

**ROLE OF GUANOSINE TRIPHOSPHATASE REGULATORS IN FIBROBLAST TRANSFORMATION
AND LYMPHOCYTE DEVELOPMENT**

by

MARK KLINGER

B.Sc., The University of British Columbia, 1996

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Medical Genetics, Faculty of Medicine)

We accept this thesis as conforming
to the required standard

The University of British Columbia

2002

© Mark Klinger, 2002

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Medical Genetics

The University of British Columbia
Vancouver, Canada

Date October 4, 2002

Abstract

Guanosine triphosphatases (GTPases) are signaling mediators involved in regulation of diverse cellular processes including regulation of the actin cytoskeleton, gene transcription, cell cycle regulation, apoptosis and transformation. Regulatory proteins including G protein coupled receptors (GPCR), GTPase activating proteins (GAP) and guanine nucleotide exchange factors (GEF) influence the activity of GTPases. Balanced regulation of GTPase activity is critical in coordinating normal cellular responses. This thesis addresses the contributions of GTPase regulators in cellular growth control, differentiation and transformation.

Over-expression of G2A or PAR-1, two GPCRs, in NIH 3T3 fibroblasts induced a full range of phenotypes characteristic of oncogenic transformation. Co-expression of dominant negative Rho or LscRGS (Lbc's second cousin regulator of G protein signaling domain), a negative regulator of $G\alpha_{12}$ and $G\alpha_{13}$ GTPases, suppressed transformation via these GPCRs. Activation of $G\alpha_{12}$, $G\alpha_{13}$ and Rho GTPases are thus required for transformation via these GPCRs. $G\alpha_{12}$ and $G\alpha_{13}$ are unique in that they are activated upstream of Rho. Moreover, Rho GTPase activity is regulated via GEFs. $G\alpha$ -mediated activation of GEFs and downstream smaller molecular weight GTPases appears to be an important mechanism of linking divergent GTPases downstream of GPCR activation.

To elucidate the role of $G\alpha_{12}$ and $G\alpha_{13}$ GTPases in lymphocyte development, transgenic mice expressing LscRGS were generated. Analyses of lymphocytes from these mice revealed that LscRGS expression did not overtly affect lymphocyte development. These results indicate that $G\alpha_{12}$ and $G\alpha_{13}$ are not required for lymphocyte development.

Rho and Cdc42 are two GTPases involved in lymphocyte development. Previous studies by others demonstrated that loss of Rho function partially blocked differentiation and survival of CD4⁺/CD8⁻ double negative (DN) thymocytes and expression of another Rho family GTPase, Cdc42, enhanced the proliferative capacity of DN thymocytes. In addition, results from other studies revealed that expression of activated Rho augments positive selection and induces CD4⁺/CD8⁻ and CD4⁺/CD8⁺ single positive (SP) thymocyte hypersensitivity to TCR-induced proliferation *in vitro*. Dbs is a Rho- and Cdc42-activating GEF normally expressed in thymus. To determine how Dbs influences lymphocyte development, transgenic mice were generated expressing an activated form of Dbs. Expression of activated Dbs in lymphocytes promoted the accumulation of early thymocytes and restricted the production of mature thymocytes. Activated Dbs expression also led to increased proliferation of DN thymocytes. The Dbs transgene caused reduced numbers of SP thymocytes and mature splenic T lymphocytes. In addition, transgenic CD4⁺/CD8⁺ double positive (DP) thymocytes expressed higher levels of T cell receptor (TCR) and were hypersensitive to apoptosis induced by injection of anti-CD3. Moreover, Dbs transgenic thymocytes displayed impaired positive selection. Thymocyte culture experiments revealed that proliferation in response to anti-CD3 was reduced in SP thymocytes from Dbs transgenic mice. Expression of activated Dbs, a positive regulator of Rho and Cdc42, promoted the accumulation of DN thymocytes; this is the opposite of the DN phenotype observed in thymocytes lacking Rho function and similar to the phenotype displayed by Cdc42 transgenic mice. Thus the accumulation of DN thymocytes is likely to occur via Rho and/or Cdc42 activation. Activated Dbs expression also caused reduced *in vitro* SP thymocyte proliferation in response to TCR cross-linking and impaired thymocyte positive selection. These results are contrary to previous reports describing transgenic mice expressing activated Rho; thus, impaired thymocyte proliferation and positive selection in Dbs transgenic mice is likely to involve a pathway independent of Rho. Results presented in this thesis provide insights into the contributions of GTPase regulators in regulation of cellular growth control, differentiation and transformation.

Table of Contents

| | |
|---|------------|
| Abstract | ii |
| List of tables | vi |
| List of figures | vii |
| List of abbreviations | ix |
| Acknowledgments | x |
| Chapter 1: Introduction | 1 |
| 1.1 Signaling via guanosine triphosphatases (GTPases) | 1 |
| 1.2 Rho family GTPases, regulators and effectors | 2 |
| 1.2.1 Basic Rho GTPase function: molecular switches | 3 |
| 1.2.2 Structure of Rho GTPases | 3 |
| 1.2.3 Regulation of Rho activation | 3 |
| 1.2.4 Manipulating Rho GTPase activity | 5 |
| 1.2.5 Cellular processes influenced by Rho family GTPases | 7 |
| 1.2.6 Signal mediation from active Rho proteins: Rho effectors and downstream components | 10 |
| 1.2.7 Dbl family proteins are GEFs for Rho GTPases | 19 |
| 1.3 G protein-coupled receptors (GPCRs) | 26 |
| 1.3.1 Structure and function of GPCRs: analogy to GEFs | 27 |
| 1.3.2 Heterotrimeric G proteins: GPCR signal mediators | 28 |
| 1.3.3 GPCR-mediated cellular responses | 31 |
| 1.4 Use of transformation assays to identify modulators of Rho activity | 36 |
| 1.5 The role of Rho family and Gα GTPases in lymphocyte development and function | 37 |
| 1.5.1 Lymphocyte development: T and B lymphocytes | 37 |
| 1.5.2 Function of Rho family GTPases in lymphocytes | 43 |
| 1.5.3 G $\alpha_{12/13}$ have the potential to influence lymphocyte development via Rho activation | 50 |
| 1.6 Rationale for studies and thesis objectives | 52 |

| | |
|---|-----------|
| Chapter 2: Materials and Methods | 54 |
| 2.1 Molecular biology | 54 |
| 2.1.1 Vector construction/modification | 54 |
| 2.1.2 cDNA modification/epitope tagging | 54 |
| 2.1.3 Transgenic fragment preparation | 55 |
| 2.1.4 RNA isolation and Northern blotting | 55 |
| 2.1.5 DNA isolation and analysis | 56 |
| 2.2 Tissue culture | 56 |
| 2.2.1 Cell lines | 56 |
| 2.2.2 Cell culture | 57 |
| 2.2.3 BOSC-23 transfection | 57 |
| 2.2.4 Retroviral transduction of NIH 3T3 fibroblasts | 57 |
| 2.3 Protein analysis | 58 |
| 2.3.1 Immunoblotting | 58 |
| 2.4 Flow cytometry | 58 |
| 2.4.1 Antibodies and flow cytometry | 58 |
| 2.4.2 Intracellular LscRGS expression by flow cytometry | 58 |
| 2.5 Cell analysis | 59 |
| 2.5.1 Fibroblast transformation assays | 59 |
| 2.5.2 <i>In vivo</i> BrdU incorporation | 59 |
| 2.5.3 Thymocyte proliferation assays | 59 |
| 2.5.4 Cell cycle status analysis | 60 |
| 2.6 Mice | 60 |
| 2.6.1 Generation of transgenic mice | 60 |
| 2.6.2 Systemic anti-CD3 administration | 61 |
| 2.6.3 Mouse breeding | 61 |

| | |
|---|----------------|
| Chapter 3: Role of RhoGEFs and $G\alpha_{12/13}$ in GPCR-induced transformation | 63 |
| 3.1 Introduction and rationale | 63 |
| 3.2 Results | 63 |
| 3.2.1 Role of RhoGEFs in GPCR-, $G\alpha_{12}$ - and $G\alpha_{13}$ -mediated transformation | 63 |
| 3.2.2 LscRGS is an inhibitor of $G\alpha_{12}$ - and $G\alpha_{13}$ -induced transformation | 67 |
| 3.2.3 Role of $G\alpha_{12}$ and $G\alpha_{13}$ in G2A and PAR-1-mediated transformation | 70 |
| 3.2.4 Distinguishing between $G\alpha_{12}$ and $G\alpha_{13}$ involvement in G2A-mediated transformation | 73 |
| 3.3 Discussion | 76 |
| 3.3.1 Model of transformation via G2A | 76 |
| 3.3.2 Model of transformation via PAR-1 | 77 |
| 3.3.3 Summary | 78 |
| Chapter 4: Generation and analysis of LscRGS transgenic mice | 80 |
| 4.1 Introduction and rationale | 80 |
| 4.2 Results | 80 |
| 4.2.1 LscRGS expression does not overtly influence lymphocyte development | 80 |
| 4.3 Discussion | 91 |
| Chapter 5: Generation and analysis of Dbs transgenic mice | 94 |
| 5.1 Introduction and rationale | 94 |
| 5.2 Results | 94 |
| 5.2.1 Increased Dbs expression in lymphocytes alters development | 94 |
| 5.3 Discussion | 122 |
| Chapter 6: Conclusion | 129 |
| References | 131 |

List of tables

| | | |
|-------------------|--|----|
| Table 1.1. | A selected list of Rho family GTPase effectors. | 12 |
| Table 1.2. | A selected list of Dbl family GEFs, their GTPase substrates and some distinguishing characteristics. | 20 |
| Table 4.1. | Proportions of DN, DP and SP thymocytes from LscRGS transgenic mice. | 86 |
| Table 4.2. | Cell cycle analysis of LscRGS transgenic thymocytes. | 88 |
| Table 4.3. | Splenic T cell proportions. | 89 |

List of figures

| | | |
|---------------------|--|-----|
| Figure 1.1. | GTPases transduce intracellular signals | 1 |
| Figure 1.2. | Rho family GTPase members. | 2 |
| Figure 1.3. | The GTPase cycle. | 4 |
| Figure 1.4. | The diversity of Dbl family proteins. | 22 |
| Figure 1.5. | T lymphocyte development. | 39 |
| Figure 1.6. | B lymphocyte development. | 42 |
| Figure 3.1. | Co-expression of dominant negative Rho, Rac and Ras with G2A and PAR-1. | 64 |
| Figure 3.2. | Co-expression of dominant negative Rho, Rac and Ras with $G\alpha_{12}$, $G\alpha_{13}$ and GEFs and Ras. | 66 |
| Figure 3.3. | Co-expression of LscRGS. | 68 |
| Figure 3.4. | Co-expression of LscRGS with G2A and PAR-1. | 71 |
| Figure 3.5. | Co-expression of $G\alpha_{12}$ and $G\alpha_{13}$ carboxyl termini. | 75 |
| Figure 4.1. | LscRGS transgenic constructs. | 81 |
| Figure 4.2. | Western blot analysis of LscRGS transgene expression. | 82 |
| Figure 4.3. | Thymocyte intracellular expression analysis. | 83 |
| Figure 4.4. | Splenic T cell intracellular expression analysis. | 84 |
| Figure 4.5. | Splenic B cell intracellular expression analysis. | 84 |
| Figure 4.6. | Bone marrow B cell intracellular expression analysis. | 85 |
| Figure 4.7. | Flow cytometric analysis of thymocytes from the LscRGS transgenic mice. | 86 |
| Figure 4.8. | Thymocyte developmental marker expression. | 87 |
| Figure 4.9. | Flow cytometric analysis of spleen B cells. | 88 |
| Figure 4.10 | Flow cytometric analysis of spleen B cells | 89 |
| Figure 4.11. | Flow cytometric analysis of bone marrow B cells. | 90 |
| Figure 5.1. | Dbc transgenic construct. | 95 |
| Figure 5.2. | Northern blot analysis of Dbc transgene expression. | 96 |
| Figure 5.3. | Total thymocyte numbers. | 97 |
| Figure 5.4. | Thymocyte analyses from 51DB and 67DB mice. | 98 |
| Figure 5.5. | Forward scatter (FSC) analysis of Dbc thymocytes. | 101 |
| Figure 5.6. | Cell cycle analysis of Dbc thymocytes. | 102 |
| Figure 5.7. | Thymocyte filamentous actin (F-actin) content. | 103 |

| | | |
|---------------------|--|-----|
| Figure 5.8. | Thymocyte F-actin content is independent of cell size. | 104 |
| Figure 5.9. | DN thymocytes analysis from Dbs transgenic mice. | 105 |
| Figure 5.10. | Thymocyte BrdU incorporation. | 107 |
| Figure 5.11. | Thymocyte bcl-2 expression. | 108 |
| Figure 5.12. | Rag2 ^{-/-} thymocytes. | 109 |
| Figure 5.13. | Thymocyte developmental marker expression. | 111 |
| Figure 5.14. | Developmental marker expression by CD4 ^{low} /CD8 ⁺ and CD4 ⁻ /CD8 ⁺ thymocytes. | 113 |
| Figure 5.15. | Anti-CD3-mediated DP thymocyte apoptosis. | 115 |
| Figure 5.16. | Transgenic TCR HY crosses. | 116 |
| Figure 5.17. | Cell cycle analysis of cultured thymocytes. | 117 |
| Figure 5.18. | CFSE analysis of cultured thymocytes. | 118 |
| Figure 5.19. | Spleen cell counts. | 119 |
| Figure 5.20. | Flow cytometric analysis of spleen cells. | 120 |
| Figure 5.21. | Flow cytometric analysis of splenic T cells. | 121 |

List of abbreviations

| | |
|---------------|---|
| AML | Acute myeloid leukemia |
| APC | Antigen presenting cell |
| BCR | B cell receptor |
| BM | bone marrow |
| C3 | bacterial toxin from <i>Clostridium botulinum</i> type C strain |
| cDNA | complimentary DNA |
| CML | Chronic myelogenous leukemia |
| DN | double negative |
| DomN | dominant negative |
| DP | double positive |
| FSC | forward scatter |
| GAP | GTPase activating protein |
| GDI | guanine nucleotide dissociation inhibitor |
| GDP | guanosine diphosphate |
| GEF | guanine nucleotide exchange factor |
| GPCR | G protein coupled receptor |
| GTP | guanosine triphosphate |
| GTPase | guanosine triphosphatase |
| HY | minor histocompatibility antigen |
| Ig | immunoglobulin |
| LN | Lymph nodes |
| MFI | mean fluorescence intensity |
| MHC | major histocompatibility complex |
| MZB | marginal zone B cells |
| PAE | porcine aortic endothelial |
| RGS | Regulator of G protein signaling |
| SCLC | Small cell lung carcinoma |
| SP | single positive |
| SRE | serum response element |
| TCR | T cell receptor |
| TCR HY | HY antigen-specific T cell receptor |
| WAS | Wiskott Aldrich Syndrome |

Acknowledgments

Thanks are due to many people:

Rob Kay, for allowing me great freedom throughout the supervision of this work.

All members of the Kay lab, for an excellent work environment. Benoit Guilbault, for sharing mouse management responsibilities. Heather Kirk, for technique advice and accompanying stories.

Rewa Grewal, for DNA microinjections and mouse handling advice.

My parents, Heather and Barry, for their support and help throughout my years as a graduate student.

Jessica, for keeping me grounded in reality and smiling.

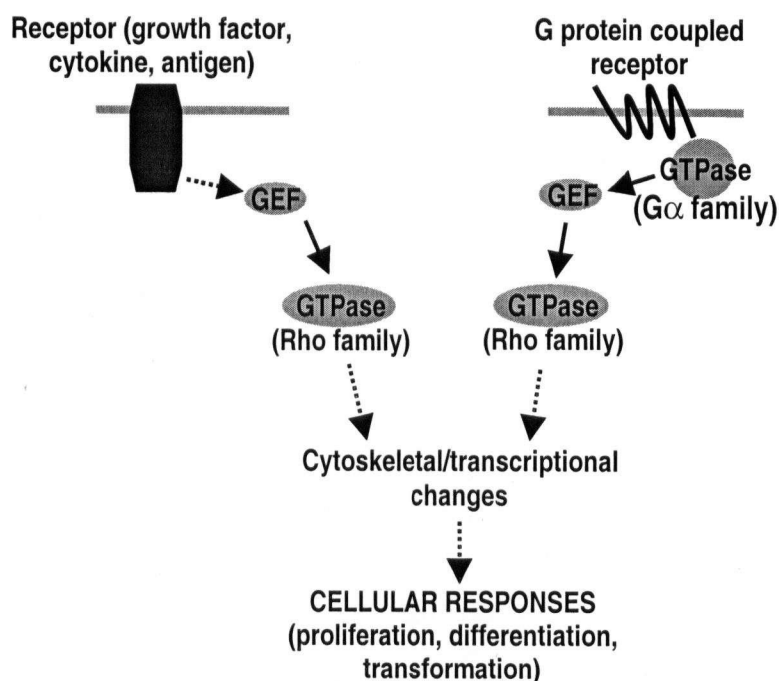
The Canadian Institutes of Health Research and the University of British Columbia for funding part of this work.

Chapter 1 – Introduction

1.1 Signaling via guanosine triphosphatases (GTPases)

Guanosine triphosphatases (GTPases) are signaling mediators involved in regulation of diverse cellular processes and are capable of interacting with numerous regulators and effectors. GTPases transduce a variety of signals downstream of growth factor, cytokine, antigen and G protein coupled receptors (Figure 1.1). These proteins function as molecular switches and activity is dependent upon the type of bound guanine nucleotide. Normal regulation of GTPase proteins is important in cellular growth control and differentiation. Altering the activity of GTPases, their regulators or effectors often leads to abnormal cellular responses and may result in cellular transformation or cancer. Therefore, characterizing the mechanisms by which cells transduce signals is of interest and importance in understanding diverse cellular processes. This thesis addresses the contributions of GTPase regulators in signal transduction and regulation of cellular growth control, differentiation and transformation.

Figure 1.1. GTPases transduce intracellular signals. Two general types of GTPases transduce signals in response to stimuli; these are referred to as the Rho and $G\alpha$ GTPase families. Some GTPases are activated in response to growth factors, cytokines or antigen (left), while others are activated downstream of G protein coupled receptors (GPCR) in response to ligand binding (chemokines, proteases, lipids and other growth factors; right). Following GTPase activation, signals are transduced to downstream effectors ultimately leading to distinct cellular responses. Guanine nucleotide exchange factors (GEF) positively regulate GTPase proteins. Aberrant activity of any of these signaling components can lead to cellular transformation.

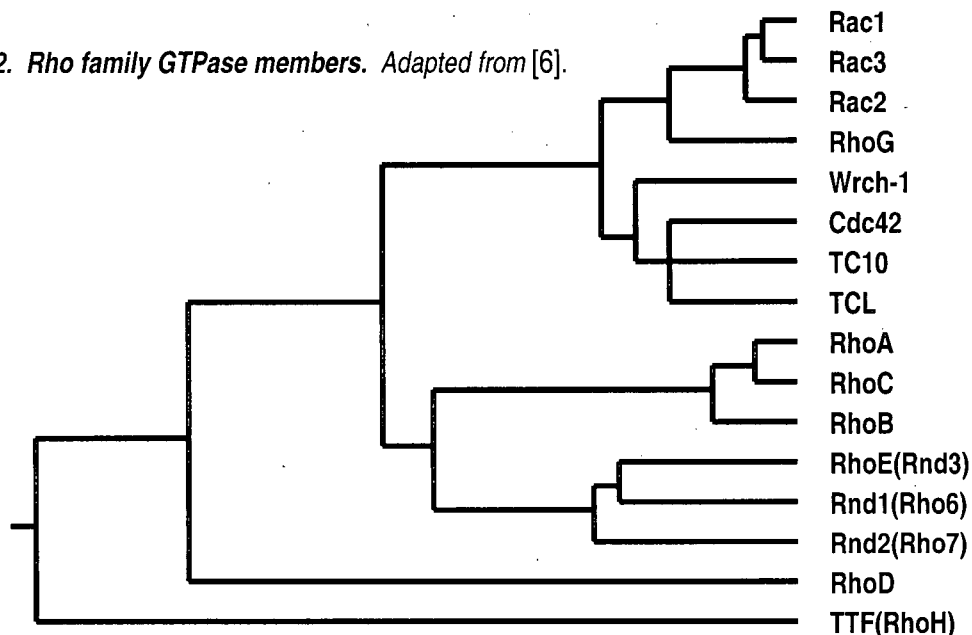


Many GTPase regulators, including the ones studied in this thesis, have been isolated in fibroblast transformation assays. Results presented in this thesis enabled the following general question to be addressed: how do the GPCRs G2A and PAR-1 mediate cellular transformation? Several GTPase regulators, in particular guanine nucleotide exchange factors (GEF; positive regulators) and GTPase activating proteins (GAP; negative regulators), were implicated in various aspects of lymphocyte development. Additional results presented in this thesis enabled this general question to be addressed: what are the roles of the GEF, Dbs and the GAP domain of Lsc in lymphocyte development? Specific thesis objectives are outlined in section 1.6. Prior to the presentation and discussion of experimental results, relevant topics will be reviewed and introduced.

1.2 Rho family GTPases, regulators and effectors

Rho family proteins comprise a major branch of the Ras superfamily of small GTPases. They are small molecular weight proteins between 20 and 30 kDa that are activated indirectly by a variety of growth factors, antigens, cytokines, adhesion molecules and some G protein coupled receptor ligands. There are at least sixteen mammalian Rho GTPase family members including: Rho (A, B and C isoforms), RhoD, RhoG, TTF/RhoH, Rac (1, 2 and 3 isoforms), Cdc42/G25K, TC10, Rnd1/Rho6, Rnd2/Rho7, Rnd3/RhoE, Wrch-1 and TCL, but the best characterized are RhoA, Rac1 and Cdc42 [1] [2] [3] (reviewed in [4] [5] [6] [7]) (**Figure 1.2**). Upon activation they coordinate signaling pathways that control a wide range of cellular processes including regulation of the actin cytoskeleton, gene transcription, cell cycle regulation, apoptosis and transformation.

Figure 1.2. *Rho family GTPase members.* Adapted from [6].



1.2.1 Basic Rho GTPase function: molecular switches

Rho GTPases act as molecular switches and their activity is dependent upon the type of bound guanine nucleotide. Rho proteins control cellular processes by cycling between active, guanosine triphosphate (GTP)-bound and inactive, guanosine diphosphate (GDP)-bound states. Conformationally distinct from the inactive form, GTP-bound Rho proteins are capable of interacting with numerous effectors and transducing signals to downstream proteins involved in cellular responses before returning to their inactive, GDP-bound states. Since Rho proteins possess an intrinsic hydrolytic capacity, they are capable of rendering themselves functionally inactive via hydrolysis of GTP to GDP.

1.2.2 Structure of Rho GTPases

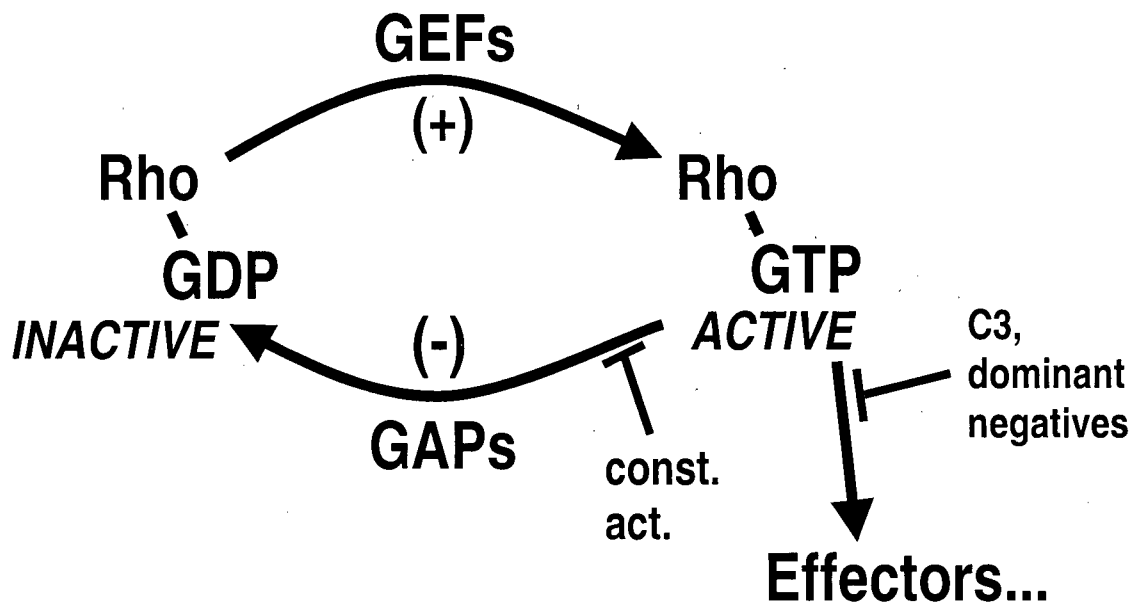
GTPases contain several domains important in interaction with regulators, effectors and nucleotides. These domains are referred to as switch I, switch II and P-loop regions. The crystal structures of Ras and Rho GTPases are similar and of the 41 conserved residues, most are located around the guanine-nucleotide binding site known as the P-loop [8]. The GTP hydrolysis switch operates in the same way in both families. Although sequence conservation exists within these GTPase families, sequences diverge considerably between families outside the P-loop, reflecting the fact that they interact with different effectors and regulators. Mutational studies and structural analyses indicate that the switch I and II regions of Rho GTPases are generally involved in guanine nucleotide exchange factor (GEF) and effector binding [9] [10] [11] [12] [13], while the P-loop is required for guanine nucleotide binding and stabilization [14]. Residues within all three (switch I, II and P-loop) regions are involved in GTPase activating protein (GAP) binding during the catalytic cycle, thus stabilizing the transition state of the GTP-hydrolysis reaction [8]. The core GTP-binding domain observed in all GTPase families is a conserved structural unit into which insertions can be built at a number of locations. Rho family proteins possess a unique thirteen-residue insertion thought to be important in target binding as well as binding to regulators [15] [16] [17].

1.2.3 Regulation of Rho activation

Rho GTPase activity is dependent upon the type of bound nucleotide. Nucleotide binding and hydrolytic cycles are slow. The activities of Rho family GTPases are regulated by three groups of proteins. GEFs regulate Rho proteins by promoting exchange of GDP for GTP, whereas GAPs

inactivate them by stimulating GTP hydrolysis. Rho guanine nucleotide dissociation inhibitors (Rho-GDI), stabilize the GDP-bound, or inactive form of the protein (**Figure 1.3**). Rho-specific GEFs belong to a family of exchange factors known as Dbl proteins.

Figure 1.3. The GTPase cycle. GTPases cycle between GDP-bound inactive and GTP-bound active states. The type of bound nucleotide is tightly regulated by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). Constitutively active (const. act.) GTPases are unable to hydrolyze GTP and remain in the GTP bound state. Dominant negative GTPases are unable to bind GTP and prevent interaction with downstream effectors. C3 transferases functionally inactivate some GTPases and also prevent effector interactions.



GTPase activation requires GEFs and inactivation requires GAPs. Normally GTPases bind guanine nucleotides with high affinity and are unstable in the nucleotide-free state. Activation via GEFs involves the formation of an initial, low-affinity GEF-GTPase-GDP ternary complex that rapidly converts to a high affinity GEF-GTPase binary complex upon expulsion of GDP and Mg^{2+} . The binary complex is stable in the absence of exogenous guanine nucleotides, however the relatively high concentration of GTP in cells favors the binding of the GTP and dissociation of the GEF resulting in activation of the GTPase (reviewed in [18]).

Rho family protein effectors and downstream targets mediate the cellular responses following Rho activation. Pathways involving Rho proteins generally involve the following components and events.

Rho protein activation via receptors or other upstream components requires activation and binding of GEFs. Upon GEF binding Rho proteins bind GTP and become activated. GTP-bound Rho proteins, in their active conformation subsequently bind to and activate effectors, thus initiating the downstream signals leading to cellular responses. Regulation of signaling intensity can occur at multiple points in this pathway.

1.2.4 Manipulating Rho GTPase activity

Interestingly, mutated forms of Rho proteins that alter the rate of nucleotide binding or hydrolysis have either been isolated or generated and used experimentally. Activating mutants display reduced GTPase activity and accumulate in the GTP-bound form. Inhibitory mutants however are unable to bind GTP and accumulate in the GDP-bound form. Furthermore, bacterial protein toxins that alter the effector binding properties of Rho proteins have also been identified. Since accumulation of either activated or inhibited forms of Rho family GTPases often lead to striking cellular consequences, use of mutant proteins or bacterial toxins experimentally has enabled characterization of Rho protein signaling pathways.

1.2.4.1 Constitutively active and fast-cycling mutants

Activation of Rho proteins is the basis for the oncogenic activity demonstrated by Dbl family GEFs and mutationally active Rho proteins are transforming when introduced into cells. Constitutively active mutants function independently of GEF activation. These mutants are generated by mutation of residues critical for GTP hydrolysis, which render the protein GTPase-defective. Notably, introduction of a GTPase-defective protein into cells leads to persistent GEF-independent signaling and an exaggerated phenotype that directly demonstrates its involvement in a particular pathway. GTPase-defective forms of RhoA (Q63L), Rac1 (G12V) and Cdc42 (G12V) are oncogenic in fibroblasts and immunocompromised mice.

In certain cell lines however, the oncogenic capacity of some GTPase-defective Rho family proteins is weak. Since proper Rho protein signaling may require a complete cycle of GTP binding and hydrolysis, fast-cycling mutants of Rho proteins have also been generated. These mutants possess enhanced intrinsic GTP-GDP exchange rates but maintain normal GTP hydrolytic activity. Expression of fast-cycling mutants of RhoA (F30L), Rac1 (F28L) and Cdc42 (F28L) also cause loss of serum dependence

and increased saturation density [19]. Thus mutational activation of Rho family proteins can be achieved through either impaired GTP hydrolysis or enhanced GTP-GDP exchange.

1.2.4.2 Dominant inhibitory/negative mutants

A useful way of studying the function of a protein is to specifically block its activity within cells via dominant inhibitory or dominant negative proteins. Notably, these mutant proteins interfere with the function of their normal cellular counterparts or with proteins that interact with them. Rho proteins have been mutationally inactivated and were generated by analogy with dominant-negative Ras (17N) [20]. Dominant negative GTPases are useful tools to determine whether GTPase pathways are involved in cellular processes including transformation. Biochemical characterization of dominant negative Ras mutants has revealed that GTPase dominant negative mutants display higher affinities for GDP than for GTP [20]. Consequently, they inefficiently bind GTP and instead remain in the GDP-bound state. GDP-bound GTPases interact with GEFs within cells. Therefore, these mutants act as dominant negatives within cells via GEF sequestration and competition with normal GTPases for binding to GEFs. The GEF-Rho dominant negative interaction forms a 'dead-end' complex and prevents the activation of endogenous Rho within cells (reviewed in [21]). Interestingly, studies involving dominant negative Ras (17N) showed that mutations within the region of Ras that interact with GEFs suppressed the inhibitory effects of dominant negative Ras [22]. Dominant negative GTPases are therefore direct inhibitors of GEFs and inhibitors of GTPase activation. Dominant negative GTPases have been useful tools in identification of GTPase pathways involved in a variety of cellular processes, including transformation.

The mechanism of dominant negative action via inhibition of GEF function however suggests that there are limitations to the interpretation of results obtained when using these mutants. Dominant negative mutants do not inhibit the GTPase itself but rather the catalytic domain of GEFs. A failure to observe an effect of dominant negative expression does not necessarily mean that signals are mediated independently of the GTPase. GTP to GDP hydrolysis, for example can also be regulated independently of GEFs via decreased GAP activity. Importantly, this regulatory route would not be influenced by dominant negative expression. Another concern relates to expression levels of the dominant negative mutant itself. GTPase expression levels vary among cell types and in each case dominant negative expression levels must be sufficiently high to achieve complete inhibition.

Analogous mutations at conserved residues of related GTPases also does not always guarantee that a

mutant generated will behave like Ras (17N). Ideally, preferential binding of GEFs or a failure to bind effectors should be demonstrated. Awareness of the limitations associated with these tools is extremely important in interpretation of results obtained when they are used experimentally. Their use has nevertheless been instrumental in defining GTPase pathways involved in many cellular processes.

1.2.4.3 Inactivation via bacterial toxins

GTPases are targets for several bacterial protein toxins and certain Rho family GTPases are ADP-ribosylated by *Clostridium botulinum* C3-like transferases and functionally inactivated. In particular, C3 transferases modify Rho (RhoA, RhoB and RhoC) but not Rac or Cdc42 at the same site at asparagine 41 in the effector region of the GTPase [23] [24] [25]. ADP-ribosylation of Rho proteins by C3 transferase increases the rate of GTP hydrolysis and either inhibits interaction of Rho with its effectors or induces sequestration of Rho-activating proteins [26]. Use of C3 transferases has also been important in determining the role of Rho signaling pathways in cellular processes.

1.2.5 Cellular processes influenced by Rho family GTPases

The Rho family of GTPases coordinate diverse cellular processes including adhesion, migration, phagocytosis, superoxide production, membrane trafficking, neurite extension and retraction, morphogenesis, polarization, growth, cell cycle progression, cytokinesis, proliferation apoptosis and invasion (reviewed in [27], [4], [5], [28], [29], [30] [31]). Rho activation occurs upon receptor stimulation via growth factors, cytokines, antigens and adhesion molecules. Coordination of cellular processes by Rho proteins arises via activation of Rho effectors and downstream signaling cascades that directly mediate the responses. Different Rho proteins exert related but distinct cellular functions due to interactions with a variety of downstream effectors. A particular cellular response is the end-result of effector interactions leading to changes in the activities of intracellular signaling mediators, gene transcription or both. Transduction of signals downstream of Rho proteins and their effectors enable Rho GTPases to regulate an array of cellular activities. The contributions of Rho GTPases to changes in the actin cytoskeleton, transformation, cell cycle progression and gene transcription are of particular relevance to this thesis and the next sections will focus on the role of Rho GTPases in these processes.

1.2.5.1 Regulation of the actin cytoskeleton

Rho family proteins were implicated in regulation of the actin cytoskeleton (reviewed in [4] [5]). In Swiss 3T3 fibroblasts, RhoA activation via extracellular ligands, including lysophosphatidic acid (LPA) led to assembly of contractile actin-myosin filaments termed stress fibers as well as associated focal adhesion complexes [32]. Other members of the Rho family of GTPases have been shown to be activated by distinct sets of agonists and induce specific actin cytoskeletal changes, distinct from those induced by active Rho. Rac for example is activated by a distinct set of agonists including platelet-derived growth factor and insulin and induces a meshwork of actin filaments at the cell periphery to produce lamellipodia and membrane ruffles [33]. More recently, Cdc42 was shown to induce actin-rich protrusions called filopodia as well as formation of multimolecular focal complexes at the plasma membrane [34] [35]. Interestingly, analysis of Cdc42 also revealed that activation of this GTPase led to the sequential activation of Rac and then Rho in Swiss 3T3 cells. In fact, Rac and Cdc42 both stimulate the assembly of multimolecular focal complexes associated with lamellipodia and filopodia that contain vinculin, paxillin and focal adhesion kinase [35]. Notably, these complexes are distinct from and formed independently of Rho-induced focal adhesion [35]. Thus in addition to their abilities to regulate stress fibers and lamellipodia, Rho and Rac also regulate the formation of focal complexes at the plasma membrane. Certain Rho family members can also antagonize the activity of others. For example, Rac signaling can antagonize Rho GTPase activity directly; thus the reciprocal balance between Rac and Rho activity influences the cellular morphology and migratory behaviour in NIH 3T3 fibroblasts [36]. Together these results suggest a molecular model for coordinated control of cell motility through members of the Rho family of GTPases and indicate that these proteins are key regulatory molecules linking surface receptors to the organization of the actin cytoskeleton.

Actin cytoskeletal changes induced by Rho proteins have been observed in many other cell types including neurons, astrocytes, epithelial and endothelial cells as well as in circulating cells such as lymphocytes, macrophages, mast cells and platelets (reviewed in [4]). These and other studies have implicated Rho, Rac and Cdc42 in morphogenetic processes involving changes in cell shape and polarity, cell movement, phagocytosis, cytokinesis, axonal guidance and membrane trafficking (secretion, endocytosis, phagocytosis and antigen transport) (reviewed in [27] [5] [28]). It is therefore likely that Rho GTPases play a role in cellular processes wherever filamentous actin is used. Members of this GTPase family are key mediators of signal transduction pathways from membrane receptors to the cytoskeleton.

1.2.5.2 Rnd proteins

Several recently identified Rho GTPases, Rnd1, Rnd2 and Rnd3/RhoE, form a distinct branch within the Rho GTPase family. Rnd1 displays a low affinity for GDP and spontaneously exchanges nucleotide rapidly *in vitro*, suggesting that this protein may be constitutively active in the GTP-bound state [37]. Notably, Rnd1 or Rnd3/RhoE protein expression in fibroblasts antagonized the formation of cytoskeletal structures by inhibiting the formation of actin stress fibers, membrane ruffles, focal adhesions and induced loss of cell substrate adhesion [37]. Thus Rnd proteins appear to inhibit the formation of cytoskeletal structures that are normally induced upon activation of Rho, Rac and Cdc42.

1.2.5.3 Rho family protein signaling pathways and cellular transformation

Transformed cells display a range of cellular phenotypes including enhanced proliferation, increased saturation density, loss of contact inhibition, anchorage-independent survival and proliferation and reduced dependence on serum. Moreover, the oncogenic potential of transformed cells is demonstrated experimentally upon injection of cells into immune-compromised mice and formation of tumors. These are the hallmarks of oncogenic transformation.

1.2.5.3.1 Rho family protein-mediated transformation

Many studies have demonstrated the transforming and oncogenic potential of Rho family GTPase signaling pathways. In fact, initial identification of oncogenes encoding exchange factors for Rho family members suggested that Rho GTPases were important in cell growth control and mitogenesis. Rho family GTPases and their regulators have since been implicated in several aspects of cell growth control. Expression of wild-type RhoA as well as constitutively active RhoA and RhoB caused transformation of NIH 3T3 fibroblasts through increased saturation density and reduced dependence on serum and anchorage [38] [39]. Injection of wild type and constitutively active RhoA-expressing fibroblasts into mice also induced tumors [40]. Furthermore, expression of activated mutants of RhoB and RhoG caused NIH 3T3 fibroblasts to grow to higher saturation density and display reduced serum and anchorage requirements for growth [39] [41]. NIH 3T3 fibroblasts expressing constitutively activated Rac1 displayed several characteristics of malignant transformation [42, 43] and caused tumors in mice following injection [44]. Expression of fast-cycling mutants of RhoA, Rac1 and Cdc42 also caused loss of serum dependence, increased saturation density and anchorage-independent growth of NIH 3T3 fibroblasts and were tumorigenic when injected into mice [19] [45]. Thus a number of studies have implicated Rho family proteins in oncogenic transformation.

1.2.5.3.2 *Rho family proteins and human cancer*

The previously described studies have implicated Rho proteins in cellular transformation. In contrast to *Ras*, no mutation in the *Rho* family genes that affect the rates of nucleotide binding or hydrolysis have been found in association with human cancer (reviewed in [46]). Nevertheless, a number of recent studies suggest involvement of Rho proteins in certain aspects of human malignancies. Expression levels of Rho, Rac and Cdc42, for example are elevated in a variety of solid tumors [47]. Moreover, increased expression of RhoC is associated with progression of human pancreatic adenocarcinoma [48]. A recently identified human Rac1 splice variant was also highly expressed in colorectal tumors at various stages of neoplastic progression when compared to adjacent tissues [49]. An important change during cancer progression is the switch from a locally growing tumor to metastatic killer. Expression of RhoC has also been associated with progression of melanoma cells to a metastatic phenotype whereas dominant negative RhoC inhibits this progression [50]. Thus in addition to their apparent role in formation of primary tumors, Rho proteins may also be involved in the acquisition of metastatic properties by these cells.

Notably, RhoH was isolated by its fusion to the *bcl-6* gene in a non-Hodgkin's lymphoma (NHL) cell line and has since been identified in a subset of NHL cases [51] [52]. RhoH expression is restricted to hemopoietic tissues [51]. Deregulated expression of either gene is likely to be a consequence of the translocation. This is the only known example of a recurrent chromosomal alteration involving a Rho family GTPase gene leading to malignancy.

Activation of Rho proteins is regulated by GEFs. A large number of Rho GEFs have been directly implicated in human malignancies. These observations indicate that Rho signaling pathways are extremely important in acquisition and progression of malignancies. GEFs and human disease are discussed in section 1.2.7.5.

1.2.6 Signal mediation from active Rho proteins: Rho effectors and downstream components

Rho family proteins influence a range of cellular responses from actin cytoskeletal rearrangements to oncogenic transformation. How do these proteins coordinate such a diversity of cellular responses?

Signals are transduced to downstream components upon binding of effectors to GTP-bound or activated Rho family proteins. Although much is known regarding the cellular processes coordinated by Rho proteins, the details of signal mediation between activation of Rho proteins and downstream components involved in the responses are less clear. The cell morphological effects induced by Rho, Rac and Cdc42 for example are clearly different in appearance [32] [33] [35] [53] [54] [55]. The mechanisms by which Rho, Rac and Cdc42 coordinate distinct cytoskeletal rearrangements, however are not well understood. The diversity of effects is the likely result of interactions of these family members with different sets of downstream effectors. Recent efforts have focused on identification and characterization of Rho effectors. A number of candidate effectors have been identified in yeast two-hybrid screens, genetic analysis or affinity column purifications (reviewed in [27] [56]). Many of these Rho effectors have been characterized and their cellular functions are beginning to be elucidated. The next few sections will summarize some of what is known regarding signal mediation downstream of activated Rho. Once again, for the purposes of this thesis emphasis will be placed on what is known regarding the particular pathways involved in actin cytoskeletal reorganization and regulation of cell growth control.

1.2.6.1 Rho effectors

Upon activation, GTP-bound Rho can bind to and activate effector proteins. Rho effectors can be divided into several categories: kinase, phosphatase, lipase and scaffold proteins (**Table 1.1**). The conformational differences between the GTP- and GDP-bound forms of RhoA are restricted primarily to two surface loops, switch regions I and II [57] [58]. Effector proteins are therefore likely to utilize these differences to discriminate between GTP- and GDP-bound forms, although they may also interact with other regions of the GTPase.

