels.

MAPK p44/42 phosphorylation was with measured phospho and total p44/42 MAPK antibodies. TNFAIP8 over-expression did not interfere with apomorphine-D2S mediated phosphorylation of p44/42 MAPK (Fig 10A). The antisense-TNFAIP8 clone, AS10, did show consistently higher basal activation of MAPK and reduced D2S-mediated activation compared to the D2S-only clone. Since Gαi2 and Gβγ are known to mediate the MAPK activation in these cells and it was shown that endogenous Gαi2 interacts with flag-TNFAIP8, it is possible endogenous and over-expressed TNFAIP8 inhibits MAPK activation keeping basal levels of activation lower than in the antisense-TNFAIP8 clone. However, once Gαi2 is activated, the effect is lost producing the greater response to apomorphine seen in these results.

Evaluating the effect of apomorphine stimulation on cAMP levels initially showed that TNFAIP8 over-expressers could not produce D2-induced adenylyl cyclase inhibition. However these clones (T4 and ΔNT3) were shown to have very low levels of D2 receptor. Subsequent experiments with clones (TB and TE) expressing higher levels of D2 showed the TNFAIP8 over-expression had no effect on cAMP levels (Fig 10B). It was observed by Ariel Wilson that these results suggested that the level of D2 receptor

was the important factor in determining the inhibition of cAMP production. Another possible consideration is the 1μM dose of apomorphine used in this experiment. It might be interesting to look at this pathway with lower doses since it is known that adenylyl cyclase inhibition is extremely sensitive even at low doses.

Calcium mobilization in response to dopamine treatment was then measured in the Balb/c-3T3 derived clones. D2S and TNFAIP8 over-expressing cells (TB and TE) had significantly decreased [Ca^{2+}] influx compared with cells transfected with D2S-only and antisense-TNFAIP8 cells (AS10). This result suggested that TNFAIP8 interactions with Gαi/o subunits might interfere with the coupling of Gβγ to phospholipase C activation (Fig 10C).

In summary, TNFAIP8 levels appear to modulate D2S pathways to calcium mobilization and MAPK activation while having no apparent effect on cAMP production.

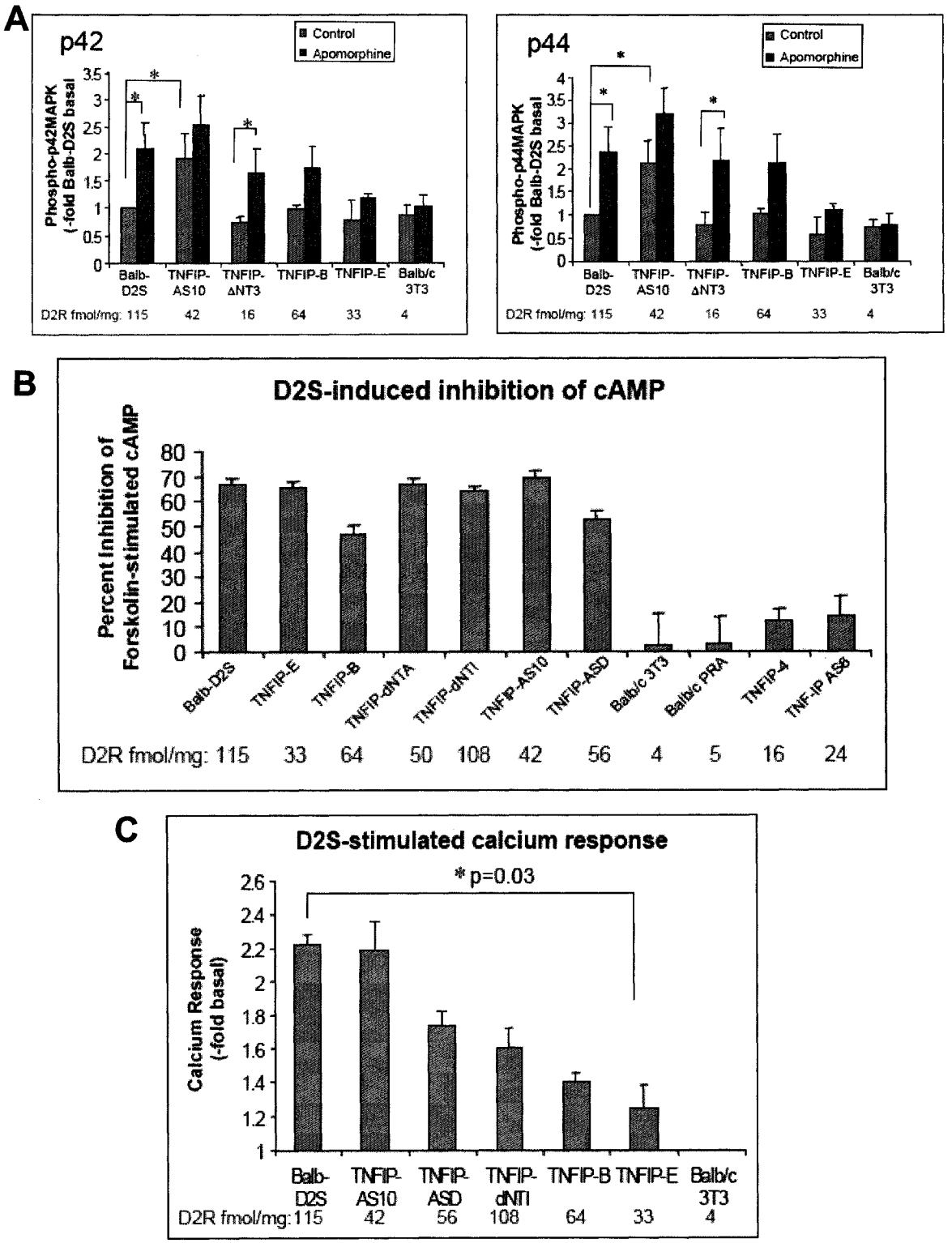


Figure 10. TNFAIP8 regulates some D2S downstream effectors (credited to Ariel Wilson). A) Balb/c-D2S clones were starved with 0.5% bovine calf serum and treated 7 minutes at 37°C with or without 1 μM apomorphine, run on a Western, and probed with phospho and total p44/42 MAPK antibodies. Densiometric analysis shows higher basal levels of MAPK activation in AS10 and no significant effect caused by TNFAIP8 overexpression. B) D2S-mediated inhibition of

cAMP in forskolin stimulated stable cells showed no significant effect caused by either knockdown or overexpression of TNFAIP8. Cells were incubated for 15 min with forskolin (10μM) with or without apomorphine (1μM). Percent inhibition of forskolin-stimulated cAMP by apomorphine is shown. C) D2S-mediated stimulation of calcium in stable cell lines showed reduced response in TNFAIP8 overexpressing TNFAIP8 E (TE) and TNFAIP8 B. Calcium mobilization in response to 1μM dopamine and 10μM ATP was measured by fluorescence of fura-2 loaded cells. Graphic representations of calcium traces show dopamine peak/ baseline ratios. The data are expressed as means +/- SEM (n=3-6) and were analyzed by student T-test (*, $p<0.05$). D2 receptor amounts are marked below each clone, and are in fmol/mg.

TNFAIP8 Identified Cancers

Since TNFAIP8's discovery, TNFα-inducible expression or endogenously elevated levels were reported in many carcinomas. Elevated expression in some cancer tissues corresponded with metastasis, chemo-resistance and radiation-resistance when compared to normal matched cancer cells. Below is a review of TNFAIP8 discoveries in cancer reported in the literature.

A study published in 1997 identified seven genes, by differential display of mRNAs and northern blot hybridizations, preferentially expressed in metastatic and radiation-resistant head and neck squamous cell carcinoma (HNSCC) when compared to non-metastatic matched tumours. One of the seven genes was TNFAIP8 (there called SCC-S2) (Patel S et al., 1997). A subsequent study, identified TNFAIP8 as over-expressed in some TNFα-treated tumor cell line (TNF-α at 20 ng/ml for 3 h: A549, ;2–9-fold; SKOV-3, ;3-fold; PCI-04A, ;3–6-fold) (Kumar D et al., 2000). Later, TNFAIP8 was again identified as a protein found in breast cancer cells (MDA-MB 231) and renal carcinoma cells (RCC-RS). Comparing human breast cancer and renal carcinoma tumor tissues samples from cancer patients to matched normal adjacent tissues TNFAIP8 had higher expression in many cases (Kumar D et al., 2004).

In addition, TNFAIP8 is an androgen-responsive gene and is upregulated over a long time course (8 to >72 hours) in four prostate cancer cell lines treated with androgen

analog, R1881. It is also downregulated by androgen depletion (DePrimo SE et al., 2002). Using a DNA promotor sequence micro-array with a protocol to provide specificity for unmethylated DNA fragments, genomic DNA of immortalized prostate epithelial cells and prostate cancer cells were compared. Of 2732 promotor sequences on the test array, 504 showed differential hybridization. Twenty-one of these genes were previously identified in prostate cancer and other cancer lines. In addition to these, some novel differentially methylated/regulated genes including TNFAIP8 were identified (Wang Y et al., 2005). In a follow-up study, TNFAIP8 was found to be methylated or reduced in copy number in androgen-dependent prostate cancer cells (LNCaP) compared to androgen-independent prostate cancer cells (PC3M) (Wang Y et al., 2005).

A comparison of mRNA expression, using a gene chip to identify genes implicated in DNA metabolism, nucleoside and nucleotide metabolism and transport, reactive oxygen species metabolism, apoptosis and response to drugs was performed in acute myeloid leukemia (AML) blasts cells taken from patients that had either blast persistence or complete remission after chemotherapy. There were 32 differentially expressed genes including upregulated TNFAIP8 in blast persistent patients. (Eisele L et al., 2007)

These findings show elevated levels of TNFAIP8 corresponding to metastasis and radiation-resistance in HNSCC as well as chemo-resistance in AML. In addition, gene regulation of TNFAIP8 by differential methylation is reported in prostate cancers and in some cases androgen receptor activation is a factor. Finally, TNFα can upregulate TNFAIP8 in some carcinomas.

TNFAIP8 in Cell Growth and Death

Since the discovery of elevated TNFAIP8 in metastatic and radiation-resistant HNSCC, a number of attempts have been made to elucidate mechanisms of TNFAIP8's actions in cancer cells. Most of these have focused on antiapoptotic functions and cell growth.

In 2000, Kumar et al.'s study also identified the inclusion of a DED2 domain in the N-terminus with significant homology to $cFLIP_L$'s DED2. However, it lacked the DED1 domain and caspase-like homology domain of $cFLIP_L$. It also showed significant anti-apoptotic effect of HeLa cells transiently transfected with flag epitope-tagged TNFAIP8 compared to vector transfected controls (Kumar D et al., 2000). An *in vitro* study of fibrosarcoma cells showed: 1) NF-κB activation by TNFα is essential for increased expression of TNFAIP8, 2) In NF-κB-null cells, transfection with TNFAIP8 inhibits TNFα-induced cell death, 3) over-expression of TNFAIP8 inhibits caspase-8 activity including cleavage of Bid and caspase-3 but not procaspase-8 processing and 4) TNFAIP8 does not affect the etoposide-activated intrinsic death pathway (You Z et al., 2001). In another study, transfection of TNFAIP8 in the breast cancer cell line MDA-MB 435 showed elevated growth rate *in vitro* compared to vector transfected controls and TNFAIP8 transfectants injected in athymic mice produced bigger tumors (Kumar D et al., 2004). These findings provided some evidence for cell growth rates and the inter-relationship between TNFα-signaling to death or survival and the role of over-expressed TNFAIP8 *in vitro*. It remains to be seen how endogenous TNFAIP8 affects death signaling *in vivo* and growth rates in cancer cells.

TNFAIP8 in Metastasis

Evidence for TNFAIP8 role in metastasis has also been investigated. In the 2004 study by Kumar et al. involving MDA-MB 435, TNFAIP8 transfectants also exhibited increased migration in collagen I compared to vector transfectants (Kumar D et al., 2004). In a subsequent study looking at TNFAIP8 ability to induce metastasis and invasion, TNFAIP8 transfected MDA MB 435 (t-transfectants) were studied *in vitro* and *in vivo*. The *in vitro* experiments showed increased invasiveness in Matrigel invasion assay of t-transfectants compared to vector-transfectants (v-transfectants). In an innoculation study of athymic mice, t-transfectants had significantly higher levels of tumor replacement and lesions in their lungs compared to control. Systemic delivery cationic liposome-entrapped TNFAIP8-antisense oligonucleotide inhibited pulmonary colonization of MDA-MB 435 transfectants (Zhang C et al., 2006).

Given that VEGFR-2 plays an important role in vascular endothelial cell growth and angiogenesis as well as tumor growth by autocrine and paracrine mechanisms, Zhang et al. next looked at *in vitro* expression of this receptor after TNFAIP8-antisense knockdown and found that it was downregulated. They next used siRNA to knockdown endogenous TNFAIP8 in MDA-MB 435 cells and observed that metalloproteinase MMP-1 and MMP-9 levels were significantly decreased, where MMPs play a role in metastasis by degrading the extracellular matrix. Taken together, these results showed that TNFAIP8 is involved in processes contributing to metastasis and invasion in cancer cells (Zhang C et al., 2006).

In the discussion of Zhang et al.'s results, they suggest some interesting mechanisms for how TNFAIP8 might mediate metastasis and regulate VEGFR-2 and

MMPs. In particular, they note that TNFAIP8 ORF has the signature sequence for vinculin family talin binding region proteins. Talin can activate integrins and is involved in cell motility, wound repair, and tumor metastasis. They therefore suggest that TNFAIP8 could act as a scaffold protein for cytoskeletal modulators (Zhang C et al., 2006). Whether or not Gαi interactions with TNFAIP8 have an influence on cytoskeletan could be interesting.

TNFAIP8 and -like Proteins in Immune Cells

A couple of studies have uncovered a role for TNFAIP8 and family member TNFAIP8-like-2 (TIPE2) in immune cell activation. In the first, viral vector expression TNFAIP8 blocked the activation of Jurkat T-cells by T-cell receptor (TCR) as assessed by expression of CD69 marker (Chu P et al., 2003). The second study uncovered a similar result for TIPE2 in T-cell and macrophage activation but went further to elucidate the mechanisms. TNFAIP8-like-2 (TIPE2) is a member of the TNFAIP8 family consisting of four members. It shares significant homology with TNFAIP8 (53% identity / 78% similarity) and is preferentially expressed in the lymphoid. Sun et al. discovered that TIPE2 is required for maintaining immune homeostasis by preventing hyperactivation of immune cells through T-cell receptor and Toll-like receptor. TIPE2 binds directly to caspase-8 through its DED domain and enhances activation-induced cell death and FasL-mediated apoptosis while blocking NF-κB activation and activating protein-1 (AP-1) activation. TIPE2 has no effect on cell death mediated by staurosporine, ultraviolet (UV), gamma irradiation, etoposide, LPS, CpG, TNFα, serum or cytokine deprivation. TIPE2 deletion leads to multi-organ failure and death in mice. TIPE2 is not

constitutively expressed in mouse fibroblast but can be induced by TNFα (Sun H et al., 2008). These results, coupled with the fact that TNFAIP8 is also highly expressed in the lymphoid and myeloid cell lines (Genomics Institute of the Novartis Research Foundation, online data: http://symatlas.gnf.org/SymAtlas/), suggest that it may have an important role in immune homeostasis. These cell lines could be interesting avenues for continued research for Gαi interactions with TNFAIP8.

TNFAIP8 in Inflammatory Disease

Using random-primed reverse transcriptase-polymerase chain reaction, TNFAIP8 was identified (and called GG2-1) in 1999 as a gene with augmented expression in vascular endothelial cells exposed to TNFα or monocyte-conditioned medium. The study was designed to identify important genes involved in atherosclerosis. TNFAIP8 expression was most elevated at 1.5 and 3 hours of TNFα treatment with fading expression through 6 and 20 hours. (Horrevoets AJ et al., 1999).

In another study using oligonucleotide microarrays, gene expression levels in independent replicate samples of rheumatoid arthritis synovial fibroblasts (RASFs) were measured after transfection with dominant negative DN-IκB (IkB sequesters NF-*k*B in the cytosol) and 3-hour treatment with 10 ng/ml TNFα. Among the genes found to be downregulated by NF-kB blockade was TNFAIP8. Inhibition of TNFAIP8 with siRNA in primary wildtype RASFs resulted in enhanced apoptosis, decreased proliferation and expression of matrix metalloproteinase (MMP) compared to vector transfected controls and wildtype cells (Zhang HG et al., 2004). MMP-1 is known to destroy connective tissues and is a key mediator of pathogenesis in arthritis (Pardo A and Selman M, 2006).

These studies suggest roles for TNFAIP8 in the pathology of inflammatory diseases in arteries and joints.

Balb/c-3T3 transfected with D2S as a model for Gi/o activation

D2 receptor was chosen for this study because it is very selective for Gi/o coupling and dopamine receptors are not expressed in fibroblast, which provides a clean background for this investigation. In addition, there is no endogenous dopamine in cells or serum eliminating the posibility of autoactivation or serum-induced activation. A study using 5HT1A receptor transfected in Balb/c-3T3 cells showed that cells transformed very rapidly due to 100nM 5-HT found media containing 10% serum (Abdel-Baset H et al., 1992)

The selection of Balb/c-3T3 cells as a model for the investigation of cell proliferation and transformation was made because this cell line is not transformed, displays contact inhibition and is transformation resistant compared to other mouse fibroblast cells (such as NIH-3T3). Gi/o signalling pathways are present in Balb/c-3T3 such that D2S mimics endogenous thrombin and lysophosphatidic acid receptors (Moolenar WH, 1995) (LaMorte VJ, 1993)(van Corven EJ, 1993).

Project Background Summary

- D2S stimulation in Balb/c-3T3 causes cell proliferation and transformation
- Activation PTX-insensitive Gαi3-mutant induces transformation in PTX challenged Balb/c-3T3
- Gαi3 interacts with TNFAIP8 in yeast mating
- Endogenous Gαi3 co-immunoprecipitates with FLAG-TNFAIP8 in Balb/c-3T3
- TNFAIP8 has a DED2 domain similar to c-FLIP, FADD and caspase-8
- TNFAIP8 inhibits caspase-8 activity and is elevated in some cancers.

Project Aims

- Show that D2S-Gαi3 activation induces transformation in Balb/c-3T3 and this is dependent on TNFAIP8.
- Show D2S-mediated inhibition of TNFα-induced cell death and caspase activity that is dependent on TNFAIP8

Hypothesis

D2S/Gαi3 activation induces transformation by inhibiting pro-apoptotic TNFα-induced death receptor signaling through TNFAIP8

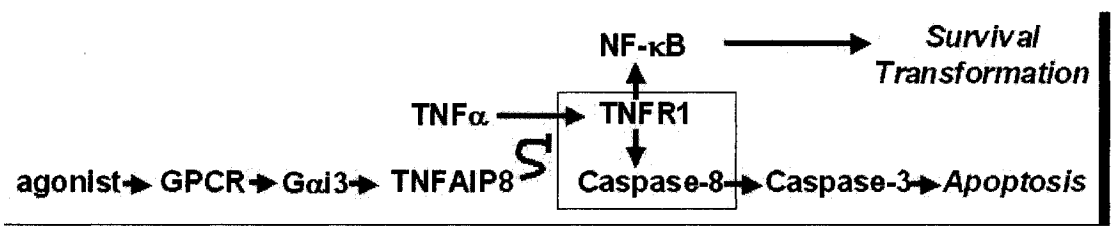


Figure 11. Hypothesis Diagram

CHAPTER 2

Materials and Methods

Cell Culture and Transfection. Wild-type BALB/c-3T3 (here called "3T3") cells and derivative clones were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 8% FBS from Wisent (matched to lot #115609), 100 μg/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37 °C, 5% CO_2 and humidity-controlled environment for all growing and experimental incubations. The stably transfected BALB/c-3T3-D2S cell line (here called "D2S") was created as per Ghahremani et al. (Ghahremani MH et al., 2000). BALB/c-3T3-D2S-TNFAIP8 (here called "T4" and "TE") and BALB/c-3T3-D2S-antisense-TNFAIP8 (here called "AS6" and "AS10") were created as per Ariel Wilson (unpublished data). In summary, vector constructs of D2S-pZEM + pY3 and TNFAIP8-pcDNA3 or antisense-TNFAIP8-pcDNA3 were cotransfected by calcium phosphate coprecipitation in BALB/c-3T3 cell. Selection for D2S-pZEM + pY3 was performed with 400 μg/ml hygromycin B and selection for TNFAIP8-pcDNA3 mutants was performed with 700 μg/ml G418. Protein levels and mRNA levels of TNFAIP8 were verified in BALB/c-3T3 clones by Western Blot analysis and RT-PCR (Appendix, p.105).

Table 1: Labels for Balb/c-3T3 derived cells and their transfected protein content

Cell Line Identifier	D2S overexpressing	Antisense-TNFAIP8 knockdown	TNFAIP8 overexpressing
3T3	No	No	No
D2S	Yes	No	No
AS6	Yes	Yes	No
AS10	Yes	Yes	No
TE	Yes	No	Yes
T4	Yes	No	Yes

D2 Binding Assay. Cells lines were grown from frozen stocks and used between passages 4 and 10 from thawing. Cells were split and grown in 10 cm dishes (as per

above) for at least 24 hours to a confluence near 100%. One hour prior to collecting cells, the DMEM containing 8% FBS was replaced with new media. Cells were placed on ice and washed with ice-cold hypotonic buffer [15 mM Tris-HCl, pH 7.4, 2.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA)]. After soaking in hypotonic buffer for 5 minutes to cause swelling, cells were scraped from plates and spun at 200g for 15 minutes at 4 °C to form pellets. Pellets were resuspended in TME buffer [75 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, 1 mM EDTA] and homogenized with a syringe and $25^{5/8}$ gauge needle by repeated aspirating and dispensing. Cell membranes were again spun down to a pellet at maximum speed for 30 minutes at 4 °C. Supernatant TME was aspirated and pellets were frozen -80 °C. Pellets were later resuspended in 700 µl hypotonic buffer and 100 µl aliquots were transferred from each sample to 6 micro-centrifuge tubes. To three total binding tubes, 200 µl of TME, 0.1% ascorbic acid and 10nM [^{3}H] spiperone and to the other 3 non-specific binding tubes, 200 µl of TME, 0.1% ascorbic acid, 10nM [^{3}H] spiperone and 10 µM apomorphine was transferred. The binding reaction was stopped after 30 minutes at room temperature with 1 ml of ice-cold 50 mM Tris at pH 7.4. Membranes were then collected on filter paper by suction and washed three times in 3ml of ice-cold 50 mM Tris at pH 7.4. Scintillation counts of the membranes were recorded and protein concentrations determined by Bradford Assay. Specific binding was calculated as (total - non-specific dpm)/specific activity ($2.5x10^5$ dpm/pmol), normalized to protein concentration.

Soft Agar Foci Formation. Cell lines were grown from frozen stocks and used between passages 4 and 10 from thawing. Cells were grown (as per above) for 48 hours from a density of 500K cells per 10cm dish prior to plating in soft agar and media was changed

24 hours prior. 6-well plates were prepared with a 1.5 ml base layer of 0.5% agar (by weight) in 85% RPMI and 15% FBS (by volume). The top layer containing 25K cells per well, 0.35% agar (by weight) 85% RPMI and 15% FBS (by volume) was added to the base layer gel. All RPMI solutions were supplemented with 100 μg/ml penicillin and 100 μg/ml streptomycin. The top layer gel of the apomorphine (APO) treatment group was supplemented with 1 μM of apomorphine. Melted agar was allowed to cool to less than 42 °C prior to mixing with cells. Cells were grown for 3 weeks in soft agar and every 2-3 days media was replaced with 200 μl treatments of 15% FBS-RPMI without and with 1 μM apomorphine in each well of control and APO treatment groups respectively. At the end of three weeks, cells were stained with 1 ml of 0.005% crystal violet for 1.5 hours. Microscopy was performed using Olympus SZ61 dissection microscope at a magnification of 0.8x and photographed with PixeLink Mega Pixel Firewire Camera. Images were analyzed and foci automatically counted using ImageJ software (colour-BMP converted to 8-bit greyscale; background subtracted using 50pixel rolling ball radius method; intensity threshold set between 135-235; foci size threshold > 4 pixels). In each 6-well plate, triplicates of control and APO wells were grown for a single cell line. These experiments were repeated independently 3 to 4 times per cell line.

Caspase Activity Assay. Cell lines were grown from frozen stocks and used between passages 4 to 10 for 3-hour starvations or 8 to 15 for 20-hour starvations from thawing. For each cell line and treatment group 300K cells were grown in 6 cm dishes for 24 hours (as per above) and then starved in 2 ml of 0.5% FBS-DMEM for either 3 hours or 20 hours (see results). To each well, drug treatments in 2 ml of DMEM containing 20 μg/ml cycloheximide (CHX) were added to produce a final concentration of 10 mg/ml CHX.

Drug treatments included mouse-TNFα (Calbiochem), APO (Sigma) and spiperone (SPP) (Sigma) (see results for specific drug doses and combinations). CHX and drug treatment were applied to cells for 3 hours in the standard incubation environment and then placed on ice. Cells were then washed twice in ice cold PBS. Cells were then collected from dishes by scraping in 100 µl of ice-cold caspase activity lysis buffer {10mM HEPES, 1mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 5 µg/ml aprotinin, 2 µg/ml, leupeptin, 0.1% NP40, 1.0 mM DTT, 0.2 mg/ml PMSF}. Lysed cells were incubated on ice for at least 15 minutes and then spun down at 14000 RPM for 10 minutes at 2 °C. Supernatants were collected and stored at -80 °C. Thawed supernatants were analyzed by Bradford assay and protein concentrations determined. In 200 µl of caspase activity assay buffer {25 mM HEPES, 10 mM DTT, 10% sucrose, 0.10% CHAPS, 15 µM Ac-DEVD-AFC (Biomol)}, 5 µg of protein was incubated at 37 °C while fluorescence intensity was measured at specified intervals in Molecular Devices SpectraMax M5. Slopes determined by linear regression of fluorescence intensity over time were used as a measure of DEVDase activity.

Live Dead Assay. Cell lines were grown from frozen stocks and used between passages 8 and 15 from thawing. For each cell line and treatment group cells were grown to 50-100% confluence in 4 wells of a 6-well plate for 24 hours (as per above) and then starved in 1.5 ml of 0.5% FBS-DMEM for 20 hours. To each well, drug treatments in 1.5 ml of DMEM containing 20 µg/ml cycloheximide (CHX) were added to produce a final concentration of 10 mg/ml CHX. Excluding control (here called "C"), all drug treatments included a final concentration of 5 ng/ml of mouse-TNFα (Calbiochem). (Drug treatments including TNFα exclusively are here called "CT"). Drug treatments including

CHX, TNFα and 0.3 μM APO (Sigma) (here called "CTA") and treatments including CHX, TNFα, APO and 1.0 μM spiperone (SPP) (Sigma) (here called "CTAS") are also included. Drug treatments were applied to cells for 3 hours in the standard incubation environment. Wells containing treated cells were aspirated to remove media. To each, 500-600 μl of 2 μM calcein-AM and 4 μM ethidium homodimer-1 were added. Cells stained quickly and were photographed for up to 30 minutes after staining. Microscopy was performed on Zeiss Axiovert S100 and photographed with Sony Power HAD 3 CCD colour video camera. Stained cells were counted manually and using ImageJ software (colour-BMP converted to 8-bit greyscale; intensity threshold set to maximize counts; foci size threshold > 1 pixel).

Active Caspase Staining Assay. Cell lines were grown from frozen stocks and used between passages 4 and 10 from thawing. For each cell line and treatment group 500K cells were grown in 4 wells of a 6-well plate on cover slips for 24 hours (as per above) and then starved in 1.5 ml of 0.5% FBS-DMEM for 16 hours. Then 1.5 ml of the four treatment solutions (C, CT, CTA and CTAS) described in the Live Dead Assay (above) were applied to the cells for 2.5 hours. Media was then removed and replaced with 350 μl of media containing 1X FLICA (or FAM-DEVD-FMK) (Chemicon Caspatag Assay Kit) for 1.5 hours incubation. After incubation, media was removed and cells were washed twice in 1 ml of wash buffer (kit). Cover slips were then mounted microscope slides with 40 μl of fixative (kit). Microscopy was performed on Zeiss Axioskop 2 MOT and photography was performed on Q-imaging QICAM FAST mono 12-bit camera. Image capturing and processing was performed with Northern Eclipse v6.0 software. Cells were counted manually.

CHAPTER 3

Results

Based on the model (Fig. 11) that TNFAIP8 links D2S receptor-Gαi3 signalling to cellular transformation in Balb/c-3T3 cells, we hypothesized that alteration in TNFAIP8 expression would affect basal or apomorphine induced foci formation. Balb-D2S cells stably transfected with sense or antisense to TNFAIP8 were examined for foci formation following three-week treatment.

1. TNFAIP8 knockdown reduces basal rates of foci formation in D2S-expressing Balb/c-3T3 cells. A focus formation assay for six Balb/c-3T3 stable cell lines (see table1) was performed. There were two treatment groups including control (15% FBS-RPMI) and a drug treatment (1 μM apomorphine in 15% FBS-RPMI). After three weeks of repeated treatment, the total numbers of foci were counted in both groups. The results showed an increase in basal rate of foci formation for D2S expressing cells (D2S, TE) that was reversed in antisense-TNFAIP8 co-expressing clones (AS6, AS10) (Fig 12A). This suggests that the constitutive activity of D2S may be sufficient to promote transformation in Balb/c-3T3. However, this effect is reversed in cells where TNFAIP8 is knocked down by antisense RNA. The fact that T4 does not show a significant increase in the basal rate of foci formation might relate to lower levels of D2S receptor expression in this cell line (see Fig 13). In summary, D2S expressing cell showed a high basal rate of foci formation compared to wild-type Balb/c-3T3 cells and this was reversed by antisense of TNFAIP8, suggesting that TNFAIP8 mediates constitutive D2S receptor-induced transformation of Balb/c-3T3 cells.