Many Rho GTPase effectors have been identified and the Rho, Rac and Cdc42 members each have at least ten known effectors (reviewed in [7]). Some effectors are specific for one family member while others are more promiscuous in terms of their binding partners. Upon interaction with Rho proteins, effectors interact with various downstream mediators and induce the range of cellular responses coordinated by these GTPases.

A common mechanism of effector activation by Rho proteins involves disruption of intra-molecular autoinhibitory interactions resulting in exposure of functional domains within the effector protein. The Rac/Cdc42 kinase target PAK1, for example possesses an intra-molecular regulatory domain that

Table 1.1. A selected list of Rho family GTPase effectors. Adapted from [7].

| Effector protein | Type of protein | Functions | Rho GTPase binding selectivity | | | References |
|----------------------|-----------------|----------------------------|--------------------------------|-----|-------|----------------|
| Rho-kinase | Ser/Thr kinase | Actin/myosin | Rho | | | [59] [60] |
| Citron kinase | Ser/Thr kinase | Cytokinesis | Rho | | | [61] [62] |
| MLK | Ser/Thr kinase | JNK | | Rac | Cdc42 | [63] [64] |
| PAK | Ser/Thr kinase | JNK/actin | | Rac | Cdc42 | [65] [66] |
| ACK | Tyr kinase | unknown | | | Cdc42 | [67] |
| PI3K | Lipid kinase | PIP ₃ levels | | Rac | Cdc42 | [68] [69] |
| PLC-β2 | Lipase | DAG/IP ₃ levels | | Rac | Cdc42 | [70] |
| Rhotekin | Scaffold | unknown | Rho | | | [71] |
| Dia | Scaffold | Actin organization | Rho | | | [72] |
| WASp | Scaffold | Actin organization | | | Cdc42 | [73] [74] [75] |
| p67phox | Scaffold | NADPH oxidase | | Rac | | [76] [77] |

inhibits kinase activity. GTPase binding displaces the inhibitory sequence enabling the kinase to bind to substrates [78]. Activated PAK leads to cytoskeletal rearrangement and c-Jun amino-terminal kinase (JNK) activation in a number of cell types (reviewed in [66]). Rho-kinase (p160^{Rock}), a Rho effector also contains an autoinhibitory region regulated by GTPase binding [79]. Rho-kinase was identified as a RhoA effector that binds GTP-bound RhoA. Mutation of residues within the effector or the switch II region of RhoA bound to GTP prevented Rho-kinase binding. GTP-bound RhoA also stimulated Rho-kinase activity [80]. Upon activation Rho kinase is able to regulate the phosphorylation of several proteins. Activation of this kinase is critical in the formation of actin stress fibers, a hallmark of Rho activation. Notably, activated Rho-kinase directly phosphorylates the myosin light chain (MLC) and inactivates the myosin phosphatase through phosphorylation of myosin-binding subunit (MBS) thus enabling stress fiber formation (reviewed in [7]).

Many Cdc42 and Rac effectors contain a binding motif referred to as the Cdc42/Rac interactive binding (CRIB). CRIB-motif-containing proteins include the serine/threonine kinase family of p21-associated kinases (PAK) and mixed-lineage kinase 2,3 (MLK-2, 3), the tyrosine kinase 1,2 (p120^{Ack}, Ack2) and WASp. Other effectors lacking the CRIB motif are also known to associate with Cdc42/Rac and include POR1 (partner of Rac1), p67^{phox} (a phagocytic cell NADPH oxidase complex component), MEK kinase 1,4 (MEKK-1,4) and phosphatidylinositol 4-phosphate 5-kinase (PI4P₅-kinase). A subset of Rho effectors also contains a conserved sequence termed Rho effector motif class 1 [71]. Effectors

containing this consensus motif include the serine/threonine kinase protein kinase N and the scaffold proteins raphilin and rhotekin [81]. Other putative Rho effectors that lack this binding motif include the scaffold protein mDia as well as three serine/threonine kinases including protein kinase N (PKN/PRK), Rho-associated coiled coil-containing protein kinase (p160^{ROCK}) and citron kinase [61]. The motifs required for recognition of Rho family GTPases by effectors are thus beginning to be characterized and others are likely to be identified in the future.

The Wiscott-Aldrich Syndrome protein (WASp) is an effector that binds activated Cdc42 [73]. The WAS gene is mutated in children with Wiscott-Aldrich syndrome (WAS), a severe X-linked inherited immune deficiency marked by bleeding, recurrent infections and eczema (reviewed in [75] [82]). WASp plays an important role in regulating the actin cytoskeleton and over-expression of the protein induces formation of polymerized actin clusters [73]. Normally, WASp is expressed in hemopoietic cells. Individuals with WAS display cytoskeletal abnormalities and impaired T cell proliferative responses to stimulation via the T cell receptor complex [83]. WASp is thought to mediate its cytoskeletal effects through direct binding of either actin monomers or the WASp-interacting protein (WIP) that binds profilin and causes actin polymerization (reviewed in [7]).

Rhotekin is another scaffold protein Rho effector that binds GTP-bound RhoA, RhoB and RhoC but not Rac1 or Cdc42 [71]. Although little is known regarding the cellular functions of Rhotekin, biochemical characterization revealed that binding of the Rho binding domain (RBD) of Rhotekin inhibits both the intrinsic and GAP-enhanced GTPase activity of endogenous Rho [71]. Interestingly, the fact that Rho effectors interact only with GTP-bound Rho has been exploited for experimental purposes. In particular, the Rho binding domain of Rhotekin has been developed as a tool to affinity-precipitate cellular GTP-Rho [84]. This assay has proved useful in determining levels of activated endogenous or exogenous Rho in cells. Despite the large number of Rho effectors identified, the details of signal mediation of Rho signals downstream of their direct effectors are fragmentary. Rhotekin for example is known to bind activated Rho, however the cellular consequences of this particular RhoGTP-effector interaction are not known.

1.2.6.2 Signal convergence on downstream targets

Signals downstream of Rho are beginning to be characterized. Given the diversity of Rho effectors, the signaling cascades downstream of these proteins are likely to converge on many targets. In efforts to

elucidate the mechanisms of cellular responses, groups have focused on particular cellular responses and analyzed components that are likely to be affected. These components are directly involved in mediating cellular responses. Approaches such as these have enabled identification of several components likely to be involved in processes coordinated by Rho proteins. Linking these downstream components to upstream effectors, however will be the focus of future works in this field. Some of the downstream components involved in cell growth control responses will be discussed in the following sections.

1.2.6.2.1 Cell cycle progression

Signal transduction pathways from Rho family GTPases play an important role in cell cycle control. Early experiments showed that cell cycle progression through the G₁ phase and subsequent DNA synthesis in Swiss 3T3 cells occurred upon injection of Rho, Rac and Cdc42. Injection of dominant negative forms of these GTPases however blocked DNA synthesis in response to serum [85]. The activity of G₁ cyclin-dependent protein kinases (Cdks) consisting of a kinase core and an associated cyclin subunit increases and decreases periodically during the growth and division of cells. The activity of these proteins determines the rate of cell cycle progression. Entry into S-phase DNA synthesis involves the G₁ Cdks that consist of Cdk4 and Cdk6 complexed with the D-type cyclins (D1, D2 and D3) and Cdk2 complexed with cyclin E (E1 and E2). Cdk activity is low in non-proliferating cells due to combined effects of low D- and E-type cyclin production and association with cyclin-dependent kinase inhibitors (CKIs) of the Cip/Kip family. Mitogenic stimuli induce production of D-type cyclins that activate Cdk4/6 and then cyclin E production, activating cyclin-E-Cdk2. Increased Cdk activity results from increased cyclin expression and sequestration of Cip/Kip inhibitors by the newly formed cyclin-D-Cdk complexes and degradation of p27^{Cip1}. G₁ Cdks regulate the phosphorylation of the retinoblastoma protein (pRb¹⁰⁵) and hypophosphorylated pRb¹⁰⁵ associates with the E2F family of transcription factors, actively repressing or suppressing E2F from genes required for entry into S-phase. A consequence of G₁ Cdk activation is the hyperphosphorylation of pRb¹⁰⁵ by G₁ Cdks, E2F release and subsequent E2F-dependent transcription of genes necessary for DNA synthesis.

Regulation of G₁ Cdk activity occurs at many levels through synthesis of cyclins, formation of cyclin/Cdk complexes, association with CKIs, phosphorylation of Cdks by activating kinases and transport of cyclin/Cdk complexes to the nucleus. Although some of these pathways are regulated upon activation of the cell cycle machinery, other events are directly regulated by intracellular signals

from the Rho family of GTPases. Activation of the Erk mitogen-activated protein kinase (MAPK) signaling pathway is a key element in induction of cyclin-D production and Rho signaling is necessary for sustained activation of ERK in fibroblasts in response to mitogenic stimulation [86]. Although inhibition of Rho blocked sustained activation of ERK, cyclin D1 was induced earlier and independently of Erk by Rac [86]. Thus Rho signaling may prevent premature cell cycle entry and maintain the correct timing of cyclin expression in G₁ phase by suppressing the ability of Rac to induce cyclin D1. Other studies have also shown the importance of Rac signaling for cyclin D1 expression. In primary endothelial cells, growth factor stimulation induced cyclin D1 expression only when the cells adhered to a matrix that supported integrin-dependent Rac activation [87]. Expression of constitutively active Rac and Cdc42 in NIH 3T3 fibroblasts potently induced E2F transcriptional activity via cyclin D1 accumulation and pRb hyperphosphorylation [88]. In addition to the effect of Rho on cyclin D1 induction, Rho activity was also required for promotion of p27^{Cip1} degradation, a cyclin-dependent kinase inhibitor, during the G₁/S transition [89] [90]. Moreover, constitutively active RhoA stimulated cyclin E/Cdk2 activity and degradation of p27^{Cip1} [91]. Thus RhoA regulated p27 degradation occurs through regulation of cyclin E/Cdk2 activity. Rho family GTPase signal transduction pathways directly regulate the cell cycle via cyclin expression, formation of cyclin/Cdk complexes and degradation of CKIs. Regulation of these components is also likely to be involved in cellular transformation via Rho family proteins. Although the cell cycle components influenced upon Rho activation are well characterized, the proteins mediating signals downstream of Rho and upstream of these components are not.

1.2.6.2.2 Induction of transcriptional target elements

RhoGTP-effector interactions lead to an array of other nuclear responses. The majority of Rho-mediated cellular responses are regulated by several proteins including members of the c-Jun amino-terminal kinase (SAPK/JNK), c-fos serum response factor (SRF) and nuclear transcription factor- κ B (NF- κ B). Although Rho-mediated responses generally do not involve activation of the mitogen-activated protein kinases (MAPK), ERK1 and ERK2, some nuclear responses are mediated via signaling pathways involving these proteins.

Expression of constitutively active Rac and Cdc42, but not Rho induced JNK and p38 activity in Cos-7 and NIH 3T3 cells and expression of dominant negative mutants of these GTPases blocked activation [92, 93]. In human 293T cells however, RhoA, RhoB, RhoC and Cdc42, but not Rac, induced JNK [94].

Rho family proteins thus appear to signal to JNK in a cell type-specific manner. More recently, expression of RhoA, but not Rac or Cdc42 stimulated *c-jun* expression and activity of the *c-jun* promoter, independently of JNK activation, through activation of Erk6, a recently identified member of the p38 family of SAPKs. Interestingly, the transforming ability of RhoA was dependent upon activation of ERK6, likely by promoting *c-jun* expression [95].

The *c-fos* serum response element (SRE) is a growth factor-regulated promoter element that is essential for the activation of immediate-early genes, such as *c-fos* and *egr-1*, by extracellular signals. The *c-fos* SRE forms a ternary complex with the SRF and ternary complex factors (TCF) and is a convergence point for several signal transduction pathways [96] [97]. TCF activity is regulated by MAP kinases (reviewed in [98] [99]). Extracellular signals can also control SRF activity in the absence of TCF binding by a pathway that involves Rho family GTPases [100] [101]. This pathway is regulated by serum, lysophosphatidic acid or intracellular activation of heterotrimeric G proteins and is critically dependent on actin polymerization [101]. Notably, the SRF is a nuclear target of Rho-mediated signaling pathways and constitutively active RhoA, Rac1, Cdc42 and TC10 activate transcription via SRF in the absence of extracellular signals; Cdc42 and Rac1 function independently of RhoA [100] [102]. Phosphatidylinositol 3-kinase (PI-3K) has been implicated in signaling to the SRF via both Rho-dependent and -independent mechanisms, although this is not detectable in all cell types [103] [104] [105]. Recently, the actin regulator LIM kinase-1 (LIMK) was identified as a potent SRF activator. More specifically, LIMK and extracellular signals cooperate to regulate the actin treadmilling cycle and SRF activity [106]. LIM kinases prevent the dissociation of actin from filament pointed ends and stabilize F-actin [107]. Thus Rho GTPases activate SRF via their ability to induce depletion of the G-actin (sequestered actin) pool. The actin treadmilling cycle represents a convergence point in the signaling pathways to SRF and reveals a direct link between cytoskeletal reorganization and gene transcription.

Rho family GTPases are also involved in activation of NF- κ B. The NF- κ B complex is composed of different homodimers and heterodimers of the Rel/NF- κ B family of transcription factors. NF- κ B, in its inactive state, is located in the cytoplasm where it is retained by the inhibitory protein, I κ B. Activation by external stimuli triggers the phosphorylation and proteolytic degradation of the I κ B protein, releasing the NF- κ B dimer, which translocates to the nucleus and binds DNA. RhoA, Rac1 and Cdc42 induced the transactivation of an NF- κ B-dependent HIV promoter in Cos-7, NIH 3T3 and Jurkat cells by

inducing the phosphorylation of I κ B α . Moreover, expression of dominant negative RhoA and Cdc42 blocked activation of NF- κ B by the physiological stimulus, tumor necrosis factor α (TNF α) [108]. Rho family proteins are important in several signal transduction pathways that modulate gene expression.

1.2.6.3 Complexity of Rho signaling

The mechanisms by which cells transduce certain Rho-induced signals leading to cellular responses often result from convergence of signaling cascades. Rho signals regulate JNK, SRF and NF- κ B proteins, for example and all of these are involved in induction of transcriptional target elements. The details of signal mediation upstream of JNK, SRF or NF- κ B are complex. JNK activation via Rac or Cdc42, for example can occur via activation of either of these Rho effectors: p21-activated kinase (PAK) [109] or mixed lineage kinase (MLK) [63]. Activation of multiple upstream effectors converging on downstream components is a recurrent theme in GTPase signaling.

As mentioned, Rho pathways also influence NF- κ B activation [108] [110]. In some cases, Rho GEF induction of the cyclin D1 promoter occurs in an NF- κ B-dependent manner [111]. Interestingly, NF- κ B activation by RhoA does not exclusively promote its nuclear translocation and binding to specific κ B sequences. NF- κ B also regulates the transcriptional activity of the c-fos SRF; SRE-dependent promoter activation via RhoA can be efficiently interfered with by expression of an I κ B α mutant that inhibits NF- κ B activity [112], thus NF- κ B is also required for regulation of the c-fos SRF. Moreover, there appeared to be a link between NF- κ B and JNK cascades since a dominant negative mutant of MEKK1 inhibited NF- κ B activation via Rac1 and Cdc42 [110]. The signaling components between Rho activation and NF- κ B activation are less clear and although NF- κ B activation in fibroblasts may involve the Rac effector PAK, activation can also occur in a PAK-independent manner [113]. The mediators of these signals are unknown. Furthermore, the effectors involved in transduction of signals converging on the SRE are poorly understood. In T cells, SRF activation upon T cell receptor (TCR) stimulation occurs through a signaling cascade consisting of Rac1/Cdc42 and the serine/threonine kinases PAK and MEK [114]. In fibroblasts, however PAK binding is dispensable for SRF activation [114], indicating that certain responses are cell type-specific.

These studies enable us to envision a general pathway for Rho-induced cellular responses. Rho activation leads to interaction with effector proteins. These effectors, whether they are scaffold, lipid, kinase or phosphatase proteins, interact with a variety of distinct downstream mediators thus initiating

signaling cascades. Ultimately, signals converge on a set of targets specifically required for a particular cellular response. The nature of the response is thus dependent on a variety of factors including Rho activators, Rho effectors, downstream targets and the regulatory proteins that act on all of these.

1.2.6.4 Cross talk with other small GTPases

Analysis of Rho and Ras proteins have revealed extensive cross talk and cooperation between GTPase-regulated signal transduction pathways. Notably, the coordinated activation and functional cooperation between members of the Ras and Rho GTPase families appears to be important in a number of processes including cell cycle progression, transformation and cytoskeletal rearrangements (reviewed in [115]).

Members of the Rho family of GTPases are known to be required for Ras transformation. In addition to their roles in regulation of the cytoskeleton, activation of downstream signaling components (lipid kinases and protein kinases, phospholipase D), and transcriptional regulation, Rho proteins are essential for transformation of cells by the related small GTPase Ras. Rho, Rac and Cdc42 were shown to synergize with Ras or Raf in focus formation assays and expression of dominant negative forms of these GTPases suppressed the ability of Ras to transform NIH 3T3 or Rat1 fibroblasts [116] [117] [42] [39] [118]. Synergistic interactions of Ras and Rho GTPases are involved in promoting cell cycle progression and this cooperation is likely to increase the proliferative capacity displayed by Ras-transformed cells. Thus signal cooperation appears to be important in Ras-induced transformation.

More detailed analysis of the cross talk between Rho and Ras have revealed that Rho suppresses an inhibitor of the cell cycle enabling Ras-induced DNA synthesis. Notably, Rho inhibition led to induction of the cyclin-dependent kinase inhibitor p21^{Cip1} and suppressed entry into S-phase by constitutively active Ras [119]. Rho activation suppressed induction of p21^{Cip1} by Ras leading to Ras-induced DNA synthesis, indicating that the primary requirement for Rho signaling, after Ras activation, was the suppression of p21^{Cip1} [119] [120]. This may in part provide a mechanism by which Rho cooperates with Ras in cellular transformation. Rho protein regulation of other aspects of oncogenic transformation including activation of the cell survival machinery, cytoskeletal rearrangements and altered adhesive interactions are also involved in oncogenic Ras transformation (reviewed in [115]). Signal integration thus appears to be important in coordination of the cellular process induced by the small GTPases. Integration can occur via branching of upstream signals or via coordinated regulation of downstream

functions. Importantly, signal convergence is likely to be the rule rather than the exception in GTPase-induced cellular responses. Regardless of the mechanism of signal integration, Rho regulators and effectors play a central role in these processes.

1.2.7 Dbl family proteins are GEFs for Rho GTPases

Abnormal Rho protein activity can alter the balance of signals within a cell leading to severe cellular consequences. Not surprisingly, the activities of these GTPases are tightly regulated. Rho GTPases are activated by GEFs that share tandem Dbl-homology (DH) and pleckstrin-homology (PH) domains. The *dbl* oncogene product was originally isolated from a diffuse B-cell lymphoma [121] [122]. Early *in vitro* experiments involving Dbl revealed that this protein stimulated the replacement of GDP bound to Rho family GTPases, with GTP [123] [124]. The DH-PH domains represent the structural module responsible for catalyzing GDP-GTP exchange and since the isolation of Dbl many other proteins have been identified that share these catalytic domains. These proteins are referred to as Dbl family GEFs.

1.2.7.1 Current members and estimates

Completed genome sequencing projects and domain-based comparative analyses have revealed that there are at least 46 Dbl family GEFs in humans [125]. Over 22 mammalian Dbl GEFs have been identified and characterized to some extent. Some of these GEFs and their properties are listed in Table 1.2. The specificities of Rho GTPase activation by different members of the Dbl family of proteins are variable and some members, including Lsc and Tiam1, are specific for a single Rho family protein [126] [43]. Other members, including Dbl and Dbs however, are more promiscuous and target multiple Rho GTPases [111] [123] [124]. Dbl family members display a range of motifs in addition to their conserved DH-PH domains and studies have revealed that Dbl proteins are multifunctional molecules that transduce diverse intracellular signals leading to activation of Rho GTPases. Activation of Rho GTPases occurs upon stimulation of cytokine receptors, growth factor receptors, cell-to-cell or extracellular matrix-to-cell adhesion receptors and G protein-coupled receptors (GPCRs). Dbl family GEFs mediate receptor-initiated intracellular signals and directly stimulate Rho GTPases. Active Rho proteins interact with effector proteins and transduce signals to downstream signaling components, resulting in diverse cellular responses.

Table 1.2. A selected list of Dbl family GEFs, their GTPase substrates and some distinguishing characteristics.

| Dbl family member | GTPase substrate | Biological activities | References |
|-------------------|------------------|---|----------------|
| Dbl | Rho | First Rho GEF isolated; proto-oncogene product isolated from <i>diffuse B</i> cell lymphoma | [122, 127] |
| Lsc / p115RhoGEF | Rho | Proto-oncogene product; interacts with G α_{12} and G α_{13} | [126, 128-131] |
| Dbp / Ost | Rho and Cdc42 | Proto-oncogene product | [132] [111] |
| Tiam1 | Rac1 | Proto-oncogene product; <i>T</i> lymphocyte invasion and metastasis | [43, 133] |
| LARG | RhoA | Proto-oncogene product; isolated as a fusion partner with mixed-lineage leukemia (LARG-MLL); interacts with G α_{12} and G α_{13} | [134, 135] |
| PDZ-RhoGEF | RhoA | Predominantly expressed in brain; interacts with G α_{12} and G α_{13} | [136] |

1.2.7.2 Primary Dbl family protein function: activation of Rho GTPases

Several lines of evidence indicate that Dbl family proteins function via activation of Rho GTPases in cells. First, the foci induced by expression of Dbl proteins are morphologically similar to those transformed by activated Rho GTPases, but distinct from those induced by other oncogenes such as Ras and Raf [121] [137] [128] [132] [132] [138] [139] [140] [141]. Second, expression of dominant negative Rho GTPase mutants suppresses the transforming abilities of Dbl family proteins [142] [128] [143]. Third, deletion or mutation of the residues within the DH domain of Dbl family proteins are unable to interact or activate Rho proteins and behave as dominant negatives in cells [128, 144] [132] [132] [145]. Fourth, the cellular responses induced by Dbl family proteins, including actin cytoskeletal reorganization [146] [147] [148] [143], stimulation of G₁ to S phase transition [149], cyclin D1 induction [150], activation of c-Jun N-terminal kinase [146] [93] [143], serum response factor [143] [151] and nuclear factor- κ B proteins [111] [110], are associated with the activation of signaling pathways mediated by Rho GTPases or their effector targets. These observations indicate that Dbl family GEFs serve as positive regulators of Rho proteins and function immediately upstream of these GTPases.

1.2.7.3 Activation and regulation of Dbl family proteins

1.2.7.3.1 Activation of Dbl proteins

Activation of Dbl proteins can occur via GTP-binding proteins, kinases and other proteins. Several types of G protein-coupled receptor stimuli are known to activate Rho GTPase pathways. Biochemical studies have identified Lsc/p115RhoGEF, LARG and PDZ-RhoGEF as a subset of GEFs containing additional domains that bind to and may be activated by $G\alpha_{13}$ [130] [136] [135]. Dbl also binds $G\alpha_{13}$ through its amino-terminal regulatory sequences, however whether this is a mechanism of activation is not known [152]. Certain Dbl family proteins including Tiam1 and Sos contain either Ras binding domains or Ras activation domains in addition to the DH-PH domains involved in Rho activation. Interestingly, Rho, Rac and Cdc42 are involved in Ras-induced transformation. Dbl proteins containing both Ras and Rho GTPase binding and activation domains may bind and activate multiple GTPase targets simultaneously or sequentially in response to stimuli.

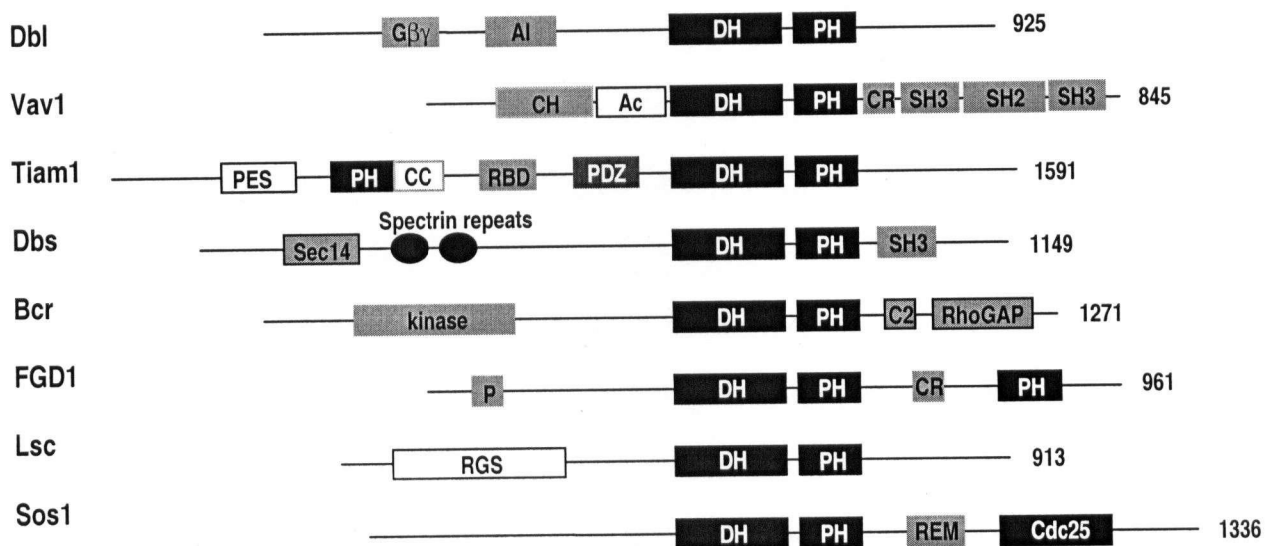
Some Dbl family proteins are phosphorylated by protein kinases in response to extracellular stimulation and this may contribute to their activation. Vav, for example is phosphorylated by Src family tyrosine kinases after receptor activation. Tyrosine phosphorylation activates Vav by relieving amino-terminal sequences from the active site of the DH domain (reviewed in [153]). Ect2, a Dbl family member involved in regulation of cytokinesis is also activated by phosphorylation [154]. As mentioned previously phosphoinositol kinases and their lipid products are capable of regulation of some Dbl members. The mechanisms of spatial and temporal regulation of Dbl proteins are only beginning to be characterized. Although Dbl activation ultimately leads to Rho GTPase substrate activation, the mechanisms by which these signals are transduced are likely to be complex and involve coordinate regulation of many proteins including RhoGAPs, RhoGDIs, in addition to the Rho-activating Dbl proteins.

1.2.7.3.2 Dbl family protein sequences diverge outside the DH-PH domains

Sequences outside of DH-PH domains of Dbl family proteins are divergent. Many Dbl family GEFs display a wide range of motifs suggesting involvement in other signaling pathways. Figure 1.4 illustrates the diversity of domains outside the DH-PH domains found in Dbl family GEFs. In addition to the invariant DH-PH domain pair, Dbl proteins display a diversity of domains including protein-protein (SH3, spectrin, RBD, PDZ), catalytic (RGS, RhoGAP, Cdc25, REM, kinase), lipid binding (C2), calcium binding (EF), immunoglobulin (Ig) and degradation motif (PEST) domains. The roles of many of these

domains within their protein contexts are only beginning to be elucidated. Two Dbl family GEFs will be described in detail in section 1.2.7.4.

Figure 1.4. The diversity of Dbl family proteins. Domain structures of representative Dbl family members. Abbreviations: **G $\beta\gamma$** , G $\beta\gamma$ binding domain; **AI**, autoinhibitory domain; **DH**, Dbl homology domain; **PH**, pleckstrin homology; **CH**, calponin homology; **Ac**, acidic amino acid rich motif; **CR**, cysteine-rich zinc butterfly motif; **SH3**, Src homology 3; **SH2**, Src homology 2; **PEST**, amino acids P, E, S and T rich, degradation motif; **CC**, coiled coil; **RBD**, Ras-binding domain; **PDZ**, DHR or GLGF domain; **Sec14**, sec14-like; **kinase**, serine/threonine kinase motif; **C2**, calcium-dependent lipid binding; **RhoGAP**, RhoGTPase-activating protein motif; **P**, proline-rich SH3-binding motif; **RGS**, regulator of G protein signaling motif; **REM**, Ras exchanger motif; **Cdc25**, RasGEF motif.



1.2.7.3.3 PH domain-mediated regulation of Dbl family proteins

Many Dbl family proteins appear to exist in an inactive or partially active state before stimulation. While the DH domain is responsible for catalytic activity, the pleckstrin homology (PH) domain appears to serve a regulatory role (reviewed in [155] [156] [157]). PH domains are likely to have a special function given their invariant location; they are always located carboxyl-terminal to the DH domain of Dbl family members. Moreover, the DH domain together with the PH domain constitutes the minimum structural unit bearing transforming function. The requirement for the DH-PH domain in transformation has been demonstrated with a number of different Dbl members including Dbl [158] Lbc [159], Lsc [128], Lfc [160] and Dbs [132].

PH domains are well known for their ability to bind phosphoinositides (reviewed in [161]). In some cases the phosphoinositide-binding site is well defined allowing specific and strong ligand binding. It is

often assumed however that all PH domains share this common function when they may instead share only the PH domain fold and form subclasses with quite different functions. It is known that some PH domains drive protein association with membranes via direct and specific recognition of phosphoinositides, however this is seen only in about ten percent of PH domain-containing proteins [161]. Interestingly, the physiological ligands of many PH domains are not restricted to polyphosphoinositides. Notably, many PH domains directly mediate protein-protein interactions.

PH domains are known to indirectly regulate the function of Dbl proteins via intracellular targeting. In fact some PH domains are capable of driving Dbl GEF recruitment to the membrane through direct recognition of phosphoinositides [162] [163]. Membrane recruitment of other Dbl family proteins via their PH domains is also essential for transforming activity [158] [159] [128] [132] [132]. Interestingly, replacement of the PH domain of Dbs with a membrane localization signal restores transforming ability [111]. PH domains are also involved in targeting of Dbl proteins to other intracellular sites. PH domains are required for localization of Lfc and Dbl to particular cytoskeletal components [158] [146]. Also, replacement of the PH domain of Dbl with a Ras membrane targeting sequence was not sufficient for transformation indicating that PH-domain-mediated recruitment to the proper sub-cellular location was essential for Dbl function. Thus PH domain-mediated targeting to specific intracellular locations is essential for proper Dbl family protein function.

Oligomerization through intermolecular interaction between DH domains also plays a role in efficient execution of GEF function. In fact, onco-Dbl protein exists in an oligomeric form *in vitro* and in cells. The ability of onco-Dbl to oligomerize allows multiple members to be recruited to the same signaling complex [164].

PH domains also regulate the activity of Dbl family proteins independently of any possible targeting role. Intra-molecular interactions between DH and PH domains of certain Dbl family members can inhibit the effect of the DH domain and regulate protein function. These interactions impose a constraint on the normal DH and/or PH domain function by masking the access of the GTPase substrate and/or intracellular targeting mediated by the PH domain. Autoinhibition via the PH domain of Dbl has been demonstrated [165] [166]. Moreover, binding of phosphoinositides by the PH domain of certain Dbl family members alleviates inhibition on the DH domain [167] [168]. Lipid products of phosphoinositol kinases may be involved in regulating intra-molecular interactions by Dbl family protein

domains. PH domains are also known to directly influence the nucleotide exchange activity of the DH domain. *In vitro* measurements of GDP release revealed that the DH-PH domain of Trio catalyzed nucleotide exchange on Rac1 one hundred-fold better than the DH domain alone [163]. Similar studies involving Dbl, Dbs and Lsc revealed a similar mode of PH-domain mediated nucleotide exchange [127] [13] [169]. Nucleotide exchange catalysis via PH domains appears to be an important regulatory mechanism employed by some Dbl family proteins. Thus PH domain-mediated autoinhibition, localization of GEFs to intracellular targets and catalysis of nucleotide exchange appear to be important mechanisms involved in regulation of Dbl protein function.

1.2.7.4 Lsc and Dbs

Lsc (*Lbc's second cousin*)/p115 RhoGEF is a Dbl family GEF that was isolated in library screens for cDNAs that cause transformation of NIH 3T3 fibroblasts [132] [128]. Full-length Lsc encompasses 920 amino acids and contains an amino-terminal regulator of G protein signaling (RGS) domain in addition to its DH-PH domains [128] [131]. This Dbl family member is a specific GEF for Rho, but not Rac or Cdc42 [126] [129]. Expression of Lsc is restricted to hemopoietic tissues [128]. The RGS domain of Lsc down-modulates heterotrimeric G proteins by acting as a GAP and stimulating the intrinsic GTPase activity of the α subunit of the trimer [131]. The Lsc RGS domain is specific toward the $G\alpha_{12}$ and $G\alpha_{13}$ subunits of G protein-coupled receptors (GPCR). In addition, the $G\alpha_{13}$ subunit induces Lsc GEF activity on Rho, possibly via binding to the RGS domain [130]. Thus, Lsc regulates the activity of Rho and is also capable of modulating signals from GPCRs to Rho. Lsc is one of several Dbl family proteins able to modulate signals from receptor-coupled $G\alpha$ subunits to Rho proteins. Other members include PDZ RhoGEF [130], LARG [130] and KIAA0380 [170].

Dbs (*Dbl's big sister*) was also isolated by its ability to transform NIH 3T3 fibroblasts [132]. Dbs expression is high in brain and lung, moderate in kidney, heart and stomach and low in thymus, bone marrow and skeletal muscle. Expression was not detected in liver or spleen [132]. Full-length Dbs encompasses 1149 amino acids and contains, in addition to the DH-PH domains, a putative amino-terminal Sec14 domain, two spectrin-like repeats and a carboxyl-terminal Src homology domain-3 (SH3) [132] [171]. Although the functional roles of the motifs outside the DH-PH domains are not yet known, they are not required for cellular transformation [132]. Notably, Dbl and Dbs exhibit high sequence similarity within the DH and PH domains. Consistent with their high degree of sequence similarity, Dbl, Dbs and Ost (the rat ortholog) are GEFs specific for Cdc42 and RhoA but not Rac [123]

[111] [172]. Dbl and Dbs also both activate multiple signaling pathways including activation of the Elk1, Jun and NF- κ B transcription factors and stimulate transcription from the cyclin D1 promoter [150] [111]. As mentioned, the PH domain of Dbs is involved in regulation of nucleotide exchange on Cdc42 and RhoA and removal of this domain results in reduced nucleotide exchange on GTPase substrates [13].

1.2.7.5 Dbl family proteins and human disease

A number of studies suggest that Dbl proteins play key roles in human disease. Notably, several Dbl members have been implicated in oncogenesis, metastasis and development. By implication, therefore Rho GTPases are likely to contribute to these processes. The Dbl family of proteins constitutes the largest single family of oncogenes (reviewed in [173]). Members have been identified for the most part via techniques designed to identify oncogenes, namely by transduction of fibroblast cell lines. In fact several Dbl members were isolated from malignant cell sources. Lbc, for example, was isolated from a lymphoid blast crisis [137] and Vav was isolated from an esophageal carcinoma [141]. Ost was isolated from an osteosarcoma cDNA expression library [172], while TIM (tumor immortalized mammary) was isolated from a mammary epithelial cDNA expression library [138]. Dbs, Lfc, and Lsc were isolated in cDNA library screens in which retroviral vectors were used to introduce hemopoietic expression into recipient cells [132] [128] [146].

Dbl family genes have been involved in chromosomal translocations from human leukemias. BCR, a Dbl member, was isolated from the breakpoint cluster region gene, which along with the ABL tyrosine kinase, is rearranged in chronic myeloid leukemia (CML) and a type of acute lymphoblastic leukemia (ALL). Chromosomal rearrangements between chromosomes 9 and 22 produce the Philadelphia chromosome (Ph') and oncogenic BCR-ABL fusion proteins. The DH and PH domains are retained only in the larger p210 form of the BCR-ABL fusion protein found in CML (reviewed in [173]). It is not known whether the DH-PH portion of the fusion contributes to the disease. Although only the first 63 amino acids of BCR are required to activate the tyrosine kinase activity of ABL, the DH domain present in the p210 fusion contributes to the stabilization of actin fibers [174] [175]. Moreover, removal of the DH domain from p210 eliminates the ability of BCR-ABL to transform Rat1 fibroblasts, mouse bone marrow and induce growth factor independence in the cytokine-dependent cell line, Ba/F3 [176]. Thus the DH portion of the BCR-ABL fusion protein is likely to contribute to CML. More recently, leukemia-associated RhoGEF (LARG) was found fused to MLL in a patient with acute myeloid leukemia (AML). The fusion was the likely result of an interstitial deletion rather than a chromosomal translocation [177].

The role of LARG in AML is not yet known. These findings illustrate the importance of Dbl protein fusions in the onset of certain human malignancies.

Studies involving another Dbl member have demonstrated the importance of Rho regulators in metastasis. TIAM-1, an oncogenic GEF for Rho, Rac and Cdc42 was isolated using an assay designed to identify invasion-inducing genes and was capable of inducing T lymphoma invasiveness and significant metastases when injected intravenously into immune-deficient mice [133] [43] [148]. Moreover, constitutively active Rac induced an invasive phenotype when expressed in T lymphoma cells [148]. Thus the metastatic effects of Tiam1 appear to be mediated via activation of Rac.

A DH domain-containing protein has been shown to be responsible for one human disease, faciogenital dysplasia (FGD). Mutations to FGD1 resulting in translational termination within the DH domain have been detected in all patients tested with this disease syndrome [178]. Thus altered Dbl protein activity can influence oncogenesis, metastasis and development, presumably via altered regulation of targeted Rho family GTPases.

1.3 G protein-coupled receptors (GPCRs)

Cell surface receptors belonging to the family of guanine nucleotide-binding (G protein) protein-coupled receptors (GPCRs), also known as seven-transmembrane, serpentine or heptahelical receptors, are involved in regulation of diverse signaling processes. These receptors comprise the largest family of transmembrane receptors in the human genome [125]. Heterotrimeric G proteins, consisting of α , β and γ subunits provide the signal coupling mechanisms to this family of receptors. Binding of diverse agonists to GPCRs generally occurs in a reversible manner and leads to activation of heterotrimeric G proteins forwarding the signal to intracellular effectors. GPCRs and the G proteins they couple to are involved in diverse physiological and pathological processes including cell growth, differentiation and apoptosis. Since this thesis addresses the roles of GPCR signals in cell growth control, transformation and lymphocyte development, their involvement in these processes will be emphasized and discussed in the following sections.

1.3.1 Structure and function of GPCRs: analogy to GEFs

Signaling via GPCRs is initiated by specific ligands that bind and activate receptors inducing conformational changes leading to stimulation of G proteins specifically coupled to the receptors. Many ligands have been shown to interact with different GPCRs including neurotransmitters, hormones, proteases, growth factors, phospholipids, photons, odorants, taste ligands, purine nucleotides and chemokines. GPCRs are characterized by a uniform molecular architecture of seven transmembrane α -helices linked by extra and intracellular peptide loops (reviewed in [179]). The amino-terminal receptor regions of GPCRs interact with ligands and the carboxyl-terminal portions of the receptors interact with the G proteins. GPCRs exist in either active or inactive states and although the inactive state is favored in most cases, some GPCRs exhibit constitutive activity under normal circumstances (reviewed in [180]).

Upon activation, GPCRs associate with distinct classes of heterotrimeric G proteins, composed of three subunits: the α subunit that has the guanine nucleotide binding site and GTPase activity and β and γ subunits that form a tightly bound dimer. $G\alpha$ proteins are another guanosine triphosphatase (GTPase) family bearing functional similarities to the family of smaller molecular weight Rho proteins. Members of both protein families are activated via GTP binding. As indicated, members of these protein families are also GTPases, retaining the intrinsic ability to hydrolyze GTP to the GDP. Thus both $G\alpha$ and Rho proteins behave as molecular switches and appear to differ only in terms of their regulatory counterparts and the effectors they interact with. Currently over 20 α subunits, 5 β subunits and 11 γ subunits have been cloned and identified (reviewed in [181] [182]). Although not all interactions are favored, multiplicity allows formation of many combinations. This, in conjunction with additional factors, like tissue specific expression of receptors and subunits, may explain how hundreds of GPCRs interact with an apparently limited repertoire of G proteins to initiate specific intracellular signals. Activated GPCRs promote the release of GDP from the inactive $G\alpha$ subunits and binding of GTP. Thus, functionally, activated GPCRs are analogous to Dbl proteins in that they serve as G protein activators. Upon binding of GTP, $G\alpha$ and $G\beta\gamma$ subunits dissociate and are able to interact with a diverse array of effector proteins (reviewed in [180]).

1.3.2 Heterotrimeric G proteins: GPCR signal mediators

1.3.2.1 Heterotrimeric G proteins: α , β and γ subunits

Heterotrimeric G protein subunits are essential for signaling via GPCRs. Like Rho proteins, $G\alpha$ subunits cycle between inactive GDP-bound and active GTP-bound states. Upon receptor activation by the appropriate signals, GPCRs catalyze the exchange of guanine nucleotides at the GDP/GTP binding sites of their coupled $G\alpha$ subunits, replacing the bound GDP with GTP. $G\alpha$ subunits, upon GTP binding dissociate from the $\beta\gamma$ subunits and both $G\alpha$ and $\beta\gamma$ subunits interact with and activate a variety of effectors thus initiating cellular responses. The $G\alpha$ subunit-effector interaction is terminated upon hydrolysis of bound GTP to GDP. As with other GTPases, hydrolysis occurs at a very low rate via the intrinsic GTPase activity of the $G\alpha$ subunit. $G\alpha$ -specific GTPase activating proteins (GAPs) called regulator of G protein signaling (RGS) proteins catalyze this reaction.

The properties of G proteins appear to be primarily determined by the identity of the $G\alpha$ subunit. Many $G\alpha$, β and γ protein subunits have been identified and characterized to some extent (reviewed in [181]). Two functional units are generated following receptor activation: the GTP-bound $G\alpha$ unit and the dissociated $\beta\gamma$ complex. Both sets of subunits, once dissociated initiate intracellular signaling pathways involved in GPCR cellular responses. Some of the properties of these subunits will be discussed in the following two sections.