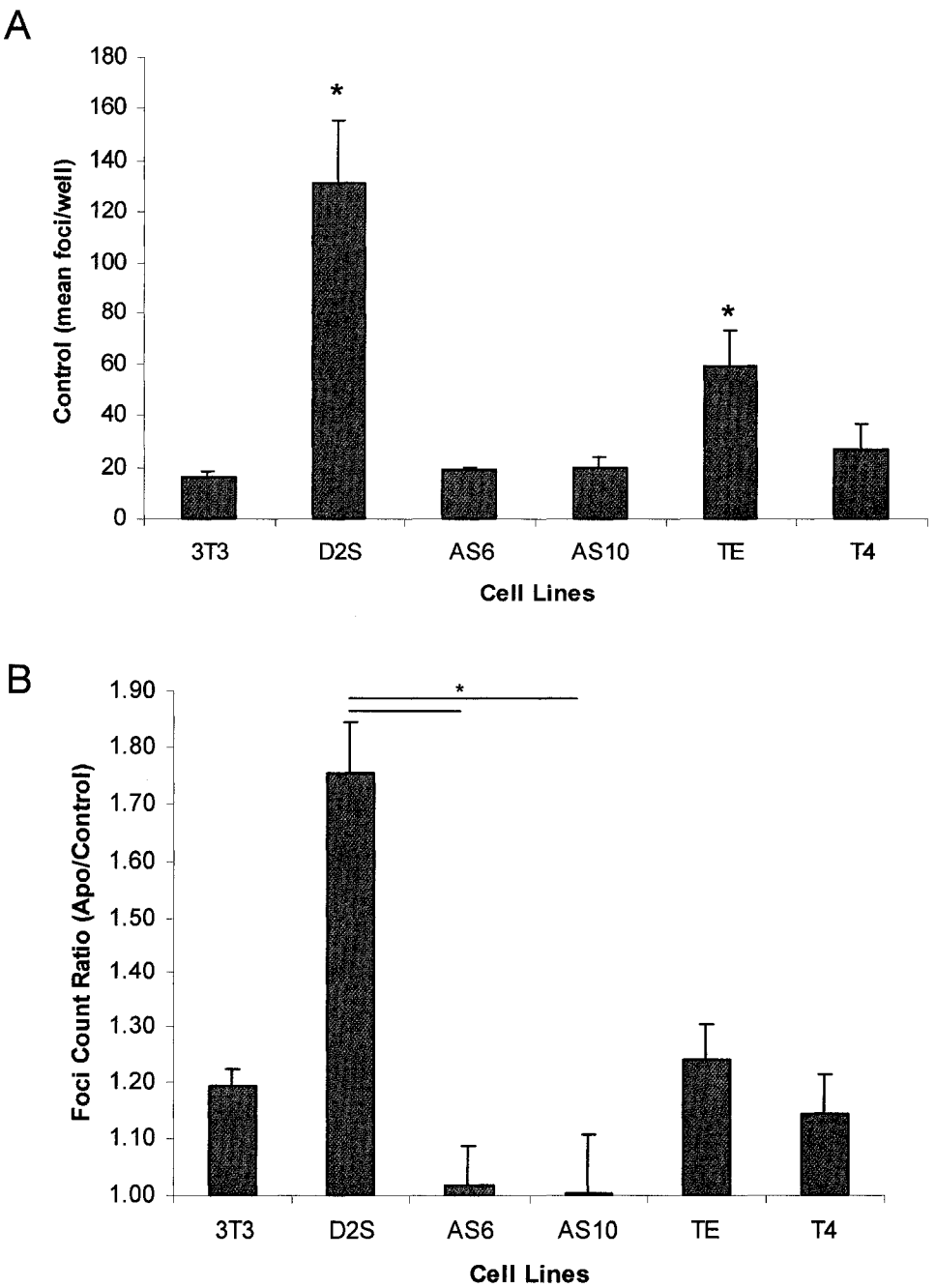


Figure 12. Basal and apomorphine stimulated foci formation in soft agar: Balb/c-3T3 clones were grown in soft agar and treated with control solution (15% FBS-RPMI) or 1 μM apomorphine (in 15% FBS-RPMI) for 3 weeks A) Mean foci/well counts for the control treatment group of various cell lines are shown. (*) significantly differ from 3T3 as determined by one-way ANOVA Dunnett test (alpha = 0.05). B) Foci count ratios of apomorphine vs control are compared between cell lines. (*) are significantly different from each other by one-way ANOVA and Bonneferroni test (alpha=0.05). {n = 4 for 3T3 and TE, n = 3 for others}. Error bars are standard error

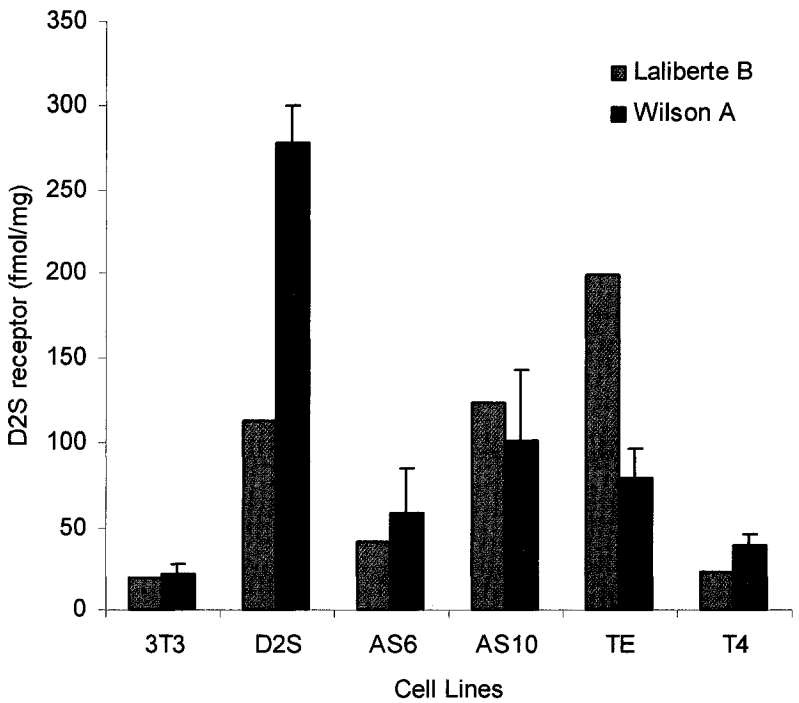
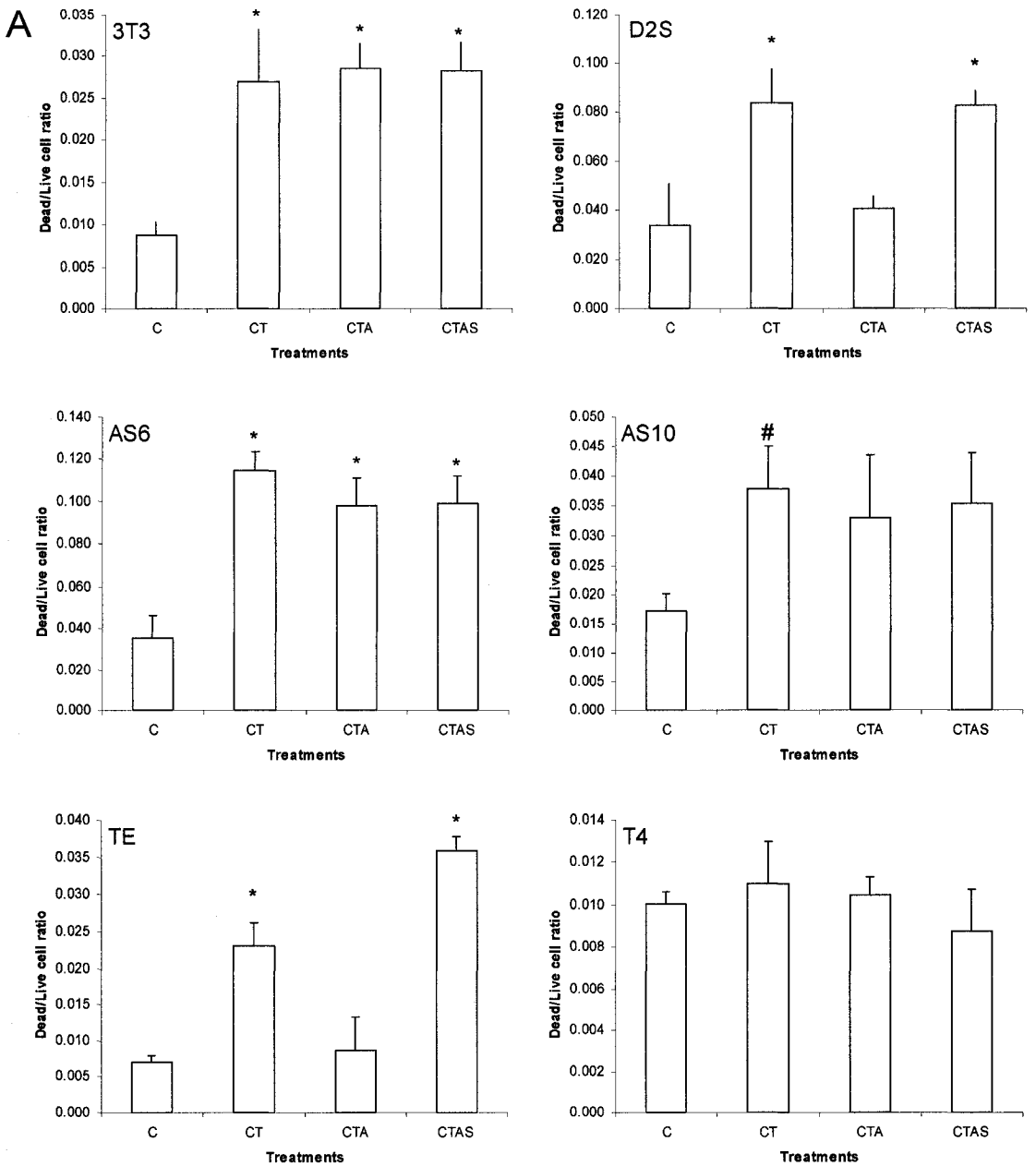


Figure 13. D2S binding assay: Assay performed on membrane preparations from the indicated cell lines. Shown is the specific [^{3}H]-spiperone binding (fmol/mg protein). The second dataset was collected by Ariel Wilson and is expressed as mean +/- SEM (n=5-9). The first dataset was collected after the experiments reported in this study were completed. I performed another D2 binding assay to recheck the levels of D2 in these cell lines. For n=2 assays done in triplicate, the results were qualitatively similar to Ariel's values with D2S, TE and AS10 binding more than 3T3, AS6 and T4 (although D2S decreased while TE increased binding.)

2. TNFAIP8 knockdown reduces apomorphine-induced foci formation in Balb-D2S cells. Foci formation following apomorphine (APO) treatment was analyzed by computing the ratio of foci in APO-treated over control plates. APO-induced foci formation in Balb-D2S cells was significantly greater than in wild-type Balb/c-3T3, AS6 and AS10 (Fig 12B). This suggests that endogenous TNFAIP8 is required for D2S-induced foci formation since TNFAIP8 knock down significantly reduces this effect. TNFAIP8 over-expression did not have a significant effect with respect to the control cell line (3T3). Over-expression of TNFAIP8 may saturate the foci formation enhancement effect or the lower D2S levels in these cell lines may limit the effect. These results suggest that TNFAIP8 is required for D2S receptor-induced transformation of Balb/c-3T3 cells.

3. D2S signalling inhibits TNFα-induced cell death in Balb/c-3T3 cells expressing endogenous or over-expressed levels of TNFAIP8. In order to address the hypothesis that D2S receptor actions to induce transformation correlate with TNFAIP8 induced inhibition of cell death, Live-Dead assays (Invitrogen) of TNFα-induced cell death were performed on the same six Balb/c-3T3 cell lines. This assay is designed to differentiate between live and dead cells based on the integrity of their membranes. Calcein-AM, used to label living cells, is membrane permeable and a fluorogenic substrate of esterases expressed ubiquitously in mammalian cells. Ethidium homodimer-1 EthD1 can only enter cells through damaged membranes where it binds nucleic acid allowing it to produce a strong fluorescent signal. As such, EthD1 labels cells in late stage apoptosis and necrosis. In this assay, cells were starved for 20 hours, and treated for 3 hours in medium containing cycloheximide without or with TNFα and APO, or spiperone.

Cycloheximide is used to block synthesis of new proteins and prevents anti-apoptotic NF-κB signalling induced by TNFα treatment, revealing the apoptotic pathway (Ogura H et al., 2008). It is also reported that cycloheximide and its more potent structural derivative acetoxycycloheximide block NF-κB by inducing TNFR1 to shed its ectodomain via the activation of p38 MAPK and ERK (Ogura H et al., 2008). Considering the control treatment of CHX only, there was no significant difference between cell lines. In wild-type Balb/c-3T3 cells, TNFα treatment increased significantly the dead/live cell ratio, and addition of APO alone or with SPP did not affect TNFα-induced cell death, consistent with the lack of D2 receptors in these cells (Fig 14A). In D2S and TE cell lines, the significant increase in TNFα-stimulated death was reversed by APO treatment and this effect was blocked by D2 antagonist, SPP. In AS6 cells, the significant increase TNFα stimulated death was unaffected by APO or SPP. The behaviour AS10 was similar to AS6 but was not statistically significant ($P<0.1$). The amount of TNFα-stimulated death in T4 cells was too low to show trends between treatment groups, consistent with an inhibitory effect of TNFAIP8 on TNFα-induced cell death. Overall, these results suggest that TNFα-induce cell death is reversed by D2S activation with APO in cells possessing endogenous or over-expressed levels of TNFAIP8. The death inhibiting effect of APO is reversed by SPP treatment and is not observed in wild-type Balb/c-3T3 cells, consistent with mediation via D2 receptors.


A
3T3
D2S
AS6
AS10
TE
T4
Dead/Live cell ratio
Treatments
C
CT
CTA
CTAS
*
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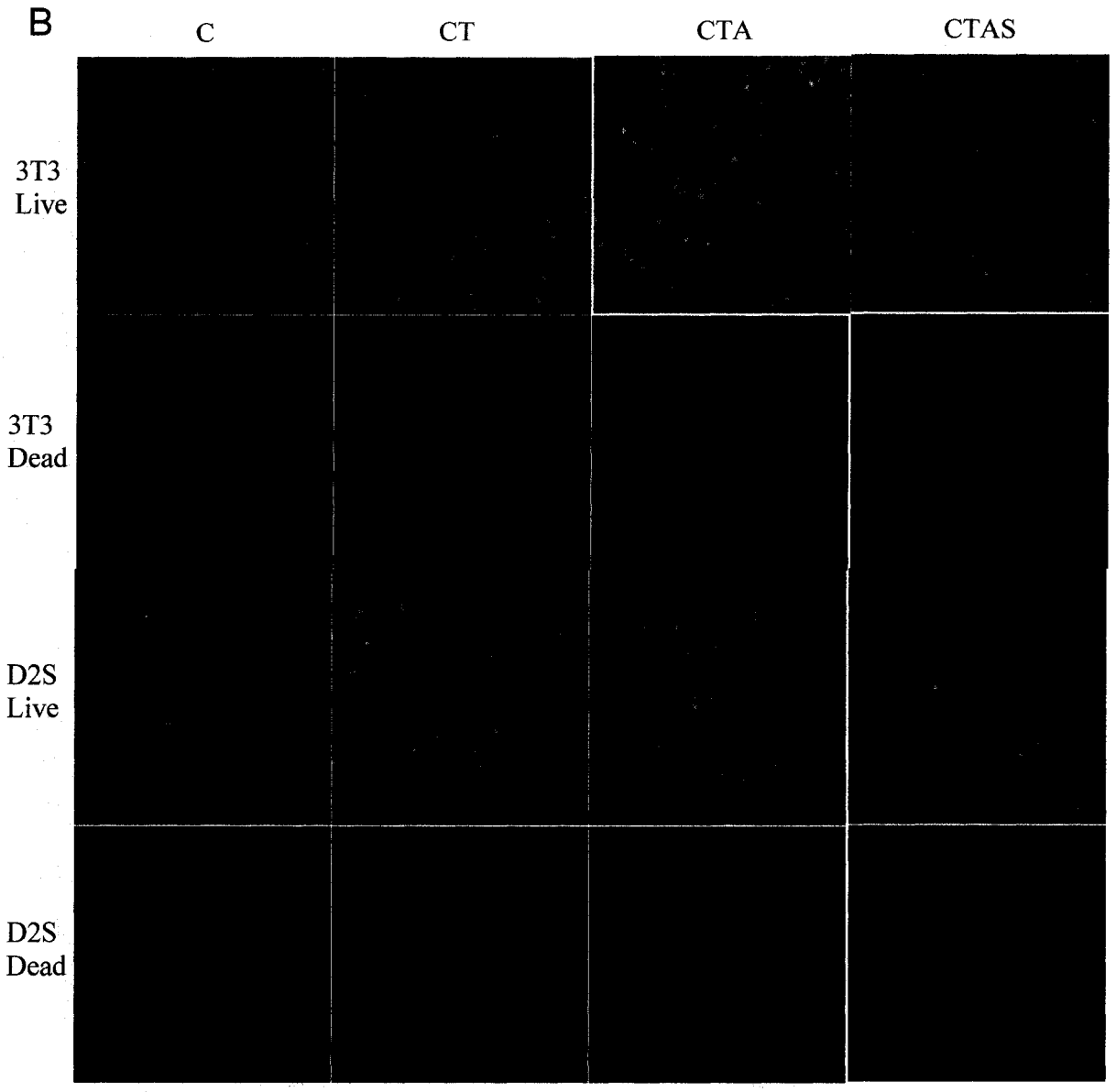


Figure 14. Invitrogen Live/Dead(R) assays were performed on Balb/c-3T3 clones starved for 20 hours in 0.5% FBS-DMEM and treated for 3 hours with cycloheximide (10 μg/ml) (C), CHX+TNFα (5 ng/ml) (CT), TNFα+ apomorphine (0.3 μM) (CTA), TNFα+APO+ spiperone (1 μM) (CTAS). A) Dead/live ratios for four treatment groups and six cell lines are shown. (*) are significantly different from control (C) by Dunnett (alpha=0.05) and (#) indicates a trend (alpha=0.10). {n = 4 for AS6, n = 3 for others}. Error bars are standard error. B) Photos of representative samples are shown [green fluorescence generated by esterase activity on calcein-AM indicates live cells while red fluorescence produced by ethidium homodimer-1 and nucleic acid binding indicates dead cells].