1.3.2.1.1 $G\alpha$ subunits

$G\alpha$ subunits are large molecular weight GTPases of approximately 45kDa. On the basis of sequence similarity, $G\alpha$ proteins are classified into four major classes, $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$. Although classification is arbitrary, there are general signaling mechanisms among members of the family. In general, stimulation of the $G\alpha_s$ subunit activates adenylyl cyclase (AC) whereas stimulation of the $G\alpha_i$ subfamily leads to its inhibition. $G\alpha_q$ stimulation activates phospholipase C (PLC), whereas stimulation of the $G\alpha_{12}$ subfamily leads to regulation of smaller molecular weight GTP binding proteins (reviewed in [180]). Most GPCRs, when activated can activate only a limited set of G proteins. In fact, most GPCRs can be broadly classified into $G\alpha_s$ -, $G\alpha_{i/o}$ -, $G\alpha_{q/11}$ - and $G\alpha_{12/13}$ -coupled receptors. Although most GPCRs are preferentially coupled to a certain subfamily of G proteins, in some cell types they also activate other classes of G proteins with reduced efficiencies (reviewed in [180]). Thus promiscuity exists in terms of the receptors that different $G\alpha$ subunits couple to.

1.3.2.1.2 $\beta\gamma$ subunits

The $\beta\gamma$ complex was initially believed to be the more passive partner of the G-protein α subunit complex. Recent studies however, have shown that $\beta\gamma$ subunits play a very important role in regulation of a number of effectors, including ion channels, ion pumps, phospholipases, adenylyl cyclases and kinases (GPCR kinases, PI3 kinase and, tyrosine kinases) (reviewed in [183]). Although many of these processes are regulated directly by the $\beta\gamma$ subunits, others appear to involve other mediators. The mechanisms for effector interaction via $\beta\gamma$ subunits are only beginning to be elucidated. Interestingly, the $\beta\gamma$ subunits do not change conformation upon dissociation from $G\alpha$ [184]. This coupled with the fact that $G\alpha$ subunits abrogate the ability of $\beta\gamma$ subunits to activate their effectors, suggests that the sites for binding $G\alpha$ and effectors are overlapping.

1.3.2.2 Regulating heterotrimeric G proteins

$G\alpha$ proteins are normally regulated by two sets of proteins: GPCRs and GAPs. $G\alpha$ proteins are positively regulated by GPCRs that, upon activation, interact with $G\alpha$ subunits and promote exchange of GTP for GDP. As with Rho proteins, GTPase activating proteins (GAPs), called RGS (regulator of G proteins signaling) proteins, negatively regulate $G\alpha$ subunits. $\beta\gamma$ subunits do not appear to have their own sets of regulators. The activities of these subunits are dependent upon whether their corresponding $G\alpha$ subunits are bound to GTP or GDP. Thus $\beta\gamma$ subunits are indirectly regulated via the guanine nucleotide binding state of their corresponding $G\alpha$ subunits.

1.3.2.2.1 $G\alpha$ -specific GAPs: and regulator of G protein signaling (RGS) proteins

GTPase activating proteins (GAPs) negatively regulate heterotrimeric $G\alpha$ proteins by increasing the rates by which they hydrolyze GTP, thus returning the $G\alpha$ subunits to their inactive state (reviewed in [185]). GAPs are important for several reasons: they enable signal termination upon removal of stimulus, signal attenuation as feedback inhibitors or in response to additional inputs, promotion of regulatory association with other proteins or signal redirection within a G protein signaling network (reviewed in [185]). $G\alpha$ -specific GAPs are called RGS proteins, and they all possess a conserved RGS domain approximately 130 amino acid residues in length. The RGS domain is capable of binding $G\alpha$ subunits and accelerating GTP hydrolysis [186] [187]. Approximately 20 mammalian RGS domains have been identified (reviewed in [185]).

Several proteins with G α GAP activity belonging to the Dbl family of GEFs have also been described [188] [170] [131] [136]. These proteins display GAP activity toward certain G α subunits and contain sequences with similarity to the RGS family of G α protein GAPs. Thus in addition to their DH-PH domains required for regulation of Rho proteins, these Dbl proteins contain RGS domains involved in regulation of G α proteins. RGS domain-containing Dbl members include Lsc/p115RhoGEF, PDZ-RhoGEF, LARG and KIAA0380 [131] [136] [170] [135]. This multi-domain Dbl family subgroup serves at least two functions: activation of Rho family GTPases via DH-PH domains and inactivation of G α subunits via RGS domains.

In contrast to GAPs for Rho proteins, structural studies revealed that the RGS domain does not make direct contact with GTP (reviewed in [185]). The mechanism of G protein GAP activity appears to be slightly different. Instead, binding of the RGS domain to the G α -GTP complex alters the conformation such that it becomes a better hydrolase (reviewed in [185]). Thus although GAPs for G α proteins functionally inactivate G α proteins upon binding, they do not directly take part in the chemistry of GTP hydrolysis.

1.3.2.2.2 Manipulating G α activity: inhibition and constitutive activation

The carboxyl-terminal region of G α subunits represents an important site of interaction between heterotrimeric G proteins and their receptors. Covalent modification, mutation of residues or binding of specific antibodies within this region uncouples G α proteins from their receptors. Synthetic peptides derived from portions of G α subunit carboxyl-termini also inhibit receptor coupling. These synthetic peptides act as dominant negatives by competing with G α proteins for receptor binding sites and enable class-specific inhibition of GPCR-initiated signals [189, 190] [191].

Many heterotrimeric G proteins transduce cell growth signals from GPCRs to intracellular effectors and thus have the potential to behave as oncogenes. In fact, several activating mutants of the G α_s subunit displaying impaired GTPase activity were isolated from endocrine tumors [192]. Analogous mutations, in other G α subunits render these proteins GTPase-deficient and hyperactive. Thus as with Rho proteins, G α activity can be manipulated experimentally via introduction of mutations that affect their intrinsic GTP hydrolysis rates. The involvement of G α subunit signaling pathways can thus be elucidated experimentally via expression or injection of constitutively active mutants of G α subunits.

1.3.3 GPCR-mediated cellular responses

Upon activation, both $G\alpha$ and $G\beta\gamma$ subunits bind a variety of effector molecules and regulate their activities. The pattern of responses of a cell to stimulation of a given GPCR is quite complex. Signaling specificity depends on the G protein subunits recognized by the receptor, specific effector molecules expressed in the cell and concentrations of the various components in the signaling pathway. Although many GPCRs have intermediary roles in metabolism and generally perform these functions in well-differentiated cells, increasing evidence indicates that GPCRs also regulate proliferative signaling pathways (reviewed in [193]).

1.3.3.1 Oncogenic potential of GPCRs and heterotrimeric G proteins

1.3.3.1.1 Some GPCRs are transforming

Studies have repeatedly demonstrated the proliferative potential of GPCR-mediated signaling pathways. The isolation of the transforming *mas* oncogene from a human epidermal carcinoma genomic DNA library provided the first evidence for the oncogenic potential of GPCRs [194]. The *mas* gene isolated in the screen did not contain any mutations within the coding sequence but did possess a rearrangement within the 5' non-coding sequences [194]. Thus, *mas* transforming activity was mediated by increased expression rather than mutational activation. The ligand for Mas is not known. Additional studies involving other GPCRs further illustrated their importance in oncogenesis. In fact, the serotonin 1C receptor, several subtypes of muscarinic acetylcholine receptors and the α_{1B} -adrenergic receptor caused agonist-dependent transformation of NIH 3T3 fibroblasts while activating point mutations induced transformation in a ligand-independent manner [195] [196] [197]. Several types of human cancer are also associated with activating GPCR point mutations. For example, approximately one-third of hyperfunctioning thyroid adenomas display mutations in the thyrotropin receptor gene [198] [199]. Activation of this receptor leads to increased intracellular concentrations of cyclic-AMP (cAMP) and can cause transformation in cell types in which cAMP functions as a mitogen. Thus some somatically mutated GPCRs can behave as proto-oncogenes via constitutive activation. Other GPCRs may contribute to tumorigenesis via paracrine and autocrine mechanisms. Gastrin-reducing peptide (GRP), for example is secreted in human small-cell lung carcinoma (SCLC) and GRP receptor antagonists can block SCLC cell growth *in vitro* and *in vivo* [200].

Recently two GPCRs, G2A and the thrombin receptor PAR-1 were isolated in library screens for cDNAs that cause transformation of NIH 3T3 fibroblasts [201] [202]. These GPCRs, when over-expressed in

fibroblasts induced a range of cellular phenotypes characteristic of oncogenic transformation including, loss of contact inhibition, anchorage-independent survival and proliferation and reduced dependence on serum. PAR-1 is a member of a family of receptors activated by the serine protease thrombin (reviewed in [203]). Although a well-known function of thrombin is in blood coagulation, it has also been shown to act as a powerful agonist capable of eliciting a variety of mitogenic responses in certain cells [204]. Thrombin receptors have also been implicated in human tumor cell invasion [205] [206]. These and other results demonstrate the oncogenic potential of GPCR-mediated signaling pathways.

1.3.3.1.2 Certain $G\alpha$ proteins are transforming

$G\alpha$ proteins are coupled to GPCRs and not surprisingly their oncogenic potential has also been demonstrated. Interestingly, in out of the twenty $G\alpha$ subunits isolated to date, ten of them, $G\alpha_s$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_o$, $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{16}$, $G\alpha_z$, $G\alpha_{12}$ and $G\alpha_{13}$ have been shown to be involved in regulation of cell growth (reviewed in [182]). Moreover, activating $G\alpha_s$ and $G\alpha_i$ mutants have been found in a subset of endocrine tumors [207]. These constitutively activated mutants, subsequently named the *gsp* ($G\alpha_s$ protein) and *gip2* ($G\alpha_{i2}$ protein) oncogenes, lead to abnormal cell growth via upregulation of distinct signaling pathways. Stimulation of the AC-cAMP-PKA pathway resulted from *gsp* mutations and led to increased DNA synthesis rates in tumor cells and cell lines [208] [209]. Constitutive activation of AC leads to persistent activation of PKA resulting in phosphorylation and activation of cAMP-responsive element-binding protein (CREBP). Binding of cAMP-responsive elements by activated CREBP leads to transcription of specific primary genes that initiate cell proliferation (reviewed in [182]). Interestingly, cell growth via *gsp* does not involve the Ras-Raf signaling since persistent activation of AC-cAMP-PKA directly inhibits Raf via phosphorylation. Cell growth via *gip2* however does involve the MEK-ERK pathway and can occur in a Ras-dependent manner [210] [211]. Interestingly, in certain cell types Gip2-mediated MEK-ERK activation, was unaffected by co-expression of dominant negative H-Ras suggesting that ERK activation also occurs independently of Ras in certain cells [212]. Gip2 can have an inhibitory effect on levels of intracellular cAMP via inhibition of AC [213] [214]. Inhibition of the AC-cAMP-PKA via *gsp2* may relieve the PKA-mediated inhibition of Raf thus enabling MEK-ERK activation independently of Ras. Expression of activated $G\alpha_q$ mutants transformed fibroblasts and induced tumors in immune compromised mice [215] [216]. $G\alpha_q$ -mediated proliferation involves activation of PLC β . Cleavage of phosphatidylinositols via activation of PLC β leads to stimulation of ERK via either Ras-dependent or Ras-independent pathways (reviewed in [182]). Activation of ERK via either of these

pathways can couple $G\alpha_q$ signals to the nucleus and regulate cell growth. Thus activating mutants of $G\alpha_s$, $G\alpha_i$ and $G\alpha_q$ appear to regulate cell growth via either activation of the ERK or CREBP downstream signaling components that directly regulate transcription of genes involved in mitogenesis.

The $G\alpha_{12}$ family of G proteins, consisting of $G\alpha_{12}$ and $G\alpha_{13}$ are the most potent transforming $G\alpha$ subunits that have been isolated so far (reviewed in [182] [193]). Wild type $G\alpha_{12}$ was isolated from a soft-tissue carcinoma and identified as a transforming oncogene [217]. Expression of the wild type form of $G\alpha_{12}$ induced several hallmarks of cellular transformation including decreased doubling time, reduced saturation density and the ability to form colonies in soft agar. $G\alpha_{12}$ -expressing cells also induced tumors in athymic nude mice. Although transformation via wild type $G\alpha_{12}$ was dependent on serum, expression of GTPase-deficient $G\alpha_{12}$ abrogated the need for serum-dependency in transformation [217] (reviewed in [182]). These results demonstrated a direct role for $G\alpha_{12}$ in regulation of cell growth. Subsequent studies have shown that $G\alpha_{12}$ and $G\alpha_{13}$ mutants potently transform other fibroblast cell lines (reviewed in [193] [182]). $G\alpha_{12}$ and $G\alpha_{13}$ appear to regulate cell growth independently of any of the conventional second messenger pathways discussed above. The effects of $G\alpha_{12}$ and $G\alpha_{13}$ are mediated primarily via activation of the small molecular weight GTPases and downstream kinase signaling pathways. These pathways will be discussed in the following section.

1.3.3.2 $G\alpha$ -mediated activation of smaller molecular weight GTPases

Many GPCR- $G\alpha$ -mediated signals activate signaling pathways independently of adenylyl cyclase (AC) and other second messengers. Instead signaling downstream of these GPCRs involves activation of Ras and Rho family GTPases and downstream kinases and do not appear to involve pathways downstream of conventional second messengers (reviewed in [218] [182]). However, it is important to note that although in most cases activation of small GTPases is regulated primarily via $G\alpha_{12}$ and $G\alpha_{13}$, other $G\alpha$ subunits may also be involved. In fact, expression of constitutively active mutants of $G\alpha_{12}$, $G\alpha_{13}$ and $G\alpha_q$ causes RhoA-dependent formation of stress fibers in certain cells, indicating that activation of Rho GTPases are not always restricted to the $G\alpha_{12}$ and $G\alpha_{13}$ subunits [219] [201]. Importantly, the smaller molecular weight GTPases regulate a variety of processes involved in cell growth control including cell cycle progression, cytoskeletal rearrangement and gene expression. Moreover, aberrant activation of these proteins can lead to uncontrolled cellular proliferation as well as

invasion and metastasis of tumor cells. GTPase activation via GPCRs is therefore consistent with their role in oncogenic transformation. Importantly, GTPase activation requires activation via GEFs, thus the minimum requirement for GPCR-mediated activation of GTPases involves GEF activation via heterotrimeric G proteins.

In some cell types $G\alpha$ subunits also appear to activate ERK via Ras activation (reviewed in [182]). Although the mechanisms of Ras activation via $G\alpha$ subunits are not clear, evidence suggests that adapter proteins may be involved. Protease-activated thrombin receptor activation of Ras via $G\alpha_{12}$ in astrocytoma cells, for example involves Shc [220]. Since Ras activation requires GEFs, in these cells the mechanism of Ras activation by $G\alpha$ subunits may involve adapter protein-mediated GEF activation.

Studies also revealed that expression of activated forms of $G\alpha_{12}$ ($G\alpha_{12}Q229L$) and $G\alpha_{13}$ ($G\alpha_{13}Q226L$) in a number of different cell lines leads to potent activation of JNKs and that activation also involved Rho, Rac, Cdc42 and Ras [221] [222] [223]. Additional growth-promoting signals transmitted by $G\alpha_{12}$ family subunits include Ras and Rac-dependent activation of ERK [224], potentiation of serum-stimulated arachidonic acid release in NIH 3T3 fibroblasts [225] and activation of several tyrosine kinases including the FAK, Tec/Bmx and Pyk-2 kinases [226] [227]. $G\alpha_{12}$ signals also mediate other growth promoting kinase pathways including PKC and the phosphatidylinositol kinases PI4K and PIP5K via activation of the small GTPases Ras, Rho, Rac and Cdc42 (reviewed in [182]).

A number of target-response elements are involved in transformation via $G\alpha_{12}$ and $G\alpha_{13}$. In fact, Rac- and Ras-mediated JNK activation via $G\alpha_{12}$ leads to enhanced *c-jun* transcriptional activity [223]. Also, activation of SRF and induction of SRE-dependent transcription via $G\alpha_{12}$ is Rho-dependent [228]. *Edg-1*, a primary response gene involved in cell proliferation and differentiation is activated by $G\alpha_{13}$ and to a lesser extent by $G\alpha_{12}$ [229]. Moreover, transcriptional activation of *cyclooxygenase-2* via $G\alpha_{12}$ provides additional growth signals [225]. Thus a number of transcriptional regulatory targets contribute to cell growth promotion via $G\alpha_{12}$ and $G\alpha_{13}$.

Many signals downstream of $G\alpha_{12}$ and $G\alpha_{13}$ are mediated by small GTPases. The mechanisms by which these $G\alpha$ subunits stimulate these GTPases are beginning to be elucidated. $G\alpha_{12/13}$ -mediated activation of the smaller GTPases can occur via several mechanisms including stimulation of specific

Dbl family proteins, competition with GDIs or inhibition of specific GAPs. In fact, both Lsc/p115RhoGEF and PDZ-RhoGEF interact with and are activated by $G\alpha_{12}$ family subunits [130] [136]. The Dbl proteins interact with these two $G\alpha$ subunits via their amino-terminal RGS domains and interaction is thought to relieve a negative inhibitory influence of the RGS domain on the DH domain of the GEF. Cell growth changes induced via $G\alpha_{13}$, $G\alpha_{12}$ and Rho can also involve receptor and non-receptor tyrosine kinases. In fact, inhibition of epidermal growth factor receptor (EGFR) signaling in fibroblasts blocks stress fiber formation via $G\alpha_{13}$, but not $G\alpha_{12}$ [230] [231]. Thus $G\alpha_{12}$ and $G\alpha_{13}$ display differential involvement in receptor-mediated stress fiber formation. Non-receptor tyrosine kinases Tec and Bmx are also involved in SRF activation via $G\alpha_{12}$ and $G\alpha_{13}$ [232]. The mechanisms of signal transduction from $G\alpha_{12/13}$ to Rho via these kinases however are not known and it is likely that kinase activation occurs downstream of Rho activation. Interestingly, JNK activation via the $G\alpha_{12}$ - or $G\alpha_{13}$ -coupled lysophosphatidic acid (LPA) receptor occurs via PKC-mediated phosphorylation of the Rac guanine nucleotide exchange factor Tiam1 [233]. These results suggest that $G\alpha_{12}$ can stimulate Rac via PKC-mediated activation of Tiam1. Thus mechanisms of $G\alpha_{12}$ - and $G\alpha_{13}$ -mediated Rho pathway activation appear to incorporate a range of signaling components.

1.3.3.3 Mas, G2A and PAR-1 transform fibroblasts via activation of Rho family GTPases

Several lines of evidence suggest that the GPCRs, Mas, G2A and PAR-1, transform fibroblasts via activation of Rho GTPases. NIH 3T3 fibroblasts transformed by these GPCRs display morphological characteristics similar to those formed by Rho family members but are distinct from those transformed with other oncogenes including Ras, Raf or Src [234] [201] [235] [202]. Moreover, like Rho proteins, Mas and PAR-1 are efficient activators of NF- κ B and SRF, but are poor activators of the Ras-Raf-ERK pathway target, Elk-1 in NIH 3T3 fibroblasts [234] [202]. G2A also activates SRF in a RhoA-dependent manner in fibroblasts [201]. In contrast to G2A and PAR-1, that appear to transform fibroblasts via RhoA, Rac is the primary mediator of Mas transformation. More specifically, Mas-transformed cells display stress fibers, focal adhesion and membrane ruffles that are mimicked by constitutively activated Rac [234]. Results in this thesis provide additional evidence that G2A- and PAR-1-mediated transformation involves certain smaller molecular weight GTPases.

What $G\alpha$ proteins mediate signals from these GPCRs to Rho? Studies involving G2A revealed that the cell morphological and cytoskeletal structures induced by this receptor are identical to those induced by

G α_{13} [201]. Moreover, G2A can efficiently induce stress fibers in mouse fibroblasts that lack G α_{12} or G $\alpha_{q/11}$, but not G α_{13} [235]. These results indicate that G α_{13} is a key regulator of RhoA activation via G2A. Additional experiments presented in this thesis provide further evidence for the involvement of G $\alpha_{12/13}$ subunits in G2A and PAR-1-mediated fibroblast transformation.

Although hyperactivity of many different G α subunits can lead to a variety of cellular responses, including transformation, G α_{12} and G α_{13} signaling pathways are distinct in that they appear to incorporate activation of the smaller GTPases rather than pathways downstream of conventional second messengers. Ultimately, G α_{12} - and G α_{13} -mediated signals converge on pathways regulated by the smaller GTPases. Activation of the Rho and Ras family GTPases requires GEFs. Thus GTPase activation and activation of downstream kinases via G α_{12} or G α_{13} likely involves activation of GEFs. Although the components of these pathways are only beginning to be characterized, the previous studies highlight the importance of Rho signaling pathways in cellular responses mediated certain G α proteins.

1.4 Use of transformation assays to identify modulators of Rho activity

Transformation assays have proven to be extremely useful in isolation and characterization of oncogenes. These assays often involve the use of fibroblast cell lines. These cells are particularly useful because they are sensitive to changes in the activities of numerous signaling pathways, especially those involved in changes in cell morphology and growth. As a result they are likely to respond to changes in the signaling intensities of pathways related to these processes. The usefulness of these cells in terms of their sensitivity to change combined with their relative ease in terms of manipulation make them extremely useful experimentally. In fact the majority of oncogenes identified to date have either been isolated in library (genomic DNA and cDNA) screens for clones capable of inducing specific cellular changes or they have been further characterized using these cells. Moreover, most of the GTPases, Dbl proteins, GPCRs and G α proteins discussed in this thesis were either isolated or characterized via fibroblast transformation. Transformation or suppression of transformation using these cells is the basis to the experimental results presented in Chapter 3 of this thesis.

1.5 The role of Rho family and G α GTPases in lymphocyte development and function

Many studies have implicated GTPases in regulation of cell survival, proliferation and differentiation during lymphocyte development. Moreover, biochemical, functional and genetic studies have shown that GTPases are important components of signal transduction via antigen, costimulatory, cytokine and chemokine receptors in regulation of the immune system. The following sections will summarize data regarding the role of Rho and G α GTPases in lymphocyte development and function.

1.5.1 Lymphocyte development: T and B lymphocytes

The goal of lymphocyte development is to generate a large repertoire of cells expressing diverse receptors that enable them to recognize and react to a broad array of foreign antigens. Both T and B lymphocyte development are characterized by positive and negative selection of cells based on their antigen receptor genes and proteins. Developing T and B lymphocytes use signaling complexes to monitor the progress of antigen receptor gene assembly referred to as pre-T cell receptors (pre-TCR) and pre-B cell receptors (pre-BCR), respectively. The next two sections will briefly summarize T and B cell development in mice.

1.5.1.1 T lymphocyte development

Differentiation of T lymphocytes is initiated in the thymus from fetal liver or bone marrow-derived hemopoietic stem cells (reviewed in [236]). Two major lineage decisions face immature T cells as they develop in the thymus (reviewed in [237]). Inherent to this process are intrinsic and extrinsic cues that modify the gene expression profiles of developing cells. At the earliest stages, cells must commit to either $\gamma\delta$ or $\alpha\beta$ lineages; once committed to the $\alpha\beta$ lineage, they commit to either a CD4⁺ or CD8⁺ fate before they can pass through the thymic medulla and exit to the periphery. The thymus, composed of stromal cells and thymocytes (T cell progenitors) provides the environment necessary to stimulate the differentiation of stem cells into antigen-reactive T lymphocytes (reviewed in [238]).

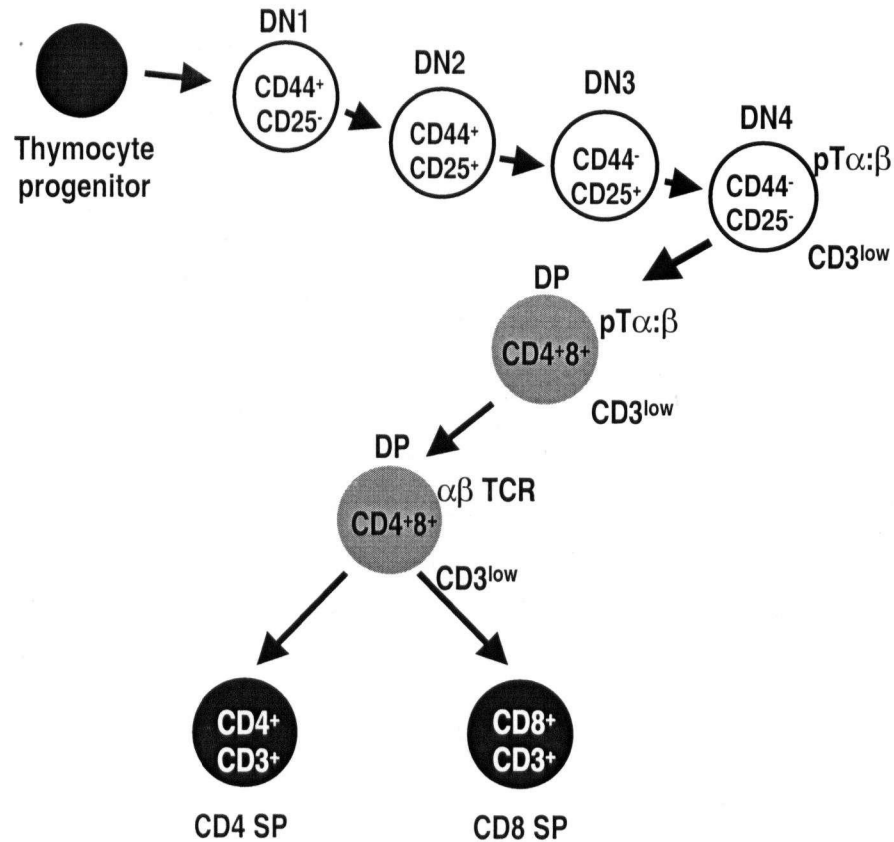
The earliest stages of T cell development occur within the thymus before surface expression of the T cell receptor (TCR). Several important events are involved in development of early thymocytes including proliferation, commitment to either $\gamma\delta$ or $\alpha\beta$ lineages and rearrangement and expression of

the TCR loci. Thymocyte survival at key developmental checkpoints is determined by signaling from cytokine receptors and the TCR. Upon expression of the TCR, thymocytes undergo rigorous selection processes. These selection events eliminate thymocytes expressing self-reactive and non-functional TCRs, allowing thymocytes with TCRs specific for self-MHC.

The earliest recognizable T cell progenitors (CD3⁺CD4^{low}CD8⁻Thy-1⁺) progressively lose their multipotentiality and become committed to the T lineage after acquiring the CD3⁺CD4⁺CD8⁻ (referred to as CD4⁺CD8⁻ double negative (DN) thymocytes) (reviewed in [236]). DN thymocytes can be subdivided into four populations according to expression of the cell surface markers CD25 (IL-2R α chain) and CD44 (Pgp-1) and define the following developmental pathway: CD25⁺CD44⁺ (DN1) \rightarrow CD25⁺CD44⁺ (DN2) \rightarrow CD25⁺CD44⁻ (DN3) \rightarrow CD25⁻CD44⁻ (DN4). Approximately ten cell divisions occur between stem cell immigration to the thymus and the DN3 stage and this is thought to generate a large pool of DN3 thymocytes prior to initiation of TCR β chain rearrangement (reviewed in [236]). During the DN2 to DN3 transition *Rag-1* and *Rag-2* genes are expressed and encode essential components of the rearrangement machinery (reviewed in [239]). Cells that successfully rearrange their TCR β chain loci express a functional pre-TCR complex. The pre-TCR complex is composed of a TCR β chain, a pre-T α surrogate α chain and the multisubunit CD3 signaling complex consisting of CD3 ϵ/γ and CD3 ϵ/δ heterodimers and a homodimer CD3 ζ (reviewed in [240]). Assembly of the pre-TCR on the cell surface of DN thymocytes promotes their survival, signals them to progress to the CD4⁺CD8⁺ DP stage of development and causes them to undergo significant clonal expansion. The pre-TCR is necessary and sufficient for sustained survival, proliferative expansion and progression of T cell progenitors via the DN4 stage into DP cortical thymocytes [241] [242] (reviewed in [243] [240]). DP thymocytes undergo TCR α gene rearrangement and TCR $\alpha\beta$ heterodimers are expressed on the cell surface (**Figure 1.5**).

DP thymocytes are faced with three choices: death by neglect, death by negative selection and life by positive selection. Most DP thymocytes die within 3-4 days by neglect because their TCR $\alpha\beta$ s cannot recognize self MHC-peptide complexes on thymic stroma (reviewed in [244]). Thymocytes expressing TCR $\alpha\beta$ become subject to immunological selection based on their TCR $\alpha\beta$ specificity (reviewed in [245]). Positive selection is the active process of rescuing MHC-restricted thymocytes from programmed cell death. DP thymocyte precursors bearing low densities of self-MHC-restricted

Figure 1.5. T lymphocyte development. The stages of thymocyte development can be distinguished based on expression of CD44 and CD25 in the earliest stages of thymocyte development (DN1 to DN4) as well as CD4 and CD8 in the later stages of thymocyte development (DP and SP).



receptors are positively selected to survive and differentiate into TCR^{hi} CD4 or CD8 SP thymocytes. DP thymocytes bearing MHC class II-specific TCR αβ will usually retain expression of CD4, the class II MHC-specific co-receptor, whereas those bearing class I MHC-specific TCR αβ will retain expression of CD8, the class I MHC co-receptor (reviewed in [244]). Negative selection refers to the deletion or inactivation of potentially autoreactive thymocytes or thymocytes with TCRs specific for self-peptides (reviewed in [246]). Following these selection events, immunologically competent SP thymocytes exit to the periphery to create a pool of T cells with diverse repertoire for antigen. Together, these processes lead to the formation of a functional mature T cell repertoire.

1.5.1.1.1 Thymocyte developmental indicators: cell surface markers CD69, CD5, HSA and CD62L

Thymocytes can be further classified by surface expression of other markers in addition to expression of CD44, CD25, TCR αβ, CD3, CD4 and CD8. TCR-dependent maturational events define the earliest

stage of positive selection. These events include up-regulation of TCR, bcl-2, CD69 and CD5 and decreased expression of Rag-1 and Rag-2 (reviewed in [244]). Additional changes in surface expression of HSA and CD62L define the latest stages of thymocyte development. These markers were used in characterization of lymphocytes from transgenic mice presented in later chapters. Changes in expression levels of some of these markers and the relevance to thymocyte development are discussed below.

CD5 functions as an inhibitor of TCR signal transduction and surface expression is regulated by TCR signal intensity during development. Notably, CD5 surface levels on mature thymocytes and T cells parallel the avidity of the positively selecting TCR/MHC/ligand interaction [247]. The inducible regulation of CD5 surface expression during thymocyte selection functions to fine tune the TCR signalling response. CD5 also acts in consort with other signalling molecules thereby ensuring that negative selection is highly efficient (reviewed in [248]).

In the thymus phenotypically and functionally mature SP thymocytes are generated from immature DP precursors through positive selection. Positive selection is a multistage process involving transition through an intermediate CD4⁺CD8⁺CD69⁺ phase. Although positive selection is a multistage process initiated by TCR-MHC interactions, continuation of this process and subsequent post-selection events are independent of ongoing TCR engagement. CD69 expression first appears on thymocytes as they begin positive selection [249] [250]. Notably, only those DP thymocytes being selected express CD69 [251]. Thus, CD69⁺ thymocytes include both TCR^{low} DP and TCR^{high} SP thymocytes and represent a population that is undergoing positive selection or has just completed that process. Additional evidence indicates that CD69 is not merely a marker for cells that have begun the selective process. In fact generation of mature SP thymocytes *in vivo* is deregulated by CD69 blockade or over-expression; thus directly implicating CD69 in the processes of thymocyte selection and maturation [252].

Heat stable antigen (HSA) acts as a co-stimulatory molecule for antigen-dependent activation of helper T cells. HSA is also expressed during the initial stages of T cell development. Expression is very high on immature DN thymocytes, reduced on DP thymocytes, very low on SP thymocytes and entirely reduced on immunologically competent CD4⁺ and CD8⁺ T lymphocytes. Importantly, down-regulation of HSA expression at the DP stage is a critical event in thymocyte development and HSA may provide signals that contribute to determining the efficiency of negative selection [253].

The homing receptor CD62L/L-selectin also defines thymocyte and peripheral T cell developmental stages. The immune system relies on the export of T cells from the thymus to create a pool of naïve T cells with diverse repertoire for antigen (reviewed in [254]). Naïve T cells, after interaction with cognate antigen give rise to memory cells that provide protection against subsequent exposure to infectious agents. Memory T cells are CD62L⁻ and preferentially target peripheral tissues. In contrast to memory T cells, naïve T cells express CD62L and selectively target to specific tissues including lymph nodes (LN) (reviewed in [254]). CD62L is the key homing receptor involved in mediating lymphocyte entry into these tissues. Notably, CD62L expression is up-regulated in SP thymocytes prior to export to the periphery. SP thymocytes, once expressing CD62L exit to the periphery to create a pool of T cells with diverse repertoire for antigen.

1.5.1.2 B lymphocyte development

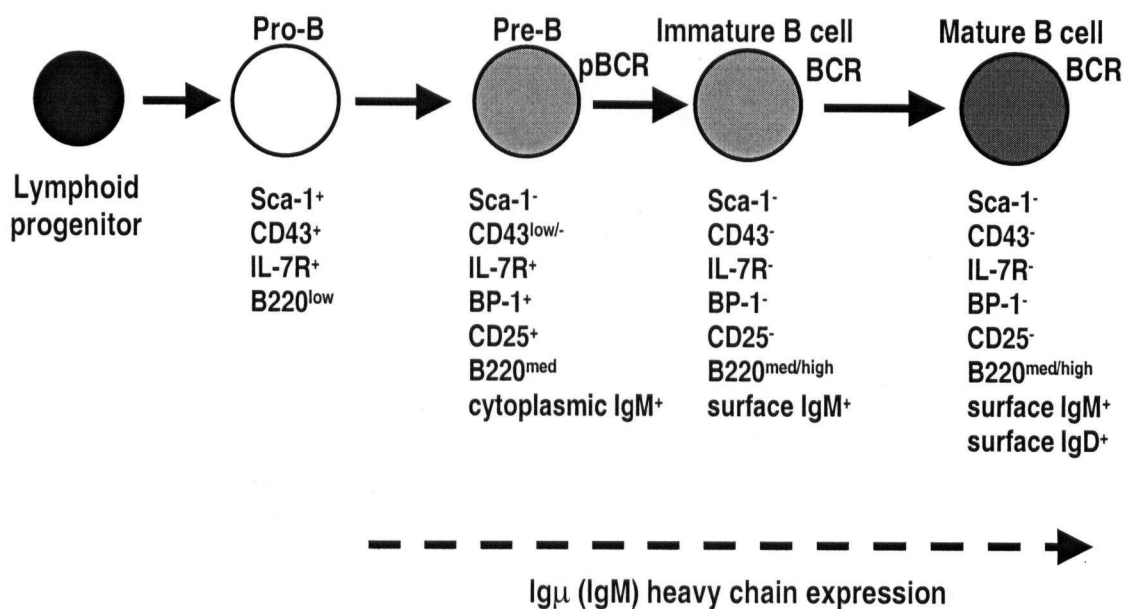
B lymphocytes are generated from hemopoietic stem cells by a complex differentiation process, in the liver before birth and in the bone marrow afterward. B lineage cell development is characterized by progression through a series of checkpoints defined primarily by rearrangement and expression of the immunoglobulin (Ig) genes. Functional immunoglobulin gene rearrangement is essential for successful B cell development. Additionally, stromal cells in the microenvironment in which the cells develop further influence progression through these checkpoints. Two major developmental checkpoints are defined based on expression of the B cell receptor (BCR) components. The earliest B lineage-committed progenitors are referred to as pro-B cells and the successful rearrangement and expression of immunoglobulin heavy chain genes in these progenitors results in their maturation into pre-B cells. The pre-BCR, consisting of the immunoglobulin μ heavy chain, surrogate immunoglobulin light chain and the Ig α /Ig β signaling heterodimer is expressed on late pro and pre-B cells. Pre-B cells, upon expression of immunoglobulin light chain protein are able to develop into immature B lymphocytes. Expression of the BCR, consisting of μ heavy chain, conventional light chain and Ig α /Ig β occurs on both immature and mature B cells (reviewed in [255]).

Extrinsic factors that regulate the growth, differentiation and survival of B lineage cells play an important role in development of B lymphocytes. In particular, association of developing precursors with non-hemopoietic stromal cells that form a supportive hemopoietic microenvironment is critical throughout development. Stromal cells mediate their effects through direct cell-to-cell contacts with the hemopoietic progenitors and secretion of cytokines that regulate lymphocyte development (reviewed in

[256]). The stromal cell-derived cytokine IL-7 for example, is indispensable for mouse B cell development and is required for the transition of pro-B cells to pre-B cells [257] (reviewed in [255]).

One way of defining the stages of pro-B cell development is by expression of CD43 and the pan-B lineage marker B220 (CD45R) [258]. The pro-B cell compartment includes a continuum of cells at various stages of development. These stages of pro-B cell development can be resolved based on expression of heat stable antigen (HSA) and BP-1 antigens. Transition to the pre-B cell stage is accompanied by down regulation of CD43. Pre-B cells develop into immature B lymphocytes that express the BCR and these cells mature into IgM+IgD+ B lymphocytes that emigrate from the bone marrow to secondary lymphoid organs including the spleen (**Figure 1.6**)

Figure 1.6. B lymphocyte development. Bone marrow B cell development can be distinguished based on expression of Sca-1, CD43, IL-7R, B220, BP-1 and CD25 in the pro- and pre-B cell stages. The stages of immature and mature B cell development can be distinguished based on expression of B220, IgM and IgD.



The BCR serves multiple functions in B lymphocytes that differ through various stages of their development (reviewed in [259] [260]). Pre-BCR and BCR complexes trigger proliferative expansion and differentiation of pre-B and mature resting B cells, respectively. Alternatively, immature B cells trigger apoptosis upon clustering of newly expressed BCRs to eliminate autoreactive B cells. Thus signaling via these receptors regulates positive and negative selection of B lymphocyte precursors. Intensity of BCR signaling in mature B cells can also regulate V(D)J recombination at the membrane

immunoglobulin locus as a mechanism to regulate the production of high affinity antibodies. Finally, antigen processing and peptide presentation to helper T cells is mediated by the BCR. Thus the BCR utilizes a complex network of signaling cascades to regulate diverse functions. B lymphocyte development progresses through several critical stages involving commitment, initial establishment of the Ig repertoire and cellular selection. Together these processes give rise to functional peripheral B lymphocyte subsets.

1.5.2 Function of Rho family GTPases in lymphocytes

A number of functional, genetic and biochemical studies have implicated Rho family GTPases in immune cell biology. In immune cells, Rho family proteins are regulated via receptor stimulation by a variety of extracellular stimuli including antigens, cytokines, co-stimulatory and adhesion molecules (reviewed in [261]). As in other cells Rho proteins can regulate gene expression and the actin cytoskeleton in immune cells that affect lymphocyte responses to receptor ligation. Notably, altered Rho family protein activity regulates many facets of lymphocyte development. The following sections will summarize the current literature regarding the roles of various Rho family GTPases in lymphocyte function and development. Where possible the involvement of downstream effector targets in lymphocytes will be discussed.

1.5.2.1 Rho pathways in lymphocytes

Perhaps the most studied Rho family GTPase member is RhoA. There is compelling evidence indicating that Rho function is important in many aspects of lymphocyte biology. Targeted expression of the C3 exoenzyme in particular has aided in understanding the cellular functions of Rho. The C3 bacterial toxin from *Clostridium botulinum* ADP-ribosylates Rho (A, B and C), but not Rac or Cdc42 proteins and functionally inactivates it by preventing interactions with downstream effector molecules (reviewed in [262]). The use of this enzyme has implicated Rho in several aspects of lymphocyte function including, integrin-mediated adhesion, migration and cytotoxicity [263] [264] [265] [266] [267]. More specifically, in T lymphocytes Rho has been shown to be important in several aspects of T cell activation including TCR-mediated cytokine production, calcium influx and lymphocyte spreading [268] [269] [270]. Rho proteins are also important in regulating cell shape and immunogenic capacity of APCs, in particular dendritic cells [271]. Thus Rho function appears to be important in many aspects of lymphocyte function.

Inactivation of Rho function in the thymus has also been achieved via targeted thymic expression of C3-transferase using specific promoters. These studies illustrated the importance of Rho function in normal T lymphocyte biology (reviewed in [261]). In thymocytes that lack Rho function from the earliest developmental stages (*lck* promoter), proliferative defects severely impair the generation of normal numbers of thymocytes and mature peripheral T cells [272]. In particular, Rho regulates the survival of early CD44⁺CD25⁺ and late CD44⁺/CD25⁺ DN thymocytes. Normally, the survival of these cells is controlled by IL-7 [273, 274]. Rho appears to be a component of the receptor signaling pathway used by IL-7 to control cell survival and the thymic phenotype caused by loss of Rho function resembles that of IL-7^{-/-} mice [272]. IL-7 is believed to regulate survival signaling pathways in thymocytes by controlling cellular levels of bcl-2 [274]. Interestingly, expression of bcl-2 can rescue the cellular deficit observed in thymocytes lacking Rho function [275]. Thus Rho may act as an intracellular link between the IL-7 receptor and events that control bcl-2 family proteins. Notably, loss of Rho function is also accompanied by the development of thymic lymphoma [276]. Lck-C3 mice develop aggressive malignant thymic lymphoblastic lymphomas between 4 and 8 months of age. Thus in addition to its effects on cell survival in DN thymocytes, inhibition of Rho function is associated with predisposition to lymphoid cell transformation.

Inactivation of Rho function in later stages of thymocyte development reveals additional Rho functions in T lymphocyte biology. In fact, expression of C3-transferase under the control of the locus control region of the CD2 gene, leads to a thymocyte differentiation block after rearrangement of the TCR β chain gene [277]. These results indicate that Rho also acts as an intracellular switch for TCR β selection, a critical thymocyte differentiation checkpoint. Thus inhibition of Rho function at different stages of thymocyte development also reveals different functions of this GTPase *in vivo*.