4. Apomorphine has caspase-independent, cytotoxic effects, at higher doses while having variable effects on caspase activity at lower doses in transfected Balb/c-3T3 cells. DEVDase (caspase-3) activity assays were performed on cells starved for 3 hours and treated with varying doses of TNFα and APO. In repeated experiments, doses of 5 ng/ml TNFα produced significant DEVDase activation in D2S and near maximal activation in 3T3 (Appendix, p. 106). Therefore, this dose was chosen for subsequent experiments. Varying doses of APO were then used on 3T3 and D2S cells treated with CHX and TNFα. Doses exceeding 0.3 μM APO produced a significant inhibition of TNFα-induced DEVDase activity in wild-type Balb/c-3T3 cells (Fig 15A). Since these cells lack dopamine receptors, this is considered a non-specific inhibition of caspase activity. Cytotoxicity of apomorphine has been reported and is characterized by the formation of green conjugates resulting from its non-specific covalent bonding to cellular proteins (El-Bacha RS et al., 1999), which were observed here at a concentration of 12.5 μM APO. In D2S cells, a similar inhibition of DEVDase activity occurs at doses exceeding 2.5 μM (Fig 15B). Paradoxically, low doses of APO (0.02 μM) produced a small but significant DEVDase activation in D2S cells, while intermediate doses (0.10-0.30 μM) produced a trend toward DEVDase inhibition. Because only a small proportion of cells undergo cell death (see Live-Dead assay) it was difficult to show a significant inhibition of total caspase-3 activity using this approach. Given these results, 0.30 μM APO treatment was chosen for the Live-Dead assay reported above.

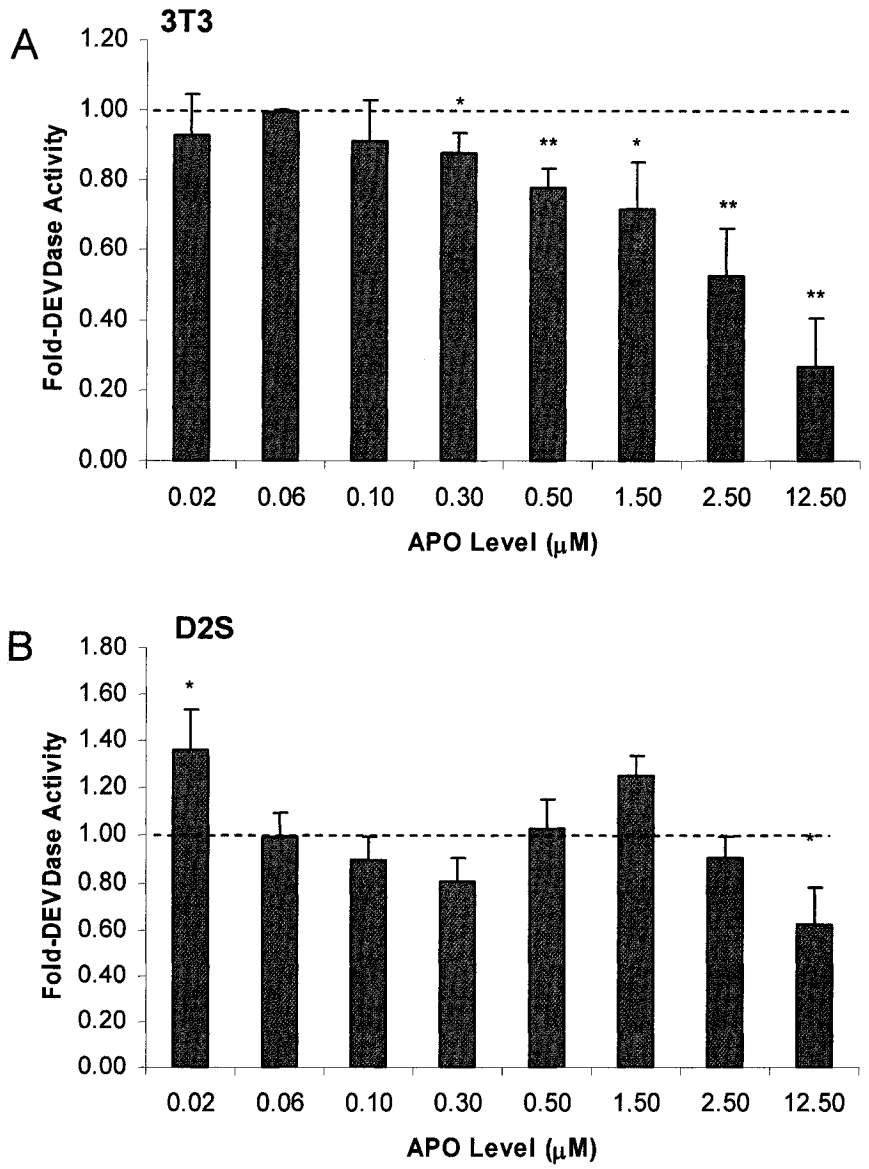


Figure 15. DEVDase activity assays were performed on 3T3 and D2S cell lines starved for 3 hours in 0.5% FBS-DMEM and treated for 3 hours with 5 ng/ml TNFα plus 10 μg/ml cycloheximide (CHX) or varying apomorphine levels plus TNFα+CHX. A) Shown are mean fold-DEVDase activity over control (CHX+TNFα) for 3T3 and B) D2S. (*) and (**) significantly differ from 1-fold as determined by confidence interval tests (alpha = 0.05 and = 0.01, respectively). {n=3 or 4 with the exception of 3T3-0.06 μM and D2S-0.06, 0.3, 1.5 μM where n=2}. Error bars are standard error

5. Apomorphine weakly inhibits TNFα-induced DEVDase activity in TNFAIP8 over-expressing clones. The six cell lines were starved for 20 hours and treated for 3 hours with varying concentrations of APO in addition to CHX and TNFα and protein lysates were used in DEVDase activity assays. The varying concentrations of APO had no significant effect of TNFα-induced DEVDase activity on Balb/c-3T3, D2S and AS6, although there was a trend toward non-specific inhibition of DEVDase activity at high APO concentration, as observed above. Interestingly, DEVDase was inhibited at low concentrations of APO in T4 and TE (Fig 16), suggesting that over-expression of TNFAIP8 may enhance D2S-induced inhibition of TNFα-induced caspase activity.

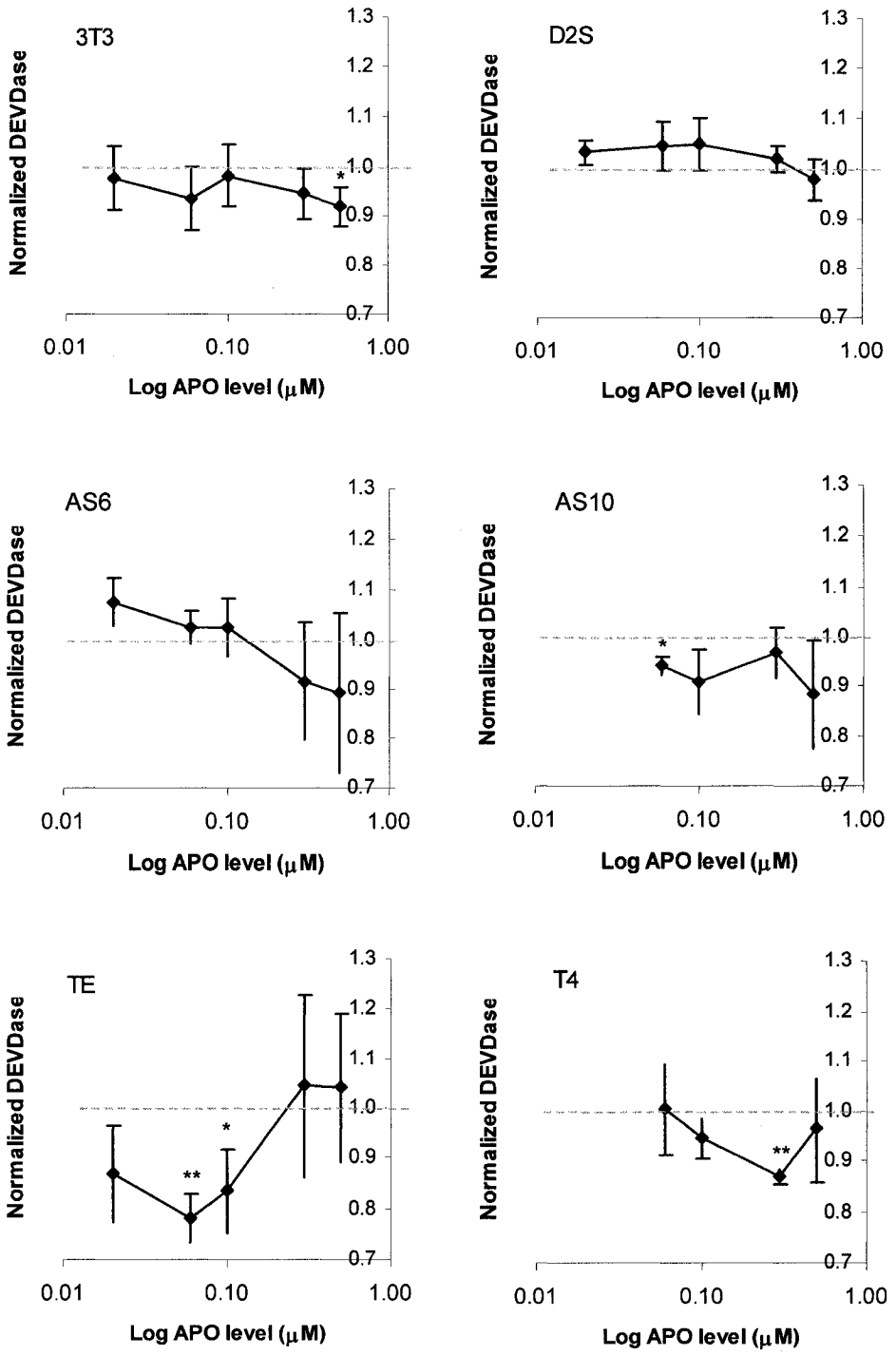
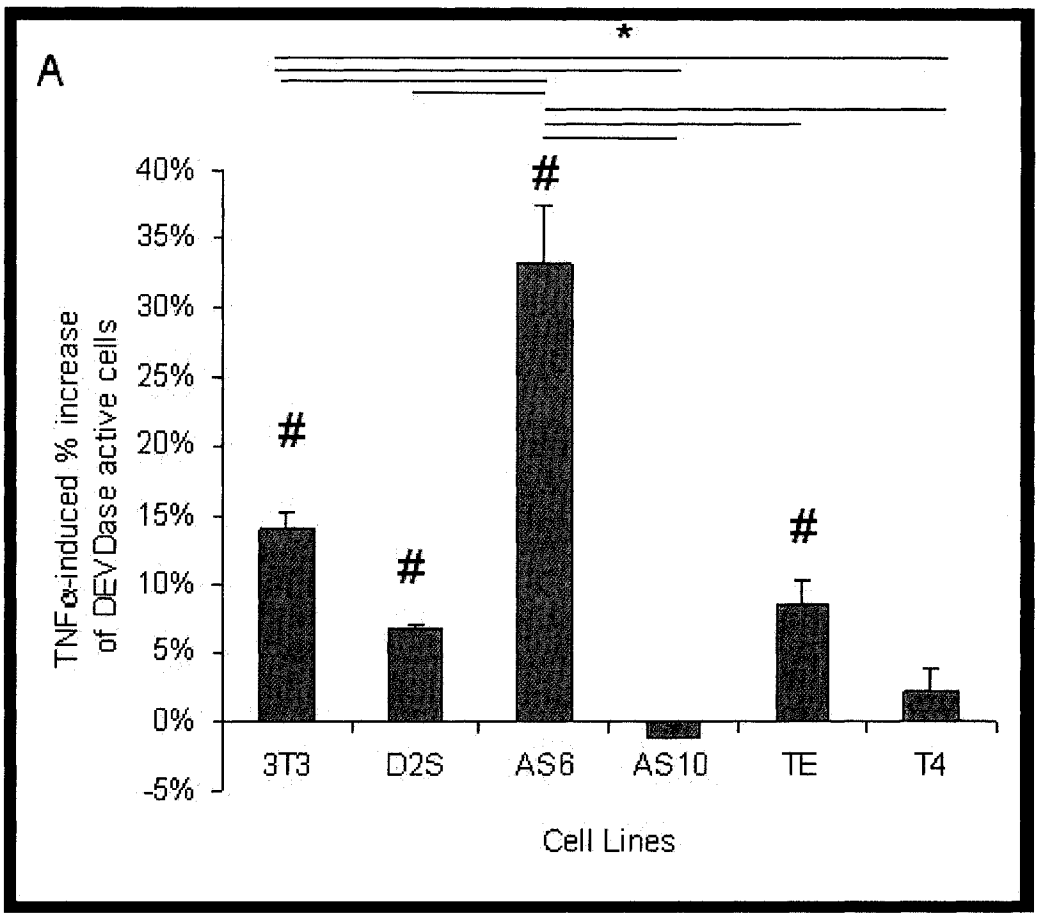


Figure 16. DEVDase activity assays (overnight serum withdrawal) of 10 μg of protein from lysates of various cell lines were measured after 20 hours starvation in 0.5% FBS-DMEM and 3 hours treatment in 10 μg/ml CHX plus 5 ng/ml TNFα and varying levels of apomorphine. Shown are the means of DEVDase activity in APO treated cells normalized to control (CHX+TNFα). (*) and (**) significantly differ from 1-fold as determined by confidence interval tests (alpha = 0.05 and = 0.01, respectively). {for 3T3, D2S, AS6, AS10, TE and T4, n = 5, 6, 4, 3, 4 and 3, respectively}. Error bars are standard error

6. Negative regulation of TNFα-induced DEVDase activation by TNFAIP8. In order to provide a more sensitive assay for DEVDase activation, Chemicon CaspaTag^{TM} assays were done to identify cells in which caspase activation was induced. Cells serum-starved for 16 hours in 0.5% FBS-DMEM and treated for 2.5 hours cycloheximide-containing medium without or with TNFα alone. Following drug treatment, cells were incubated with a non-cytotoxic, membrane permeable and fluorescein-labelled inhibitor of caspase-3/7 that selectively and covalently labels active caspases with substrate specificity for DEVD peptide sequences (Ekert PG et al., 1999), and fluorescent cells were counted. Comparing the TNFα response, two cell lines failed to show any significant increase in the number of DEVDase-active cells. The AS10 cell line had an elevated basal level of DEVDase active cells in the control condition (Fig 17C) as well as fewer total cells compared to all other cell lines (Table 2), and suggesting a higher level of basal apoptotic signalling in these cells that may have occluded the TNFα signal in this assay. By contrast, the AS6 cells retained the strongest TNFα response, consistent with the inhibitory role of TNFAIP8 in caspase signalling (Fig 17A). Conversely, the T4 cell line failed to show a TNFα response, which is consistent with the lack of response in the Live-Dead assay that may be the result of TNFAIP8-induced inhibition of TNFα signalling. Taken together, these results suggest that TNFAIP8 negatively regulates TNFα-induced caspase activation.