Loss of Rho function blocks pre-T cell differentiation and survival, indicating that this GTPase is a critical signaling molecule during early thymocyte development. Gain of function studies revealed that RhoA is also important in determining the fate of mature T cells. Transgenic mice expressing an activated mutant of RhoA did not appear to display defects in early thymocyte development, however they were hyperresponsive to TCR stimulation and showed augmented positive selection [278]. Thus in addition to its effects on DN thymocyte survival and differentiation, RhoA is involved in determining the fate of mature T cells. Also unlike Rac1, RhoA could not initiate changes in actin dynamics

necessary for DN thymocyte development in the absence of functional TCR. In fact, activated RhoA was unable to drive pre-T cell differentiation in the *Rag2^{-/-}* background [278]. Together, the loss and gain of function studies involving Rho proteins *in vivo* have revealed multiple roles for Rho GTPases in several aspects of lymphocyte biology.

1.5.2.2 *Rac pathways in lymphocytes*

Many studies have highlighted the importance of Rac function in lymphocytes. Induction of cytokine gene expression is an important component of lymphocyte function. Ras signals in mature lymphocytes are mediated by transcription factors of the NFAT (nuclear factor of activated T cells) family (reviewed in [279]). NFAT molecules are regulatory targets for antigen receptors in T and B cells as well as the FcεR1 in mast cells and control activation of cytokine genes including IL-2, IL-4, GM-CSF or TNF-α upon stimulation [280] [281]. Moreover, NFAT activation requires the coordinated interaction of receptor-induced Ras signaling pathways and receptor-induced calcium/calcineurin signaling pathways [282]. Interestingly activation of NFAT also requires the coordinated action of multiple Ras effector pathways. Notably, experiments with activated and inhibitory mutants of Rac revealed that transcriptional activity of AP-1 and NFAT by Ras is dependent on Rac function [280]. Thus it appears that Rac is an effector molecule that couples Ras to the signaling pathways that regulate AP-1 and NFAT.

Studies involving the Rac exchange factor Vav1, in particular, have shown that Rac signals play a critical role in other aspects of lymphocyte activation. Vav1 is a Dbl family GEF that specifically activates Rac1 and is regulated by phosphorylation (reviewed in [261]). Vav1 is rapidly and transiently tyrosine phosphorylated in response to antigen receptor ligation in T and B cells [283] [284] [284, 285]. Moreover, Vav1-deficient mice display defects in thymocyte positive and negative selection that result in reduced numbers of SP thymocytes [286] [284]. Antigen receptor signaling and cytokine expression by peripheral T cells are also impaired in these mice [287] [288]. These results indicate that Vav1-Rac signals are important in lymphocyte function.

Other studies have directly implicated Rac in lymphocyte function and development. For example, deficiency of the hemopoietically expressed Rac2 protein results in impaired T cell, signaling, proliferation and actin polymerization [289]. B lymphocytes from *Rac2^{-/-}* mice are also reduced in numbers and exhibit impaired B cell immunoglobulin secretion and responses to antigen and

chemokines [290]. Also, expression of an activated mutant of Rac1 was shown to regulate pre-T cell differentiation via proliferation of DN thymocytes at the point of TCR β selection [291]. Interestingly, these effects are retained when a mutant form of activated Rac1 that is unable to bind PAKs or downstream kinases is expressed [291]. Thus, PAK-mediated kinase signaling cascades are not essential for Rac1-induced pre-T proliferation. Activated Rac1 was also able to drive a small number of pre-T cells to differentiate into the DP subset in Rag^{-/-} mice [291]. Rac1 signals however were not sufficient to drive T cell proliferation and restore thymic cellularity in Rag2^{-/-} mice. These results are consistent with a model in which the main function of Rac1 is to potentiate signals generated by the pre-TCR complex. Rac1 signals were unable to regenerate SP thymocytes in the Vav1^{-/-} background [291]. Although these results suggest that Rac1 is unable to substitute for Vav1 in TCR regulation of positive and negative selection, enhanced TCR signaling via activated Rac1 may also lead to impaired production of SP thymocytes in the Vav1^{-/-} background. These and other studies illustrate the importance in Rac signaling in lymphoid and non-lymphoid cells.

1.5.2.3 Cdc42 pathways in lymphocytes

Signaling pathways via Cdc42 have also been implicated in immune cell function. Motility and T cell interactions with epithelial cells and antigen presenting cells (APCs) require coordinated regulation of the actin cytoskeleton. Studies with activated and inhibitory mutants of Cdc42 indicate that this GTPase is important in cytoskeletal regulation in many cells including lymphocytes. In T cells in particular Cdc42 regulates cytoskeletal polarization toward APCs but is not involved in other T cell signaling processes including cytokine production [292].

Extensive studies involving the Cdc42 effector WASp have shown that Cdc42-mediated signals are important in lymphocyte function. WASp interacts with Cdc42 upon activation and patients with WAS that lack WASp display cytoskeletal and cell activation abnormalities in lymphocytes [293] [294]. In addition, defective T cell-APC interactions in WASp-deficient cells may result from reduced microvilli on the cell surface and poor responses to protein antigens in WAS patients (reviewed in [74]). WASp interacts with numerous signaling molecules known to alter the actin cytoskeleton. Recently the effect of a gain of function mutation in WASp was reported in a family affected with X-linked severe congenital neutropenia (XLN) [295]. These patients displayed increased numbers of activated peripheral T lymphocytes. Moreover, this WASp mutant was capable of Cdc42-independent activation of actin polymerization *in vitro* [295]. Thus activating and inactivating mutations in WASp cause distinct

hereditary disorders. Notably, WASp provides a link between Cdc42 and the actin cytoskeleton and impaired or enhanced activity of this protein may explain the cellular defects underlying WAS and XLN, respectively.

Cdc42 was directly implicated in thymocyte development and T cell function upon characterization of transgenic mice expressing an activated mutant of Cdc42 (Q61L). In particular, expression of activated Cdc42 in transgenic mice induced T cell apoptosis in thymus and peripheral lymph organs [296]. These mice displayed an overall reduction in the number of thymocytes and peripheral T cells. Analysis of thymocyte subsets revealed that DP and SP thymocyte numbers were reduced while the numbers of late DN thymocytes were increased. Interestingly, a high proportion of thymocytes and T cells were apoptotic, explaining the reduced thymocyte and peripheral T cell numbers. Further analysis revealed that Cdc42 triggered distinct apoptotic pathways in thymocytes and peripheral T cells [296].

1.5.2.4 Multiple Rho, Rac and Cdc42 effector pathways mediate lymphocyte responses

As indicated there is an abundance of evidence implicating Rho family GTPases in many aspects of immune cell function and development including regulation of cell survival, cell cycle progression, adhesion and cytokine gene expression in response to antigen or costimulatory receptor stimulation. Rho family proteins are known to bind to and activate a variety of effectors including kinases, phosphatases, lipases and scaffold proteins to initiate cellular responses. The majority of these effector interactions have been identified and characterized in non-lymphoid cells. Although it is possible that many of these effectors are involved in cellular responses of Rho GTPases in lymphocytes, their exact roles in these cells are not well understood. Nevertheless, there are Rho family targets potentially involved in the cellular responses of Rho proteins in lymphocytes including the actin cytoskeleton and protein kinases. Regulation of these effector pathway targets by Rho proteins would have a major impact on lymphocyte biology.

The first biological function ascribed to Rho GTPases was regulation of the actin cytoskeleton in fibroblasts. Current fibroblast models allow Cdc42, Rac and Rho to have unique functions in control of the actin cytoskeleton but importantly link these GTPases in a linear cascade in which Cdc42 stimulates Rac responses, which in turn initiate Rho responses in controlling the organization of the actin cytoskeleton [35]. As mentioned above, there is strong genetic evidence indicating that these GTPases regulate the actin cytoskeleton in lymphocytes. Moreover fibroblast models would predict

that Cdc42 would initiate Rac and Rho-controlled cytoskeletal changes in lymphocytes. Despite evidence indicating that Rho proteins mediate morphological changes upon lymphocyte activation, integrin-mediated adhesion and cell-mediated cytotoxicity [297] (reviewed in [279]), a general model for GTPase control of the actin cytoskeleton in these cells has not yet been established. Rho GTPases regulate a variety of other cellular responses in addition to the cytoskeleton including gene transcription and cell cycle progression. The roles of Rho GTPases in those responses are likely to be distinct from those maintaining the actin cytoskeleton. It is important to note that although multiple effectors exist for Rho, Rac and Cdc42, the effectors involved in regulating the cytoskeleton appear to be quite different than those involved in other cellular responses.

Additional effectors important for lymphocyte biology are protein kinases. As mentioned, Rac is important for cytokine gene regulation in lymphocytes and the SAP kinases JNK/p38 appear to be important in these processes. JNK/p38 are activated by cytokines in lymphocytes, FcεR1 in mast cells and antigen receptors in T and B cells. The transcription factors ATF-2, c-jun and Ets family proteins are substrates for these kinases. Interestingly, there is evidence for functional coupling of Rac to JNK in lymphocytes; notably, active mutants of Rac can induce the JNK/p38 pathway [298]. These studies suggest a preliminary model that links antigen receptors to Rac, then to JNK, and finally to transcription factors involved in cytokine gene induction. The details of this model however need to be resolved. In fact activation of this pathway may depend on the exact nature of the receptor signals or cells since transcription factor activation upon T cell activation does not always involve JNK activation. Rho-mediated AP-1 activation in Jurkat T lymphocytes upon activation occurs via a MAPK-independent pathway involving PKCα [299]. Moreover CD46/CD3 co-stimulation of human T cells induces activation of extracellular signal-regulated kinase (ERK) [297]. As in other cell types, there are multiple routes to transcriptional target activation via Rho family protein activation. In fact, Rac and Cdc42 GTPases both mediate T cell antigen receptor-induced NF-κB activation. In this pathway, NF-κB activation and cytokine gene induction downstream of the Rho family GTPases occurs via phosphorylation of IκB kinases by mixed-lineage kinase 3 (MLK3) [300]. Thus MLK3 is an activator of NF-κB-mediated transcriptional activation that also leads to AP-1-dependent cytokine gene induction. Together these results indicate that a number of kinase signaling cascades are involved in Rho family T lymphocyte responses.

Regulation of inositol lipid metabolism via Rho proteins may also have a major impact on lymphocyte biology. Evidence in non-lymphoid cells indicates that Rho proteins regulate inositol lipid metabolism via a mechanism regulated by PI4P5 and PI 3-kinases [68] [301] (reviewed in [56]). More specifically, Rho and Rac signals regulate cellular levels of D-5 phosphoinositides, particularly phosphatidylinositol 4,5-bisphosphate (PI[4,5]P₂) via PI4P5 kinase (reviewed in [56]). This lipid is an important molecule in antigen receptor signaling in lymphocytes. In particular, hydrolysis of PI[4,5]P₂ by phospholipase C members generates inositol 1,4,5-trisphosphate (IP₃) and diacylglycerols that regulate intracellular calcium and protein kinase C, respectively. Also, phosphorylation of PI[4,5]P₂ by PI 3-kinase generates PI[3,4,5]P₃ that can in turn be dephosphorylated to produce phosphatidylinositol 3,4-bisphosphate (PI[3,4]P₂), another important modifier of intracellular signal transduction pathways. Regulation of these phosphoinositides and their products by Rho family GTPases may be an important mechanism of lymphocyte responses. Interestingly, defective calcium responses leading to impaired induction of cyclin D2 of Vav1^{-/-} B cells are responsible for their impaired proliferative responses to antigen stimulation [302]. Altered inositol lipid production in the absence of Vav-Rac signaling may explain the proliferative defects in these cells. Although there is no evidence for direct coupling between Rho proteins and lipid kinases in lymphocytes, this will be an important area of future research.

Rho GTPases are thus important for the regulation of different cellular responses essential for immune function. Knowledge regarding the biological function of the effector pathways that mediate lymphocyte responses however is fragmentary. A fundamental feature of the biology of Rho GTPases is that receptors do not uniformly activate all cellular pathways mediated by a certain GTPase. In fact, different Rho GTPase effectors can function independently. This is highlighted by the observations that altered Rho GTPase activity can have rather unique responses in different cell populations within the thymus. This is likely to be a common theme in Rho-mediated lymphocyte signaling. As in other cells, the nature of the cellular response is dependent upon the Rho proteins activating signals as well as the temporal and spatial regulation of Rho effector molecules by adapters, anchoring and scaffold proteins. Thus the outcome of Rho GTPase activation by various immune stimuli is likely to be determined, at least in part, by the expression patterns of different Rho effectors in lymphocytes.

1.5.3 $G\alpha_{12/13}$ have the potential to influence lymphocyte development via Rho activation

The roles of $G\alpha$ proteins in lymphocytes are beginning to be elucidated. The majority of studies have involved chemokines, chemokine receptors and their roles in lymphocyte migration. Chemokine receptors are a major subset of GPCRs that couple to a number of different $G\alpha$ subunits (reviewed in [303]). Genetic studies indicate that some $G\alpha$ proteins are required for lymphocyte development and function. In fact, $G\alpha_{12}$ -deficient mice display a number of lymphocyte defects including decreased numbers of mature CD4 and CD8 SP thymocytes, defective lymphocyte homing and impaired T cell trafficking processes [304]. $G\alpha_{15}$ is a hemopoietically expressed $G\alpha$ subunit [305]. Expression of $G\alpha_{16}$, the human equivalent of the mouse $G\alpha_{15}$, is up and down-regulated following T cell activation and disruption of this regulation impairs activation-induced responses [306]. Interestingly, $G\alpha_{15}$ -deficient mice do not display any defects in hemopoiesis or inflammatory responses, despite discrete signaling defects in some of these cells [307]. Although these studies implicate several $G\alpha$ subunits in lymphocyte development, the role of others is much less obvious.

Although both $G\alpha_{12}$ and $G\alpha_{13}$ are expressed in most tissues including thymus and spleen, evidence implicating these particular $G\alpha$ subunits in lymphocyte signaling is limited [308]. Characterization of these $G\alpha$ subunits in non-lymphoid cell systems, however indicate that they are capable of influencing Rho signaling pathways. The influence of Rho proteins in lymphocyte development is well established. Therefore by implication, $G\alpha_{12}$ and $G\alpha_{13}$ are potential mediators of lymphocyte signaling. Interestingly $G\alpha_{12}$ -deficient mice do not display any obvious developmental defects whereas $G\alpha_{13}$ -deficient mice die by embryonic day 9.5 [309] [181].

Several lines of evidence from studies with potential $G\alpha_{12}$ and $G\alpha_{13}$ regulators suggest roles for these $G\alpha$ subunits in lymphocyte signaling. RGS1 is a GAP for $G\alpha_i$ and $G\alpha_q$ but not $G\alpha_{12}$, however it efficiently binds GDP- or GTP-bound $G\alpha_{12}$ [310]. Consequently RGS1, although not a GAP, is a $G\alpha_{12}$ effector antagonist. RGS1 expression in fact, impairs downstream signaling via activated $G\alpha_{12}$ in cell lines [310]. Thus RGS1 retains the ability to regulate signals from several $G\alpha$ subunits. RGS1 expression also impairs B lymphocyte migratory responses to a number of chemokines, including stromal-derived factor-1 (SDF1) [310] [311]. Interestingly, germinal center B lymphocytes are refractory to SDF-1-triggered migratory responses and express high levels of RGS1 [310]. In addition, following

in vivo activation by antigen, B cells rapidly up-regulate expression of certain RGS proteins, including RGS1 [311]. RGS1-mediated antagonism of $G\alpha_{12}$ activity may contribute to the refractory migratory responses of B lymphocytes, indicating a role for $G\alpha_{12}$ in lymphocyte signaling. Together these results indicate that RGS proteins can profoundly affect the directed migration of lymphoid cells. Antigen receptor-mediated changes in RGS molecule expression may also be involved in the mechanism by which BCR signaling regulates B cell migration within lymphoid tissues.

Recent characterization of Lsc-deficient mice further implicates $G\alpha_{12/13}$ in lymphocyte function. Lsc may have a role in modulation of signals that emanate from GPCRs by down-regulating $G\alpha_{12}$ and $G\alpha_{13}$ via the RGS domain while it transmits signals to Rho through the GEF domain. In fact, Lsc is required for marginal zone B (MZB) cells, regulation of lymphocyte motility and immune responses [312]. Splenocytes from Lsc^{-/-} mice displayed reduced levels of actin polymerization via agonists implicated in activation of $G\alpha_{12}$ and $G\alpha_{13}$, including lysophosphatidic acid (LPA) and thromboxane A₂ (TXA₂) [312]. Lsc was also required for normal numbers of MZB cells and humoral responses to antigens and inhibition of basal T cell proliferation [312]. These findings identify Lsc as essential to immune responses and implicate $G\alpha_{12}$ and $G\alpha_{13}$ subunits in lymphocyte functions.

Additional evidence has also implicated $G\alpha_{13}$ in lymphocyte function. G2A is a hemopoietically expressed GPCR that transforms fibroblasts and is induced in B lymphocytes following exposure to genotoxic agents [313] [201]. In fibroblasts, G2A is coupled to $G\alpha_{13}$ and mediates stress fiber formation via RhoA activation [201] [235]. Thus, G2A is a potential regulator of a number of cellular processes including proliferation and integration of extracellular signals with cytoskeletal reorganization. Ablation of G2A in mice has revealed that this receptor plays a critical role in controlling peripheral lymphocyte homeostasis. In particular, G2A^{-/-} mice develop a novel, late-onset autoimmune syndrome [314]. Notably, T cells from these mice display enhanced proliferative responses to TCR cross-linking and co-stimulation even though lymphocytes appear to develop normally [314]. Although it is not known whether G2A couples to $G\alpha_{13}$ in lymphocytes, it is possible. Thus the molecular basis to these responses still needs to be addressed. Nevertheless, analyses involving Lsc- and G2A-deficient lymphocytes reveal important potential roles for $G\alpha_{12}$ and $G\alpha_{13}$ in lymphocyte signaling.

1.6 Rationale for studies and thesis objectives

Upon activation, both $G\alpha_{12/13}$ and Rho family GTPases coordinate a wide range of cellular process primarily through reorganization of the actin cytoskeleton and regulation of gene transcription. The pathways they coordinate are diverse and deregulated GTPase activity can adversely affect cells. Enhancing GTPase activity via increased expression, GEF expression or mutational activation, in fact often leads to cellular transformation or cancer. Balanced regulation of GTPase activity is thus critical in coordinating normal cellular responses. Understanding the regulation of these GTPases is therefore important in understanding both their normal cellular functions and the mechanisms by which they mediate abnormal cellular responses.

This thesis addresses the contributions of GTPase regulators in cell growth control and transformation in fibroblasts as well as in lymphocyte development and function. Several GTPase regulators were utilized in the studies described in this thesis: the Rho-activating G protein-coupled receptors (GPCR), G2A and PAR-1, and the Dbl family guanine nucleotide exchange factors (GEFs), Lsc and Dbs. Interestingly, all of these GTPase regulators were isolated in library screens for cDNAs that cause transformation of fibroblasts.

It was clear that G2A and PAR-1 were sufficient to cause transformation of fibroblasts. Thus, I was interested in addressing the following general question regarding these two GPCRs: **How do the GPCRs G2A and PAR-1 mediate cellular transformation?** Initial experiments revealed that transformed fibroblasts expressing G2A or PAR-1 displayed cell morphological characteristics similar to those induced by Rho and distinct from those induced by Ras. It appeared that Rho activation was important in G2A and PAR-1-mediated transformation. Was Rho required for transformation via these GPCRs? If so, how did signals from these GPCRs lead to Rho activation? Results presented in this thesis suggest a model by which G2A and PAR-1 transform fibroblasts.

The Dbl GEFs, Dbs and Lsc also transform fibroblasts via Rho family GTPase activation. Interestingly, these GEFs are normally expressed in lymphoid tissues. Also, in addition to the DH and PH domains, Lsc contains an RGS domain that acts as a GAP toward certain $G\alpha$ proteins. Many studies have illustrated the importance of both Rho and $G\alpha$ family GTPases in lymphocyte biology. Since Lsc and Dbs are regulators of GTPase activity, I was interested in addressing the following question: **What are the roles of Dbs and Lsc in lymphocyte development?** Results in this thesis suggest a role for Dbs

in lymphocyte biology. Several approaches were utilized to address the above questions and experiments can be divided into those involving cell lines (Chapter 3) and those involving lymphocytes from transgenic mice (Chapters 4 and 5). Listed below are my four thesis objectives.

1.6.1 To determine the role of RhoGEFs in G2A-, PAR-1 and G $\alpha_{12/13}$ -induced transformation

1.6.2 To determine the contribution of G α_{12} or G α_{13} to G2A- or PAR-1-induced transformation

1.6.3 To determine the effect of LscRGS, a GAP for G $\alpha_{12/13}$, on lymphocyte development

1.6.4 To determine the effect of expression of activated Dbs on lymphocyte development

Chapter 2 – Materials and Methods

2.1 Molecular biology

2.1.1 Vector construction/modification

Dominant negative GTPase retroviral constructs were generated by sub-cloning cDNAs encoding RhoA (19N), Rac1 (17N) and H-Ras (17N) into CTV83 vectors. Wild type $G\alpha_{12}$ and $G\alpha_{13}$ retroviral constructs were generated by sub-cloning the Mlu-Sall fragment of TL18-54-c1 and TL37-2-c1, respectively, into CTV81 vectors. Constitutively activated mutants of $G\alpha_{12}$ (pCDNA3- $G\alpha_{12}$ (Q229L)) and $G\alpha_{13}$ (pCDNA3- $G\alpha_{13}$ (Q226L)) were provided by Henry Bourne [315]. These $G\alpha$ mutants were also sub-cloned into CTV81 retroviral vectors. G2A retroviral constructs were generated by sub-cloning TL37-5c1 into a CTV81 retroviral vector. The human PAR-1 cDNA was provided by Ellen Van Obberghen-Schilling (Centre de Biochimie, CNRS, France). Mouse PAR-1 retroviral constructs were generated by sub-cloning TL18-8-c6 into a CTV81 vector. PAR-1 retroviral constructs were generated by sub-cloning cDNAs encoding human and mouse PAR-1 into CTV81.

2.1.2 cDNA modification/epitope tagging

2.1.2.1 $G\alpha$ dominant negative (*DomN*) peptide construction

Short carboxyl-terminal oligonucleotides were generated by sub-cloning the AflIII-DraI fragment of wild-type $G\alpha_{12}$ and the Eco47III-BglI fragment of the wild-type $G\alpha_{13}$ cDNA into CTV83 vectors.

2.1.2.2 *LscRGS* construction

The RGS domain of Lsc consisted of the first 283 amino acids of Lsc (MGEVAGGAAP...NRGEPSAPDC) fused to a hemagglutinin (HA; PYPYDVPDYASG) epitope-tag at the carboxyl terminus expressed within a CTV83 retroviral vector. Lsc [128] is identical to human p115 RhoGEF in 122 of 126 amino acids in the RGS domain, with all four differences being structurally conserved.

2.1.2.3 *Dbp-HA* construction

The amino- and carboxyl-terminally truncated form of Dbp (MSEPRQGRTS...AEGLWYVRDL) (Dbp-D13) containing 482 amino acids of the full-length cDNA was generated as described in [132]. HA-tagged Dbp-D13 (Dbp-D13/HA) was generated by sub-cloning the ApaI-DraI fragment into an HA fusion

CTV retroviral vector. Subsequent sequencing analysis revealed a truncating single base-pair deletion at base pair 3290 of Dbs-D13/HA between the PH and SH3 domains of the Dbs transgenic construct. This deletion created a premature stop codon at amino acid 386 of Dbs-D13/HA prior to the SH3 domain and HA tag within the LIT2 transgenic construct and the error was discovered after the three Dbs transgenic lines were generated.

2.1.3 Transgenic fragment preparation

Approximately 20 µg of transgenic vector was digested with NotI for one-half hour. The NotI digested gel fragment was resolved and cut out following agarose gel electrophoresis in the absence of ultraviolet illumination. The NotI DNA fragment was further purified without the use of gloves using filter-sterilized solutions prepared with milliQ water. Agarose gel purified DNA was further purified using a QIAEX II gel purification kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. After eluting the DNA, it was further purified using Z-spin columns (Gelman Sciences). Purified DNA was ethanol precipitated, dried and suspended in low TE (5mM Tris; 0.1 mM EDTA; pH 7.45). The transgenic DNA fragments were quantified by agarose gel electrophoresis (final concentration: 6-8 ng/µl). DNA was stored at -70° Celsius.

2.1.4 RNA isolation and Northern blotting

Total RNA was isolated from thymus, spleen and bone marrow of 8 week old non-transgenic or Dbs transgenic mice, separated on 5% formaldehyde agarose gels (4 µg total RNA per lane) and transferred to Hybond N⁺ nylon membrane (Amersham). Hybridization and high stringency washing were performed as described [316] using an Apal fragment of the full-length mouse Dbs cDNA [132] and labeled with ³²P-ATP by extension of random sequence primers. Molecular weight sizes were estimated from pictures of ethidium bromide-stained gels containing RNA ladder (Gibco BRL).

2.1.5 DNA isolation and analysis

2.1.5.1 Genomic DNA isolation

Mouse genomic DNA was isolated from tail clippings. Tails (approximately 1.5 mm long) were incubated at 50° in 200µl tail buffer (0.5 % SDS, 10 mM EDTA, 10 mM Tris (pH 8.0), 50 mM NaCl) for 2 hours. After digestion, an equal volume of phenol was added and samples were mixed. Polymerase chain reactions were performed on 1 µl of the aqueous layer following centrifugation.

2.1.5.2 Mouse genotype analysis by PCR

The genotype of LscRGS and Dbs transgenic mice were identified by PCR of tail genomic DNA using primers for HSA (5'-ACCAAACATCTGTTGCACCGTTTCC-3' and 5'-ACCTGTGCCCCAATTTCAAGTGAGAG-3') and human growth hormone (hGH) (5'-TTCAAGCAGACCTACAGGAAGTTCG-3' and 5'-GCACTGGAGTGGCAACTTCCAG-3'). The Rag2 genotype of mice was identified by PCR of tail genomic DNA using PCR primers for Rag 2 (5'-CCAGCTGATAACCCACCCACAA-3' and 5'-GTATAGTCGAGGGAAAAGCAT-3') and neomycin phosphotransferase (5-TGGGATCGGCCATTGAACAAG-3' and 5'-CACGGGTAGCCAACGCTA TGT-3'). Standard PCR conditions were used (denaturation: 95°/40 seconds; annealing: 60°/40 seconds; extension: 72°/60 seconds; 30 cycles) and products were visualized by agarose gel electrophoresis.

2.1.5.3 DNA sequencing

The LscRGS/HA construct was sequenced with an ABI 377 DNA Sequencer using FS Taq Dye Terminators (Nucleic Acid-Protein Service Unit, University of British Columbia).

2.2 Tissue culture

2.2.1 Cell lines

BOSC23 packaging cells were used for production of retroviruses [317]. NIH3T3 fibroblasts used for the transformation assays were obtained from American Type Culture Collection (Manassas, Virginia).

2.2.2 Cell culture

NIH3T3 fibroblasts were maintained at low density in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. BOSC-23 retroviral packaging cells were cultured in DMEM supplemented with 10% fetal bovine serum.

2.2.3 BOSC-23 transfection

Retroviral plasmids containing the constructs described in 2.1.1 were converted into retroviruses using the BOSC-23 ecotropic virus packaging cell line. For each retroviral plasmid construct, 5 μ g was transfected as described previously [128].

2.2.4 Retroviral transduction of NIH 3T3 fibroblasts

As described previously [127], NIH3T3 cells were infected with retroviruses produced by transfection of the BOSC-23 packaging cell line with CTV81 or CTV83 retroviral vectors carrying resistance for puromycin (Sigma) or G418 (Geneticin, Invitrogen), respectively. The infection efficiencies of the cells expressing control or construct viruses were identical as determined by the number of drug-resistant colonies following low volume provirus infection. Approximately 7×10^5 NIH 3T3 fibroblasts were plated into each wells of a 6-well plate the night before retroviral infection. NIH 3T3 fibroblasts were transduced with 1 ml of proviral supernatant. Following infection, cells were selected in growth medium supplemented with 900 mg/ml G418 for 7 days. Vector-, RhoA (19N)-, Rac1 (17N)-, H-Ras (17N)-, LscRGS-, G α_{12} (DomN) and G α_{13} (DomN)-expressing NIH 3T3 fibroblasts were established by pooling multiple G418-resistant colonies. Low density NIH 3T3 fibroblasts expressing vector, RhoA (19N), Rac1 (17N), H-Ras (17N), LscRGS, G α_{12} (DomN) or G α_{13} (DomN) constructs were transduced with vector, G2A, mPAR-1, hPAR-1, G α_{12} (Q229L), G α_{13} (Q226L), Lsc, Vav, H-Ras (12V), G α_{12} , G α_{13} or TL37-5c1 proviruses. Transduced cells were maintained in DMEM supplemented with 10% calf serum and grown for up to 3 weeks.

2.3 Protein analysis

2.3.1 Immunoblotting

Cells from thymus, spleen and bone marrow of non-transgenic and LscRGS littermates were isolated by disaggregation through a wire mesh. Cells were then washed with 4°C PBS and solubilized by boiling for 2 min with SDS-sample buffer. For protein total cell lysates 10^6 cell equivalents were separated by SDS-PAGE on a 10% gel, transferred to an Immobilon-P nitrocellulose transfer membrane (Millipore), and immunoblotted using the mouse monoclonal anti-HA antibody HA.11 (Covance/Berkeley Antibody Co., Richmond CA), in PBS 0.05% Tween-20 with 5% BSA. Bound antibodies were detected with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) and enhanced chemiluminescence according to the manufacturer's instructions (Amersham Life Science, Buckinghamshire, England).

2.4 Flow cytometry

2.4.1 Antibodies and flow cytometry

The following mAb conjugates were from BD Pharmingen (San Diego, CA): biotinylated monoclonal antibodies to mouse CD25, anti-CD3 ϵ -FITC, anti-CD44-PE, anti-CD25-PE, anti-CD4-PE, anti-CD8 α -APC, anti-CD24-FITC (HSA) and anti-CD69-FITC. Following mAb staining, cells were suspended in Hank's Balanced Salt Solution/2% fetal bovine serum/(HBSS/2%FBS), analyzed using a FACScalibur flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA).

2.4.2 Intracellular LscRGS expression by flow cytometry

Thymocytes from transgenic and non-transgenic littermate mice from the TL41 and DL5 lines were isolated, rinsed once with HBSS/2% FBS and incubated with anti-CD4-APC and anti-CD8-PE for 30 minutes. Thymocytes were then rinsed twice with HBSS/2%FBS and fixed with cytofix/cytoperm solution (BD Pharmingen) for 20 minutes. Fixed thymocytes were rinsed twice with 1X cytoperm/wash solution and incubated with FITC-conjugated anti-HA antibody (Santa Cruz Biotechnology) for 30 minutes. Stained thymocytes were rinsed twice with 1X cytoperm/wash, suspended in HBSS/2% FBS and analyzed by flow cytometry.

2.5 Cell analysis

2.5.1 Fibroblast transformation assays

For focus formation assays, NIH 3T3 fibroblasts expressing vector, RhoA (19N), Rac1 (17N), H-Ras (17N), LscRGS, G α_{12} (DomN) or G α_{13} (DomN) constructs and either another control vector or cDNA (G2A, mPAR-1, hPAR-1, G α_{12} (Q229L), G α_{13} (Q226L), Lsc, Vav, H-Ras (12V), G α_{12} , G α_{13} or TL37-5c1) were grown to confluence in DMEM supplemented with 10% calf serum. At 10 days post-confluence cells were stained with 0.1% methylene blue and foci were quantified by visual inspection. Two weeks after confluence, pictures were taken of live cells. Graphed results in figures are from at least three experiments and, unless otherwise stated, error bars represent standard error of the mean from multiple experiments.

2.5.2 *In vivo* BrdU incorporation

Dbs transgenic or non-transgenic littermate mice were injected intra-peritoneally with 2 x 1 mg injections of BrdU, given 2 hours apart. 18 hours later, thymocytes were isolated, incubated with antibodies directed against anti-CD4 (CyChrome) and anti-CD8 α (PE), fixed, permeabilized and incubated with an FITC-conjugated antibody directed against BrdU (Becton Dickinson) according to manufacturers instructions (BrdU flow kit; Becton Dickinson, San Jose, CA).

2.5.3 Thymocyte proliferation assays

For analysis of *in vitro* proliferation using carboxyfluorescein succinimidyl ester (CFSE), thymuses were isolated, forced through a cell strainer and thymocytes were suspended in RPMI-1640 containing 10% FBS (HyClone, Logan, Utah), 2 mM glutamine, 25mM HEPES, 50 μ M 2-ME, 100 U/ml penicillin, and 100 μ g/ml streptomycin (RPMI 1640 media). Thymocytes (10^8 per ml) were labeled with 2.5 μ M CFSE in PBS (Molecular Probes, Eugene, Oregon) for 10 min at 37 °C. Cells were washed 3x with ice cold RPMI-1640 media, re-counted and incubated in 12-well plates at 2×10^6 cells/ml of RPMI-1640 media. For anti-CD3 ϵ stimulation, wells were previously incubated overnight at 4°C with 50 μ g/ml rabbit anti-hamster IgG (Sigma Chemical Corp., St. Louis, MO) in PBS, washed with PBS, followed by affinity purified anti-CD3 ϵ [318], at the indicated concentration in PBS for 2 hours at 37 °C. After 48 and 72 hours, cells were incubated with antibodies directed against CD4 (CyChrome) and anti-CD8 α (APC) and analyzed by flow cytometry.

2.5.4 Cell cycle status analysis

Thymocytes were isolated, rinsed in HBSS/2%FBS and fixed with ice-cold 70% ethanol and left at 4°C overnight. Fixed thymocytes were rinsed twice with HBSS and incubated with propidium iodide (5µg/ml) at 4°C for at least 4 hours and analyzed by flow cytometry.

2.6 Mice

All mice were housed under specific pathogen-free conditions in the Joint Animal Facility (JAF) at the British Columbia Cancer Research Centre (BCCRC). C57BL/6 mice were from Taconic Farms (Germantown, NY). C3H mice were from Jackson Laboratory (Bar Harbor, Maine).

2.6.1 Generation of transgenic mice

2.6.1.1 DNA microinjection

Rewa Grewal performed the transgenic DNA microinjections. Founder mice were obtained by microinjecting the NotI fragment from LIT2-Dbs-D13/HA, 1017D-LscRGS/HA or TXV-20 LscRGS/HA into (C57BL/6 x C3H) x C57BL/6 F₁ embryos, and bred to C57BL/6 mice to establish lines. Transgene positive mice were identified by PCR amplification of tail genomic DNA using the human growth hormone primers.

2.6.1.2 LscRGS transgenic mice

The transgenic expression vector p1017D was derived from the Lck proximal promoter vector p1017 [319] by removing multiple introns of the human growth hormone gene (retaining the growth hormone polyadenylation signal, bp 1361-2154, Genbank accession number M13438), and inserting the second intron of the rabbit β -globin gene (bp 551-1242 Genbank number V00878) downstream of the Lck promoter sequence.

The LscRGS-p1017D transgene expression construct was generated by adding a HA (hemagglutinin) epitope tag to the 3' end of a C-terminally truncated mouse Lsc cDNA as described in [201]. The encoded protein has the C-terminal sequence APDC**PYPYDVPDYASG** (hemagglutinin in bold). The LscRGS/HA cDNA was inserted into p1017D at the MluI and SalI sites between the β -globin intron and the human growth hormone polyadenylation signal. LscRGS/HA founder mice were obtained by

microinjecting the NotI fragment from p1017D-LscRGS/HA into (C57BL/6 x C3H) x C57BL/6 F₁ zygotes, and bred to C57BL/6 mice to establish lines. Transgene positive mice were identified by PCR of tail DNA using the human growth hormone primers.

2.6.1.3 Dbs transgenic mice

The LIT2-Dbs (LIT2-Dbs-D13/HA) transgenic expression vector was generated by sub-cloning the HA-tagged Dbs construct into the LIT2 vector. The transgenic expression vector LIT2 was generated as described [253]. Briefly, a 270-bp fragment from the HSA cDNA encompassing the complete open reading frame was placed under the transcriptional control of the *TCR V β* promoter and *immunoglobulin μ* enhancer. Sequences upstream of the *lck* gene proximal promoter were also included, as this region was suspected to function as a locus-activating region. The *human growth hormone (hGH)* gene with a frame shift mutation in the coding region was inserted 3' of the Dbs-D13/HA cDNA to provide introns that appear to enhance transgene expression. The transgene was injected into (C57BL/6 X C3H) X C57BL/6 F₁ hybrid zygotes and bred to C57BL/6 mice to establish lines.

2.6.2 Systemic anti-CD3 administration

Dbs transgenic and non-transgenic littermates of varying ages were injected intra-peritoneally with 20 μ g of anti-CD3 (clone 145-2C11; Southern Biotechnology Associates, Birmingham, AL) [320]. 48 hours later thymocytes were isolated, incubated with antibodies directed against CD4 and CD8 and analyzed by flow cytometry.

2.6.3 Mouse breeding

2.6.3.1 Rag 2^{-/-} crosses

Dbs transgenic mice (51DB line) were bred with Rag 2^{-/-} mice [321] (Taconic Farms, Germantown, New York). Dbs transgenic/Rag 2^{+/-} progeny were then crossed to Rag 2^{-/-} mice for analysis of offspring between 6 and 12 weeks of age.

2.6.3.2 TCR HY crosses

Dbs transgenic mice (51DB line) were bred with Rag2^{-/-}/TCR HY mice (Taconic farms, Germantown, New York). Rag2^{-/-}/TCR HY (H-2D^b/H-2D^d heterozygotes) offspring with and without the Dbs transgene were analyzed between 6 and 10 weeks of age.

Chapter 3 – Role of RhoGEFs and $G_{\alpha_{12/13}}$ in GPCR-induced transformation

3.1 Introduction and rationale

G2A and PAR-1 are two GPCRs isolated in cDNA library screens for clones that cause oncogenic transformation of NIH 3T3 fibroblasts (refer to section 1.3.3.3 for details). Increased expression of G2A or PAR-1 in NIH 3T3 fibroblasts induced a full range of phenotypes characteristic of oncogenic transformation, including focus formation, loss of contact inhibition, anchorage-independent survival and proliferation and reduced dependence on serum [201] [202]. Foci of transformed cells induced by G2A or PAR-1 were similar to those induced by activated RhoA and distinct from those induced by Ras. Rho-transformed cells are distinct from those of Ras-transformed cells in that they are non-refractory, while Ras transformed cells are elongated (spindle-like) and refractile in appearance when examined by microscopy. Results presented in this chapter provide a model of G2A- and PAR-1-mediated cellular transformation. The results presented in this chapter are published [201] [202].

3.2 Results

3.2.1 Role of RhoGEFs in GPCR-, $G_{\alpha_{12}}$ - and $G_{\alpha_{13}}$ -mediated transformation

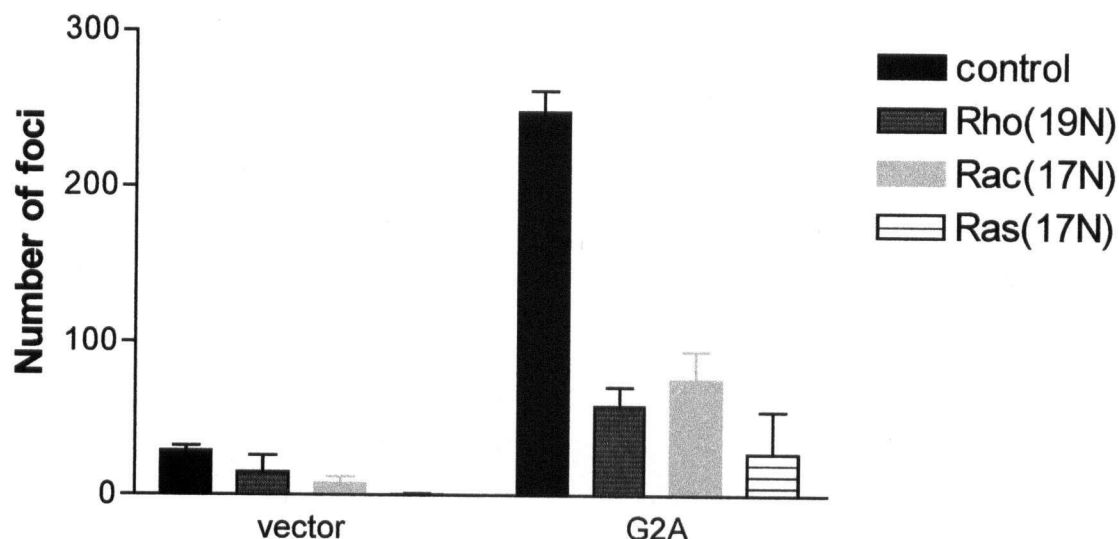
Rho activation, via RhoGEFs downstream of G2A, PAR-1 and $G_{\alpha_{12/13}}$ may be sufficient to cause transformation of NIH3T3 fibroblasts. Dominant negative GTPases are unable to bind GTP and thus remain in their inactive GDP-bound state. These mutant GTPases act as dominant negatives by failing to interact with their effectors and sequestering GEFs. Expressing dominant negative GTPases with oncogenes is useful in determining the role of GTPase signaling pathways in cellular transformation.

To examine whether Rho activation is required for transformation via these GPCRs and heterotrimeric G proteins, dominant negative forms of RhoA (19N), Rac1 (17N) and H-Ras (17N) were co-expressed with either the GPCRs or G_{α} proteins and the effects on transformation were determined. Expression of dominant negative RhoA (19N) suppressed transformation by G2A and both mouse and human PAR-1 (**Figure 3.1**). RhoA (19N) strongly suppressed the transforming ability of constitutively active, GTPase-deficient $G_{\alpha_{13}}$ ($G_{\alpha_{13}}$ (Q226L)) and partially suppressed transformation via constitutively active $G_{\alpha_{12}}$ ($G_{\alpha_{12}}$ (Q229L)) (**Figure 3.2**). Transformation via G2A, PAR-1, $G_{\alpha_{12}}$ and $G_{\alpha_{13}}$, thus occurred via signals mediated by RhoA. Dominant negative RhoA (19N) expression however did not affect

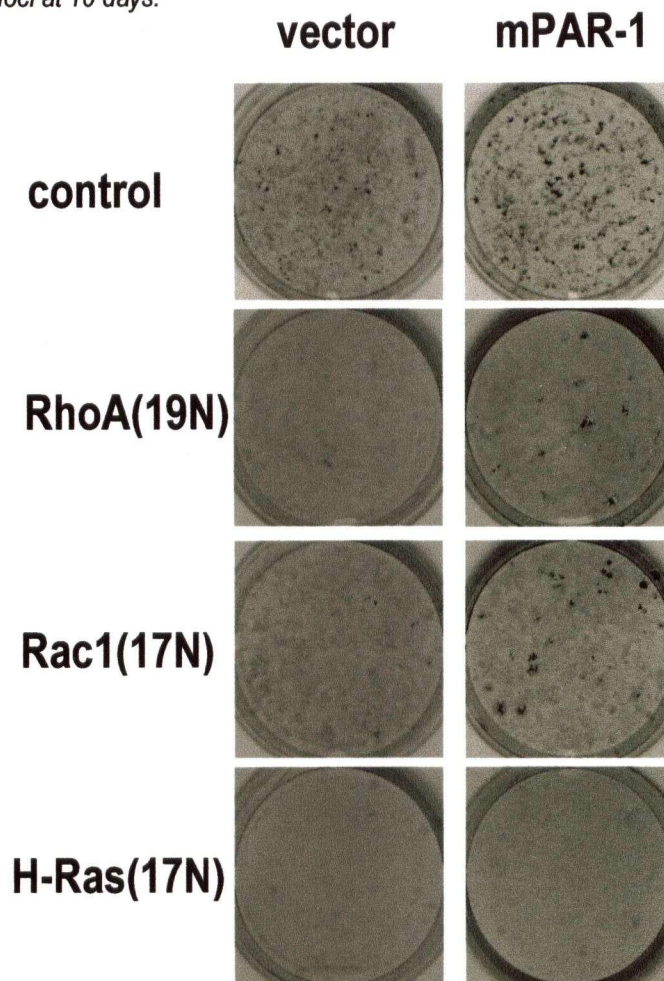
transformation via the Rho exchange factor Lsc, the Rac exchange factor Vav and constitutively active H-Ras (V12) (**Figure 3.2**). Although the cellular consequences of dominant negative RhoA (19N) are thought to result from a failure to bind GTP and sequestration of Rho-specific GEFs, over-expression of Lsc overrode the effect of RhoA (19N). Lsc strongly promotes GDP dissociation from RhoA, but not Rac1, Cdc42 or Ras [126]. The failure of this dominant negative GTPase to suppress Lsc-mediated transformation may reflect differences in the relative expression levels of the RhoA (19N) versus Lsc. G2A-, PAR-1-, $G\alpha_{12}$ (Q229L)-, $G\alpha_{13}$ (Q226L)-, Lsc-, and Vav-induced transformation were also suppressed by dominant negative forms of Rac1 (17N) and H-Ras (17N) (**Figure 3.2**), thus implicating Rac1 and H-Ras in transformation via these oncogenes. Transformation via constitutively active H-Ras (V12), however was unaffected by co-expression of dominant negative Rac1 (17N) (**Figure 3.2**), suggesting that H-Ras (V12)-induced transformation does not involve downstream signals mediated by Rac1. Together these results implicate RhoA, Rac1 and H-Ras in transformation via G2A, PAR-1, $G\alpha_{12}$ and $G\alpha_{13}$.

Figure 3.1. Co-expression of dominant negative Rho, Rac and Ras with G2A and PAR-1. Low density NIH 3T3 fibroblasts expressing RhoA (19N), Rac1 (17N) and H-Ras (17N) were infected with a CTV vector encoding a drug resistance gene, or a CTV vector encoding G2A or PAR-1 as well as a drug resistance gene. Transduced cells were selected, grown to confluence and foci were scored 10 days later.

a) Co-expression of dominant negative Rho, Rac and Ras with G2A. Bars represent the number of foci at 10 days in control, Rho (19N)-, Rac1 (17N)- or H-Ras (17N)-expressing NIH 3T3 fibroblasts following infection with either control vector or G2A.



b) Co-expression of dominant negative Rho, Rac and Ras with PAR-1.
 Pictures represent foci at 10 days.



c) Co-expression of dominant negative Rho, Rac and Ras with PAR-1. Bars represent the number of foci at 10 days in control, Rho (19N)-, Rac1 (17N)- or H-Ras (17N)-expressing NIH 3T3 fibroblasts following infection with control vector, mouse PAR-1 (mPAR-1) or human PAR-1 (hPAR-1).

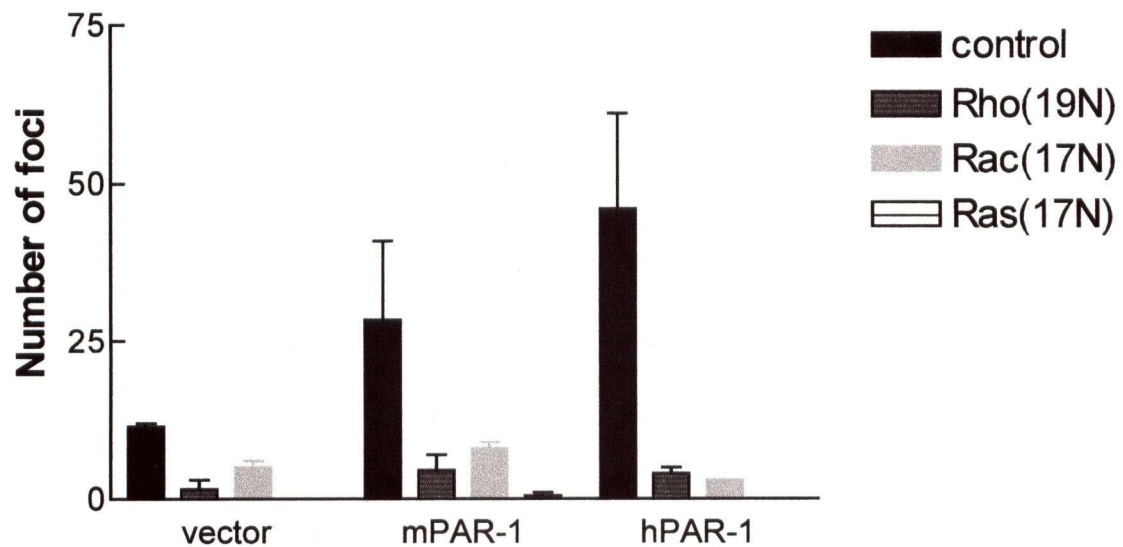
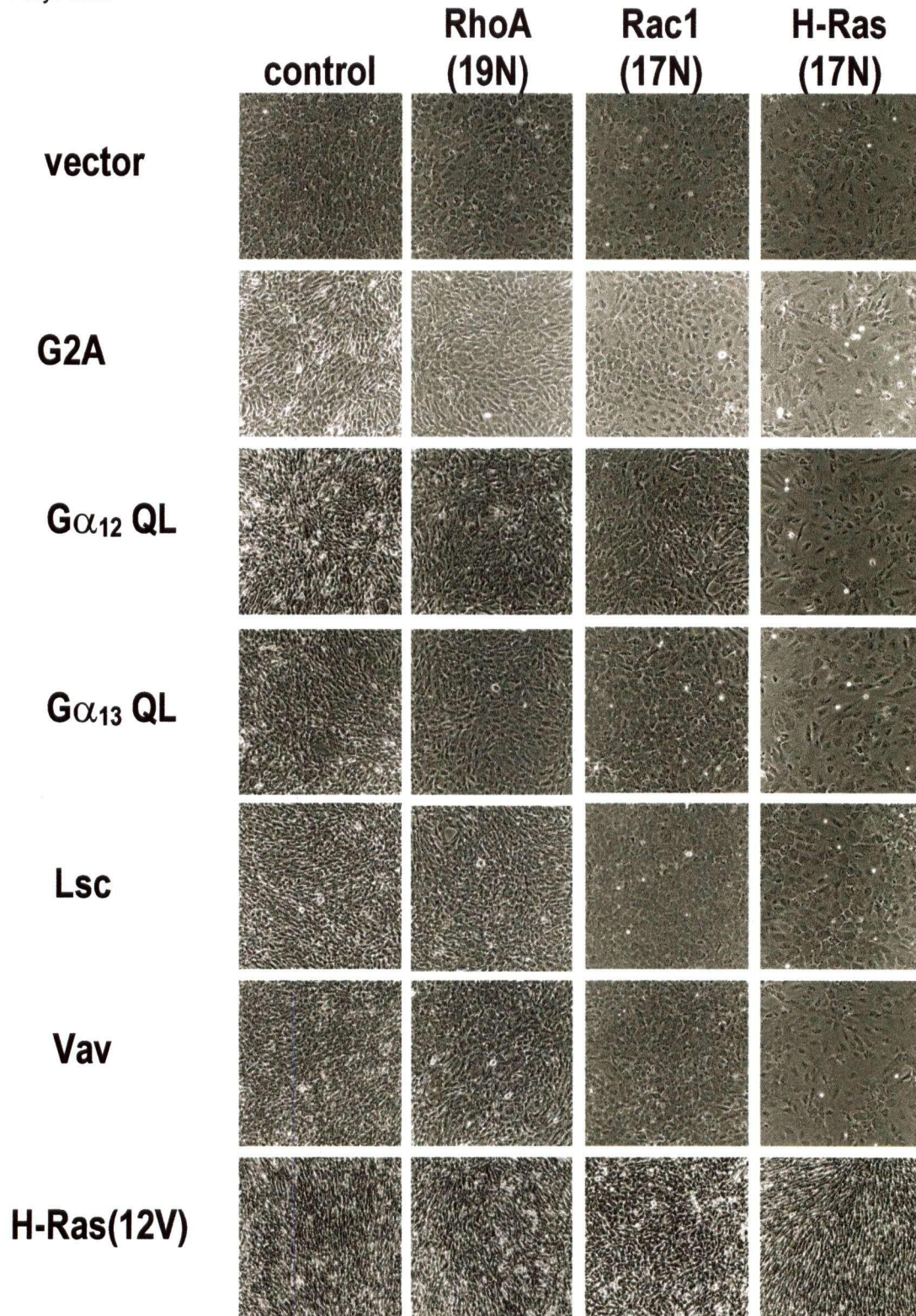


Figure 3.2. Co-expression of dominant negative Rho, Rac and Ras with $G\alpha_{12}$, $G\alpha_{13}$, GEFs and Ras. Low density NIH 3T3 fibroblasts expressing a control vector (control), RhoA (19N), Rac1 (17N) and H-Ras (17N) were infected with a CTV vector encoding a drug resistance gene (vector), or a CTV vector encoding G2A, GTPase-deficient $G\alpha_{12}$ (Q229L) or $G\alpha_{13}$ (Q226L), the Dbl proteins Lsc and Vav or activated H-Ras (12V), as well as a drug resistance gene. Transduced cells were selected, grown to confluence and pictures were taken 14 days later.



The above results reveal that inhibition of RhoA activation suppresses transformation via G2A, PAR-1, $G\alpha_{12}$ and $G\alpha_{13}$; however, Rac1 (17N) and H-Ras (17N) also suppressed transformation via G2A, PAR-1, Lsc (a Rho-specific exchange factor) as well as activated forms of $G\alpha_{12}$ and $G\alpha_{13}$. There are two possible explanations for these results. The first is that these Rho activators may also activate Rac and Ras under these experimental conditions, although the cytoskeletal structures of G2A- PAR-1-, $G\alpha_{12}$ - and $G\alpha_{13}$ -expressing cells indicate that this is not the case [201] [202]. The second explanation is that Rho activation is necessary for transformation via these GPCRs and $G\alpha$ subunits, but transformation cannot be attained if the basal levels of Ras or Rac activation provided by growth factors in serum are suppressed. Although co-expression experiments do not enable discrimination between these two possibilities, limited evidence suggests that this may be the case. The spontaneous rate of transformation was strongly suppressed by expression of Rac1 (17N) and H-Ras (19N) and saturation densities were variably affected by the three different dominant negative GTPases. This indicates that a variety of growth characteristics can be differentially perturbed by co-expression of these dominant negatives (**Figures 3.1 and 3.2**). Although the above results illustrate the limitations associated with the use of dominant negatives to define signaling pathways contributing to a complex event such as cellular transformation, they do reveal that transformation signals induced by G2A and PAR-1 are sensitive to inhibition of these small GTPases. Results from other experiments including the ones outlined below suggest that Rho is the primary signaling mediator of transformation via these GPCRs and $G\alpha$ proteins, implying that active Rac and Ras are required for, but not directly involved in transformation by these proteins.

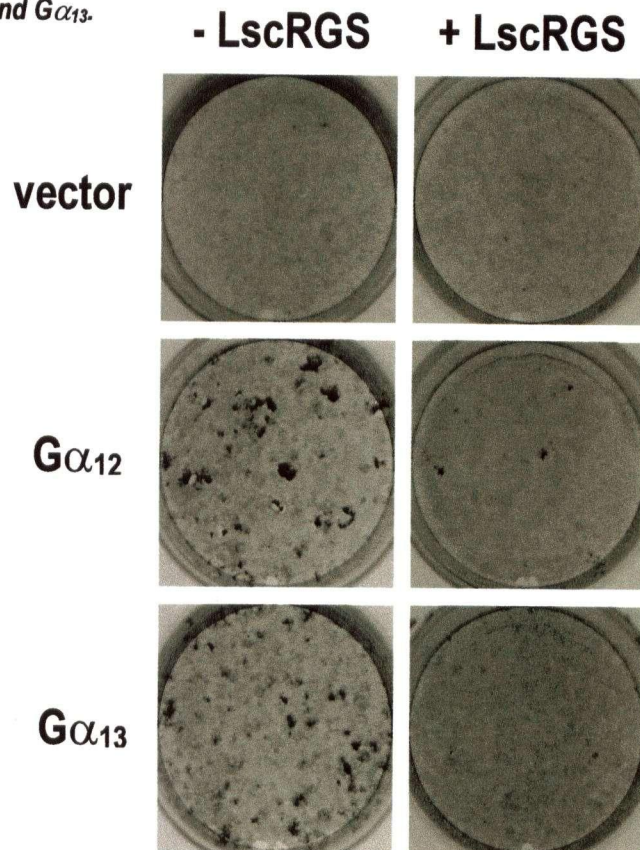
3.2.2 LscRGS is an inhibitor of $G\alpha_{12}$ - and $G\alpha_{13}$ -induced transformation

Several studies revealed that activation of $G\alpha_{12}$ or $G\alpha_{13}$ leads to activation of Rho (refer to section 1.3.3). The RGS domain of Lsc/p115RhoGEF (LscRGS) specifically abrogates signaling through $G\alpha_{12}$ and $G\alpha_{13}$ by stimulating the intrinsic GTPase activities of these $G\alpha$ subunits. The RGS domain has no effect on $G\alpha_q$, $G\alpha_{i1}$, $G\alpha_z$ or $G\alpha_s$ [131]. Since LscRGS inhibits the enzymatic activity of $G\alpha_{12}$ and $G\alpha_{13}$, I determined if LscRGS blocked transformation via $G\alpha_{12}$ or $G\alpha_{13}$. LscRGS was co-expressed with wild type and constitutively active mutants of $G\alpha_{12}$ or $G\alpha_{13}$ and the effects on transformation were determined (**Figure 3.3**). Expression of LscRGS suppressed the ability of wild type $G\alpha_{12}$ and $G\alpha_{13}$ to transform fibroblasts but had no effect on transformation induced by expression of GTPase-deficient

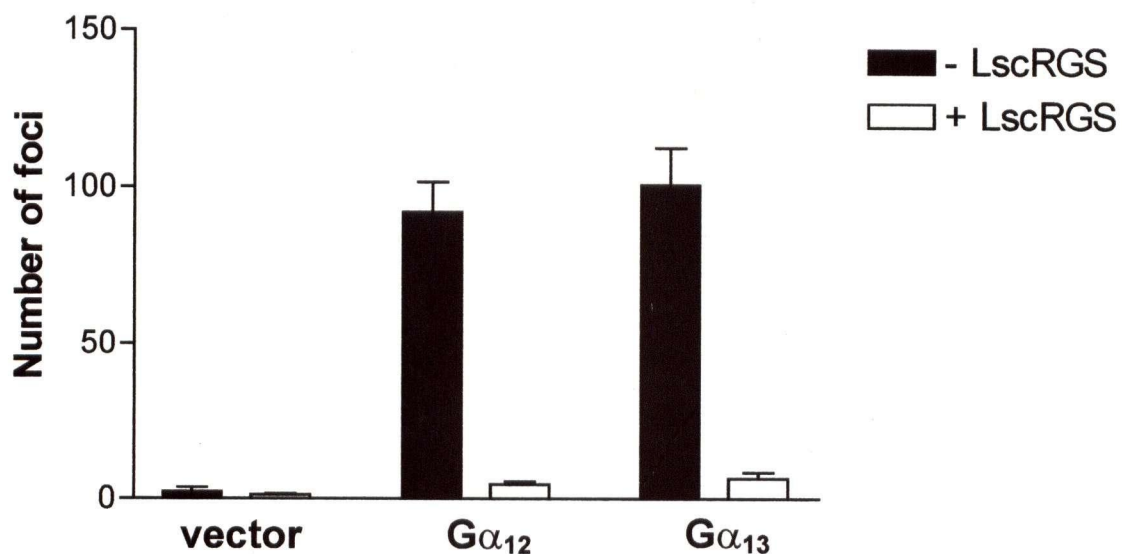
Figure 3.3. Co-expression of LscRGS.

a) Co-expression of LscRGS with $G\alpha_{12}$ and $G\alpha_{13}$.

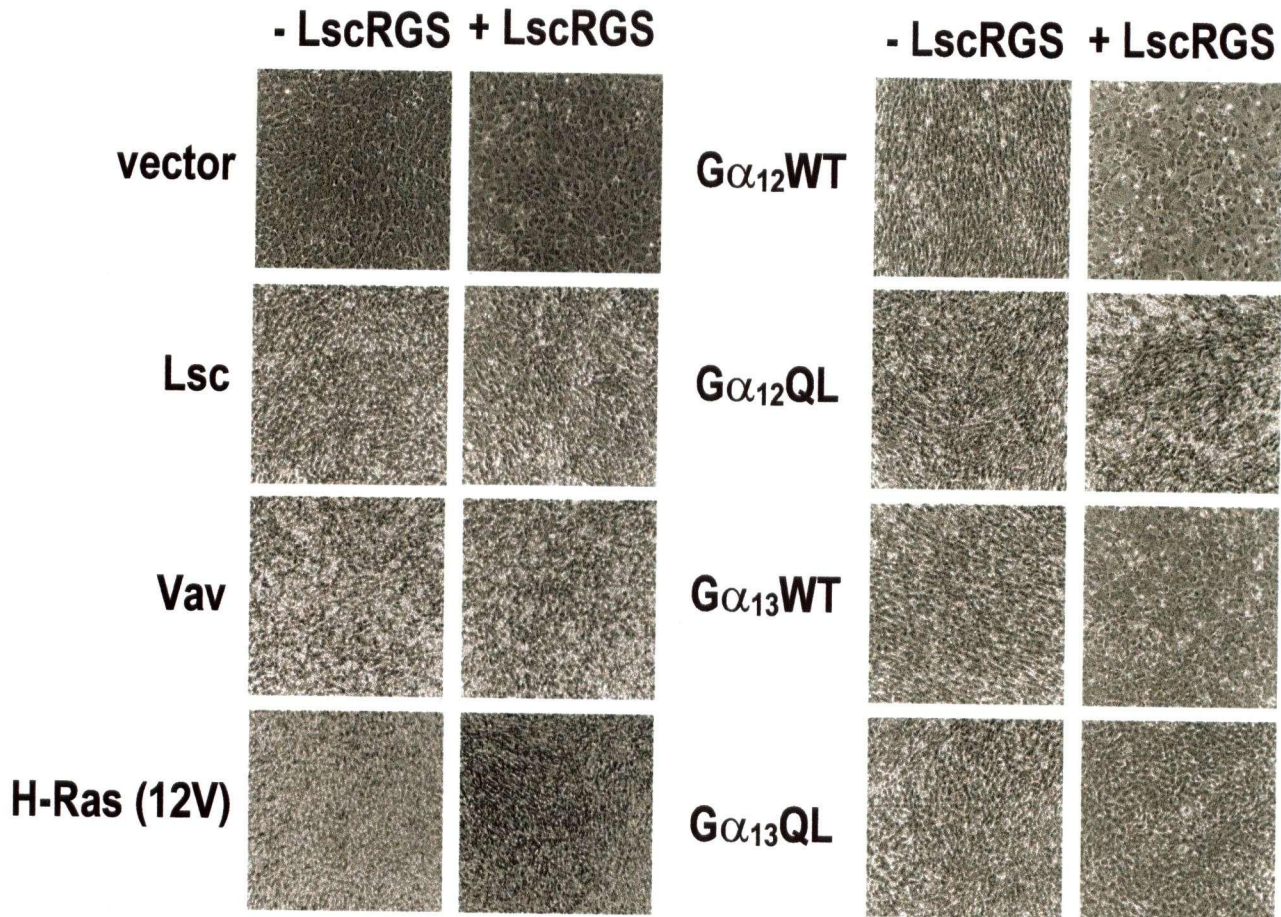
Control (-LscRGS) or LscRGS-expressing (+LscRGS) NIH 3T3 fibroblasts were infected at low density with a CTV vector encoding a drug resistance gene (vector), or a CTV vector encoding $G\alpha_{12}$ or $G\alpha_{13}$ as well as a drug resistance gene. Transduced cells were selected, grown to confluence and foci were scored 10 days later.



b) Co-expression of LscRGS with $G\alpha_{12}$ and $G\alpha_{13}$. Bars represent the number of foci at 10 days in either control (-LscRGS) or LscRGS-expressing (+LscRGS) NIH 3T3 fibroblast monolayers following infection with control vector, $G\alpha_{12}$ - or $G\alpha_{13}$ -expressing vectors.



c) Co-expression of LscRGS with $G\alpha_{12}$, $G\alpha_{13}$, GEFs and Ras. Low density NIH 3T3 fibroblasts expressing either a control vector (-LscRGS) or LscRGS (+LscRGS) were infected with a CTV vector encoding a drug resistance gene (vector), or a CTV vector encoding wild-type (WT) $G\alpha_{12}$ and $G\alpha_{13}$, GTPase-defective $G\alpha_{12}$ (Q229L) and $G\alpha_{13}$ (Q226L), the Dbl proteins Lsc and Vav or activated H-Ras (12V) as well as a drug resistance gene. Transduced cells were selected, grown to confluence and pictures were taken 14 days later.



forms of $G\alpha_{12}$ and $G\alpha_{13}$ suggesting that inhibition of transformation occurred via the GAP activity of LscRGS (**Figure 3.3**). The effects of LscRGS expression on transformation via other groups of $G\alpha$ subunits were not determined, since expression of wild type forms of $G\alpha_s$, $G\alpha_i$ or $G\alpha_q$ does not lead to fibroblast transformation. LscRGS expression also had no effect on transformation induced via Lsc, Vav, activated Ras and Raf (**Figure 3.3**). These results demonstrate the high specificity of LscRGS for suppressing only $G\alpha_{12}$ or $G\alpha_{13}$ -mediated transformation.

The fibroblast experiments outlined above involving co-expression of the RGS domain of Lsc with transforming cDNAs, in addition to the biochemical studies involving this domain [131], suggest that LscRGS may be a useful inhibitor of $G\alpha_{12}$ and $G\alpha_{13}$. Moreover, co-expression of LscRGS with transforming GPCRs may enable characterization of the $G\alpha$ subunits required for transformation.

3.2.3 Role of $G\alpha_{12}$ and $G\alpha_{13}$ in G2A and PAR-1-mediated transformation

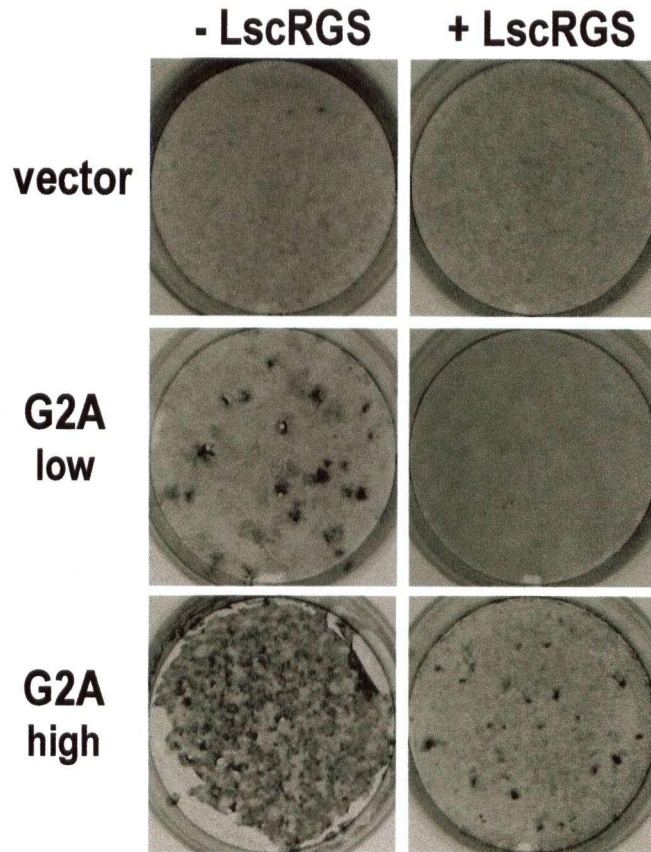
The GPCRs, G2A and PAR-1 as well as $G\alpha_q$, $G\alpha_{12}$ and $G\alpha_{13}$ can activate Rho family GTPases. Cytoskeletal and cell morphological changes induced by G2A and PAR-1 are identical to those of Rho. What proteins mediate signals generated via G2A and PAR-1 upstream of Rho? NIH 3T3 fibroblast transformation via these GPCRs may involve $G\alpha_{12/13}$ activation downstream of the receptor and upstream of Rho.

To test the hypothesis that $G\alpha_{12}$ or $G\alpha_{13}$ mediates transformation signals from the GPCRs, G2A and PAR-1, LscRGS was co-expressed with each GPCR and the effect on transformation was determined. Expression of LscRGS suppressed the abilities of G2A, mouse PAR-1 and human PAR-1 to transform fibroblasts (**Figure 3.4**). Thus $G\alpha_{12}$ or $G\alpha_{13}$ upstream of Rho mediates G2A- and PAR-1-induced NIH 3T3 fibroblast transformation.

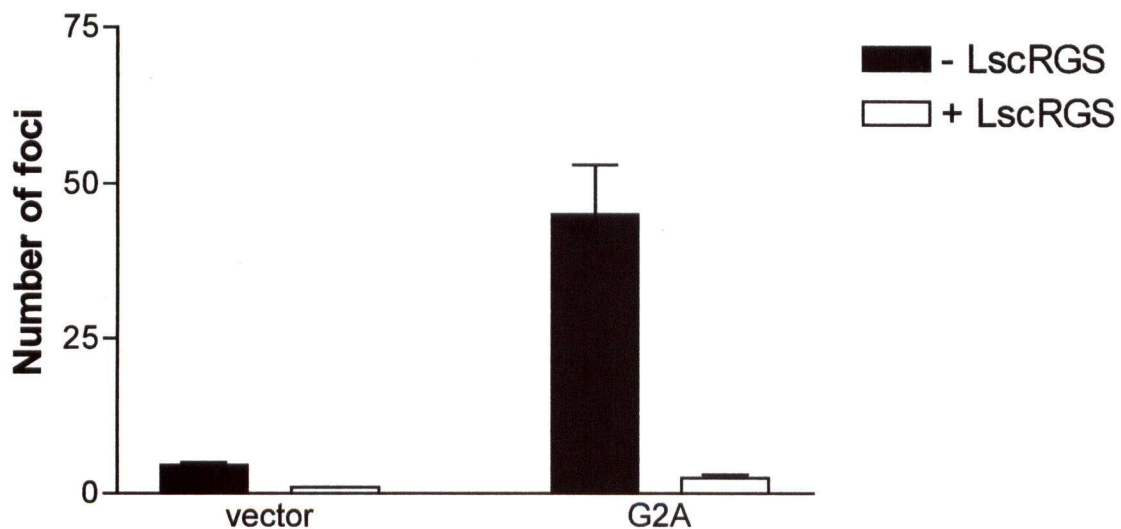
Co-expression of LscRGS with either G2A or PAR-1 resulted in complete reversion of the G2A-expressing cells to a non-transformed morphology. These results implicate $G\alpha_{12}$ and/or $G\alpha_{13}$ in transformation via these two GPCRs.

Figure 3.4. Co-expression of LscRGS with G2A and PAR-1.

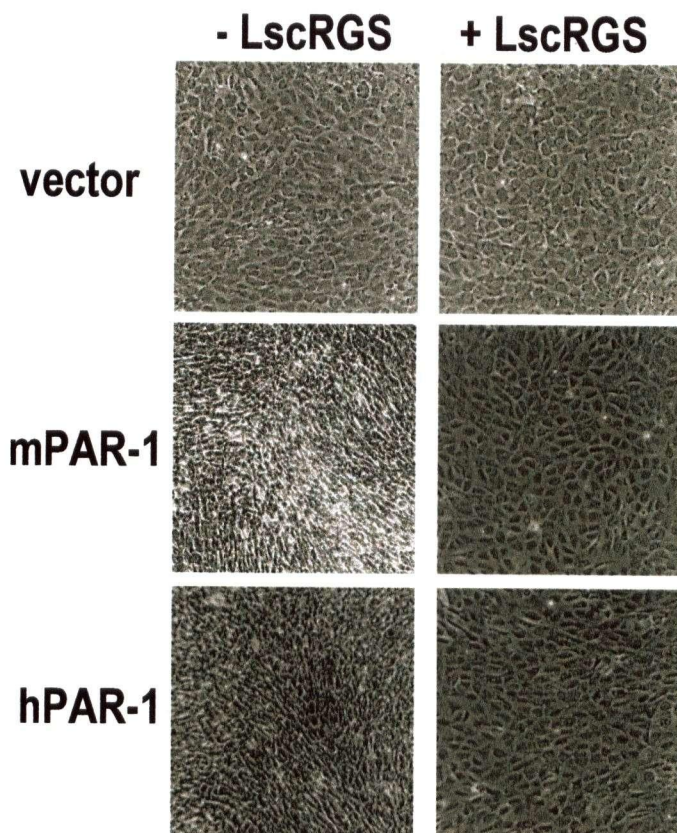
a) Co-expression of LscRGS with G2A. Control (-LscRGS) or LscRGS-expressing (+LscRGS) NIH 3T3 fibroblasts were infected at low density with either a CTV vector encoding a drug resistance gene (vector) or a CTV vector encoding G2A (both low and high supernatant volumes). Transduced cells were selected, grown to confluence and foci were scored 10 days later.



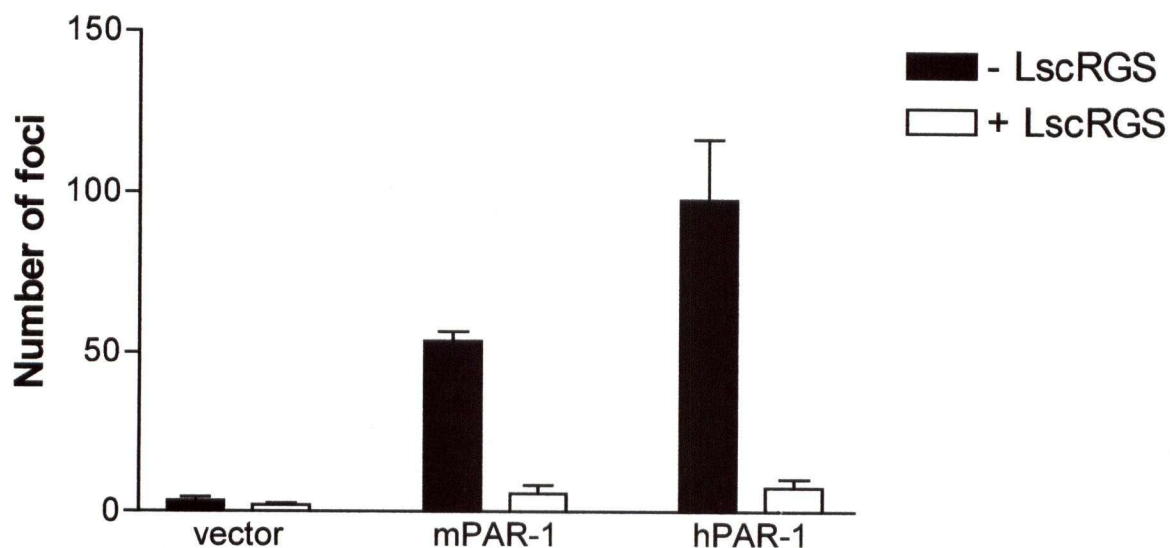
b) Co-expression of LscRGS with G2A. Bars represent the number of foci at 10 days in either control (-LscRGS) or LscRGS-expressing (+LscRGS) NIH 3T3 fibroblast monolayers following infection with either control vector or G2A-expressing vectors (low volume supernatant).



c) Co-expression of LscRGS with PAR-1. Vector expressing (-LscRGS) or LscRGS-expressing (+LscRGS) NIH 3T3 fibroblasts were infected at low density with a CTV vector encoding a drug resistance gene (vector) or a CTV vector encoding mouse (mPAR-1) or human PAR-1 (hPAR-1). Transduced cells were selected, grown to confluence and pictures were taken 14 days later.



d) Co-expression of LscRGS with PAR-1. Bars represent the number of foci at 10 days in vector-expressing (-LscRGS) or LscRGS-expressing (+LscRGS) NIH 3T3 fibroblast monolayers following infection with either control vector or PAR-1-expressing vectors.



3.2.4 Distinguishing between $G\alpha_{12}$ and $G\alpha_{13}$ involvement in G2A-mediated transformation

The above results indicate that G2A-mediated transformation involves $G\alpha_{12}$, $G\alpha_{13}$ or both. The carboxyl-terminal regions of $G\alpha$ subunits represent important sites of interaction between heterotrimeric G proteins and their receptors. Synthetic peptides derived from portions of $G\alpha$ subunit carboxyl-termini inhibit receptor coupling by stabilizing the high affinity (agonist occupied) state of the receptor [189] [322] [190]. These synthetic peptides act as dominant negatives by competing with $G\alpha$ proteins for receptor binding sites and expression of these peptides may enable class-specific inhibition of GPCR-mediated transformation.

Early characterization of the transforming properties of $G\alpha_{12}$, indicated that expression of wild type $G\alpha_{12}$ was sufficient to transform NIH 3T3 fibroblasts, however transformation was dependent upon the presence of serum in the growth medium since serum starvation inhibited the transforming ability of wild type $G\alpha_{12}$ [217]. Interestingly, expression of a GTPase-deficient, constitutively active mutant of $G\alpha_{12}$ ($G\alpha_{12}$ (Q229L)) abrogated the requirement for serum-dependency for transformation [323]. Although wild type and mutationally activated $G\alpha_{12}$ expression were sufficient to transform cells, transformation via wild-type $G\alpha_{12}$ was serum and presumably receptor agonist-dependent. Moreover, the wild type GTPase may have required agonist-occupied high affinity receptors to stimulate exchange of GDP for GTP and activate the $G\alpha$ subunit. I predicted, based on these studies that transformation via wild type $G\alpha_{12}$ and $G\alpha_{13}$ would be suppressed, if $G\alpha$ dominant negative peptides were co-expressed and competing for high affinity receptors. The peptide presence would limit the number of wild type $G\alpha$ subunits from binding high affinity receptors and would result in impaired $G\alpha$ activation. I also predicted that, GTPase-deficient $G\alpha_{12}$ -mediated transformation would be unaffected by expression of $G\alpha_{12}$ dominant negative peptide, due to a lack of dependence on serum and receptor-mediated activation.

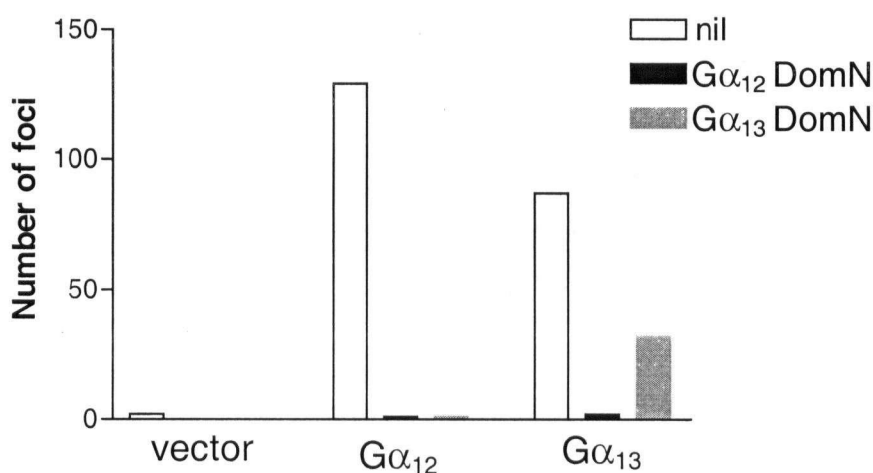
In order to determine whether $G\alpha_{12}$ - or $G\alpha_{13}$ -mediated transformation was inhibited via inhibition of $G\alpha_{12}$ or $G\alpha_{13}$ receptor coupling, peptides derived from the carboxyl-termini of these two $G\alpha$ subunits were generated, co-expressed with $G\alpha$ subunits and the effect on transformation was determined. Expression of $G\alpha_{12}$ dominant negative peptide suppressed transformation via both $G\alpha_{12}$ and $G\alpha_{13}$

(Figure 3.5). $G\alpha_{13}$ dominant negative peptide expression strongly suppressed $G\alpha_{12}$ -mediated transformation, but suppressed $G\alpha_{13}$ -mediated transformation to a lesser extent. Transformation via constitutively activated $G\alpha_{12}$ was unaffected by co-expression of $G\alpha_{12}$ or $G\alpha_{13}$ dominant negative peptides (Figure 3.5). Although $G\alpha_{12}$ dominant negative and $G\alpha_{13}$ dominant negative peptide expression were sufficient to impair transformation to varying extents via both $G\alpha_{12}$ and $G\alpha_{13}$ presumably through high affinity receptor binding, class-specific inhibition of transformation was not demonstrated. The usefulness of $G\alpha_{12}$ and $G\alpha_{13}$ dominant negative peptides as tools in determining the specific contribution of $G\alpha_{12}$ or $G\alpha_{13}$ to G2A-mediated transformation was therefore limited.

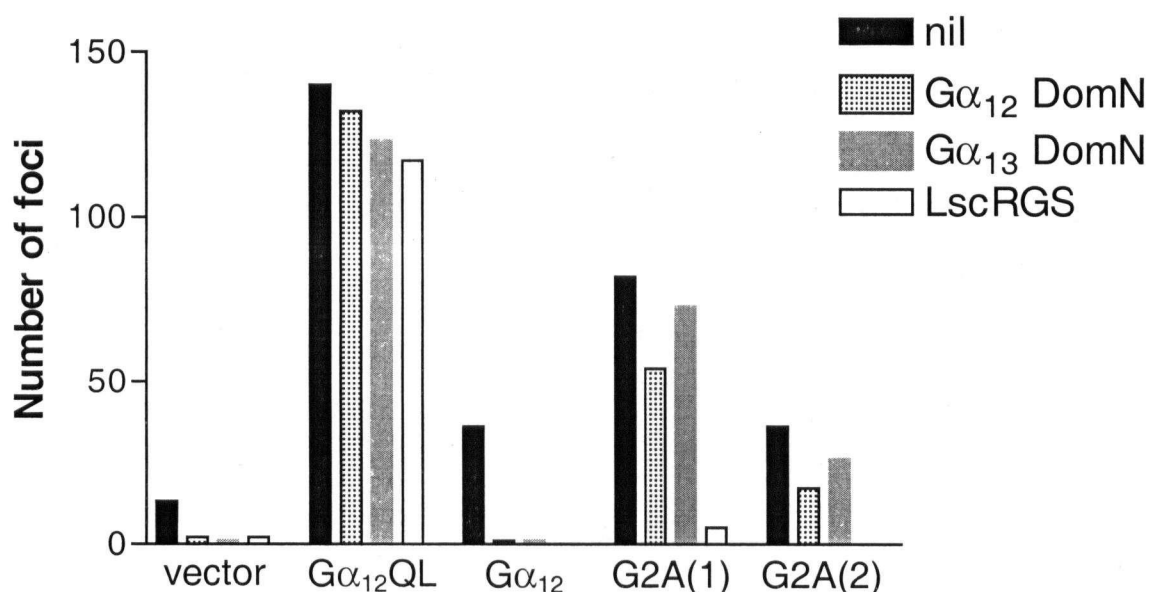
Despite these limitations, I determined whether G2A-mediated transformation was suppressed at all by expression of these dominant negative peptides by co-expressing them with G2A and determining the effect on transformation. Expression of $G\alpha_{12}$ dominant negative peptide suppressed transformation to a limited extent via two independent G2A clones (G2A and TL37-5c1) and $G\alpha_{13}$ dominant negative peptide expression had a very limited suppressive effect on transformation via either of these G2A clones (Figure 3.5). Thus, $G\alpha_{12}$ dominant negative peptide expression completely suppressed transformation via both $G\alpha_{12}$ and $G\alpha_{13}$, but had a limited effect on transformation via G2A, which is thought to signal through one or both of these $G\alpha$ subunits. There are a number of explanations for these results. LscRGS may suppress G2A-mediated transformation via inactivation of other $G\alpha$ subunits in addition to $G\alpha_{12/13}$. Consequently, G2A-transformation would be the result of activation of $G\alpha_{12/13}$ and another $G\alpha$ subunit whose cycling rate is unaffected by expression of $G\alpha_{12}$ dominant negative peptide expression. Although this scenario cannot be excluded, it is unlikely. Biochemical results indicate that LscRGS is a specific GAP for $G\alpha_{12}$ and $G\alpha_{13}$ and has no effect on the GTP hydrolysis rate by other $G\alpha$ subunits including, $G\alpha_q$, $G\alpha_{i1}$, $G\alpha_z$ or $G\alpha_s$ [131]. Also, wild type $G\alpha_{12}$ and $G\alpha_{13}$ are the only $G\alpha$ subunits that are capable of transforming cells [217] [324] and $G\alpha_s$, $G\alpha_{i2}$ and $G\alpha_q$ are either non-transforming or weakly transforming when mutated to the GTPase-deficient state and expressed in fibroblasts [325] [326] [212] [215] [216]. Also, the cell morphological and cytoskeletal structures induced by G2A expression in fibroblasts were equivalent to those induced by $G\alpha_{13}$ and expression of dominant negative RhoA suppressed transformation via G2A [201]. Thus if any other $G\alpha$ subunit in addition to $G\alpha_{12/13}$ was involved in G2A transformation, this contribution would be minor. Presumably the activity of a non- $G\alpha_{12/13}$ subunit would not result in the high number of foci induced by G2A in the presence of inhibitors of GPCR- $G\alpha$ interactions (Figure 3.5).