7. Apomorphine-induced inhibition of TNFα-induced DEVDase activation in D2S-expressing Balb/c-3T3 cells is sensitive to TNFAIP8 expression. We next examined the effect of D2S receptor activation on TNFα-induced responses in the TNFAIP8 clones by CaspaTag^{TM} assay in the presence or absence of APO or APO and spiperone (Fig

17C). In wild-type Balb/c-3T3 cells, the proportion of TNFα -induced caspase-positive cells was not altered by APO or spiperone treatment, consistent with the lack of D2 receptors in these cells. By contrast, in D2S-expressing D2S cells, APO inhibited TNFα-induced increase in DEVDase active cells, and this was reversed by spiperone, indicating that the D2S receptor couples to inhibition of TNFα-mediated caspase activation. In the TNFAIP8 depleted AS6 cells, spiperone-sensitive D2S-induced inhibition was observed. This suggests that there is still enough residual TNFAIP8 in these cells to mediate caspase inhibition, or that the D2S receptor may couple to alternate TNFAIP8-independent pathways to inhibit caspase activation. As described above the AS10 clone did not respond to TNF but had higher basal level of caspase positive cells, and this was not inhibited by APO, suggesting a TNFAIP8-sensitive pathway. Spiperone slightly induced caspase in these sensitive cells, perhaps through non-specific toxicity. Finally in TNFAIP8-overexpressing TE cells, APO inhibited TNFα action, but this was incompletely reversed by spiperone. Since the D2S-negative Balb/c-3T3 cells fail to respond to APO, it seems likely that spiperone inhibition was incomplete (perhaps due to inactivation) and that the stronger coupling of D2S receptors due to over-expression of TNFAIP8 was not inhibited. Taken together these data show that the D2S receptor inhibits TNFα-induced caspase activation, and that this effect is dependent in part on TNFAIP8.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*
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3T3
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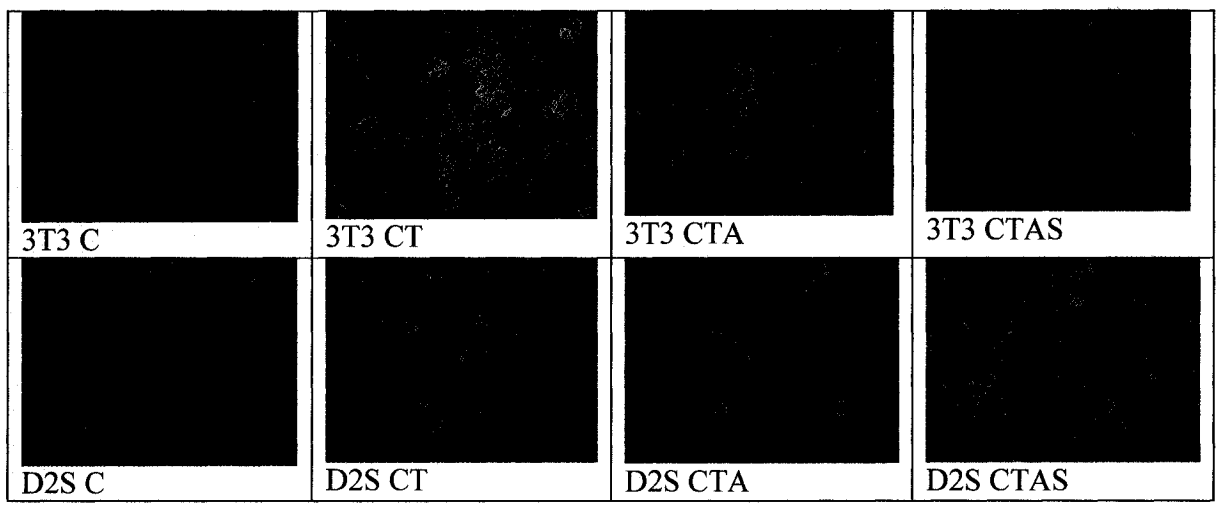
TE
T4
Cell Lines

B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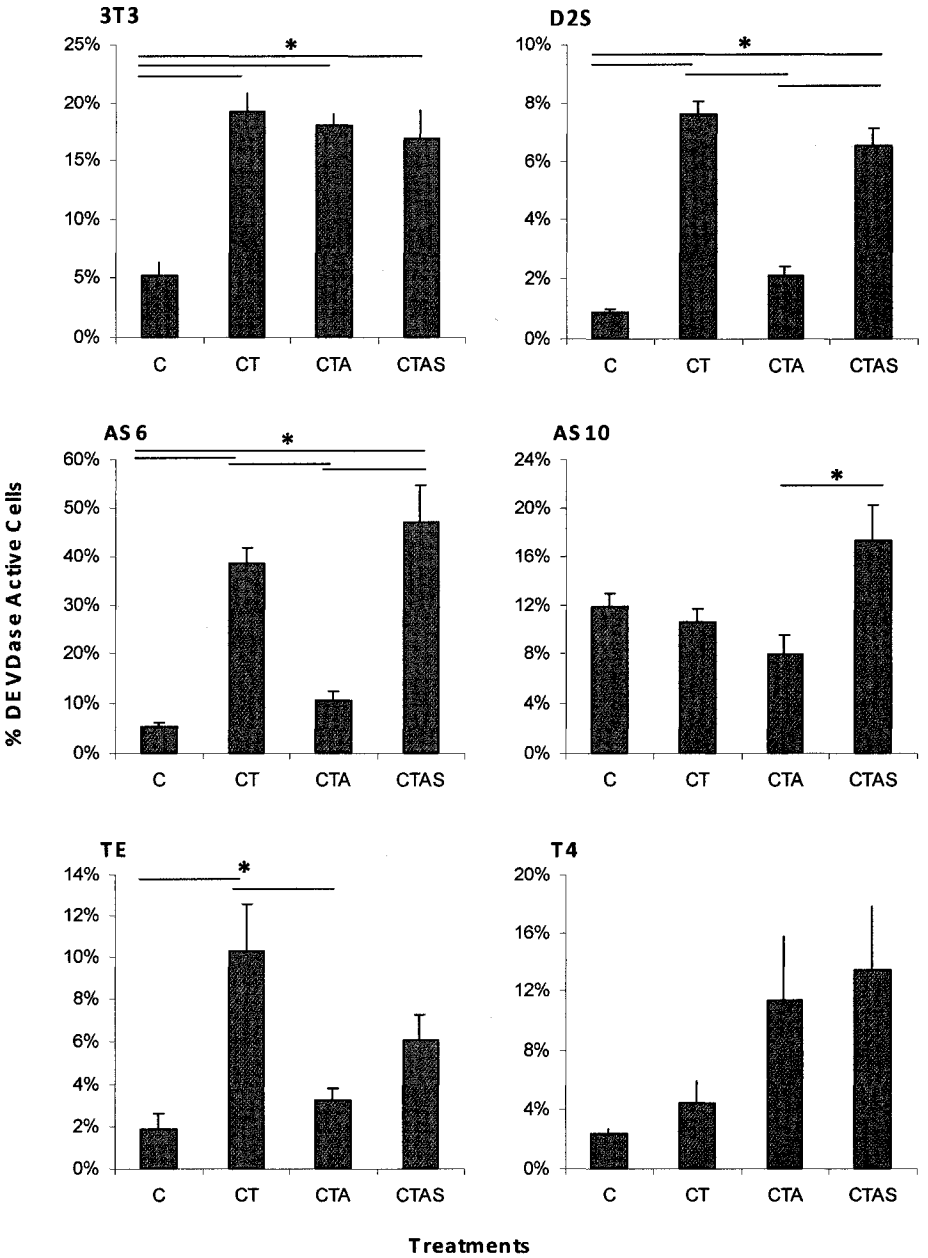

Figure 17. Chemicon's CaspaTag™ Caspase-3/7 *In situ* assay kits were used on balb/c-3T3 clones starved for 16 hours in 0.5% FBS-DMEM and treated for 2.5 hours with cycloheximide (10 μg/ml) ***(C),*** CHX+TNFα (5 ng/ml) ***(CT)***TNFα+ apomorphine (0.3 μM) ***(CTA)***, TNFα+APO+ spiperone (1 μM) ***(CTAS)***. This was followed by incubation with a fluorescein-labelled fluorochrome inhibitor of caspase-3/7 (FAM-DEVD-FMK) in media for 1.5 hours under normal growing conditions. Cells were photographed and counted using fluorescence microscopy. A) The difference between the percentage of caspase active cells in ***(CT)*** and ***(C)*** for six cell lines are shown. B) Merged photographs from phase and fluorescence microscopy of select samples are shown for two cell lines and four treatment groups. C) The percentage of total cells that are DEVDase-active for four treatment groups and six cell lines are shown. (*) are significantly different from each other by one-way ANOVA and Bonferroni test (alpha=0.05) while (#) are significantly stimulated by TNFα by confidence interval test (alpha=0.05). {n = 4 for TE, n = 3 for others}. Error bars are standard error

Table 2: CaspaTag™ cell counts for 6 cell lines given one of 4 treatments

Cell Line	Treatment	Total Cells	SE	DEVDase active cells	SE
3T3[a]	C[g]	442.0	49.2	23.2	6.7
	CT[h]	265.3	37.8	49.8	10.2
	CTA[i]	267.0	34.5	48.0	6.4
	CTAS[j]	275.5	42.6	48.5	12.5
D2S[b]	C	1004.0	97.1	9.0	1.6
	CT	491.2	49.2	36.2	5.2
	CTA	728.8	89.8	15.8	3.6
	CTAS	563.7	28.3	36.5	2.2
AS6[c]	C	571.0	16.1	30.3	5.5
	CT	226.3	12.3	87.3	2.6
	CTA	241.7	11.7	25.5	3.8
	CTAS	190.7	24.9	90.2	21.7
AS10[d]	C	371.0	11.2	43.8	5.0
	CT	350.8	18.7	36.0	2.6
	CTA	365.8	15.2	28.7	4.2
	CTAS	333.7	9.3	57.5	10.8
TE[e]	C	813.1	62.6	16.3	11.4
	CT	489.6	47.0	49.8	14.2
	CTA	555.6	29.8	17.9	3.4
	CTAS	547.4	19.1	33.1	9.6
T4[f]	C	495.6	28.4	11.3	1.2
	CT	421.4	33.3	18.0	4.3
	CTA	383.7	15.6	42.3	15.7
	CTAS	447.8	45.0	59.4	19.0

a 3T3 cells are wild type Balb/c-3T3
b D2S cells are wild type Balb/c-3T3 transfected with D2S receptor
c AS6 cells are D2S cells transfected with anti-sense-TNFAIP8
d AS10 cells are D2S cells transfected with anti-sense-TNFAIP8
e TE cells are D2S cells transfected with TNFAIP8
f T4 cells are D2S cells transfected with TNFAIP8
g "C" is a treatment of 10 μg/ml of cycloheximide for 2.5 hours
h "CT" is treatment "C" plus 5 ng/ml TNFα for 2.5 hours
i "CTA" is treatment "CT" plus 0.3 μM apomorphine for 2.5 hours
j "CTAS" is treatment "CTA" plus 1μM spiperone for 2.5 hours

CHAPTER 4

Discussion

Through the application of foci formation assays, cell death assays and caspase activation assays, the role of D2S-mediated transformation has been investigated with respect to TNFAIP8 at endogenous levels, over-expression and antisense knockdown. The results show that D2S mediated inhibition of caspase activity and death resulting in transformation has dependence on TNFAIP8.

TNFAIP8, a cytosolic protein elevated in some cancers, is proposed to inhibit death receptor signalling via the protein-protein interaction domain, DED, also found in DISC proteins, caspase-8, FADD and cFLIP (Kumar D et al., 2000)(You Z et al., 2001). In addition, work done in the Albert lab has shown that TNFAIP8 interacts in yeast mating assays with constitutively active Gαi/o mutants as well as wild-type Gαi3 (unpublished data). They further confirmed the interaction by detecting endogenous Gαi proteins by co-immunoprecipitation of FLAG-TNFAIP8 in Balb/c-3T3 (unpublished data). This information combined with the identification of active Gαi3 as a mediator of cellular transformation in D2S transfected Balb/c-3T3 cells (Ghahremani MH et al., 2000) lead us to hypothesize a link between Gαi3 regulation of TNFAIP8 and inhibition of the extrinsic death pathway resulting in transformation and/or cancer survival. While previous work by Ariel Wilson investigated a role for TNFAIP8 in D2S signalling (unpublished data), this study shows its role in transformation, cell death and caspase inhibition. Experiments were performed in wild-type Balb/c-3T3 cells and cells stably transfected with D2S alone or in combination with TNFAIP8 overexpression or full-length TNFAIP8-antisense.

TNFAIP8 knockdown reduces basal rates of foci formation in D2S-expressing Balb/c-3T3 cells: To study transformation, cells were grown in soft agar for three weeks in two groups, where one group was exposed to D2S agonist, *apomorphine*, and the other *control* group was not. First the basal rate of foci formation between cell lines was compared in the untreated *control* group. Cells stably transfected with D2S and expressing endogenous or plasmid-TNFAIP8, had significantly more foci than wild-type cells or cells transfected with antisense-TNFAIP8 (exceptional cell line, T4, is discussed below). This suggested anchorage-independent cell growth could be enhanced by D2S expression independent of receptor stimulation provided there were at least endogenous levels of TNFAIP8.

The mechanism by which this may occur is not fully understood and shall require further investigation. One possibility is that D2S has constitutive activity and this activity is sufficient to mediate an anti-apoptotic effect through TNFAIP8 resulting in increased transformation. The existence of constitutive activity of D2S receptors in heterologous expression systems and the ability of inverse agonist to reverse it has been shown (Nilsson CL et al., 1993) (Hall DA and Strange PG, 1997). Given D2S constitutive activity and the hypothesis that D2S activity stimulates TNFAIP8's anti-apoptotic effect, the enhanced foci formation result is consistent. Another foci formation assay using an inverse agonist treatment group could be used to see if anchorage-independent growth is attributed to D2S constitutive activity or some other property.

TNFAIP8 knockdown reduces apomorphine-induced foci formation in Balb-D2S cells: The next question considered in the foci formation experiment was the effect of apomorphine stimulation on transformation. The foci ratios of the apomorphine

treated group over control were compared between cell lines. In this analysis, we see that TNFAIP8 is required for D2S receptor-induced transformation of Balb/c-3T3 cells since antisense-TNFAIP8 clones are significantly less responsive. The hypothesis is that D2S-Gαi3-TNFAIP8-mediated inhibition of the death receptor pathway prevents cell death thus enabling cells to survive and form foci. As a result, it follows that cells expressing both TNFAIP8 and D2S should have more foci in response to apomorphine than cells without D2S or having knocked-down levels of TNFAIP8.

In fact, we also see that there is no significant difference between TNFAIP8 over-expressing cells and wildtype cells (without D2S receptors). This unexpected observation can be reconciled by considering that the basal level of activation was already significantly higher in TE compared to 3T3 and that over-expression of TNFAIP8 may have saturated the anti-apoptotic effect regardless of D2S-activation (i.e. the anti-apoptotic switch is always on). If this is the case, constitutive activity of D2S in TNFAIP8 over-expressing cells may be over-powered by elevated levels of TNFAIP8. This suggests that for elevated basal foci formation to occur, the constitutive activity of D2S in the presence of endogenous TNFAIP8 is sufficient; however, elevated TNFAIP8 alone increases basal foci formation, regardless of D2S activity. Therefore, it follows that in apomorphine stimulated foci formation, D2S receptor and no more or less than endogenous levels of TNFAIP8 are necessary. Again, repeating this experiment with a D2S inverse agonist could help confirm these determinations.