Figure 3.5. Co-expression of $G\alpha_{12}$ and $G\alpha_{13}$ carboxyl termini.

a) Co-expression of $G\alpha_{12}$ and $G\alpha_{13}$ carboxyl termini with $G\alpha_{12}$ and $G\alpha_{13}$. NIH 3T3 fibroblasts expressing either empty vector (nil) or carboxyl terminal peptide sequences derived from $G\alpha_{12}$ ($G\alpha_{12}DomN$) or $G\alpha_{13}$ ($G\alpha_{13}DomN$) were infected at low density with a CTV vector encoding a drug resistance gene (vector) or a CTV vector encoding $G\alpha_{12}$ or $G\alpha_{13}$ as well as a drug resistance gene. Transduced cells were selected, grown to confluence and scored for foci greater than 1mm 10 days later.



b) Co-expression of $G\alpha_{12}$ and $G\alpha_{13}$ carboxyl termini with $G\alpha_{12}$ and G2A. NIH 3T3 fibroblasts expressing empty vector (nil), LscRGS or carboxyl terminal peptide sequences derived from either $G\alpha_{12}$ ($G\alpha_{12}DomN$) or $G\alpha_{13}$ ($G\alpha_{13}DomN$) were infected at low density with a CTV vector encoding a drug resistance gene (vector) or a CTV vector encoding wild type $G\alpha_{12}$, GTPase-deficient $G\alpha_{12}$ (Q229L) or G2A (2 different vector types) as well as a drug resistance gene. Transduced cells were selected, grown to confluence and scored for foci greater than 1mm 10 days later.



Another explanation to the previous results highlights an inherent limitation to these experiments. Altering the amounts of different components (receptors versus G α subunits) involved in GPCR signaling pathways and observing the effects on transformation are useful only when neither of the components are limiting. Competition resulting from either increased numbers of GPCRs or G α subunits with introduced G α dominant negative peptides will be reflected in the numbers of foci generated. If receptors are a limiting component, which may be the case when over-expressing G α_{12} and G α_{13} with dominant negative peptides, then the result of competition for a limited number of receptors between G α peptides and subunits will be reflected in terms of the number of foci generated. On the other hand, if G α subunits are the limiting component, which may be the case when over-expressing G2A with G α dominant negative peptides, then the result of competition for an unlimited number of receptors will also be limited in terms of the number of foci generated. Consequently, the extent of transformation suppression would be less. Thus limiting the number of receptor binding sites via expression of G α dominant negative peptides and determining the extent of transformation suppression is dependent upon whether receptors or G α subunits are a limiting factor. This may explain why G α_{12} dominant negative peptide suppressed both G α_{12} and G α_{13} -mediated transformation but had a limited effect on transformation via G2A (Figure 3.5).

3.3 Discussion

The results described above show that G2A and PAR-1 expression leads to NIH 3T3 fibroblast transformation downstream of Rho and that transformation via these two GPCRs requires activation of either G α_{12} or G α_{13} . These results and others were published in two papers characterizing transformation via G2A [201] and PAR-1 [202] and have enabled us to propose models of G2A- and PAR-1-mediated transformation.

3.3.1 Model of transformation via G2A

The co-expression studies outlined above involving dominant negative GTPases and the RGS domain of Lsc revealed that G2A transforms fibroblasts via Rho downstream of G α_{12} or G α_{13} . The complete inhibition by RhoA (19N) of the abilities of G2A and G α_{13} to induce NIH 3T3 fibroblast transformation as well as experiments by collaborators showing that the equivalent alterations induced in the

cytoskeleton of porcine aortic endothelial (PAE) cells by G2A and $G\alpha_{13}$ expression, indicate that G2A may be activating $G\alpha_{13}$ but not $G\alpha_{12}$ in both these cell types. More recent genetic studies have revealed that G2A-mediated cytoskeletal changes are dependent upon $G\alpha_{13}$ but not $G\alpha_{12}$ [235]. The ability of $G\alpha_{13}$ to activate Rho on its own suggests a linear relationship of activation from G2A to $G\alpha_{13}$ to Rho. The simplest mechanism of Rho activation involves direct $G\alpha_{13}$ coupling to G2A, activation of a Dbl family member by $G\alpha_{13}$ upon activation and consequent Rho activation by the Dbl GEF. Several Dbl proteins have recently been described as being activated by $G\alpha_{12}$ family subunits and include Lsc, LARG, PDZ-RhoGEF and KIAA0380 (refer to section 1.2.7.6). Also, several of the components of the above-mentioned pathway are expressed in NIH 3T3 cells. Lsc in particular is a likely mediator of signals downstream of G2A since both of these proteins are expressed in the same lymphoid tissues and cell lines [128] [201]. Rho and $G\alpha_{13}$, the other components of this pathway, are expressed in all cell types [327] [328]. It remains to be determined if the $G\alpha_{13}$ -Lsc pathway is involved in or required for transformation via G2A or any other oncogenic GPCR.

It is not known whether the ability of G2A to activate $G\alpha_{13}$ is dependent on ligand binding.

Lysophosphatidylcholine (LPC) has recently been described as a ligand for G2A [329]. The role of LPC in G2A mediated fibroblast transformation also remains to be determined.

The serum response factor (SRF) is important in signaling downstream of Rho and G2A-mediated signals downstream of Rho activation may involve the SRF. In fact, G2A expression caused Rho-dependent activation of the SRF in NIH 3T3 fibroblasts [201]. It is not known whether the SRF is required for transformation via G2A. Moreover, the role of other transcriptional regulators in G2A-mediated fibroblast transformation has not been determined.

3.3.2 Model of transformation via PAR-1

PAR-1 was also isolated in library screens for cDNAs that cause transformation of NIH 3T3 fibroblasts. Like G2A, PAR-1-expressing cells displayed characteristics similar to those expressing activated RhoA, but not H-Ras [202]. The results of co-expression studies described in this chapter involving dominant negative GTPases and the RGS domain of Lsc suggest that PAR-1 transforms fibroblasts via Rho downstream of $G\alpha_{12}$ or $G\alpha_{13}$. These results and others conducted by our collaborators showed that

PAR-1 has signaling and transforming activities similar to those induced by activated RhoA [202]. Moreover, PAR-1 like RhoA and G2A induced the formation of stress fibers and dominant negative RhoA, Rac1 and H-Ras blocked PAR-1 transformation. Thrombin stimulation has been shown to stimulate signaling pathways that promote the activation of RhoA and Ras [330] (reviewed in [331]). As with G2A, results from the dominant negative co-expression experiments alone do not exclude the possibility of direct involvement of Rac1 and H-Ras in PAR-1-mediated fibroblast transformation. Cell morphological characteristics of PAR-1-expressing cells however suggest that the primary mediator of signals downstream of receptor activation is RhoA. PAR-1-mediated transformation may not be attained if the basal levels of Ras or Rac activation provided by growth factors in serum are suppressed.

Unlike G2A, multiple G α subunits are involved in PAR-1-mediated transformation. Interestingly, PAR-1 was sensitive to both G $\alpha_{i/o}$ inhibition via pertussis toxin and down-regulation by the RGS domain of Lsc that is specific for G α_{12} family members. Thus a variety of growth promoting G α subunits may mediate PAR-1-induced fibroblast transformation.

PAR-1 mediated transformation is dependent upon ligand engagement and cleavage, however the relevant ligand required for transformation does not appear to be thrombin [202]. The protease involved in PAR-1-mediated fibroblast transformation remains to be determined.

Elevated expression of the thrombin receptor has been reported in some human cancers and associated with increased tumor cell invasiveness [332] [205] [206]. Although most studies of thrombin have concentrated on its normal physiological role in wound healing and blood clotting, our studies illustrate the broad oncogenic potential of this receptor when it is expressed in the inappropriate context.

3.3.3 Summary

The GPCRs G2A and PAR-1 transform fibroblasts through activation of Dbl GEFs, leading to activation of Rho GTPases. G2A, PAR-1 and Mas are all transforming GPCRs known to signal through Rho proteins. Interestingly, transformation via both G2A and PAR-1, upstream of the Dbl GEFs, involves members of the G α_{12} family of GTPases. In this respect G2A and PAR-1 differ in terms of their G α

specificities; G2A transformation, for example, appears to be mediated primarily via $G\alpha_{13}$, whereas transformation via PAR-1 involves $G\alpha_{12}$ and/or $G\alpha_{13}$ as well as $G\alpha_{40}$ members. G2A and PAR-1 are two examples of a small number of GPCRs that mediate signals via activation of both large and small molecular weight GTPases. Moreover, $G\alpha$ -mediated activation of GEFs and ultimately, the smaller molecular weight GTPases, appears to be an important mechanism of linking divergent GTPases downstream of GPCR activation. To date, $G\alpha_{12}$ and $G\alpha_{13}$ are the only known $G\alpha$ proteins that activate downstream signaling components via direct activation of GEFs. G2A and PAR-1 are two GPCRs that couple to $G\alpha_{12}$ and $G\alpha_{13}$ and activate Rho, presumably via Rho GEF activation, to transform fibroblasts. The extent to which $G\alpha$ protein-mediated activation of GEFs and, ultimately, GTPases, is involved in signaling via other GPCRs is not known.

Chapter 4 – Generation and analysis of LscRGS transgenic mice

4.1 Introduction and rationale

LscRGS specifically stimulates the hydrolysis of GTP to GDP by $G\alpha_{12}$ and $G\alpha_{13}$ but has no effect on hydrolysis rates by other $G\alpha$ subunit members including, $G\alpha_q$, $G\alpha_{i1}$, $G\alpha_z$ or $G\alpha_s$ (refer to section 1.2.7.4 for details) [131]. Moreover, expression of LscRGS suppresses both $G\alpha_{12}$ - and $G\alpha_{13}$ -mediated fibroblast transformation but has no effect on transformation induced by GTPase-deficient mutants of these two $G\alpha$ subunits [201] (section 3.2.2). Thus, LscRGS specifically inhibits the biochemical activity of $G\alpha_{12}$ and $G\alpha_{13}$ by acting as a GAP and has the potential to suppress the cellular responses of cells signaling through $G\alpha_{12}$ - and $G\alpha_{13}$ -coupled GPCRs.

$G\alpha_{12}$ and $G\alpha_{13}$ are expressed in many tissues, including those involved in lymphocyte development [308]. The effects of homozygous inactivation of $G\alpha_{12}$ and $G\alpha_{13}$ are variable and $G\alpha_{12}^{-/-}$ mice do not display any obvious defects [181], while $G\alpha_{13}^{-/-}$ embryos do not survive past embryonic day 9.5 due to defective embryonic angiogenesis [309]. G2A is a GPCR that activates Rho through activation of $G\alpha_{13}$ [201] [235] and is expressed in lymphoid cells [201] [313]. The roles of $G\alpha_{12}$ and $G\alpha_{13}$ in signal mediation from GPCRs in lymphocytes are unknown. LscRGS-mediated inhibition of $G\alpha_{12}$ and $G\alpha_{13}$ activity via GAP activation is one approach toward understanding the role of these $G\alpha$ proteins in lymphocyte development.

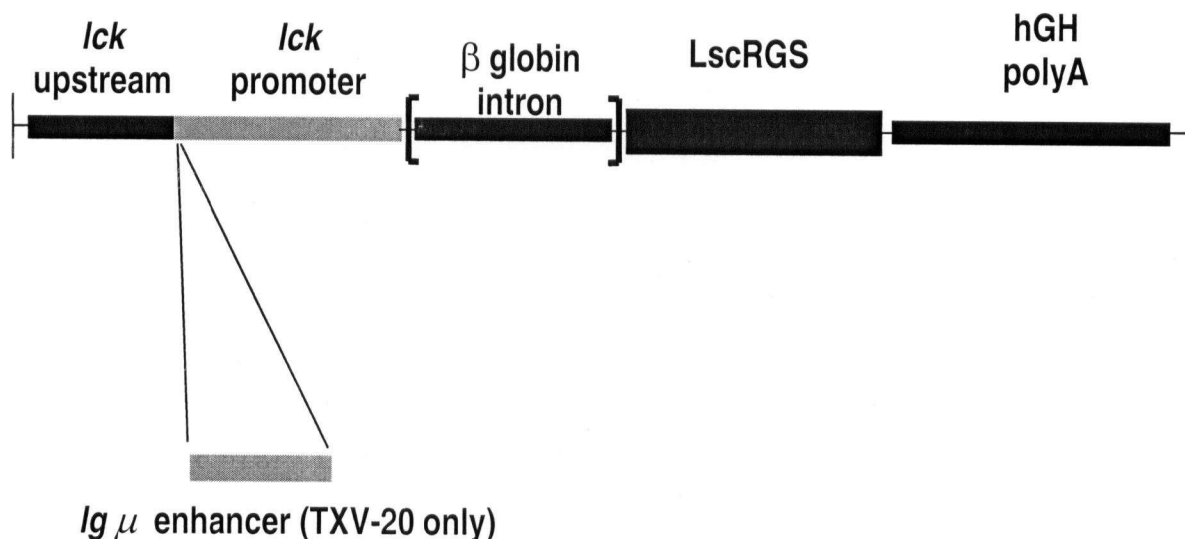
4.2 Results

4.2.1 LscRGS expression does not overtly influence lymphocyte development

To determine the effect of LscRGS expression on lymphocyte development, I generated transgenic mice expressing LscRGS within lymphoid tissues. Two vectors were utilized, TXV-20 and 1017D, both of which contained a cDNA encoding a carboxyl-terminally hemagglutinin (HA) epitope-tagged LscRGS transcribed from the *lck* proximal promoter (**Figure 4.1**). These vectors also contained downstream introns and a polyadenylation site derived from the human growth hormone gene. The difference between the two vectors was that TXV20 contained an additional immunoglobulin μ heavy chain enhancer region within the *lck* promoter, enabling both T and B lymphoid expression. Six LscRGS transgene-positive founders were obtained from blastocyst injections using both TXV-20 (lines

designated TL18, TL37, TL38 and TL41) and 1017D (lines designated DL5 and DL23) as expression vectors. The LscRGS transgene was expressed at variable levels in lymphoid tissues from all of the lines and the three lines, DL5, TL37 and TL41 were chosen for further study.

Figure 4.1. LscRGS transgenic constructs. Illustration of 1017D and TXV-20 vectors.



Immunoblot analyses with anti-HA antibody showed variable expression of transgenic LscRGS in thymus, spleen and bone marrow from the TL37, TL41 and DL5 lines (**Figure 4.2**). Expression of LscRGS in thymus was highest in the TL41 line, moderate in the DL5 line and lowest in the TL37 line. Spleen expression of LscRGS was high in all three lines, whereas LscRGS expression in the bone marrow was low in both TL37 and TL41 and moderate in DL5. To determine which lymphocyte populations from the DL5 and TL41 lines expressed the LscRGS transgene, lymphocytes were stained with antibodies directed against cell surface markers, fixed, permeabilized, incubated with FITC-conjugated anti-HA antibody and analyzed by flow cytometry. HA-tagged LscRGS was detected in each of the DN, DP, CD4 SP and CD8 SP thymocyte populations (**Figure 4.3**) as well as in CD4 and CD8 splenic T cells from both TL41 and DL5 lines (**Figure 4.4**), however expression was high in all four thymocyte populations from the TL41 line and CD8 T cells from the DL5 line (**Figures 4.3 and 4.4**). To determine which B lymphocyte populations from the transgenic mice express LscRGS, spleen and bone marrow cells were analyzed for intracellular expression of the HA-tagged protein. The DL5 line was derived from the 1017D vector and did not contain the immunoglobulin enhancer region and consequently, LscRGS was not detected in spleen or bone marrow B lymphocytes from this line

(Figures 4.5). LscRGS however was expressed within B cell populations from the TL41 line and was detected in B220⁺ and B220⁺/IgD⁺ populations from the spleen as well as pre-B (B220^{low}/IL-2R⁺), immature (B220^{high}/IgD^{low}) and mature B (B220^{high}/IgD⁺) lymphocytes from the bone marrow (Figures 4.5 and 4.6). The above results indicate that the LscRGS transgene was expressed in lymphocytes from transgenic mice.

Figure 4.2. Western blot analysis of LscRGS transgene expression. Cell lysates from thymus, spleen and bone marrow from non-transgenic and LscRGS transgenic mice from the TL37, TL41 and DL5 transgenic lines were incubated with an antibody directed against the epitope tag (hemagglutinin; HA) portion of the LscRGS transgene.

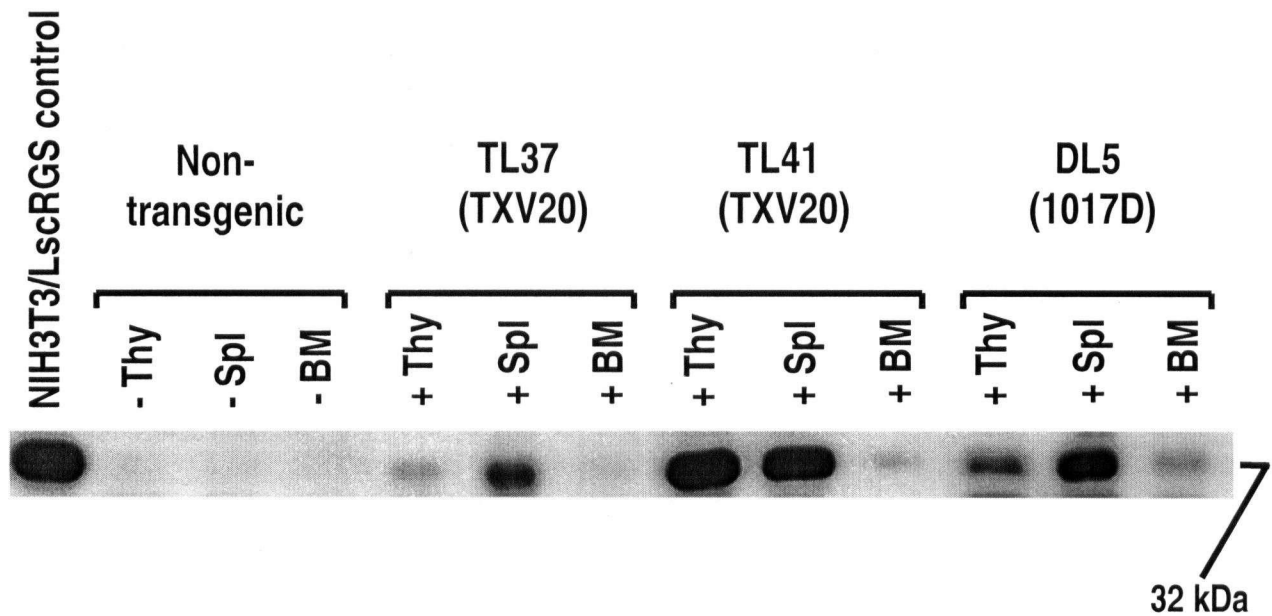


Figure 4.3. Thymocyte intracellular expression analysis. Thymocytes from non-transgenic and LscRGS transgenic littermates from the TL41 and DL5 transgenic lines were incubated with antibodies directed against CD4 and CD8, fixed, permeabilized, incubated with antibodies directed against the HA-tagged portion of the LscRGS transgenic protein and analyzed by flow cytometry. Histogram overlays represent the fluorescence intensities of non-transgenic (gray line) and LscRGS transgenic (black line) cells from each thymocyte subset (DN, DP and SP). Numbers indicate the mean fluorescence intensity of each histogram.

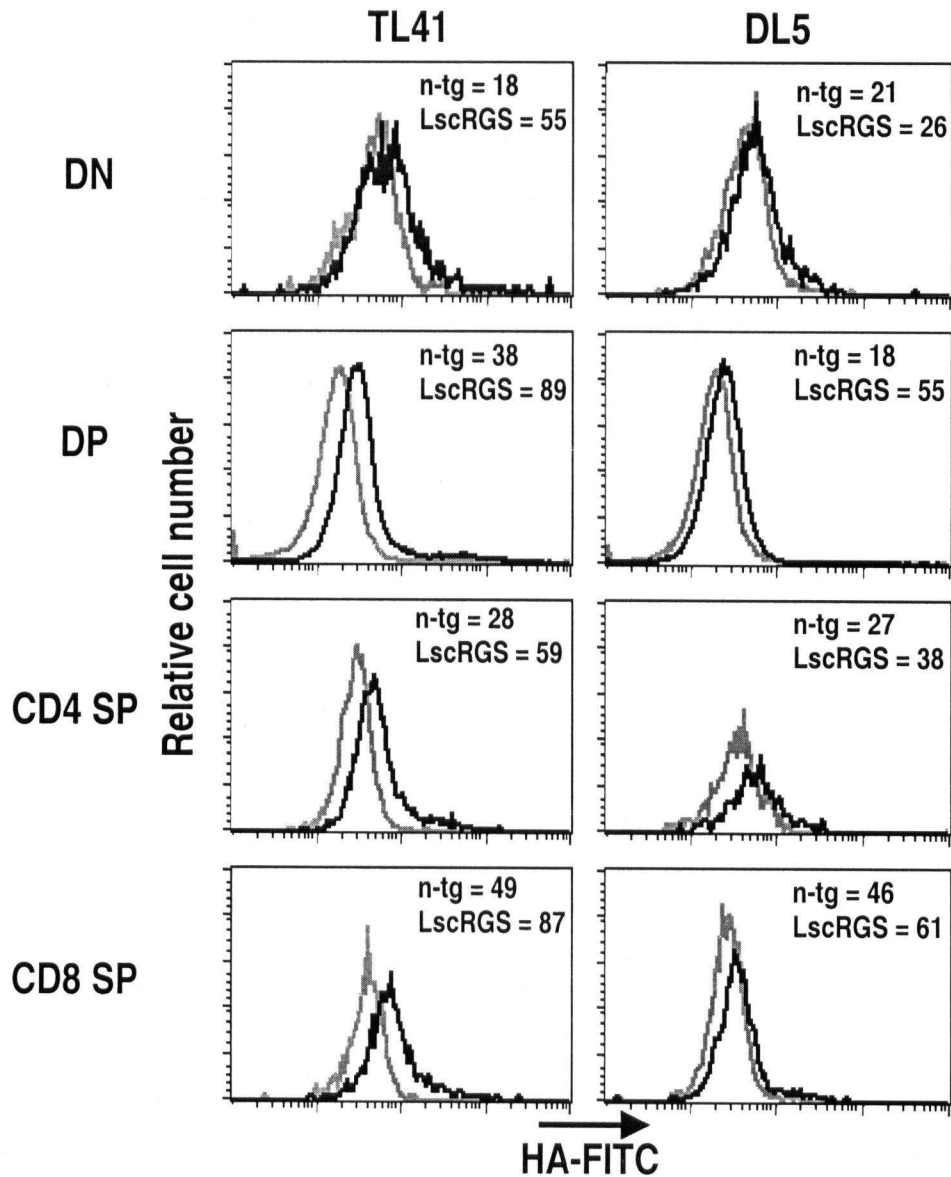


Figure 4.4. Splenic T cell intracellular expression analysis. Spleen cells from non-transgenic and LscRGS transgenic littermates from the TL41 and DL5 transgenic lines were incubated with antibodies directed against CD4 and CD8, fixed, permeabilized, incubated with antibodies directed against the HA-tagged portion of the LscRGS transgenic protein and analyzed by flow cytometry. Histogram overlays represent the fluorescence intensities of non-transgenic (gray line) and LscRGS transgenic (black line) cells from either CD4⁺ or CD8⁺ splenic T cells. Numbers indicate the mean fluorescence intensity of each histogram.

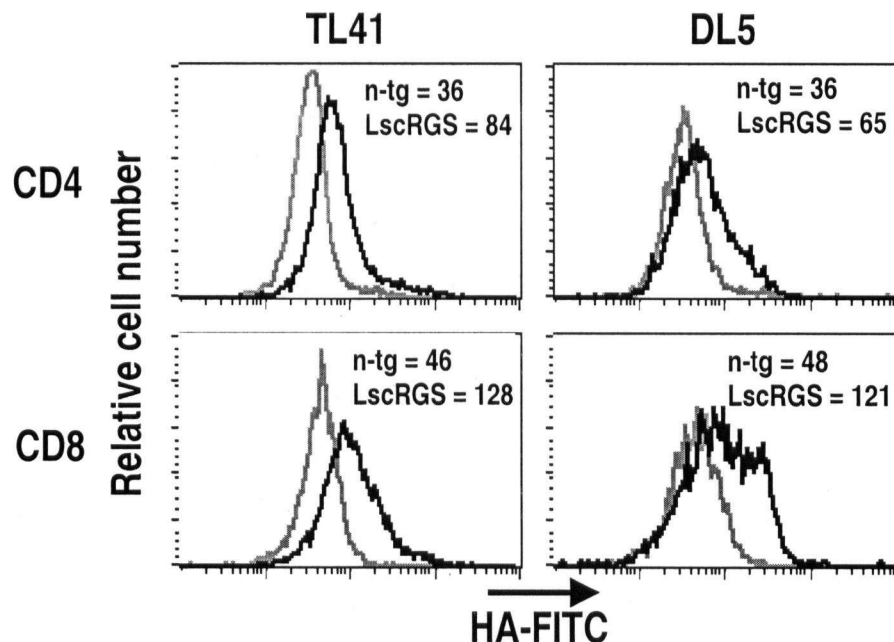


Figure 4.5. Splenic B cell intracellular expression analysis. Spleen cells from non-transgenic and LscRGS transgenic littermates from the TL41 and DL5 transgenic lines were incubated with antibodies directed against B220 and IgD, fixed, permeabilized, incubated with antibodies directed against the HA-tagged portion of the LscRGS transgenic protein and analyzed by flow cytometry. Histogram overlays represent the fluorescence intensities of non-transgenic (gray line) and LscRGS transgenic (black line) cells from either B220⁺/IgD⁺ or B220⁺/IgD⁻ splenic B cells. Numbers indicate the mean fluorescence intensity of each histogram.

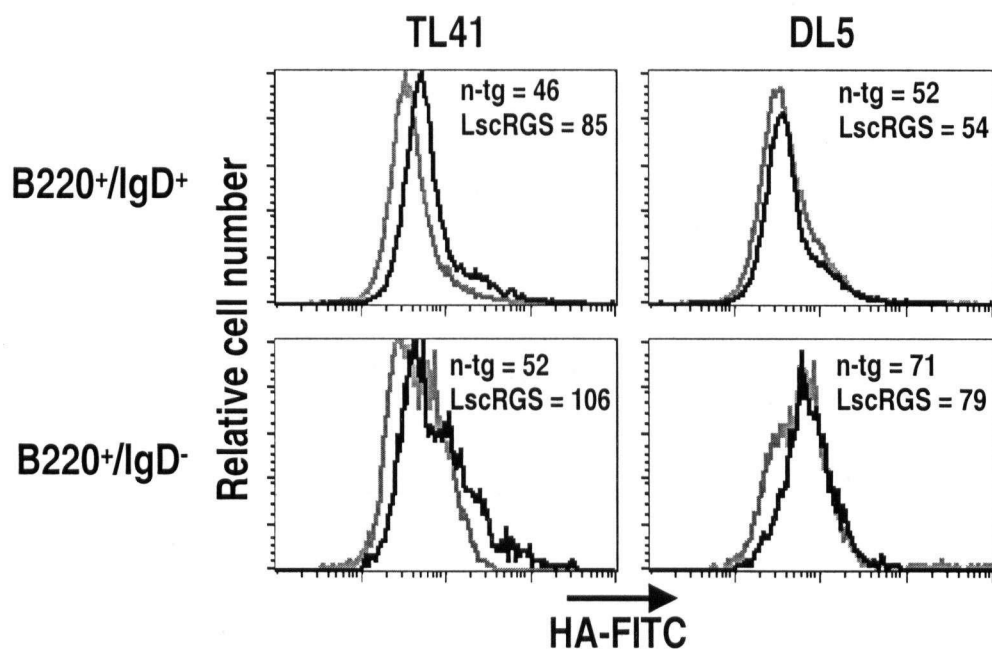
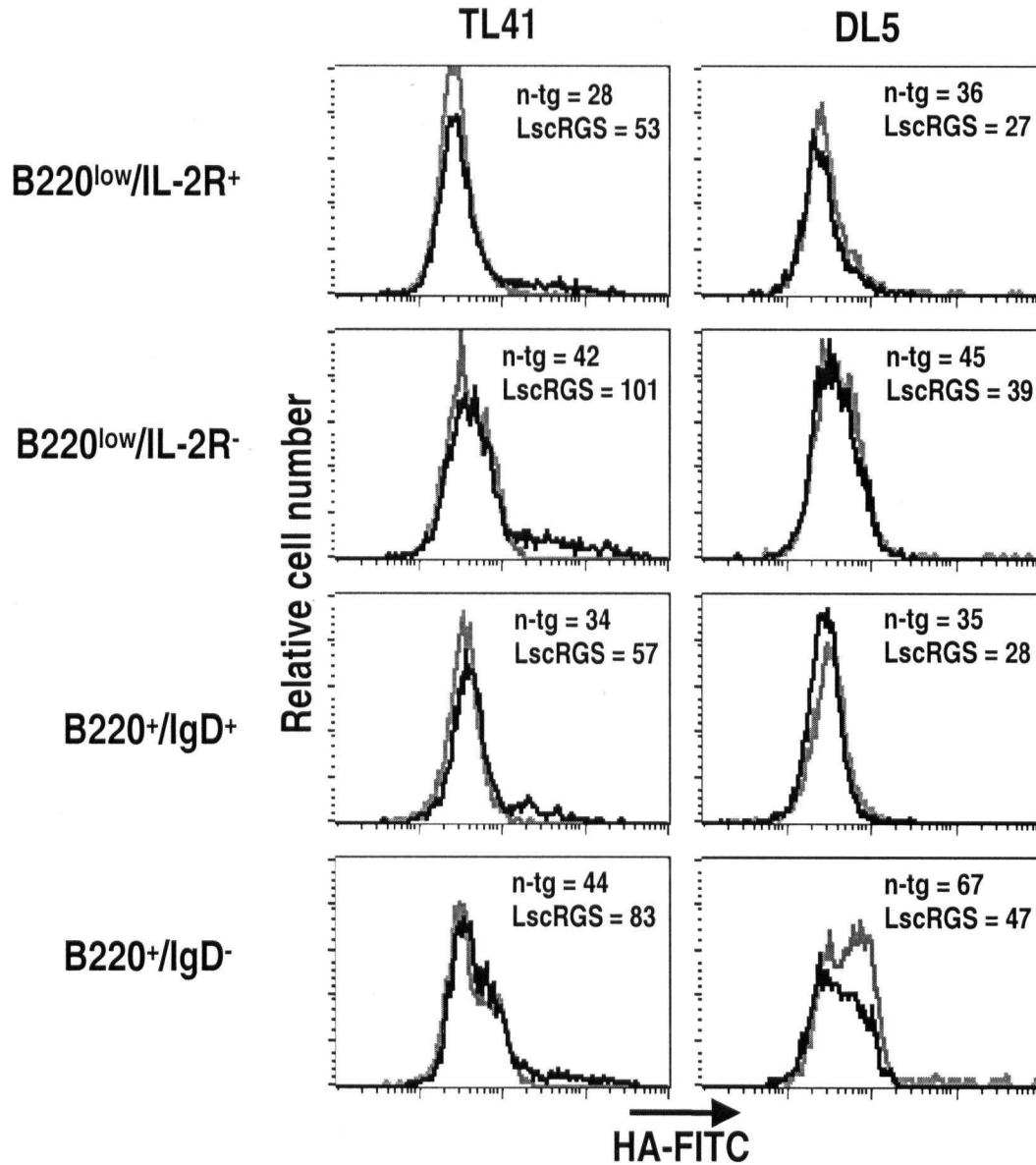


Figure 4.6. Bone marrow B cell intracellular expression analysis. Cells from bone marrow from non-transgenic and LscRGS transgenic littermates from the TL41 and DL5 transgenic lines were incubated with antibodies directed against either B220 and IL-2R or B220 and IgD, fixed, permeabilized, incubated with antibodies directed against the HA-tagged portion of the LscRGS transgenic protein and analyzed by flow cytometry. Histogram overlays represent the fluorescence intensities of non-transgenic (gray line) and LscRGS transgenic (black line) cells from B220^{low}/IL-2R⁺, B220^{low}/IL-2R⁻, B220⁺/IgD⁺ or B220⁺/IgD⁻ bone marrow B cells. Numbers indicate the mean fluorescence intensity of each histogram.



To determine whether LscRGS expression alters lymphocyte development, thymocytes from the TL37, TL41 and DL5 lines were isolated, incubated with antibodies directed against the cell surface markers CD4 and CD8 and analyzed by flow cytometry. The proportions of DN, DP, CD4 SP and CD8 SP thymocytes were equivalent to those of non-transgenic controls in the three lines examined (**Figure 4.7** and **Table 4.1**).

Figure 4.7. Flow cytometric analysis of thymocytes from the LscRGS transgenic mice. Thymocytes from LscRGS transgenic and non-transgenic littermate mice from the TL37 transgenic line were isolated and incubated with antibodies directed against CD4 and CD8 and analyzed by flow cytometry.

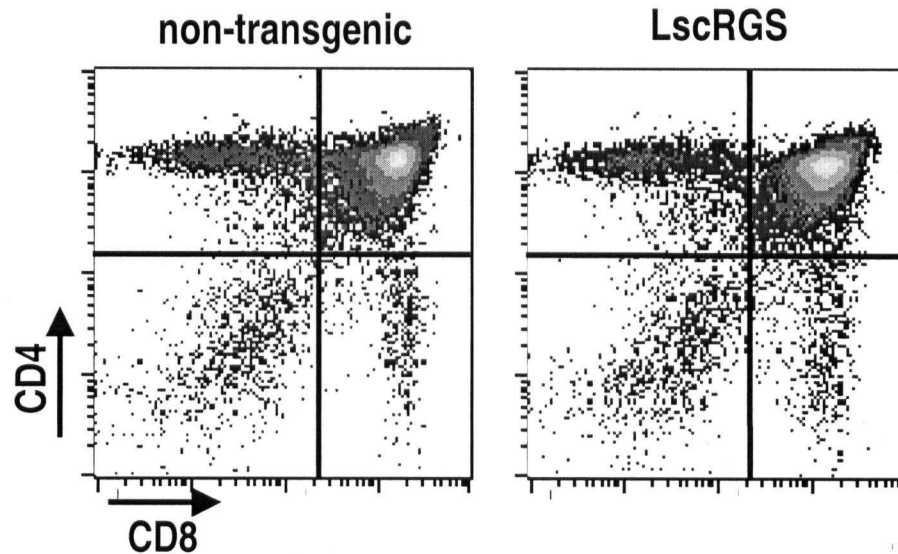
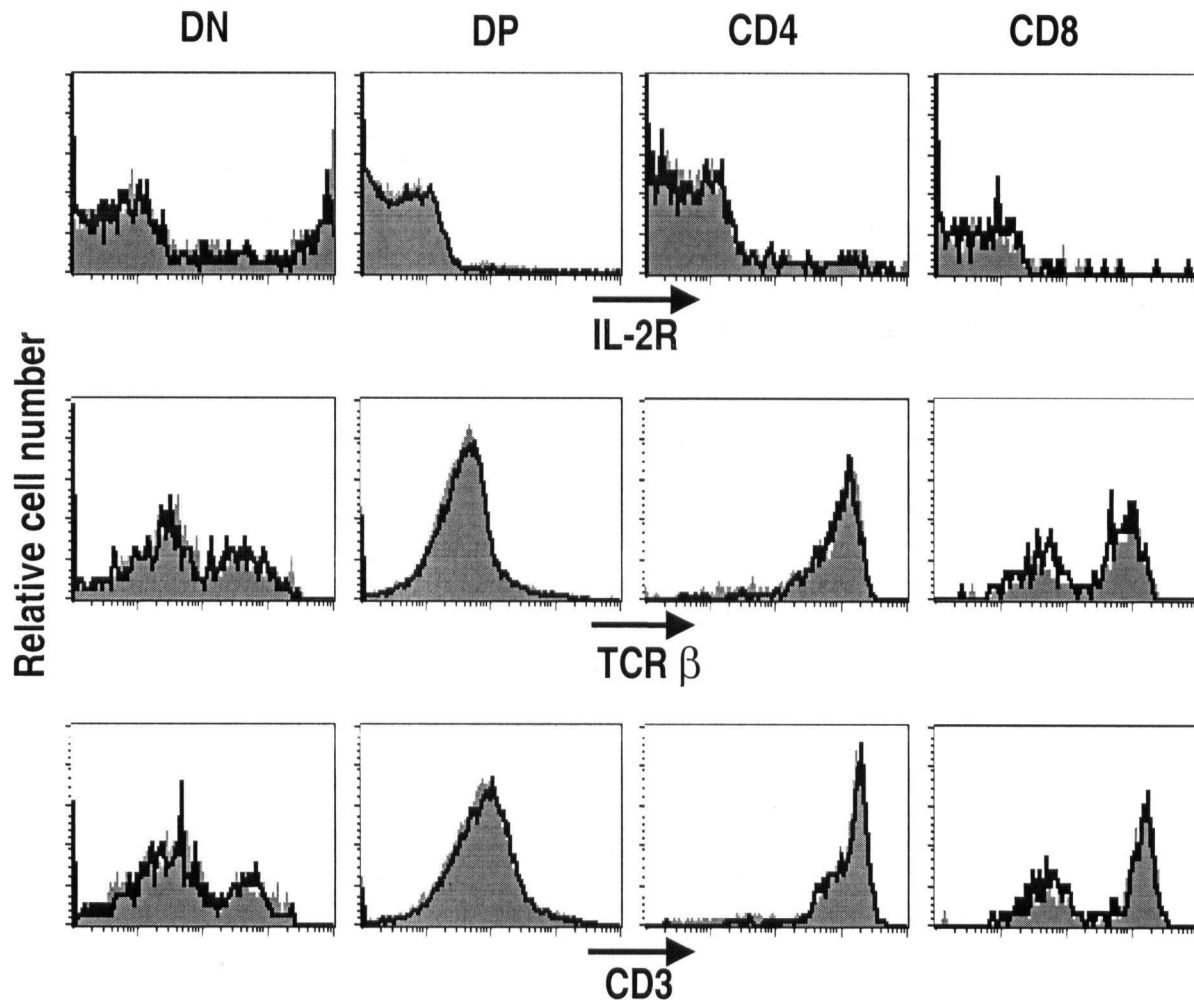


Table 4.1. Proportions of DN, DP and SP thymocytes from LscRGS transgenic mice. Proportions of live gated thymocytes from non-transgenic (-) and LscRGS transgenic (+) littermates from the TL37, TL41 and DL5 transgenic lines are indicated in the table. TL37 littermates indicated in bold are represented in the above density plots.

| | | Thymocyte population | | | | | | | |
|------|-------------|----------------------|------------|-------------|-------------|------------|------------|------------|------------|
| | | DN | | DP | | CD4 SP | | CD8 SP | |
| Line | Age (weeks) | - | + | - | + | - | + | - | + |
| TL37 | 12 | 2.6 | 3.2 | 89.1 | 84.6 | 6.3 | 9.6 | 2 | 2.6 |
| | 34 | 2.9 | 2.9 | 90.2 | 89.2 | 5.4 | 5.8 | 1.5 | 2.1 |
| TL41 | 7 | 2.6 | 3.4 | 86 | 84.8 | 8.7 | 8.9 | 2.7 | 3 |
| | 26 | 3.2 | 3 | 87.8 | 90 | 7.6 | 6.2 | 1.3 | 1.3 |
| DL5 | 10 | 2.9 | 3 | 87.9 | 87.1 | 7 | 7.4 | 2.2 | 2.5 |
| | 60 | 3.5 | 5.4 | 87.3 | 86.2 | 7.4 | 6.8 | 1.9 | 1.7 |

To evaluate the maturity of transgenic thymocytes, DN, DP, CD8 SP and CD4 SP thymocytes from TL37 and DL5 transgenic mice were isolated and analyzed for cell surface expression of TCR β , CD3 and IL-2R by flow cytometry. Expression levels of TCR β , CD3 and IL-2R by transgenic DN, DP, CD8 SP and CD4 SP thymocytes were also equivalent to those of non-transgenic controls (**Figure 4.8**).

Figure 4.8. Thymocyte developmental marker expression. Thymocytes from *LscRGS* transgenic and non-transgenic littermate mice from the TL37 transgenic line were isolated, incubated with antibodies directed against CD4, CD8 and CD25/IL-2R, TCR β or CD3 and analyzed by flow cytometry. Histogram overlays indicate fluorescence intensities of non-transgenic (solid gray) and *LscRGS* transgenic (black line) thymocytes within each thymocyte subset.



To determine whether *LscRGS* expression influences the cell cycle status of developing thymocytes, TL41 thymocytes were fixed, stained with PI and analyzed by flow cytometry. The proportion of cells with $<2n$ and $2n - 4n$ DNA content were similar to those of littermate controls (**Table 4.2**), suggesting that proliferation of transgenic thymocytes was unchanged in *LscRGS* transgenic mice.

Table 4.2. Cell cycle analysis of LscRGS transgenic thymocytes. Thymocytes from non-transgenic and LscRGS transgenic littermate mice from the TL41 line were isolated, fixed, incubated with propidium iodide and analyzed by flow cytometry. Proportions of thymocytes with <2n and >2n DNA are indicated in the table below.

| | DNA content | | | |
|--------|-------------|--------|--------|--------|
| | <2n | | >2n | |
| | non-tg | LscRGS | non-tg | LscRGS |
| Thymus | 0.3 | 0.3 | 5.9 | 5.0 |

HA-tagged LscRGS was also detected in splenic CD4 and CD8 T cells. To examine whether LscRGS expression altered the proportion of CD4 or CD8 T cells within the spleen, splenocytes were isolated, incubated with antibodies directed against CD4 and CD8 and analyzed by flow cytometry. The proportions of CD4 and CD8 T cells from LscRGS transgenic mice were equivalent to those from non-transgenic controls (**Figure 4.9** and **Table 4.3**). These results indicate that LscRGS expression does not overtly influence thymocyte or T cell development.