Anoikis, survival and proliferation mechanisms in soft agar: In soft agar, the knockdown of TNFAIP8 is preventing D2S-stimulated anchorage-independent growth by an unknown mechanism. We propose that it is by inhibition of the extrinsic death

pathway and there is evidence that this is activated in soft agar. Loss of contact with the extracellular matrix (ECM) disrupts integrin-dependent signals to cell proliferation and survival resulting in activation of the extrinsic and intrinsic death pathways leading to apoptosis by *anoikis* (Chiarugi P and Giannoni E, 2008). In the soft agar experiment, there is no external source of death ligand, so the extrinsic pathway could be activated in this integrin-dependent manner. This is an important consideration since our hypothesis relies on death receptor pathway inhibition.

Another possible *anoikis* pathway for TNFAIP8 is based on the existence of a signature sequence for vinculin family talin binding region in its ORF (Zhang C et al., 2006). Talin is an integrin anchoring protein that can recruit many other signalling proteins. Therefore, it appears as though the cytoskeletal machinery associated with integrins may also interact with TNFAIP8 suggesting crosstalk between Gαi3, extrinsic death pathway and cytoskeletal signalling complexes. Therefore, it is not clear if the enhanced foci formation mediated by D2S and TNFAIP8 involes caspase inhibition and enhanced survival, or modulation of integrin signalling to survival. Since a role for TNFAIP8 in caspase inhibition has been shown here and by others, it is likely an important contributor. But a TNFAIP8-integrin-dependent pathway contributing to enhance foci formation in soft agar cannot be ruled out.

D2S signalling inhibits TNFα-induced cell death in Balb/c-3T3 cells expressing endogenous or over-expressed levels of TNFAIP8: In Live/Dead$^{(R)}$ assays, the viability of cells is determined based on the permeability of the plasma membrane. Plasma membranes become permeable in the late stages of apoptosis or in necrosis.

Therefore, this assay provides a useful test for the ability of D2S and TNFAIP8 signalling to inhibit cell death in response to the death ligand, TNFα.

The results showed significant increases in the ratio of dead to live cells in all cell lines tested with the exception of T4 and only a trend for increased death in AS10. Treatments involving stimulation or inhibition of D2S produced no effect on TNFα-induced death in TNFAIP8-antisense clone (AS6) (trend also seen in AS10) nor did it produce and effect in wild-type (3T3). However, apomorphine treatment in the D2S expressing clone (D2S) reduced cell death ratios and this was reversed by spiperone. These results show that apomorphine action to inhibit cell death is mediated by D2 receptors since it is block by the D2 antagonist. These results are consistent with the soft agar findings and suggest that D2S signalling in the presence of endogenous TNFAIP8 levels are necessary for apomorphine-mediated survival, which could lead to rescue of pre-oncogenic cells, and ultimately to increased formation of foci of transformed cells.

On the other hand, if D2S receptors have constitutive activity for enhancing survival, as suggested by the soft agar assay, one might expect that TNFα + spiperone (a D2 inverse agonist) would induce significantly more death than TNFα alone. This was not observed and may have to do with significant differences between soft agar conditions promoting death through *anoikis* and the Live/Dead$^{(R)}$ condition, where cells were serum deprived and treated with cycloheximide prior to and during the activation of the death pathway. The effect of D2S constitutive activity could possibly be verified in a Live/Dead$^{(R)}$ experiment that would not rely on serum withdrawal and cycloheximide. Such an experiment would require longer treatments or stronger stimulation of TNFR1, which appear to be limiting in the Live-Dead assay. Perhaps cell transfected to over-

express TNFR1 would provide a better system to examine constitutive actions of D2S receptors on TNFα-induced cell death.

In the soft agar assay, the results suggested that the over-expression of TNFAIP8 alone could be sufficient for enhanced survival (basal foci formation) given a lack of response to D2S receptor stimulation. Therefore, it might be expected that TNFAIP8 over-expressing cells would not respond to D2S stimulation or inhibition. This was not the case for TE where significant inhibition of death was observed in apomorphine treatment, which was reversed by spiperone. Again, the differences between soft agar assay and the Live/Dead$^{(R)}$ assay are probably important. The constant stimulation of D2S receptor in soft agar likely leads to desensitization and reduced signalling potency that becomes undetectable in the presence of over-expressed TNFAIP8. In the Live/Dead$^{(R)}$ protocol, apomorphine treatment is applied for only three hours. Although D2S desensitization and GRK2/5-dependent internalization has been shown to occur within 2 hours (Ito K et al., 1999), overall strength of the signal in the early stages of TNFα application must be quite strong and very important to the results in this short assay. Therefore, it is reasonable to see D2S stimulated inhibition of cell death in TNFAIP8 over-expressing cells in this assay.

With the exception of T4, discussed later, the results of the soft agar assay and Live/Dead$^{(R)}$ assay support the hypothesis that D2S stimulation of TNFAIP8 inhibits TNFα-induced cell death and this effect enhances cellular transformation.

Mechanisms of D2S-TNFAIP8 inhibition TNFR1 death signalling: As mentioned previously, a number of studies have shown the anti-apoptotic effect of TNFAIP8 on death signalling and it is speculated that this occurs via DED domain

interactions at FADD or caspase-8 (Kumar D et al., 2000)(You Z et al., 2001)(Zhang H-G et al., 2004). Work in our lab and with collaborators has shown Gαi3 mediates transformation in Balb/c-3T3 and that TNFAIP8 preferentially binds to inactive Gαi3 compared to constitutively active RC/QL-mutant Gαi3. It is therefore possible that TNFAIP8 co-localizes with inactive Gαi3 at the D2S receptor complex. This of course needs to be verified by techniques such as immunohistochemistry and/or co-immunoprecipitation experiments on endogenous proteins. However, if this hypothesis is correct, then activation of Gαi3 may liberate TNFAIP8 to induce its anti-apoptotic effect. This interaction probably occurs in the early stages of receptor activation and prior to GRK and β-arrestin mediated internalization. Where TNFAIP8 localizes throughout the receptor endocytic process and how it is deactivated are important questions that have yet to be answered.

In this study, the inverse agonist spiperone is used to block the effect of apomorphine. Using the extended ternary complex model, spiperone has recently been shown to have higher affinity for the G-protein uncoupled receptor, which shifts the equilibrium away from the G-protein coupled state (Roberts DJ and Strange PG, 2005). Whether or not TNFAIP8's activation is dependent on the G-protein/receptor coupling state or GTP/GDP binding to G-protein is not clear. However, results in Live/Dead$^{(R)}$ and in CaspaTag^{TM} (discussed below), where spiperone effectively reverses the effects of apomorphine, may suggest that GTP binding to Gα proteins is the determining factor in TNFAIP8's activity. This can be deduced because while apomorphine induces receptor activation and active GTP-Gα complex, spiperone maintains the decoupled state and favours the inactive GDP-Gα complex.

Both TNFR1 and D2S receptors internalize via clathrin-coated pits (CCP) upon agonist stimulation. Internalization of the TNFR1 membrane complex I is necessary for switching from cell survival signalling to death signalling of the cytosolic complex II (Mischeau O and Tschopp J, 2003)(Schutze S et al., 2008). Therefore, an interesting question is whether or not D2S and TNFR1 internalize in the same CCPs and does TNFAIP8 localize with them. Recent studies have shown that different GPCRs can be segregated to specific subsets of CCPs (Puthenveedu M and von Zastrow M, 2006)(Mundell SJ et al., 2006), although there is currently no information on the CCP specificity of TNFR1. If both D2S and TNFR1 internalize in the same CCP, then they may continue to regulate each other at the receptosome level. However, If D2S releases TNFAIP8 and then undergoes GRK phosphorylation and internalization in distinct CCP populations; then this mechanism may serve to regulate TNFAIP8 activity by sequestration of the inactivating D2S complex. Since TNFR1 must be internalized for caspase-8 activation, then it follows that TNFAIP8 must be present at the TNFR1 receptosome to inhibit its activity. While many of these mechanistic points remain to be investigated, this study did look at the inhibitory effect of D2S signalling on executioner caspase activation by TNFα, which may be a primary consequence of TNFAIP8 activation on cell death and transformation.

Apomorphine's variable effects on caspase activity at lower doses in transfected Balb-D2S cells: Using an optimal CHX-TNFα treatment for inducing DEVDase activity, varying doses of apomorphine were evaluated in 3T3 and D2S cells. High doses of apomorphine produced cell death in a caspase-independent fashion. This has been reported and is characterized by apomorphine binding non-specifically to

cellular proteins forming green conjugates (El-Bacha RS et al., 1999), which I observed at a dose of 12.5μM. Therefore, the discussion will concentrate on the low end of the dose scale between 0.02-0.3 μM, which had little effect on wild-type cell survival or DEVDase activity.

At 0.3μM apomorphine, DEVDase activity trended towards inhibition. Potentially, TNFAIP8 activation is responsible for DEVDase inhibition at the dose of 0.3 μM. Hence, this was the dose chosen for the Live/Dead and subsequent DEVD substrate assays including activity assays and CaspaTag^{TM} assays. Ultimately, arguments can be made that this dose does in fact inhibit DEVDase activity through TNFAIP8 given the results of these experiments with respect to over-expressing and knockdown clones.

At the extreme low end (0.02μM APO), DEVDase activity was increased in D2S cells. A perplexing question is why does a low dose of TNFα stimulate DEVDase activity. There are a few possible explanations: 1) At low doses, DED containing protein, c-FLIP$_L$, is reported to enhance caspase-8 activation and apoptosis, while at higher doses (like in cancer), its role is anti-apoptotic (Chang DW et al., 2002). Therefore, if TNFAIP8 were to be functionally similar to c-FLIP, then it may also have a biphasic effect depending on the level of active protein. A low level of D2S stimulation may activate TNFAIP8 to the first pro-apoptotic phase, while a stronger stimulation induces the shift to the anti-apoptotic phase. 2) D2S has many effector pathways and regulatory interactions that are dependent on D2S stimulation strength and duration. It is possible that at low doses one of the effector or regulatory pathways is differentially stimulated while the TNFAIP8 activation pathway is not. For instance, inhibition of cAMP is strongly coupled to D2S with an EC_{50} of 8 nM giving 50-70% inhibition in transfected

GH4 cells (Albert PR et al., 1990). 3) Finally, a novel D2S pathway that couples directly to caspase-8 or -3 activation may be responsible. For instance, the activation of Gαi2 splice variant (sGαi2) by D2S is reported to activate caspase-3 (Lopez-Aranda et al., 2008). D2S activation may have to reach a threshold before anti-apoptotic TNFAIP8 can dominate.

The most important finding from this DEVDase experiment was the appropriate selection of agonist dose given the variable dose response dynamics.

Apomorphine weakly inhibits TNFα-induced DEVDase activity in TNFAIP8 over-expressing clones. Due to the increasing number of cell lines planned for this DEVDase activity experiment (6 instead of 2), a slightly different protocol was designed to allow more flexibility for cell management. The new protocol closely resembled the protocol for Live/Dead$^{(R)}$ with overnight starvation. The results failed to show meaningful apomorphine-induced inhibition of DEVDase activity. Some weak yet significant inhibition was shown in TNFAIP8 over-expressing cells but not in the D2S cell line.

From this, it was determined that the number of cells with active caspases in total population of cells was too small to accurately detect a signal in a cell lysate activity assay. Therefore, the CaspaTag^{TM} assay that labels individual cells containing active caspase-3 was selected for the next experiment.

Negative regulation of TNFα-induced DEVDase activation by TNFAIP8. Looking at the increased percentage of caspase-3 positive cells following by TNFα treatment in CaspaTag^{TM} assays, the effect of TNFAIP8 over-expression and knockdown were observed. Knockdown of TNFAIP8 relates to reduced cell viability and increased

responsiveness to TNFα. AS6 responded very strongly to TNFα compared to wild-type cells and cells over-expressing TNFAIP8. T4 (discussed below) and AS10 cell lines failed to show any increased activation. AS10 however, has the highest basal rate of DEVDase-active cells and the lowest number of cells attached in the basal condition. Therefore, it is possible that TNFα could not activate the death pathway anymore than is already the case in the basal condition without cells detaching and this could be due in part to knockdown of TNFAIP8. Reduced cell viability is reported in unchallenged Human Lung Microvascular Endothelial Cells transfected with TNFAIP8 antisense-oligonucleotide (Zhang C et al., 2006), and this could similarly explain what is happening to AS10. These results show TNFAIP8's basal protective effect with respect cell viability and response to TNFα.

Apomorphine-induced inhibition of TNFα-induced DEVDase activation in D2S-expressing Balb/c-3T3 cells is sensitive to TNFAIP8 expression. The CaspaTag™ experiment with the usual four treatment groups including TNFα, apomorphine and spiperone produced results supporting the proposed role of D2S and TNFAIP8 in caspase inhibition. In a similar pattern to the Live/Dead(R) experiments, D2S stimulation caused decreased TNFα-induced DEVDase-active cells and this was blocked by spiperone in the D2S cell line but not wild-type cells (which has no D2S receptors).

AS6 and AS10 show differential responses to apomorphine and spiperone. This is not expected in cells with significant knockdown of TNFAIP8. However, for this assay AS6 and AS10 cells used for all my previous experiments after reselection in G418 would not grow from frozen stocks and this might be related to freezer problems we had

in the lab. As a result, I had to use old stocks from a predecessor which could have been at a late passage and losing their expression of antisense-TNFAIP8. In general, it is recognized that antisense-knockdown strategies require strong expression of the antisense vector to effectively block a gene. Therefore, it is possible that while these cells show elevated basal DEVDase-active cell counts (AS10) or elevated TNFα-induced DEVDase-active cell counts (AS6), the protein levels of TNFAIP8 may have increased enough to produce significant D2S-mediated effects. An antibody to TNFAIP8 generated in rabbit successfully showed TNFAIP8 knockdown in AS10 (Wilson AM, unpublished data) and a new commercial antibody (Abcam) is now available. These could be used to assess the levels of TNFAIP8 in AS6 and AS10 compared to wild type. However, it may be more effective to develop siRNA techniques for future experiments.

In TNFAIP8 and D2S co-overexpressing cells, TE responded the apomorphine with decreased DEVDase activity, however, spiperone failed to reverse this significantly in contrast to D2S cells. The spiperone treatment group was also not significantly different from any other treatment group indicating that whatever the signalling effect (if any), it is ambiguous. The variance for these measurements was probably too high for the small sample size such that additional measurements may reveal the action of spiperone in these cells. Finally, T4 failed to respond to all stimuli as usual and is discussed below.

In this experiment, the results show a role for D2S-mediated inhibition of caspase activity and considering that AS6 had the strongest response to TNFα while AS10 had the highest basal level caspase activity, there is support for the role of TNFAIP8 in inhibition of caspase activity. Taken together with Live/Dead$^{(R)}$ and foci formation

results, the D2S mediated inhibition of caspase activity and death resulting in transformation shows strong dependence on TNFAIP8.

Cancer and Immunology: TNFAIP8 has been identified as protein elevated in some cancer tissues as well as being elevated in tumour tissues that are radiation resistant, chemoresistant and metastatic compared to matched-tumours. In rheumatoid arthritis synovial fibroblasts, it is related to increased cell survival and expression of matrix metalloproteinase (MMP) causing pathogenesis. Despite being elevated in disease, TNFAIP8 is highly expressed in normal myeloid and lymphoid tissues compared to all other tissues and a TNFAIP8 family member (TIPE2) is reported to be a vitally important regulator of immune homeostasis.

These findings indicate that while elevated TNFAIP8 may have a pathological role in some tissues, it likely also has a normal physiological role in other tissues such as immune cells. Therefore, further research to differentiate its normal function from aberrant function shall be necessary to determine opportunities for new therapies.

Tissue of exceptional behaviour: *Through out these experiments, one cell line has failed to respond to just about every stimulus in every condition. This is of course, T4. A possible explanation for this is that the cell line has low D2S levels, which prevents apomorphine/spiperone signalling and the over-expression of TNFAIP8 may also completely block TNFα response. The only hole in this logic comes from its insignificant basal foci count. Cells over-expressing TNFAIP8 do not respond to apomorphine stimulus with respect to foci formation (see TE). Yet TE has elevated basal foci formation which based on the last observation is not likely due to D2S constitutive*

activity but rather TNFAIP8 over-expression. Therefore T4 with TNFAIP8 over-expression should also show elevated basal foci counts, which it does not. The only response to stimuli shown by this cell line was in a DEVDase assay that was considered inaccurate (see above). This observed response to apomorphine is also likely inaccurate given the low D2S levels found in this cell line. Hence, this cell line does not appear to respond normally to any of the stimuli given here, and therefore, I propose that this cell line is transformed and should be abandoned in future experiments.