Figure 4.9. Flow cytometric analysis of spleen T cells. Spleen cells were isolated and incubated with antibodies directed against CD4 and CD8 and analyzed by flow cytometry. Numbers indicate the percent total CD4⁺ and CD8⁺ T cells within spleens from non-transgenic and LscRGS transgenic mice from the TL41 line.

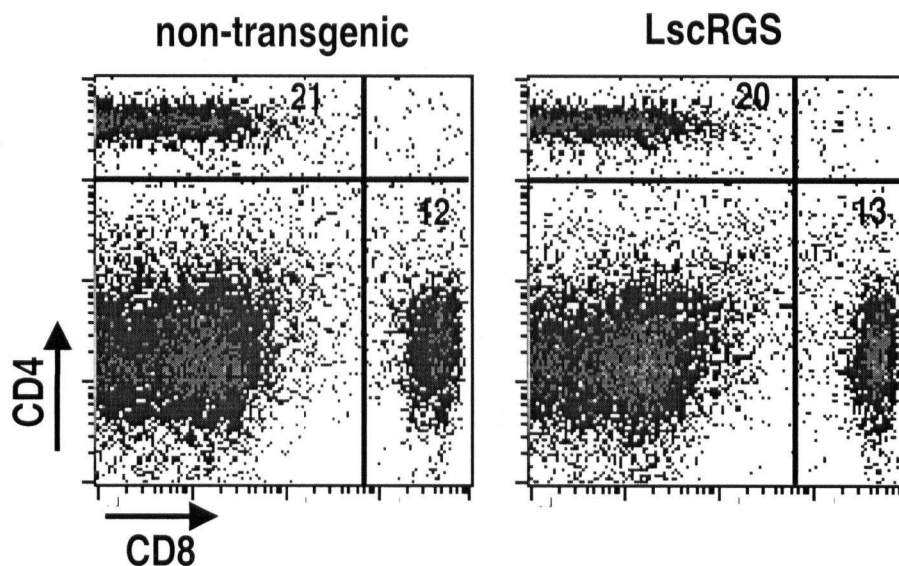


Table 4.3. Splenic T cell proportions. Numbers in the table represent the percent total splenic CD4⁺ and CD8⁺ T cells from non-transgenic and LscRGS transgenic littermates from the TL37, TL41 and DL5 lines. TL41 littermates indicated in bold are represented in the above density plots.

| | | Splenic T cell population | | | |
|-------------|-------------|---------------------------|-------------|-------------|-------------|
| | | CD4 | | CD8 | |
| Line | Age (weeks) | - | + | - | + |
| TL37 | 12 | 17.8 | 15.5 | 13.2 | 11.42 |
| | 34 | 11.7 | 13 | 7.3 | 9.4 |
| TL41 | 7 | 21 | 20.1 | 12.1 | 12.5 |
| | 26 | 17.6 | 20.6 | 9.4 | 9.0 |
| DL5 | 10 | 17.7 | 20.9 | 14.6 | 13.7 |
| | 60 | 13 | 11.2 | 8 | 4.2 |

B lymphocytes from the TL37 and TL41 transgenic lines expressed HA-tagged LscRGS. To determine whether LscRGS expression altered B lymphocyte development, cells from spleen and bone marrow were analyzed for expression of cell surface B lymphocyte developmental markers by flow cytometry. Splenic B lymphocyte (IgM⁺/IgD⁻ or IgM⁺/IgD⁺) proportions were normal in the TL37 and TL41 lines (**Figure 4.10**). Proportions pre-B (B220^{low}/IL-2R⁺), immature B (B220^{high}/IgM^{low}) and mature B (B220^{high}/IgM^{high}/IgD⁺) lymphocytes from transgenic mice were also equivalent to those of non-transgenic controls (**Figure 4.11**). Together, these results indicate that LscRGS expression does not overtly influence B lymphocyte development.

Figure 4.10. Flow cytometric analysis of spleen B cells. Spleen cells were isolated, incubated with antibodies directed against IgM and IgD and analyzed by flow cytometry. Numbers indicate the percent total IgM⁺/IgD⁺ and IgM⁺/IgD⁻ spleen B cells from non-transgenic and LscRGS transgenic littermates from the TL37 line.

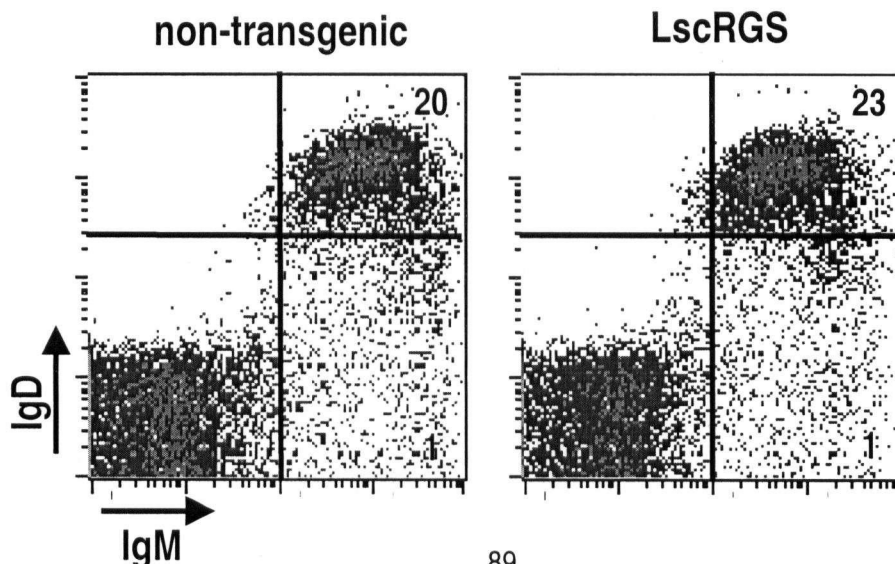
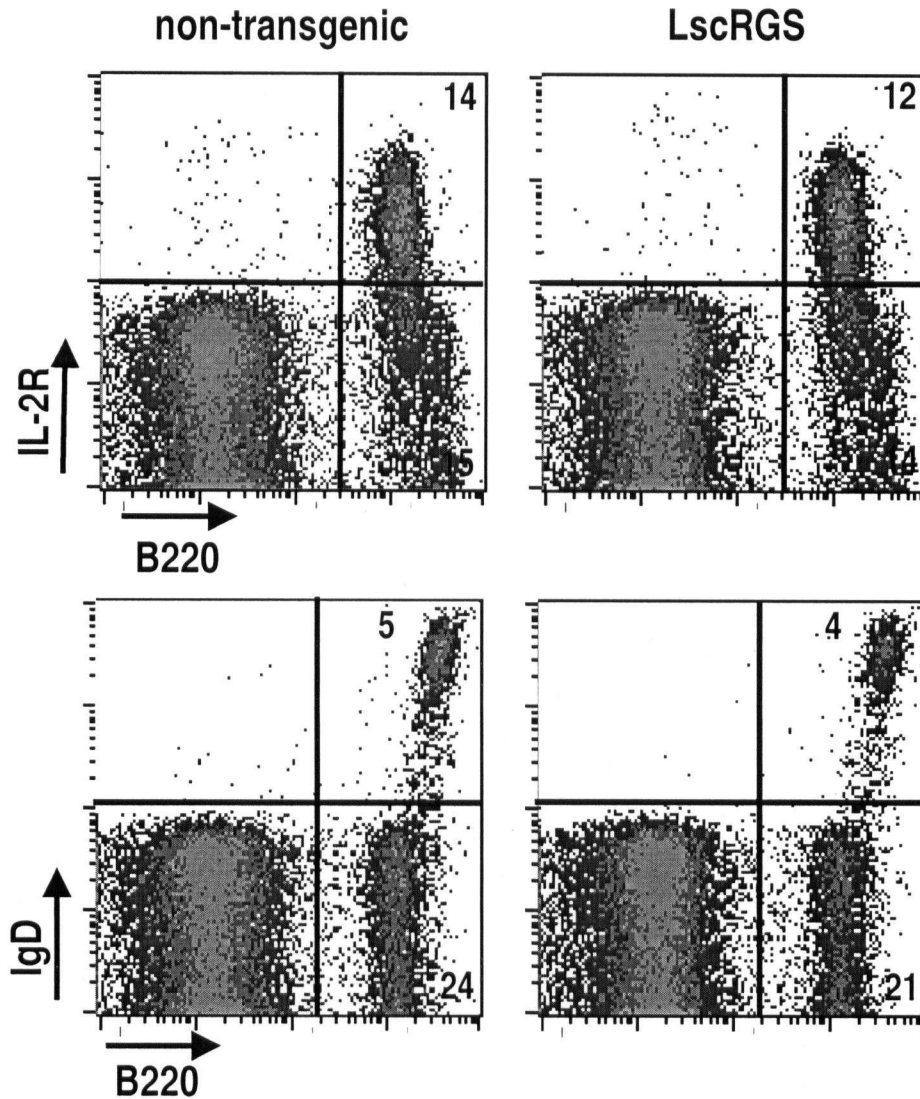


Figure 4.11. Flow cytometric analysis of bone marrow B cells. Bone marrow cells from non-transgenic and LscRGS transgenic littermates from the TL41 transgenic line were incubated with antibodies directed against either B220 and IL-2R or B220 and IgD, and analyzed by flow cytometry. Numbers indicate the percent gated IL-2R⁺/B220^{low} and IL-2R⁺/B220^{low/high} (upper panel) IgD⁺/B220⁺ and IgD⁺/B220⁺ (lower panel) bone marrow B cells from non-transgenic and LscRGS transgenic littermates.



4.3 Discussion

In an attempt to implicate $G\alpha_{12}$ and $G\alpha_{13}$ in lymphocyte development, transgenic mice expressing LscRGS, a negative regulator or GAP for these $G\alpha$ proteins, were generated. Although analyses of lymphocytes from these mice revealed that LscRGS expression did not overtly affect development, I was unable to conclude that $G\alpha_{12}$ or $G\alpha_{13}$ do not play a role in lymphocyte development. At the time the experiments with LscRGS transgenic mice were conducted very little was known regarding $G\alpha_{12}$ and $G\alpha_{13}$ within the context of lymphocytes (refer to section 1.5.3); as a result, rationale for experiments was often limited. Upon completion of my experiments with these mice and while I was consumed with another transgenic project (described in Chapter 5), work from two groups involving known regulators and effectors of $G\alpha_{12}$ and $G\alpha_{13}$, were published, highlighting the importance of these $G\alpha$ subunits in lymphocyte development [314] [312]. Results from their studies provide a strong basis and justification for future experiments involving LscRGS transgenic mice that, to date, do not show any obvious defects in lymphocyte development.

Recently, the effects of homozygous inactivation of G2A, a $G\alpha_{13}$ -coupled GPCR expressed in lymphocytes, were described and the receptor ligand was identified. G2A^{-/-} mice developed a late onset autoimmune syndrome and T lymphocytes were hyperresponsive to antigen receptor stimulation *in vitro* [314]. Lysophosphatidylcholine (LPC) is a high-affinity ligand for G2A and activation of G2A by LPC increased intracellular calcium concentration, induced receptor internalization, activated Erk and modified migratory responses of Jurkat T lymphocytes [329]. Although it is known that G2A couples to $G\alpha_{13}$ in fibroblasts, it is not known whether this receptor couples to $G\alpha_{13}$ in lymphocytes. G2A-mediated signals are mediated via $G\alpha_{13}$ activation in NIH 3T3 cells, endothelial cells and embryonic fibroblasts (section 3.2) [201] [235]. Both $G\alpha_{13}$ and G2A are also expressed in several of the same tissues, including thymus and spleen [308] [201]. Overlapping expression patterns and the observation that G2A couples to $G\alpha_{13}$ in several different cell types suggest that $G\alpha_{13}$ may mediate G2A signals in lymphocytes. Interestingly, impaired Rho activation also leads to a loss of sustained increase in calcium and Erk activation in Jurkat cells [269]. Thus it is possible that some of the defects displayed by G2A^{-/-} mice are the result of impaired Rho activation via $G\alpha_{13}$ and G2A. Moreover, LscRGS expression may impair antigen- or LPC-mediated ERK activation, calcium release or migratory responses of transgenic T lymphocytes. The effect of LscRGS expression in responses of transgenic T lymphocytes to LPC or antigen stimulation remains to be determined.

Many reports have illustrated the importance of chemokine receptors, a large group of GPCRs, in lymphocyte migration. Inflammatory chemokines are expressed transiently in inflamed tissues by resident or infiltrated cells upon stimulation by pro-inflammatory cytokines during contact with pathogens; these chemokines are specialized for the recruitment of specialized cells, including effector T cells. Homeostatic chemokines are produced in discrete microenvironments within non-lymphoid or lymphoid tissues and maintain traffic and positioning of cells during hemopoiesis, antigen sampling and immune surveillance. Although the predominant role of chemokines is in lymphocyte traffic control, some chemokines are also involved in migration-independent responses including differentiation and lymphocyte effector function (reviewed in [333] [334]). Prior to chemokine-induced migratory responses, cells polarize, forming lamellipodia at the leading edge and uropods at the trailing edge, allowing them to convert cytoskeletal forces into net cell-body displacement [335]. Thereafter, chemokines provide directional cues for cell motility, enabling migration along the chemokine gradient. Many signaling molecules are involved in these processes. Perhaps not surprisingly, members of the Rho family regulate the cytoskeletal responses underlying cell polarization and migration (reviewed in [335]). Although $G\alpha_{12}$ and $G\alpha_{13}$ may mediate Rho family protein activation downstream of chemokine receptors, the majority of studies to date have not focussed on identifying the $G\alpha$ proteins required for chemokine receptor-induced responses. LscRGS is a negative regulator of $G\alpha_{12}$ and $G\alpha_{13}$ and is expressed in mature T lymphocytes from LscRGS transgenic mice. The effect of LscRGS expression on lymphocyte chemokine responses has not been addressed.

The most compelling evidence implicating $G\alpha_{12}$ and $G\alpha_{13}$ in lymphocyte migratory responses was illustrated upon characterization of Lsc deficient mice. Lsc is a Dbl family GEF that specifically activates Rho upon activation by $G\alpha_{13}$ [126] [130]. Lsc mediates signals downstream of $G\alpha_{13}$ and upstream of Rho. As indicated previously, in addition to its ability to activate Rho, Lsc contains an RGS domain that specifically inactivates $G\alpha_{12}$ and $G\alpha_{13}$; thus Lsc has the potential to regulate the activity of $G\alpha_{12}$, $G\alpha_{13}$ and Rho GTPases [131]. Characterization of Lsc^{-/-} lymphocytes revealed that actin polymerization via lysophosphatidic acid (LPA) and thromboxane A₂ (TXA₂) was impaired. Lsc was also required for normal numbers of marginal zone B (MZB) cells, full humoral responses to both thymus-dependent and -independent antigens and the inhibition of basal T cell proliferation. Importantly, Lsc was also an important regulator of cell motility and Lsc^{-/-} lymphocytes displayed reduced basal cell motility [312]. Together these findings implicate $G\alpha_{12}$ and $G\alpha_{13}$ proteins in several aspects of lymphocyte function and immune responses. As a negative regulator of $G\alpha_{12}$ and $G\alpha_{13}$,

LscRGS expression may impair lymphocyte responses to $G\alpha_{12}$ or $G\alpha_{13}$ activating stimuli, such as LPA and TXA_2 . The migratory responses of LscRGS transgenic lymphocytes to LPA, TXA_2 or serum have also not been examined and would be an interesting project in the future.

Within the context of my experiments, LscRGS expression did not appear to influence lymphocyte development. Although it is possible that, when expressed in transgenic mice, LscRGS is not at sufficient levels to stimulate the GTPase activity of $G\alpha_{12}$ and $G\alpha_{13}$, this scenario is unlikely. LscRGS expression levels in lymphocytes from LscRGS transgenic mice were comparable to levels of expression required for suppression of $G\alpha_{12}$ - or $G\alpha_{13}$ - mediated transformation in NIH 3T3 fibroblasts. Regulatory mechanisms may be different in lymphocytes than in fibroblasts; thus is it possible that LscRGS may be negatively regulated when expressed in lymphocytes of transgenic mice. Several mechanisms are known to influence the activity of RGS proteins. $G\alpha$ phosphorylation, for example can inhibit the GAP activity of some RGS proteins [336]. Phosphorylation and protein binding also regulate RGS proteins. In particular, the phosphoserine-binding protein 14-3-3 serves as a scavenger of RGS3, regulating the amounts of RGS3 available for binding $G\alpha$ proteins [337]. Lipid-protein interactions are also known to regulate RGS proteins. Notably, the inhibitory effect of RGS4 was reduced by phosphatidylinositol-3,4,5,- trisphosphate ($PI[3,4,5]P_3$) [338]. These studies reveal additional levels in the regulation of G protein signaling, in which the inhibitors of $G\alpha$ proteins, RGS proteins, are regulated by both protein and lipid interactions. Although regulatory mechanisms of RGS proteins in lymphocytes have not yet been reported, they are likely to be important. Therefore, despite high LscRGS expression, RGS GAP activity may be more tightly regulated in lymphocytes than in fibroblasts.

Chapter 5 – Generation and analysis of Dbs transgenic mice

5.1 Introduction and rationale

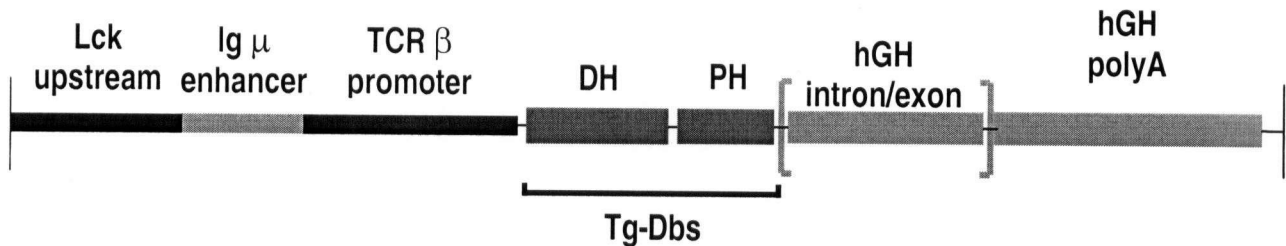
As indicated in chapter 1 (section 1.5), altered GTPase activity can influence lymphocyte development. Interestingly, the majority of GTPase studies in lymphocytes have utilized mutationally activated, inactivated or bacterial toxins that impair the normal function of the GTPase. While these approaches have been extremely useful in implicating GTPases in lymphocyte development, the role of GTPase regulators is not as well understood. GTPase activity is normally tightly regulated by GEFs and GAPs. Dbl family proteins regulate the activity of Rho proteins and several of these GEFs, including Dbs, have been isolated in screens for cDNAs that cause transformation of NIH 3T3 fibroblasts. Moreover, an activated, amino- and carboxyl-terminally truncated form of Dbs (herein referred to as activated Dbs), aggressively transforms fibroblasts [132]. Subsequent experiments revealed that Dbs is a Rho and Cdc42 GEF expressed in the thymus [132] [111]. Since Dbs is an activator of Rho and Cdc42 and is expressed in lymphoid tissues, I wanted to determine the effect of activated Dbs expression on lymphocyte development.

5.2 Results

5.2.1 Increased Dbs expression in lymphocytes alters development

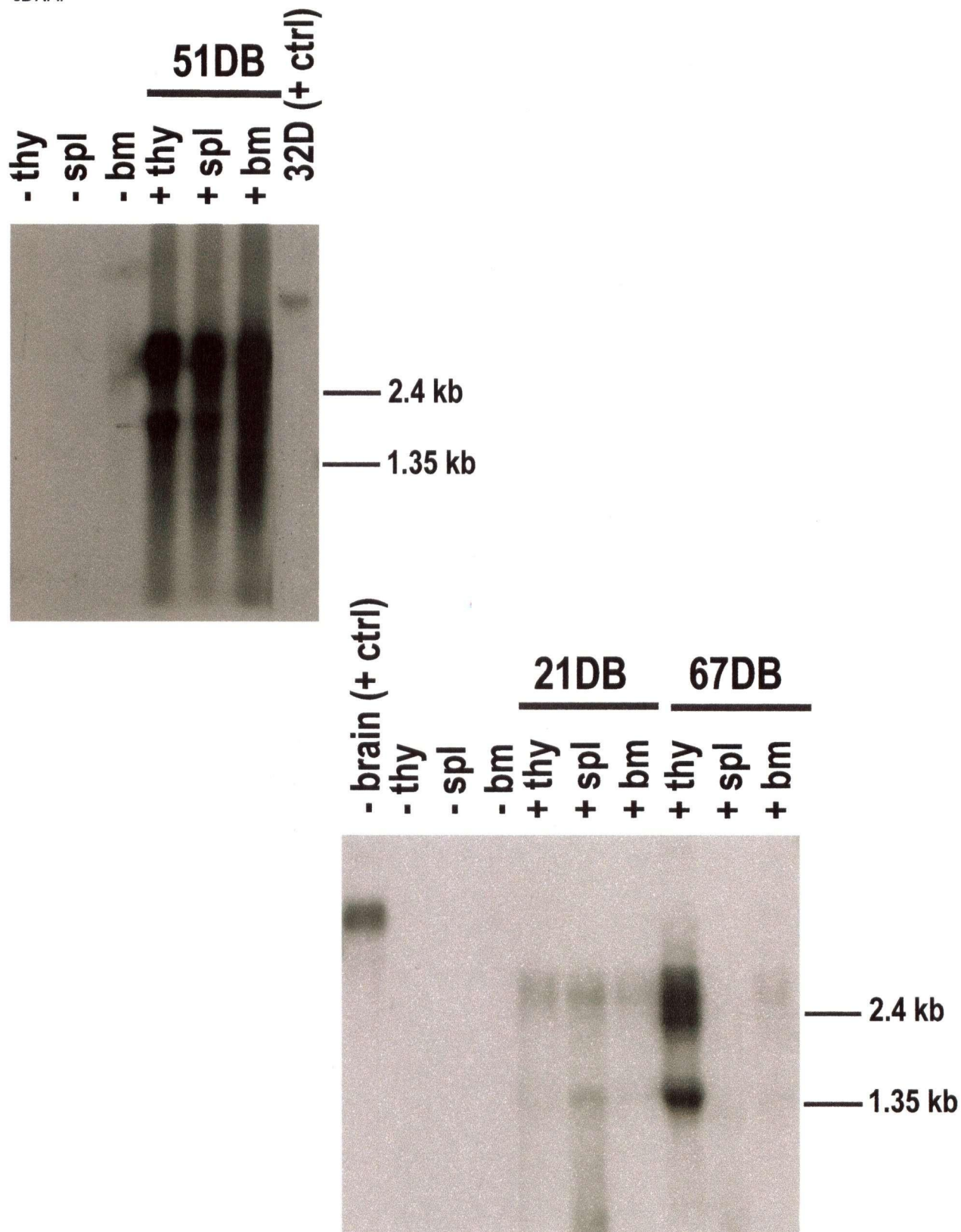
To determine how Dbs affects lymphocyte development, we generated transgenic mice expressing an activated form of Dbs within lymphoid tissues. The LIT2 vector was utilized and contained a cDNA encoding a truncated form of Dbs (Dbs-D13/HA; refer to section 2.1.2.3 for construct details) transcribed from the *TCR β* promoter (**Figure 5.1**). The LIT2 vector also contained an *immunoglobulin μ heavy chain* enhancer and sequences upstream of the *lck* gene proximal promoter, enabling both T and B lymphoid-restricted expression. Three Dbs transgene-positive founders were obtained from blastocyst injections using LIT2 as an expression vector (lines designated 21DB, 51DB and 67DB). Activated Dbs was expressed at variable levels in lymphoid tissues from these three transgenic lines. Expression of the Dbs transgene was highest in the 51DB and 67DB transgenic lines and these two were chosen for further study.

Figure 5.1. Dbs transgenic construct. The LIT2 vector.



The predicted transgenic messenger RNA (mRNA) size is 2.5 kb ((1450 bp (Dbs) + 850 bp (hGH) + 200 bp (polyA)). Analysis of Northern blots revealed multiple transcripts in addition to the predicted transcript. While the higher molecular weight cluster at approximately 3.0 kb was the likely result of alternative splicing of the intronic sequences within the hGH region of the transgene, the lower molecular weight transcripts (at approximately 1.4 kb) were the likely result of alternative splicing of the intronic sequences of the hGH region with cryptic sites within the Dbs cDNA. Normally, Dbs is expressed at relatively high levels in brain, moderate levels in spleen and low levels in thymus [132]. Northern blot analysis of tissues isolated from 51DB, 21DB and 67DB transgenic mice, using the Apal fragment of Dbs as a probe, showed variable transgene expression in thymus, spleen and bone marrow (**Figure 5.2**). Dbs transgene was expressed at high levels in thymus, spleen and bone marrow from mice from the 51DB line. Expression of activated Dbs was moderate in thymus and very low in spleen and bone marrow from 67DB mice. Activated Dbs was detected at extremely low levels in thymus, spleen and bone marrow from the 21DB line. Although Dbs transgene was expressed at variable levels in the three lines generated, expression was highest in the 51DB line.

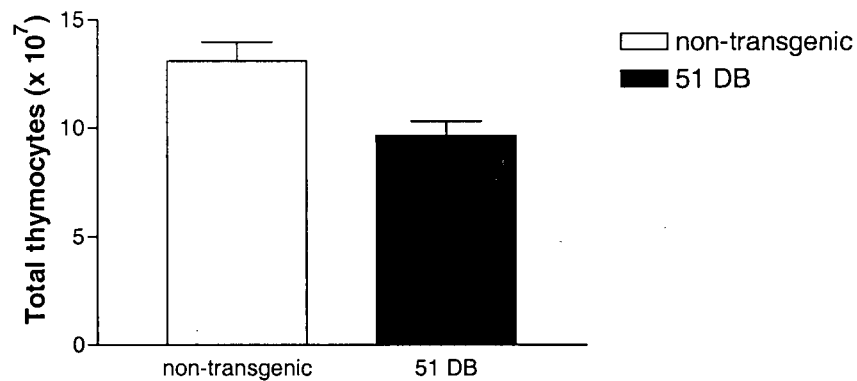
Figure 5.2. Northern blot analysis of *Db*s transgene expression. Total RNA was isolated from cells from thymus, spleen and bone marrow from mice from the 51DB, 21DB and 67DB lines. Total RNA was separated by electrophoresis (4 μ g/lane), transferred to nylon membranes and hybridized with an *Ap*I probe from the *Db*s cDNA.



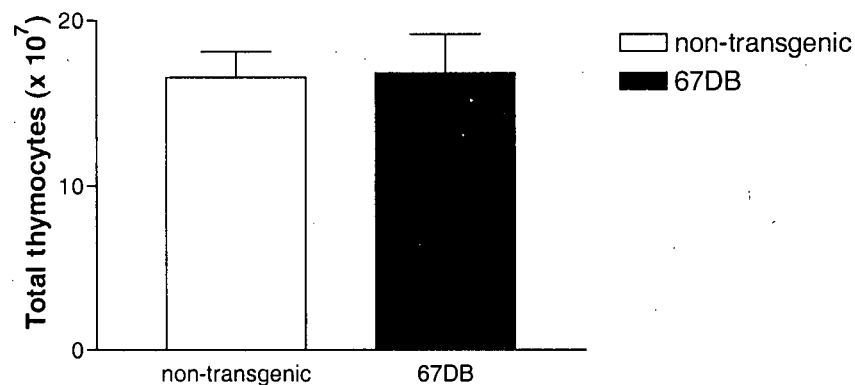
To determine whether activated Dbs expression alters total thymocyte numbers, thymocytes from 51DB and 67DB mice were counted. Total thymocytes were reduced by about twenty percent in mice from the 51DB line when compared to non-transgenic controls (**Figure 5.3**). Total thymocytes from 67DB mice, however were equivalent to those of non-transgenic controls (**Figure 5.3**).

Figure 5.3. Total thymocyte numbers.

a) Total thymocytes from 7-10 week old mice from *Dbs* transgenic and non-transgenic littermates from the 51DB line were isolated and counted.



b) Total thymocytes from 9-16 week old mice from *Dbs* transgenic and non-transgenic littermates from the 67DB line were isolated and counted.

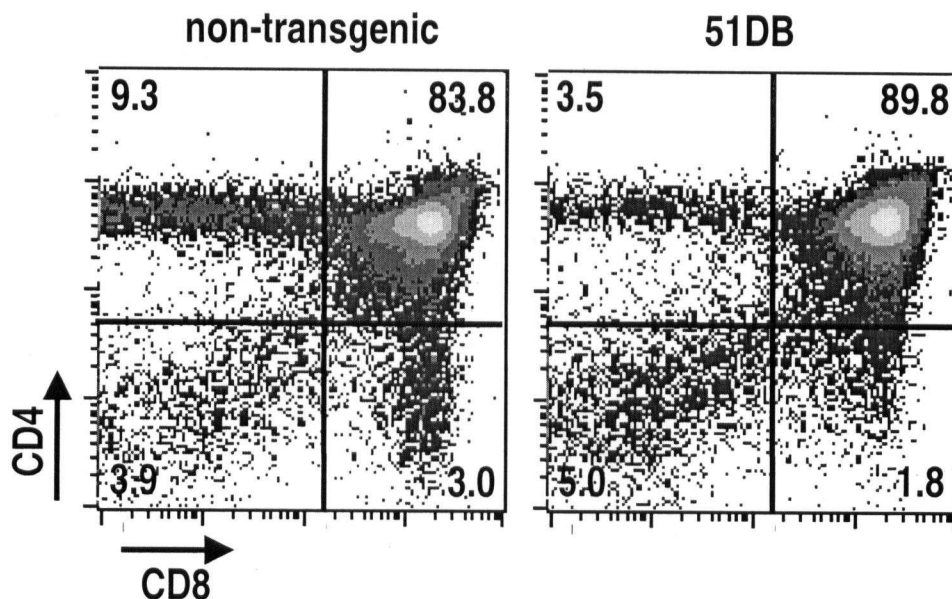


To determine whether activated Dbs expression alters thymocyte development, cells from 51DB and 67DB thymuses were isolated, incubated with antibodies directed against the cell surface markers CD4 and CD8 and analyzed by flow cytometry. The proportion and total numbers of DN thymocytes from 51DB transgenic mice were increased when compared to non-transgenic controls (**Figure 5.4**). DP

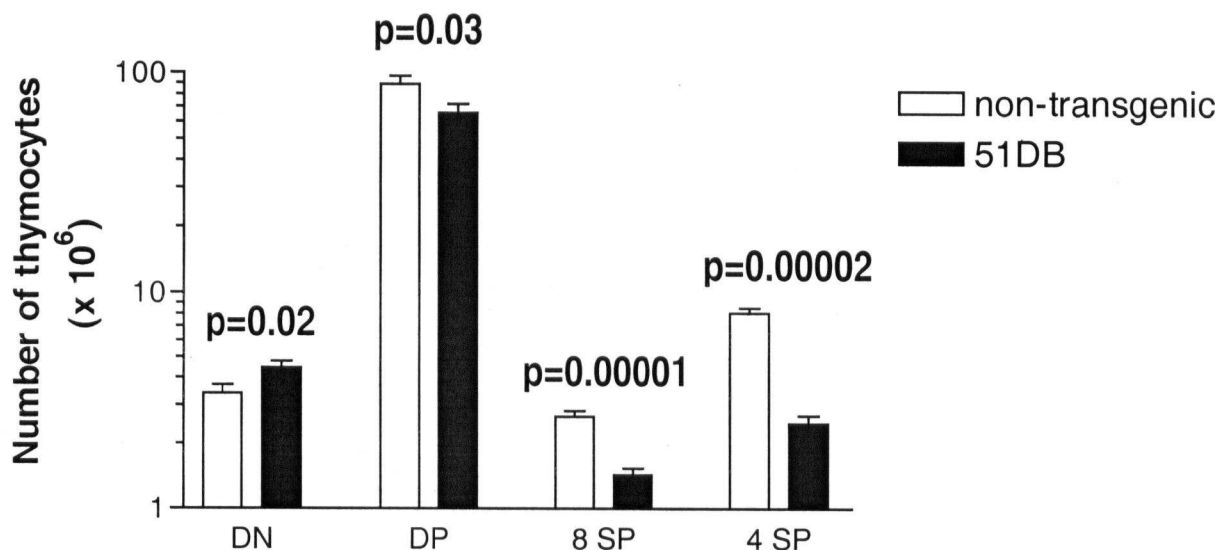
thymocytes from 51DB transgenic mice were reduced slightly compared to non-transgenic controls, while the proportions of CD4 and CD8 SP thymocytes were approximately half those of non-transgenic thymocytes (**Figure 5.4**).

Figure 5.4. Thymocyte analyses from 51DB and 67DB mice.

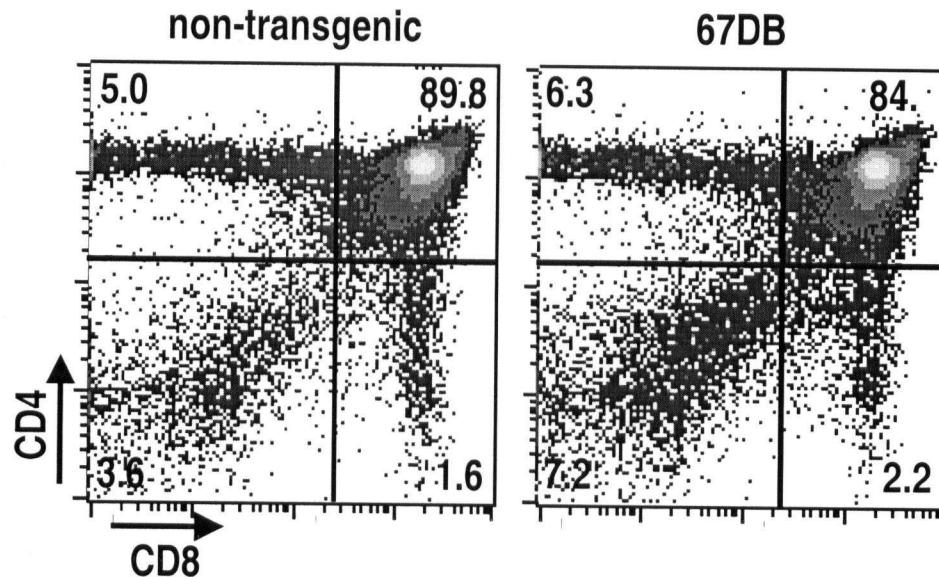
a) Flow cytometric analysis of thymocytes from the 51DB transgenic line. Thymocytes from *Db*s transgenic and non-transgenic littermate mice from the 51DB transgenic line were isolated and incubated with antibodies directed against CD4 and CD8 and analyzed by flow cytometry. Quadrant numbers represent proportions of live-gated DN, DP and SP thymocyte populations.



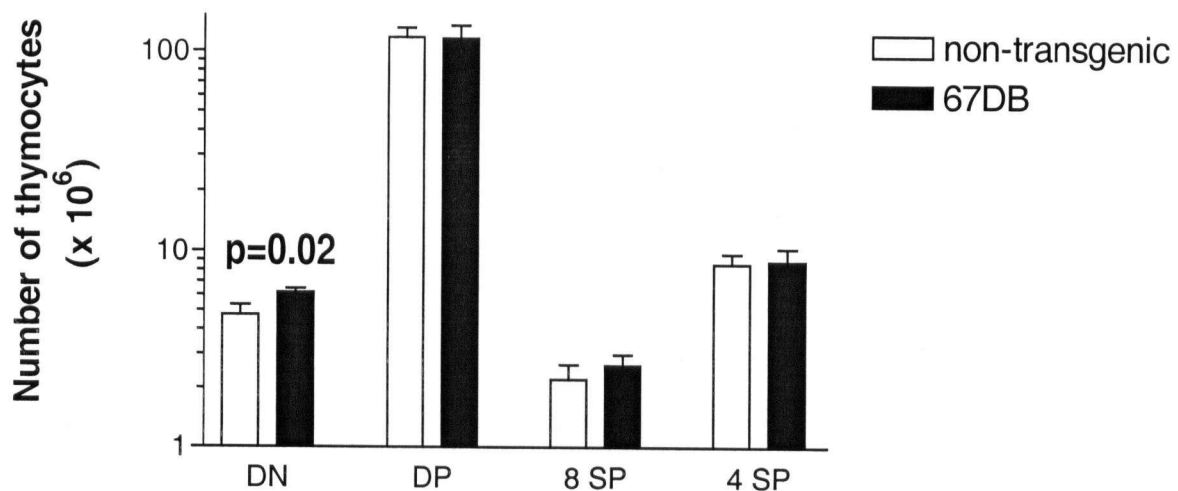
b) Total DN, DP and SP thymocytes from mice from the 51DB line. Values were calculated by multiplying proportions of DN, DP and SP thymocytes by the number of total cells. Numbers indicate the probability value associated with a paired t-test.



c) **Flow cytometric analysis of thymocytes from the 67DB transgenic line.** Thymocytes from *Db*s transgenic and non-transgenic littermate mice from the 67DB transgenic line were isolated and incubated with antibodies directed against CD4 and CD8 and analyzed by flow cytometry. Quadrant numbers represent proportions of live-gated DN, DP and SP thymocyte populations.



d) **Total DN, DP and SP thymocytes from mice from the 67DB line.** Values were calculated by multiplying proportions of DN, DP and SP thymocytes by the total number of cells. The number indicates the probability value associated with a paired t-test.

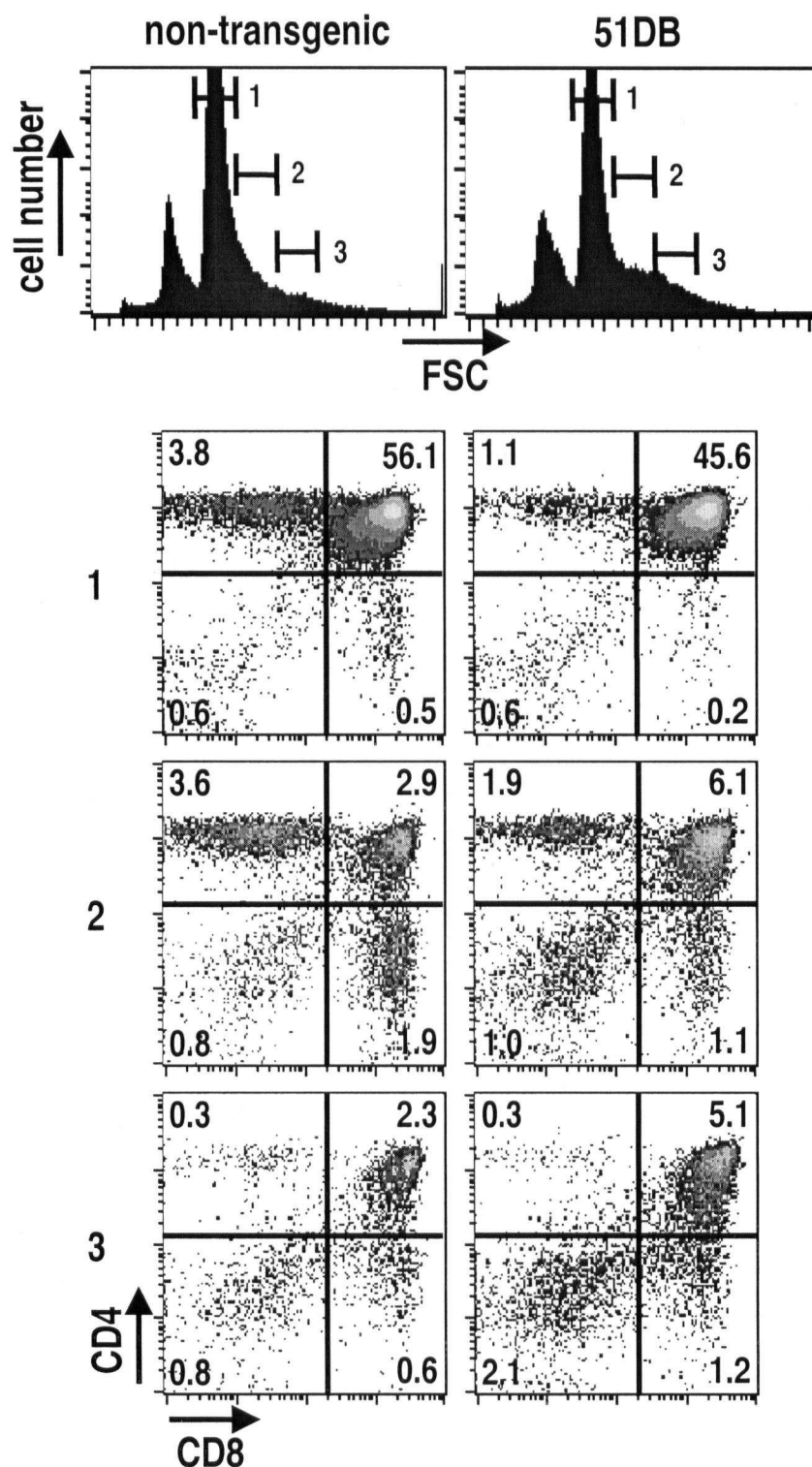


Positively selected DP thymocytes differentiate into CD8 SP thymocytes via down-regulation of CD4 and the most mature CD8 SP thymocytes express the lowest levels of CD4. Although 51DB transgenic mice displayed an overall reduction in CD8 SP thymocytes, transgenic thymocytes that expressed the lowest levels of CD4 were depleted more than those expressing intermediate levels of CD4. Thus the reduction in CD8 SP thymocytes observed in 51DB transgenic mice was due mainly to reduced frequencies of the most mature CD8 SP thymocytes expressing the lowest levels of CD4. Although the proportion and numbers of DN thymocytes from 67DB mice were often increased when compared to non-transgenic controls, numbers of DP and SP thymocytes from 67DB mice were equivalent to those of non-transgenic controls (**Figure 5.4**). The extent of the DN increase was variable in mice from the 67DB line and out of five pairs analyzed, the increase in the proportion of DN thymocytes compared to non-transgenic controls was unchanged in one, moderate (1.2-fold) in another and approximately two-fold in the remaining three.