Conclusion

The hypothesis for this project proposed that TNFAIP8 is activated in a Gαi3 dependent manner and this activation results in the inhibition of the extrinsic death pathway resulting in enhanced cell survival and transformation. Using Balb/c-3T3 cells transfected with D2S receptors and derived cell lines with over-expressed TNFAIP8 or with antisense-knockdown of TNFAIP8, assays measuring anchorage-independent growth, cell death and caspase-3 activation were performed. D2S-Gαi3-activation resulted in inhibition of caspase activation and cell death in D2S expressing cells with endogenous or over-expressed TNFAIP8. D2S constitutive activity coupled with TNFAIP8 expression and TNFAIP8 over-expression resulted in agonist-independent increases in foci formation. D2S-activation in cells with endogenous TNFAIP8 resulted in increased foci formation compared to non-activated cells. TNFAIP8 knockdown resulted in lowered foci formation in D2S expressing cells and no D2S mediated inhibition of cell death.

Taken together, these results show that D2S mediated inhibition of caspase activity and death resulting in transformation is dependent on TNFAIP8. This protein represents an important target for continued research that may lead to the development of cancer and inflammatory disease therapies.

CHAPTER 5

References

Abdel-Baset H, Bozovic V, Szyf M, Albert PR. 1992. Conditional transformation mediated via a pertussis toxin-sensitive receptor signalling pathway. Mol Endocrinol. 6(5):730-40.

Albert PR, Neve KA, Bunzow JR, Civelli O. 1990. Coupling of a cloned rat dopamine-D2 receptor to inhibition of adenylyl cyclase and prolactin secretion. J Biol Chem. 265(4):2098-104.

Bao Q, Shi Y. 2007. Apoptosome: a platform for the activation of initiator caspases. Cell Death Differ. 14(1):56-65.

Benoit-Marand M, Borrelli E, Gonon F. 2001. Inhibition of dopamine release via presynaptic D2 receptors: time course and functional characteristics in vivo. J Neurosci. 21(23):9134-41.

Benzing T, Köttgen M, Johnson M, Schermer B, Zentgraf H, Walz G, Kim E. 2002. Interaction of 14-3-3 protein with regulator of G protein signaling 7 is dynamically regulated by tumor necrosis factor-alpha. Benzing T, Köttgen M, Johnson M, Schermer B, Zentgraf H, Walz G, Kim E. J Biol Chem. 277(36):32954-62.

Blum D, Torch S, Nissou MF, Verna JM. 2000. A cautionary note on the use of stable transformed cells. Apoptosis. 5(2):115-6.

Boatright KM, Renatus M, Scott FL, Sperandio S, Shin H, Pedersen IM, Ricci JE, Edris WA, Sutherlin DP, Green DR, Salvesen GS. 2003. A unified model for apical caspase activation. Mol Cell. 11(2):529-41.

Böhm SK, Grady EF, Bunnett NW. 1997. Regulatory mechanisms that modulate signalling by G-protein-coupled receptors. Biochem J. 15;322 (Pt 1):1-18.

Byung-S. Youn, Young J. Kim, Charlie Mantel, Kang-Yeol Yu, and Hal E. Broxmeyer 2001. Blocking of c-FLIPL–independent cycloheximide-induced apoptosis or Fas-mediated apoptosis by the CC chemokine receptor 9/TECK interaction. BLOOD, 15 98:4.

Callier S, Snapyan M, Le Crom S, Prou D, Vincent JD, Vernier P. 2003. Evolution and cell biology of dopamine receptors in vertebrates. Biol Cell. 95(7):489-502.

Chiarugi P, Giannoni E. 2008. Anoikis: A necessary death program for anchorage-dependent cells. Biochem Pharmacol.

Cho DI, Beom S, Van Tol HH, Caron MG, Kim KM. 2006. Characterization of the desensitization properties of five dopamine receptor subtypes and alternatively spliced variants of dopamine D2 and D4 receptors. Biochem Biophys Res Commun. 350(3):634-40.

Chang DW, Xing Z, Pan Y, Algeciras-Schimnich A, Barnhart BC, Yaish-Ohad S, Peter ME, Yang X. 2002. c-FLIP(L) is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. EMBO J. 21(14):3704-14.

Chu P, Pardo J, Zhao H, Li CC, Pali E, Shen MM, Qu K, Yu SX, Huang BC, Yu P, Masuda ES, Molineaux SM, Kolbinger F, Aversa G, de Vries J, Payan DG, Liao XC. 2003. Systematic identification of regulatory proteins critical for T-cell activation. J Biol. 2(3):21.

Deng Y, Ren X, Yang L, Lin Y, Wu X. 2003. A JNK-dependent pathway is required for TNFalpha-induced apoptosis. Cell. 3;115(1):61-70.

DePrimo SE, Diehn M, Nelson JB, Reiter RE, Matese J, Fero M, Tibshirani R, Brown PO, Brooks JD. 2002. Transcriptional programs activated by exposure of human prostate cancer cells to androgen. Genome Biol. 3(7):RESEARCH0032.

Donepudi M, Mac Sweeney A, Briand C, Grütter MG. 2003.Insights into the regulatory mechanism for caspase-8 activation. Mol Cell. 11(2):543-9.

El-Bachá RS, Netter P, Minn A. 1999. Mechanisms of apomorphine cytoxicity towards rat glioma C6 cells: protection by bovine serum albumin and formation of apomorphine-protein conjugates. Neurosci Lett. 263(1):25-8

Fadeel B, Ottosson A, Pervaiz S. 2008. Big wheel keeps on turning: apoptosome regulation and its role in chemoresistance. Cell Death Differ. 15(3):443-52.

Filmore D. 2004. It's a GPCR world. Modern Drug Discovery.

Fulda S, Debatin KM. 2006. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene. 25(34):4798-811.

Garcia-Calvo M, Peterson EP, Leiting B, Ruel R, Nicholson DW, Thornberry NA. 1998. Inhibition of human caspases by peptide-based and macromolecular inhibitors. J Biol Chem. 273(49):32608-13.

Geserick P, Drewniok C, Hupe M, Haas TL, Diessenbacher P, Sprick MR, Schön MP, Henkler F, Gollnick H, Walczak H, Leverkus M. 2008. Suppression of cFLIP is sufficient to sensitize human melanoma cells to TRAIL-and CD95L-mediated apoptosis. Oncogene. 27(22):3211-20.

Ghahremani MH, Forget C, Albert PR. 2000. Distinct roles for Galpha(i)2 and Gbetagamma in signaling to DNA synthesis and Galpha(i)3 in cellular transformation by dopamine D2S receptor activation in BALB/c 3T3 cells. Mol Cell Biol. 20(5):1497-506.

Gogvadze V, Orrenius S, Zhivotovsky B. 2006. Multiple pathways of cytochrome c release from mitochondria in apoptosis. Biochim Biophys Acta. 1757(5-6):639-47.

Goldmacher VS. 2005. Cell death suppression by cytomegaloviruses. Apoptosis. 10(2):251-65.
Guillermet-Guibert J, Saint-Laurent N, Davenne L, Rochaix P, Cuvillier O, Culler MD, Pradayrol L, Buscail L, Susini C, Bousquet C. 2007. Novel synergistic mechanism for sst2 somatostatin and TNFalpha receptors to induce apoptosis: crosstalk between NF-kappaB and JNK pathways. Cell Death Differ. (2):197-208.

Hall DA, Strange PG. 1997. Evidence that antipsychotic drugs are inverse agonists at D2 dopamine receptors. Br J Pharmacol. 121(4):731-6.

Hao C, Beguinot F, Condorelli G, Trencia A, Van Meir EG, Yong VW, Parney IF, Roa WH, Petruk KC. 2001. Induction and intracellular regulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated apotosis in human malignant glioma cells. Cancer Res. 61(3):1162-70.

Horrevoets AJ, Fontijn RD, van Zonneveld AJ, de Vries CJ, ten Cate JW, Pannekoek H. 1999. Vascular endothelial genes that are responsive to tumor necrosis factor-alpha in vitro are expressed in atherosclerotic lesions, including inhibitor of apoptosis protein-1, stannin, and two novel genes. Blood. 93(10):3418-31.

Ito K, Haga T, Lameh J, Sadée W. 1999. Sequestration of dopamine D2 receptors depends on coexpression of G-protein-coupled receptor kinases 2 or 5. Eur J Biochem. 260(1):112-9.

Jeanneteau F, Guillin O, Diaz J, Griffon N, Sokoloff P. 2004. GIPC recruits GAIP (RGS19) to attenuate dopamine D2 receptor signaling. Mol Biol Cell. 15(11):4926-37.

Jomphe C, Tiberi M, Trudeau LE. 2006. Expression of D2 receptor isoforms in cultured neurons reveals equipotent autoreceptor function. Neuropharmacology. 50(5):595-605.

Kelly E, Bailey CP, Henderson G. 2008. Agonist-selective mechanisms of GPCR desensitization. Br J Pharmacol.153 Suppl 1:S379-88.

Kim J, Ahn S, Ren XR, Whalen EJ, Reiter E, Wei H, Lefkowitz RJ. 2005. Functional antagonism of different G protein-coupled receptor kinases for beta-arrestin-mediated angiotensin II receptor signaling. Proc Natl Acad Sci U S A. 102(5):1442-7.

Kim SJ, Kim MY, Lee EJ, Ahn YS, Baik JH. 2004. Distinct regulation of internalization and mitogen-activated protein kinase activation by two isoforms of the dopamine D2 receptor. Mol Endocrinol. 18(3):640-52.

Kohlhaas SL, Craxton A, Sun XM, Pinkoski MJ, Cohen GM. 2007. Receptor-mediated endocytosis is not required for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. J Biol Chem. 282(17):12831-41.

Kumar D, Gokhale P, Broustas C, Chakravarty D, Ahmad I, Kasid U. 2004. Expression of SCC-S2, an antiapoptotic molecule, correlates with enhanced proliferation and tumorigenicity of MDA-MB 435 cells. Oncogene. 23(2):612-6.

Kumar D, Whiteside TL, Kasid U. 2000. Identification of a novel tumor necrosis factor-alpha-inducible gene, SCC-S2, containing the consensus sequence of a death effector domain of fas-associated death domain-like interleukin- 1beta-converting enzyme-inhibitory protein. J Biol Chem. 275(4):2973-8.

LaMorte VJ, Harootunian AT, Spiegel AM, Tsien RY, Feramisco JR. 1993. Mediation of growth factor induced DNA synthesis and calcium mobilization by Gq and Gi2. J Cell Biol. 121(1):91-9.

Lee JW, Lee KF, Hsu HY, Hsu LP, Shih WL, Chu YC, Hsiao WT, Liu PF. 2007. Protein expression and intracellular localization of prostate apoptosis response-4 (Par-4) are associated with apoptosis induction in nasopharyngeal carcinoma cell lines. Cancer Lett. 257(2):252-62.

Lee TJ, Lee JT, Kim SH, Choi YH, Song KS, Park JW, Kwon TK. 2008. Overexpression of Par-4 enhances thapsigargin-induced apoptosis via down-regulation of XIAP and inactivation of Akt in human renal cancer cells. : J Cell Biochem. 103(2):358-68.

Li FY, Jeffrey PD, Yu JW, Shi Y. 2006. Crystal structure of a viral FLIP: insights into FLIP-mediated inhibition of death receptor signaling. J Biol Chem. 281(5):2960-8.

Liu G, Ghahremani MH, Banihashemi B, Albert PR. 2003. Diacylglycerol and ceramide formation induced by dopamine D2S receptors via Gbeta gamma -subunits in Balb/c-3T3 cells. Am J Physiol Cell Physiol. 284(3):C640-8

Liu J, Lin A. 2005. Role of JNK activation in apoptosis: a double-edged sword. Cell Res. 15(1):36-42.

López-Aranda MF, Navarro-Lobato I, López-Téllez JF, Blanco E, Masmudi-Martín M, Khan ZU. 2008. Activation of caspase-3 pathway by expression of sG_i2 protein in BHK cells. Neurosci Lett. 439(1):37-41.

Lorenzo HK, Susin SA. 2007. Therapeutic potential of AIF-mediated caspase-independent programmed cell death. Drug Resist Updat. 10(6):235-55.

Marabese M, Mazzoletti M, Vikhanskaya F, Broggini M. 2008. HtrA2 enhances the apoptotic functions of p73 on bax. Cell Death Differ. 15(5):849-58.

Marsden CA. 2006. Dopamine: the rewarding years. Br J Pharmacol. 147 Suppl 1:S136-44.

McDonald 3rd ER and El-Deiry WS, eds. Death Receptors in Cancer Therapy: Mammalian Death Pathways. Totowa NJ: Humana Press, 2005.

Micheau O, Thome M, Schneider P, Holler N, Tschopp J, Nicholson DW, Briand C, Grütter MG. 2002. The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. J Biol Chem. 277(47):45162-71.

Moolenaar WH. 1995. Lysophosphatidic acid, a multifunctional phospholipid messenger. J Biol Chem. 270(22):12949-52.

Morris SJ, Van-Ham II, Daigle M, Robillard L, Sajedi N, Albert PR. 2007. Differential desensitization of dopamine D2 receptor isoforms by protein kinase C: the importance of receptor phosphorylation and pseudosubstrate sites. Eur J Pharmacol. 577(1-3):44-53

Mosselmans R, Hepburn A, Dumont JE, Fiers W, Galand P. 1988. Endocytic pathway of recombinant murine tumor necrosis factor in L-929 cells. J Immunol. 141(9):3096-100.

Mundell SJ, Luo J, Benovic JL, Conley PB, Poole AW. 2006. Distinct clathrin-coated pits sort different G protein-coupled receptor cargo. Traffic. 7(10):1420-31.

Neve KA, Neve RL, eds. The Dopamine Receptors. Totowa NJ: Humana Press, 1997.

Neve KA, Seamans JK, Trantham-Davidson H. 2004. Dopamine Receptor Signaling. J Recept Signal Transduct Res. 24(3):165-205.

Nilsson CL, Eriksson E. 1993. Haloperidol increases prolactin release and cyclic AMP formation in vitro: inverse agonism at dopamine D2 receptors? J Neural Transm Gen Sect. 92(2-3):213-20.

Ogura H, Tsukumo Y, Sugimoto H, Igarashi M, Nagai K, Kataoka T. 2008. ERK and p38 MAP kinase are involved in downregulation of cell surface TNF receptor 1 induced by acetoxycycloheximide. Int Immunopharmacol. 8(6) 922-926.

Pardo A, Selman M. 2005. Molecules in focus MMP-1: the elder of the family. 2005. The International Journal of Biochemistry & Cell Biology 37 283–288.

Patel S, Wang FH, Whiteside TL, Kasid U. 1997. Identification of seven differentially displayed transcripts in human primary and matched metastatic head and neck squamous cell carcinoma cell lines: implications in metastasis and/or radiation response. Oral Oncol. 33(3):197-203.

Pirger Z, Rácz B, Kiss T. 2008. Dopamine-induced programmed cell death is associated with cytochrome-c release and caspase-3 activation in snail salivary gland cells. Biol Cell

Puthenveedu MA, von Zastrow M. 2006. Cargo regulates clathrin-coated pit dynamics. Cell. 127(1):113-24.

Rashid AJ, So CH, Kong MM, Furtak T, El-Ghundi M, Cheng R, O'Dowd BF, George SR. 2007. D1–D2 dopamine receptor heterooligomers with unique pharmacology are coupled to rapid activation of Gq/11 in the striatum. Proc Natl Acad Sci U S A. Jan 9;104(2):654-9.

Recillas-Targa F. 2006. Multiple Strategies for Gene Transfer, Expression, Knockdown, and Chromatin Influence in Mammalian Cell Lines and Transgenic Animals. Mol Biotechnol. 34(3):337-54.

Ren XR, Reiter E, Ahn S, Kim J, Chen W, Lefkowitz RJ. 2005. Different G protein-coupled receptor kinases govern G protein and beta-arrestin-mediated signaling of V2 vasopressin receptor. Proc Natl Acad Sci U S A 102(5):1448-53.

Renganathan H, Vaidyanathan H, Knapinska A, Ramos JW. 2005. Phosphorylation of PEA-15 switches its binding specificity from ERK/MAPK to FADD. Biochem J. 390(Pt 3):729-35.

Roberts DJ, Strange PG. 2005. Mechanisms of inverse agonist action at D2 dopamine receptors. Br J Pharmacol. 145(1):34-42.

Sharp DA, Lawrence DA, Ashkenazi A. 2005. Selective Knockdown of the Long Variant of Cellular FLICE Inhibitory Protein Augments Death Receptor-mediated Caspase-8 Activation and Apoptosis. J. Biol. Chem., 280(19):19401-09.

Schneider-Brachert W, Tchikov V, Merkel O, Jakob M, Hallas C, Kruse ML, Groitl P, Lehn A, Hildt E, Held-Feindt J, Dobner T, Kabelitz D, Krönke M, Schütze S. 2006. Inhibition of TNF receptor 1 internalization by adenovirus 14.7K as a novel immune escape mechanism. J Clin Invest. 116(11):2901-13.

Schütze S, Tchikov V, Schneider-Brachert W. 2008. Regulation of TNFR1 and CD95 signalling by receptor compartmentalization. Nat Rev Mol Cell 9(8):655-62.

Shen HM, Pervaiz S. 2006. TNF receptor superfamily-induced cell death: redox-dependent execution. FASEB J. 20(10):1589-98.

Sheridan C, Delivani P, Cullen SP, Martin SJ. 2008. Bax- or Bak-induced mitochondrial fission can be uncoupled from cytochrome C release. Mol Cell. 31(4):570-85.

Siderovski DP, Willard FS. 2005. The GAPs, GEFs, and GDIs of heterotrimeric G-protein alpha subunits. Int J Biol Sci. 1(2):51-66.

So CH, Verma V, O'Dowd BF, George SR. 2007. Desensitization of the Dopamine D1 and D2 Receptor Hetero-Oligomer Mediated Calcium Signal by Agonist Occupancy of Either Receptor. Mol Pharmacol. 72(2):450-62.

Srinivasula SM, Ashwell JD. 2008. IAPs: what's in a name? Mol Cell. 30(2):123-35.

Strange PG. 1999.Agonism and inverse agonism at dopamine D2-like receptors. Clin Exp Pharmacol Physiol Suppl. 26:S3-9.

Sun H, Gong S, Carmody RJ, Hilliard A, Li L, Sun J, Kong L, Xu L, Hilliard B, Hu S, Shen H, Yang X, Chen YH. 2008. TIPE2, a negative regulator of innate and adaptive immunity that maintains immune homeostasis. Cell.133(3):415-26.

Swanson R, Raghavendra MP, Zhang W, Froelich C, Gettins PG, Olson ST. 2007. Serine and cysteine proteases are translocated to similar extents upon formation of covalent complexes with serpins. Fluorescence perturbation and fluorescence resonance energy transfer mapping of the protease binding site in CrmA complexes with granzyme B and caspase-1. J Biol Chem. 282(4):2305-13.

Thome M, Tschopp J. 2001. Regulation of lymphocyte proliferation and death by FLIP. Nat Rev Immunol. 1(1):50-8.

Thon L, Möhlig H, Mathieu S, Lange A, Bulanova E, Winoto-Morbach S, Schütze S, Bulfone-Paus S, Adam D. 2005. Ceramide mediates caspase-independent programmed cell death. FASEB J. 19(14):1945-56.

Usiello A, Baik JH, Rougé-Pont F, Picetti R, Dierich A, LeMeur M, Piazza PV, Borrelli E. 2000. Distinct functions of the two isoforms of dopamine D2 receptors. Nature 408(6809):199-203.

Ueffing N, Keil E, Freund C, Kühne R, Schulze-Osthoff K, Schmitz I. 2008. Mutational analyses of c-FLIPR, the only murine short FLIP isoform, reveal requirements for DISC recruitment. Cell Death Differ. 15(4): 773-782

van Corven EJ, Hordijk PL, Medema RH, Bos JL, Moolenaar WH. 1993. Pertussis toxin-sensitive activation of p21ras by G protein-coupled receptor agonists in fibroblasts. Proc Natl Acad Sci U S A. 90(4):1257-61.

Van-Ham II, Banihashemi B, Wilson AM, Jacobsen KX, Czesak M, Albert PR. 2007. Differential signaling of dopamine-D2S and -D2L receptors to inhibit ERK1/2 phosphorylation. J Neurochem. 102(6):1796-804.

Yamashita K, Takahashi A, Kobayashi S, Hirata H, Mesner PW Jr, Kaufmann SH, Yonehara S, Yamamoto K, Uchiyama T, Sasada M. 1999. Caspases mediate tumor necrosis factor-alpha-induced neutrophil apoptosis and downregulation of reactive oxygen production. Blood. 93(2):674-85.

You Z, Ouyang H, Lopatin D, Polver PJ, Wang CY. 2001. Nuclear factor-kappa B-inducible death effector domain-containing protein suppresses tumor necrosis factor-mediated apoptosis by inhibiting caspase-8 activity. J Biol Chem. 276(28):26398-404.

Wallach D, Kang TB, Kovalenko A. 2008. The extrinsic cell death pathway and the élan mortel. Cell Death Differ. 15(10):1533-41.

Wang Y, Hayakawa J, Long F, Yu Q, Cho AH, Rondeau G, Welsh J, Mittal S, De Belle I, Adamson E, McClelland M, Mercola D. 2005. "Promoter array" studies identify cohorts of genes directly regulated by methylation, copy number change, or transcription factor binding in human cancer cells. Ann N Y Acad Sci. 1058:162-85.

Wang Y, Xu R, Sasaoka T, Tonegawa S, Kung MP, Sankoorikal EB. 2000. Dopamine D2 Long Receptor-Deficient Mice Display Alterations in Striatum-Dependent Functions. J Neurosci. 20(22):8305-14.

Wang Y, Yu Q, Cho AH, Rondeau G, Welsh J, Adamson E, Mercola D, McClelland M. 2005. Survey of differentially methylated promoters in prostate cancer cell lines. Neoplasia. 7(8):748-60.

Webb CK, McCudden CR, Willard FS, Kimple RJ, Siderovski DP, Oxford GS. 2005. D2 dopamine receptor activation of potassium channels is selectively decoupled by Galpha-specific GoLoco motif peptides. J Neurochem. 92(6):1408-18

Yoon K, Jung EJ, Lee SR, Kim J, Choi Y, Lee SY. 2008. TRAF6 deficiency promotes TNF-induced cell death through inactivation of GSK3beta. Cell Death Differ. 15(4):730-8.

Zhang C, Chakravarty D, Sakabe I, Mewani RR, Boudreau HE, Kumar D, Ahmad I, Kasid UN. 2006. Role of SCC-S2 in experimental metastasis and modulation of VEGFR-2, MMP-1, and MMP-9 expression. Mol Ther. 13(5):947-55.

Zhang HG, Hyde K, Page GP, Brand JP, Zhou J, Yu S, Allison DB, Hsu HC, Mountz JD. 2004. Novel tumor necrosis factor alpha-regulated genes in rheumatoid arthritis. Arthritis Rheum. 50(2):420-31.

Zheng LT, Hwang J, Ock J, Lee MG, Lee WH, Suk K. 2008. The antipsychotic spiperone attenuates inflammatory response in cultured microglia via the reduction of proinflammatory cytokine expression and nitric oxide production. J Neurochem.

APPENDIX

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Figure 1

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Figure 2, 3, 4

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Figure 5

Publication:	Journal of Biological Chemistry
Order Detail ID:	23355774
ISBN/ISSN:	00219258
Publication Year:	2004
Publisher:	American Society for Biochemistry and Molecular Biology
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Figure 7

License Number:	2018530734046
Date:	Aug 29, 2008
Liscensed Content Publisher:	American Society for Microbiology
Liscensed Content Publication:	Microbiology and Cellular Biology
Liscenced Content Title:	Distinct Roles for Gai2 and Gbg in Signaling to DNA Synthesis and Gai3 in Cellular Transformation by Dopamine D2S Receptor Activation in BALB/c 3T3 Cells
Liscenced Content Author:	Ghahremani MH, Forget C, Albert PR
Volume number	20
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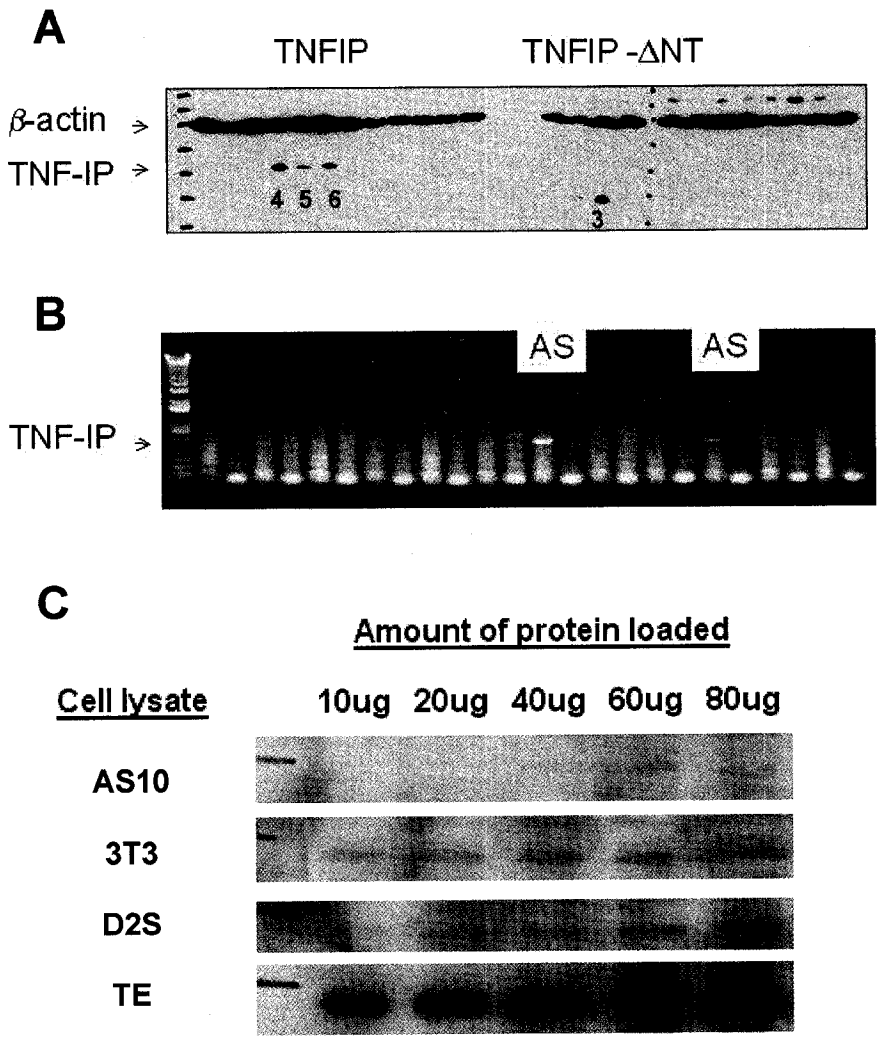
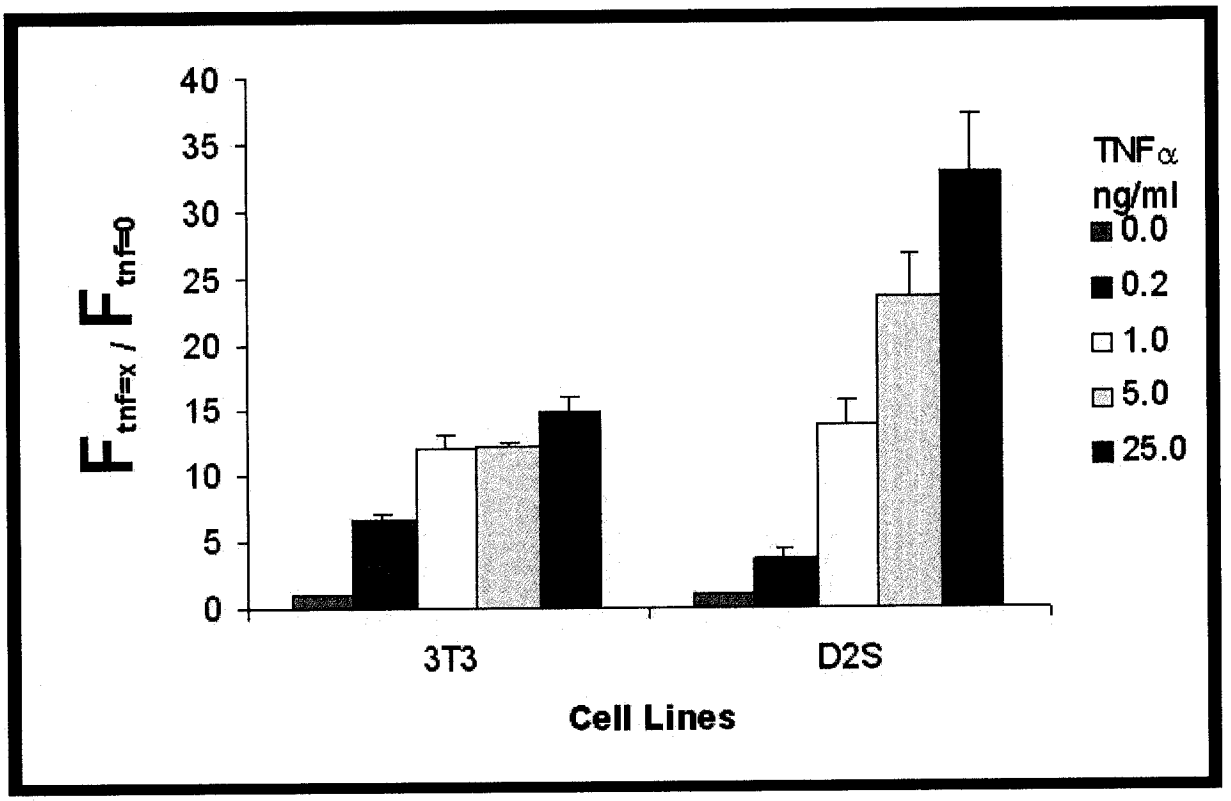
Year of Publication	2000
Portion used	Figures / Tables



Generation of stable TNFAIP8 (sense/antisense) cell lines. (A) Western blot (anti-Flag /β-Actin) of Flag-TNFAIP8 and Flag-TNFAIP8-ΔNT (faster migrating) stably transfected in D2S cells (credited to Helen Mao). (B) RT-PCR (A: antisense/ S: sense) of RNA from clones of D2S stably transfected with antisense TNFAIP8 construct. The indicated clones were positive (credited to Helen Mao). (C) Anti-TNFAIP8 serum was tested on lysates of cells overexpressing TNFAIP8 (TE), on endogenous TNFAIP8 (3T3 and D2S) and on cell with knockdown of TNFAIP8 (AS10). Increasing protein amounts (10-80μg) were loaded as indicated to detect endogenous levels of the protein. (1:50,000 dilution of serum) (credited to Ariel Wilson).



Caspase-3/-7 activity measurements in response to increasing doses of TNFα were performed on 3T3 and D2S cell lines starved for 3 hours in 0.5% FBS-DMEM and treated for 3 hours with 10 μg/ml cycloheximide (CHX). Shown is the mean ratio of TNFα-induced caspase activity (Ac-DEVD-AFC fluorescence) over control (where TNFα = 0 ng/ml) for 3T3 and D2S. {n=3 to 4}. Error bars are standard error