Although transgene expression in the thymus was at least two-fold higher in 51DB mice than 67DB mice and the extent of the increase in 67DB DN thymocytes was variable, there was overlap in terms of the effect of activated Dbs expression on DN thymocytes. Activated Dbs expression thus altered the numbers and proportions of developing thymocyte subsets in mice. Since expression in the 51DB line was highest, the majority of experiments performed were with mice from this line.

Analysis of FSC and SSC characteristics by flow cytometry is useful in estimating cell size and normally high FSC cells are DP and DN thymocytes. 51DB transgenic thymocytes were characteristically high FSC, indicating a higher proportion of large cells (**Figure 5.5**). Although total thymocyte numbers from 51DB mice were reduced, the proportions of high FSC DN and DP thymocytes were about twice those of non-transgenic controls (**Figure 5.5**). Therefore a large proportion of the high FSC thymocytes from 51DB transgenic mice were DN and DP thymocytes.

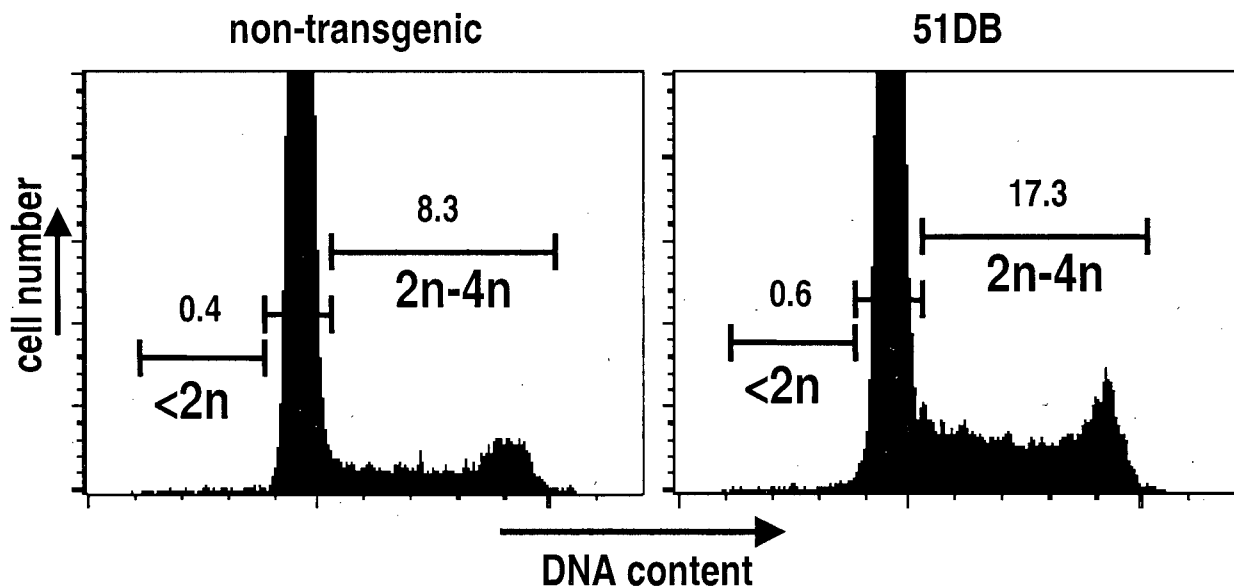
Figure 5.5. Forward scatter (FSC) analysis of *Db*s thymocytes. Thymocytes from *Db*s transgenic and non-transgenic littermate mice from the 51DB transgenic line were isolated and incubated with antibodies directed against CD4 and CD8 and analyzed by flow cytometry. Small, medium and large thymocytes were discriminated based on FSC intensity (1, 2 and 3, respectively; upper panel). Proportions of live-gated low (1), medium (2) and high (3) FSC DN, DP and SP thymocytes are indicated in each quadrant.



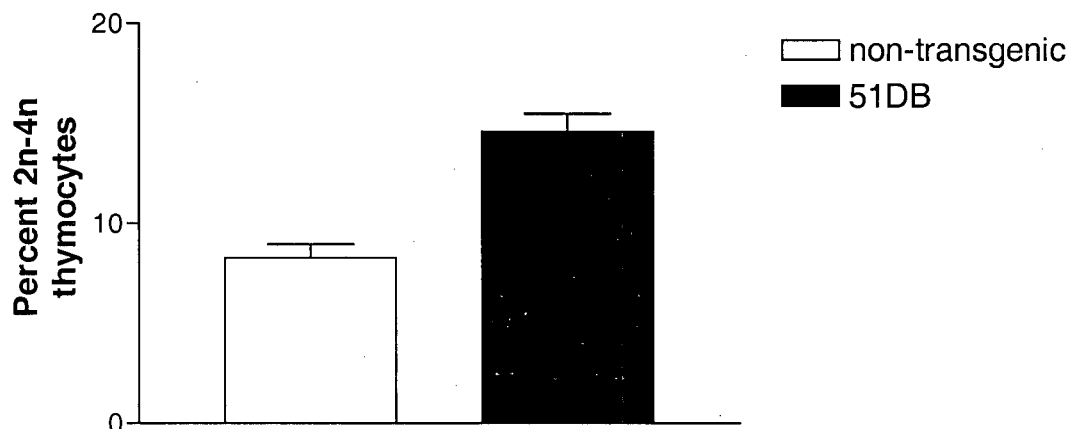
The scatter results indicated that Dbs transgenic thymocytes were larger and potentially hyperproliferative. To determine whether activated Dbs expression influences the cell cycle status of developing thymocytes, cells from 51DB thymuses were fixed, stained with PI and analyzed by flow cytometry. While the proportion of cells with $<2n$ DNA was similar to that of littermate controls, the proportion of thymocytes with $>2n$ DNA was about twice that of non-transgenic controls (**Figure 5.6**). These results suggest that thymocyte proliferation is elevated in thymocytes from Dbs transgenic mice.

Figure 5.6. Cell cycle analysis of Dbs thymocytes.

a) Thymocytes from non-transgenic and Dbs transgenic littermate mice from the 51DB line were isolated, fixed, incubated with propidium iodide and analyzed by flow cytometry. Proportions of thymocytes with $<2n$ and $2n-4n$ DNA are indicated.



a) Proportions of thymocytes with 2n-4n DNA from multiple experiments.



Rho family GTPases are involved in reorganization of the actin cytoskeleton. Rho family members induce specific filamentous actin (F-actin) cytoskeletal changes and as a result, Rho proteins are likely to play a role in cellular processes wherever F-actin is used. Measurement of F-actin content is an indirect measure of Rho family GTPase activity and phalloidin is a fungal toxin that irreversibly binds F-actin. To test the hypothesis that activated Dbs expression causes increased levels of F-actin within cells, thymocytes were incubated with antibodies directed against cell surface antigens, fixed, permeabilized and incubated with FITC-phalloidin and analyzed by flow cytometry. The mean fluorescence intensities (MFI) of DN, DP and SP thymocyte populations from mice from the 51DB line were higher than non-transgenic controls (**Figure 5.7**). To determine whether the increased MFI values were simply the result of a higher frequency of large (high FSC) cells in Dbs transgenic thymuses, low (small; gate 1) and high (large; gate 2) FSC cells from 51DB and non-transgenic thymuses were gated and their MFI values were measured. Small and large FSC DP, CD4 SP and CD8 SP thymocytes from Dbs transgenic mice displayed higher MFI values (**Figure 5.8**). Thus, Dbs transgenic thymocytes contained increased levels of filamentous actin and this was independent of cell size.

Figure 5.7. Thymocyte filamentous actin (F-actin) content. Thymocytes from Dbs transgenic and non-transgenic littermate mice from the 51DB transgenic line were isolated, incubated with antibodies directed against CD4 and CD8, fixed, permeabilized, incubated with FITC-phalloidin and analyzed by flow cytometry. Mean fluorescence intensities of DN, DP and SP thymocyte subsets are shown in the graph.

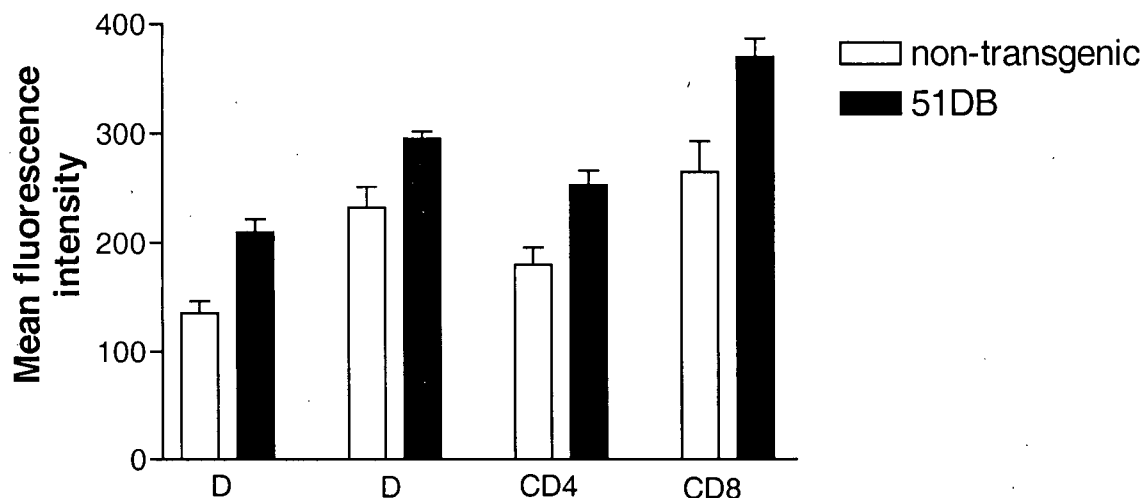
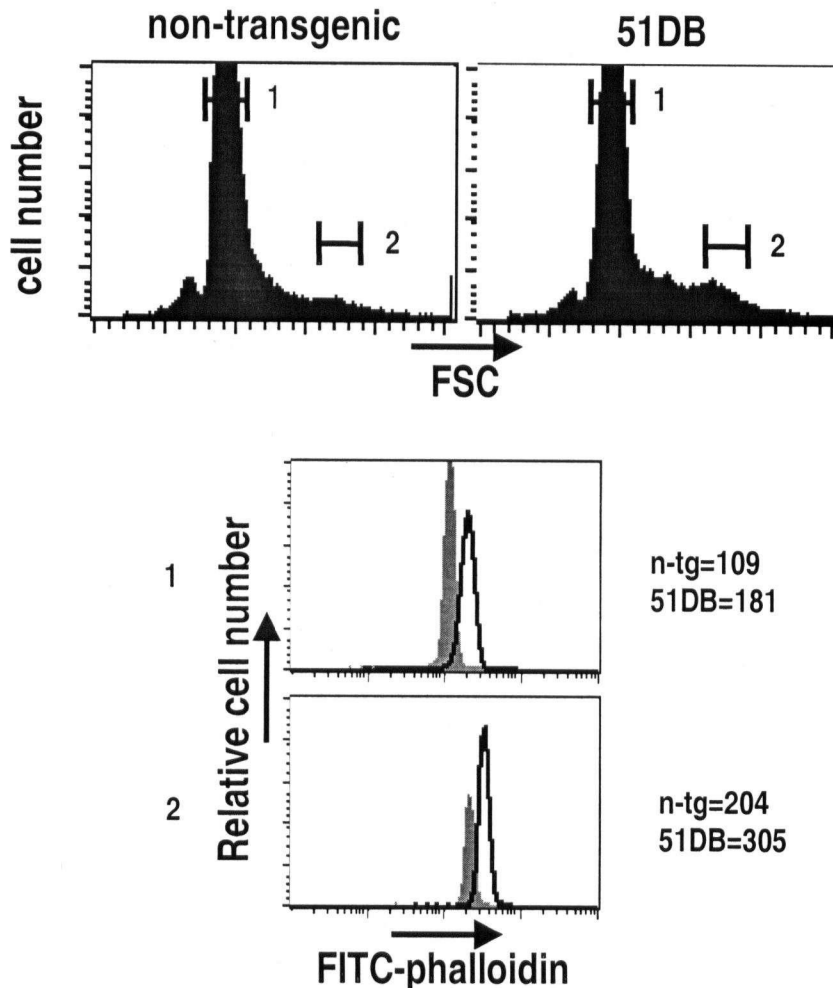


Figure 5.8. Thymocyte F-actin content is independent of cell size. Thymocytes from Dbs transgenic and non-transgenic littermate mice from the 51DB transgenic line were isolated, incubated with antibodies directed against CD4 and CD8, fixed, permeabilized, incubated with FITC-phalloidin and analyzed by flow cytometry. Low (1) and high (2) FSC thymocytes representing small and large cells (upper panel), respectively, were gated and F-actin content measured by flow cytometry (Lower panel). Histogram overlays indicate fluorescence intensities of non-transgenic (solid gray) and Dbs transgenic (black line) thymocytes after gating on either low (1) or high (2) FSC cells (lower panel). Numbers indicate histogram peak mean fluorescence intensities.

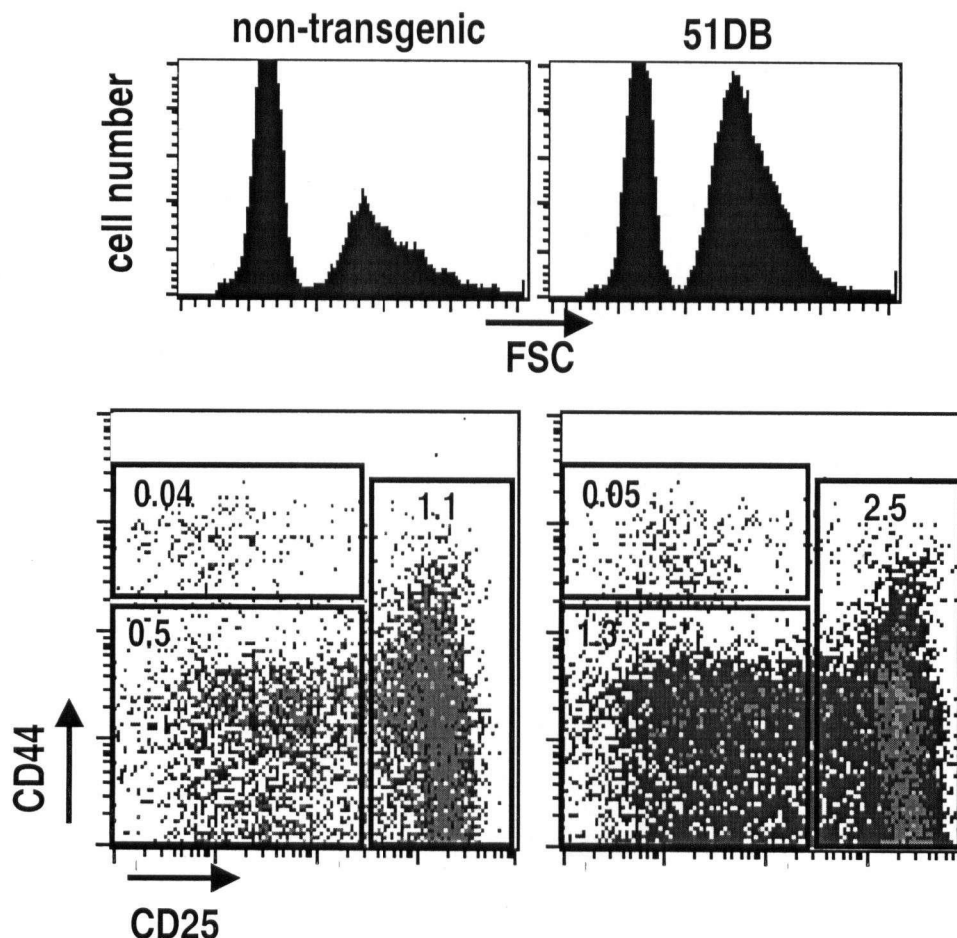


Inhibition of Rho function causes a developmental block in DN thymocytes. DN thymocytes are negative for CD4 and CD8 antigens but differentially express CD44 and CD25 antigens. The earliest DN thymocytes are CD44⁺ and CD25⁻. The next DN developmental subset is characterized by an up-regulation of CD25 (CD44⁺CD25⁺) and the transition to the next developmental stage is marked by down-regulation of CD44 and rearrangement of TCR β . Upon successful TCR β rearrangement, DN thymocytes down-regulate CD25 and differentiate into DP thymocytes through expression of CD4 and CD8. Dbs transgenic mice display an increased proportion of DN thymocytes. To determine the effect

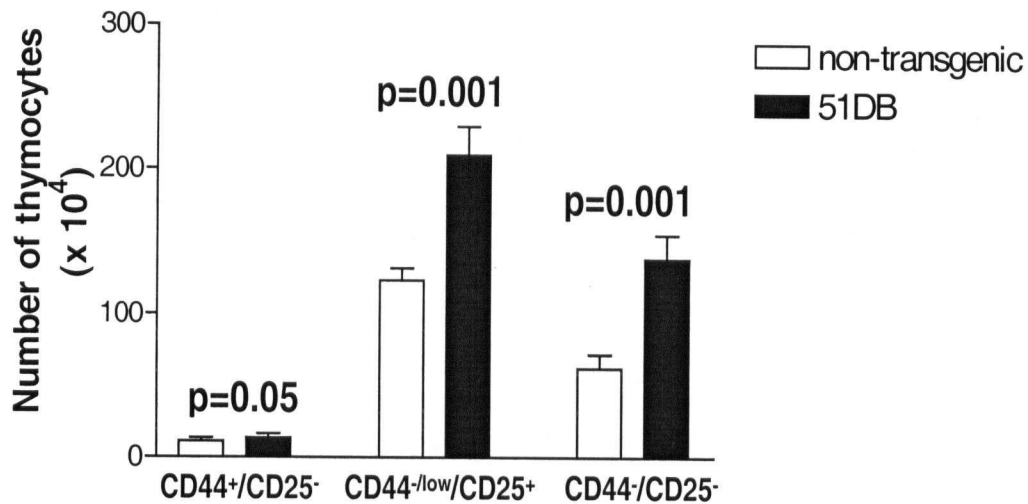
of activated Dbs expression on DN thymocyte development, cells from 51DB and 67DB mice were isolated and incubated with antibodies directed against markers for DP and SP thymocytes, non-T lymphocyte lineages and CD44 and CD25 and analyzed by flow cytometry. DN thymocytes from Dbs transgenic mice were characteristically high FSC (**Figure 5.9**). After gating out non-DN thymocyte populations, the proportions and numbers of CD44⁺/CD25⁻ were equivalent to those of non-transgenic thymocytes (**Figure 5.9**). Proportions and numbers of the CD44^{low}/CD25⁺ and CD44⁻/CD25⁻ DN thymocytes however were twice that of controls (**Figure 5.9**). Thus, activated Dbs expression increased the numbers of late stage DN thymocytes.

Figure 5.9. DN thymocytes analysis from Dbs transgenic mice.

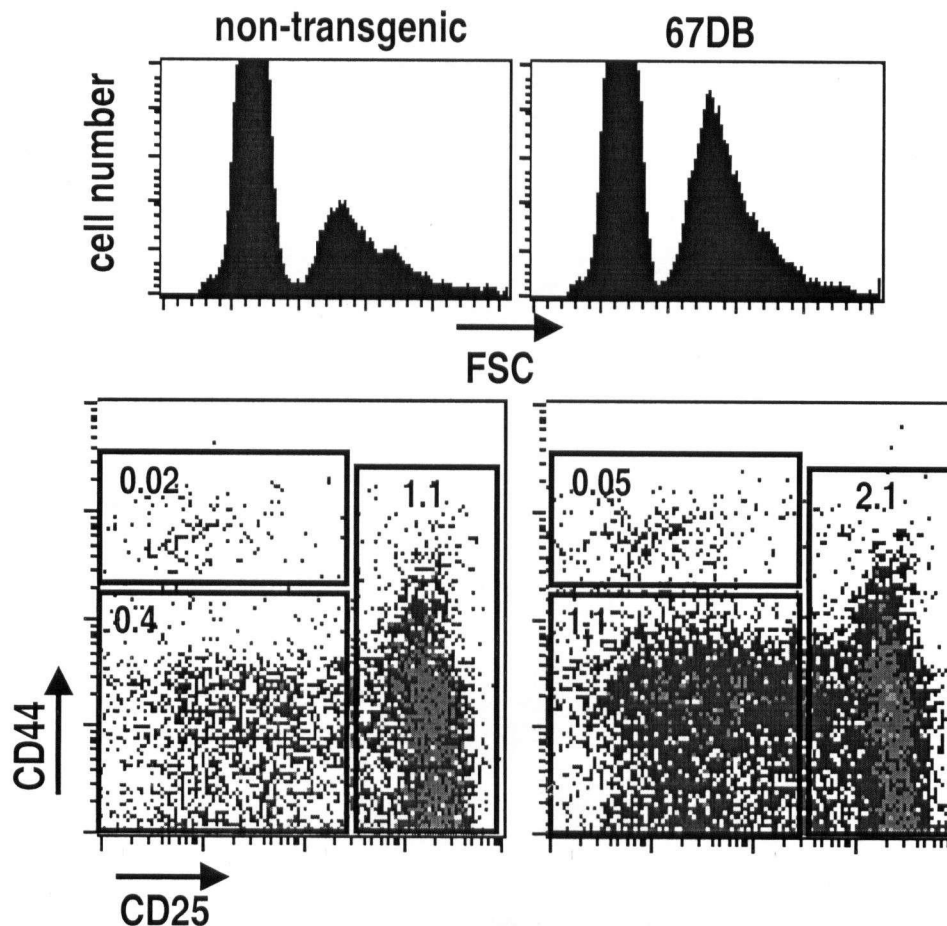
a) Flow cytometric analysis of DN thymocytes from the 51DB transgenic line. Thymocytes from Dbs transgenic and non-transgenic littermate mice from the 51DB transgenic line were isolated and incubated with antibodies directed against non-DN thymocytes (CD4, CD8, CD3, B220, Mac-1, Gr-1), CD44 and CD25 and analyzed by flow cytometry. DN thymocyte (lineage; CD4⁻, CD8⁻, CD3⁻, B220⁻, Mac-1⁻, Gr-1⁻) FSC measurements are shown in the histograms (upper panel). CD44 and CD25 expression by DN thymocytes (lineage⁻) are shown in the density plots (lower panel). Numbers indicate percent live-gated thymocytes.



b) Total CD44⁺/CD25⁻, CD44^{low/-}/CD25⁺, CD44⁻/CD25⁻ DN thymocytes from the 51DB transgenic line. Numbers were calculated by multiplying proportions of DN subsets by the total thymocytes. Numbers indicate the probability value associated with a paired t-test.

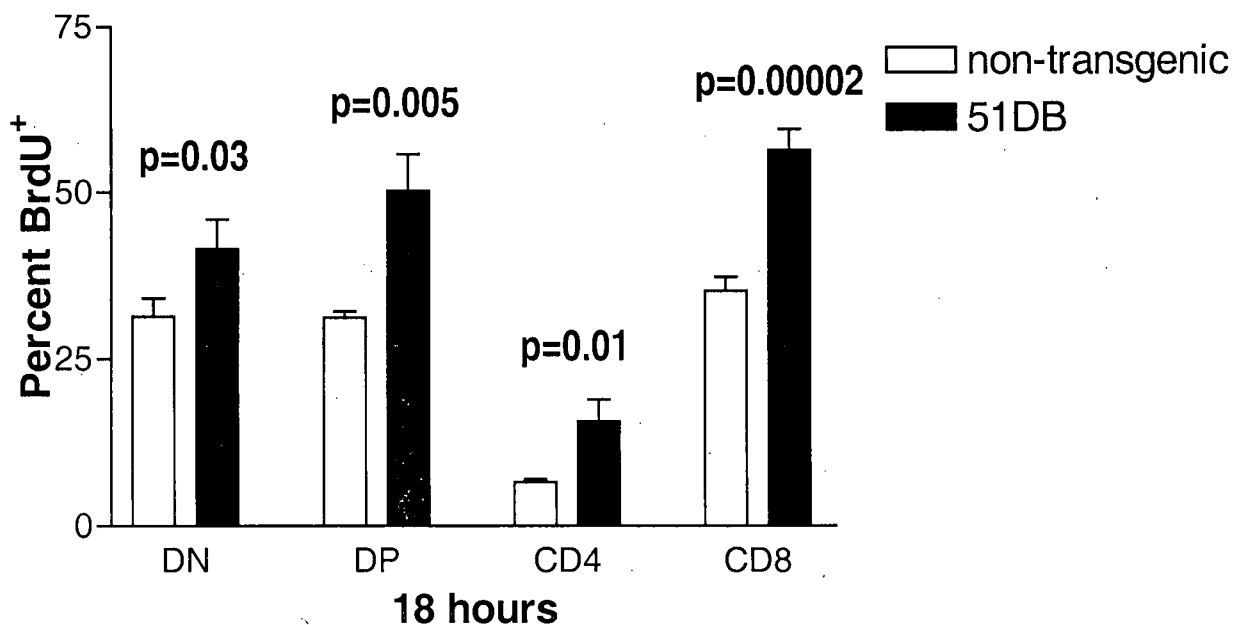


c) Flow cytometric analysis of DN thymocytes from the 67DB transgenic line. Thymocytes from Dbs transgenic and non-transgenic littermate mice from the 67DB transgenic line were isolated and incubated with antibodies directed against non-DN thymocytes (CD4, CD8, CD3, B220, Mac-1, Gr-1), CD44 and CD25 and analyzed by flow cytometry. DN thymocyte (lineage⁻; CD4⁻, CD8⁻, CD3⁻, B220⁻, Mac-1⁻, Gr-1⁻) FSC measurements are shown in the histograms (upper panel). CD44 and CD25 expression by DN thymocytes (lineage⁻) are shown in the density plots (lower panel). Numbers indicate percent live-gated thymocytes.



DN thymocytes from Dbs transgenic mice are increased in numbers and accumulation may be the result of increased proliferation and/or enhanced survival of these cells. As indicated previously, Dbs transgenic thymocytes are characteristically high FSC and a larger proportion of total thymocytes from Dbs transgenic mice contain 2n-4n DNA. Accumulated Dbs transgenic DN thymocytes may therefore be a result of increased proliferation of cells within this subset. To test the hypothesis that the Dbs transgene increases DN thymocyte proliferation, mice were injected with BrdU and thymocyte BrdU incorporation was measured by flow cytometry 18 hours later. Proliferating cells accumulate BrdU into their DNA upon each cell division. BrdU accumulation at 18 hours was elevated by about 10% in DN thymocytes from 51DB mice (**Figure 5.10**). Moreover, the proportions of BrdU⁺ DP, CD4 and CD8 SP thymocytes were increased by about two-fold in 51DB mice (**Figure 5.10**). Together the cell cycle and BrdU incorporation experiments reveal that thymocytes are proliferating more rapidly in Dbs transgenic mice and that proliferation is not restricted to the DN subset.

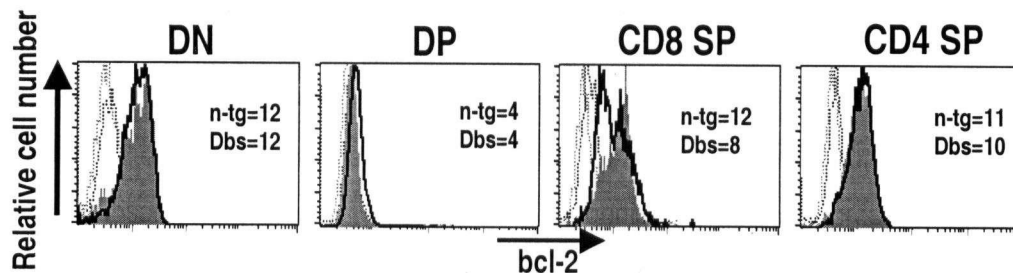
Figure 5.10. Thymocyte BrdU incorporation. Non-transgenic and transgenic mice from the 51DB transgenic line were injected with BrdU and 18 hours later thymocytes were isolated, incubated with antibodies directed against CD4 and CD8, fixed, permeabilized and incubated with antibodies directed against BrdU and analyzed by flow cytometry. The percent BrdU⁺ cells within each thymocyte subset are represented in the graph. Numbers indicate the probability value associated with a paired t-test.



Dbs transgenic thymocytes may also display defects in survival. Thymocyte and peripheral T cell susceptibility to apoptosis is influenced by expression of bcl-2 family members, some of which are expressed in a developmentally patterned manner. Bcl-2 is an anti-apoptotic protein expressed in DN

and SP, but not DP thymocytes [339]. Moreover, increased expression of *bcl-2* has been shown to enhance survival of thymocytes [340] [341]. To test the hypothesis that activated *Db*s expression increases *bcl-2* expression in transgenic thymocytes, *bcl-2* levels were measured by flow cytometry in thymocyte subsets from non-transgenic and *Db*s transgenic mice. *Bcl-2* expression levels in *Db*s transgenic DN, DP and CD4 SP thymocytes were equivalent to those of non-transgenic controls. CD8 SP thymocytes, however expressed lower levels of *bcl-2* (**Figure 5.11**).

Figure 5.11. Thymocyte *bcl-2* expression. Thymocytes from non-transgenic and *Db*s transgenic littermates were isolated, incubated with antibodies directed against CD4 and CD8, fixed, permeabilized, incubated with either antibodies directed against *bcl-2* or an isotype control, incubated with a fluorochrome conjugated secondary antibody and analyzed by flow cytometry. Histogram overlays indicate fluorescence intensities of anti-*bcl-2*-incubated non-transgenic (solid gray) and *Db*s transgenic (black line) thymocytes within each thymocyte subset. Numbers indicate mean fluorescence intensities of solid gray (non-transgenic; n-tg) and *Db*s transgenic (black line; *Db*s) histogram peaks. Broken line histograms indicate fluorescence intensities of isotype control-incubated non-transgenic (gray broken line) and *Db*s transgenic (black broken line) thymocytes.

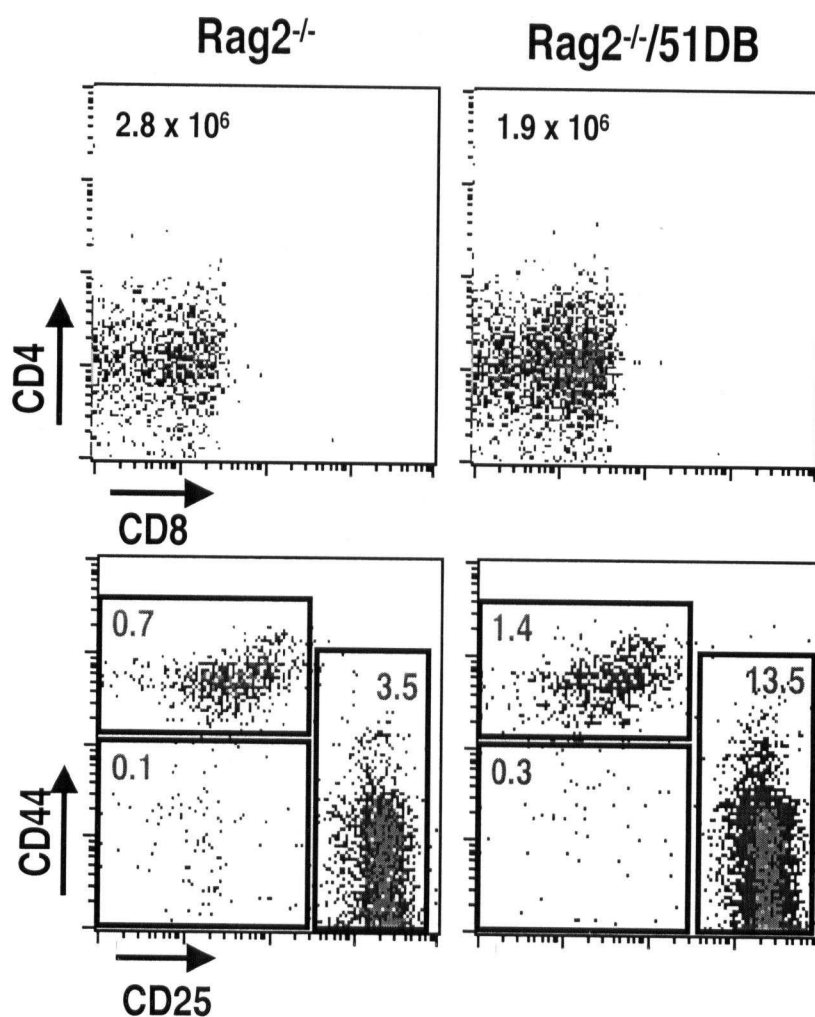


TCR β gene rearrangements normally begin at the CD44^{low}/CD25⁺ stage of DN thymocyte development. Upon successful TCR β gene rearrangements, DN thymocytes express a pre-TCR, down-regulate CD25 and differentiate into DP thymocytes. Accumulation of DN thymocytes in thymuses from *Db*s transgenic mice appears to occur during and after TCR β gene rearrangement. To determine if activated *Db*s expression contributes to the differentiation signal provided by pre-TCR signaling, I tested the ability of the *Db*s transgene to over-ride the DN to DP differentiation block that occurs in *Rag2*^{-/-} mice. *Rag2*^{-/-} lymphocytes do not initiate V(D)J rearrangement; consequently, thymocytes are blocked at the DN3 stage of thymocyte development, prior to rearrangement of the TCR β gene [321]. *Db*s transgenic mice were crossed with *Rag2*^{-/-} and *Db*s/*Rag2*^{+/-} offspring were backcrossed to *Rag2*^{-/-} mice. Thymocytes from *Rag2*^{-/-} mice with or without the *Db*s transgene were analyzed. Because of their inability to express the TCR β chain of the pre-TCR complex, *Rag2*^{-/-} mice have virtually no DP thymocytes. *Db*s transgenic *Rag2*^{-/-} mice also do not have any DP thymocytes

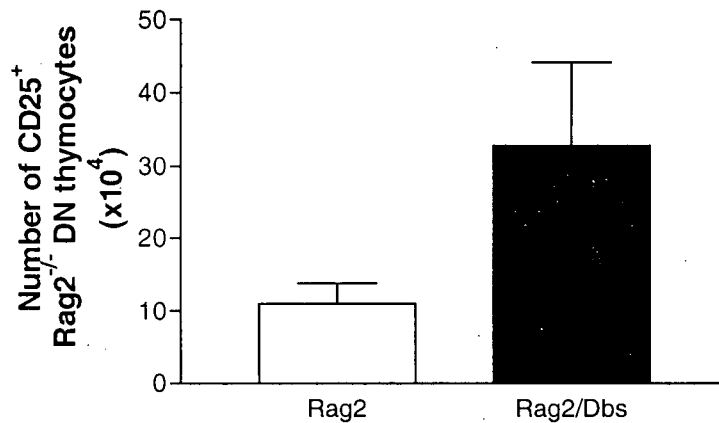
(Figure 5.12). Thus the Dbs transgene does not promote differentiation of DN thymocytes to DP thymocytes in the absence of functional pre-TCR. Although the developmental block is maintained in the presence of the Dbs transgene, the proportions and numbers of CD44^{low}/CD25⁺ thymocytes are elevated in Dbs transgenic Rag2^{-/-} mice (Figure 5.12). The Dbs transgene therefore enables accumulation of DN thymocytes prior to initiation of TCR β gene rearrangement, however it does not over-ride the requirement for functional pre-TCR. Thus differentiation beyond the DN3 stage of thymocyte development is unaffected by the presence of the Dbs transgene.

Figure 5.12. Rag2^{-/-} thymocytes.

a) Flow cytometric analysis. Thymocytes from Rag2^{-/-} and 51DB/Rag2^{-/-} littermates were isolated and incubated with antibodies directed against CD4 and CD8 and analyzed by flow cytometry (upper panel). Total thymocytes from each mouse are indicated in the upper left corner. Thymocytes were also incubated with antibodies directed against non-DN thymocytes (lineage⁺; CD4⁺, CD8⁺, CD3⁺, B220⁺, Mac-1⁺, Gr-1⁺), CD44 and CD25 and analyzed by flow cytometry. CD44 and CD25 expression by DN thymocytes (lineage⁻) are shown in the density plots (lower panel). Numbers in rectangles indicate percent total thymocytes.

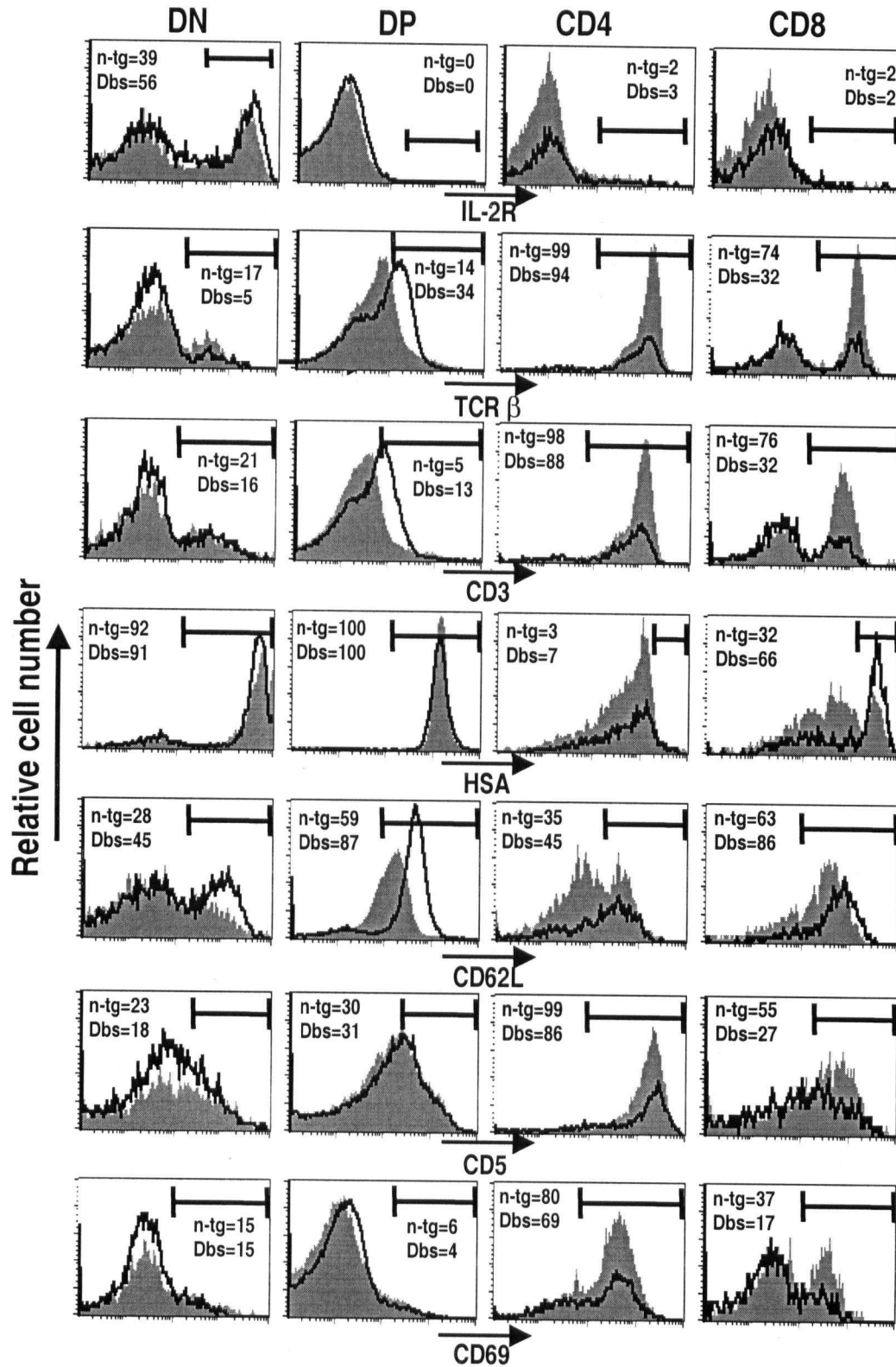


b) Total $CD44^{low/-}/CD25^{+}$ DN thymocytes from the $Rag2^{-/-}$ and $51DB/Rag2^{-/-}$ mice. Numbers were calculated by multiplying proportions of $CD44^{low/-}/CD25^{+}$ DN thymocytes subsets by total thymocytes.



To evaluate the maturity of transgenic thymocytes, DN, DP, CD8 SP and CD4 SP thymocytes from 51DB mice were isolated and analyzed for cell surface expression of CD25 (IL-2R), TCR β , CD3, HSA (CD24), CD62L, CD5 and CD69 by flow cytometry. CD25 is expressed by a subset of DN thymocytes during TCR β rearrangement and mature T cells following activation. As indicated previously, a higher proportion of Dbs transgenic DN thymocytes express CD25 (**Figures 5.9 and 5.13**). DP and SP thymocytes do not normally express CD25 and expression levels of this marker by these populations were equivalent to non-transgenic controls (**Figure 5.13**). TCR β and CD3 ϵ are components of the TCR and CD3 ϵ is expressed at low levels prior to and after TCR β chain rearrangement in DN thymocytes. Importantly, TCR β is expressed with CD3 ϵ by DN thymocytes at very low levels upon productive TCR β gene rearrangement and expression of pre-T α : β receptors. The proportions of TCR β - and CD3 ϵ -expressing DN thymocytes are lower in Dbs transgenic mice. Upon differentiation into DP thymocytes however, TCR β and CD3 ϵ are expressed at intermediate levels. Expression of these two markers by Dbs transgenic DP thymocytes, as indicated by histogram peak fluorescence intensities and percent positive cells (**Figure 5.13**), were elevated when compared with non-transgenic controls. Normally, TCR β and CD3 ϵ levels increase on thymocytes with maturation from the DP to SP developmental stages. Despite increased TCR marker expression by DP thymocytes, the proportion and expression levels of TCR β and CD3 ϵ were normal on CD4 SP thymocytes and reduced slightly on CD8 SP thymocytes when compared to non-transgenic control thymocytes (**Figure 5.13**).

Figure 5.13. Thymocyte developmental marker expression. Thymocytes from *Db*s transgenic and non-transgenic littermate mice from the 51DB transgenic line were isolated, incubated with antibodies directed against CD4, CD8 and CD25/IL-2R, TCR β , CD3, HSA, CD62L, CD5 or CD69 and analyzed by flow cytometry. Histogram overlays indicate fluorescence intensities of non-transgenic (solid gray) and *Db*s transgenic (black line) thymocytes within each thymocyte subset. Numbers indicate percent of thymocytes within each marker region.



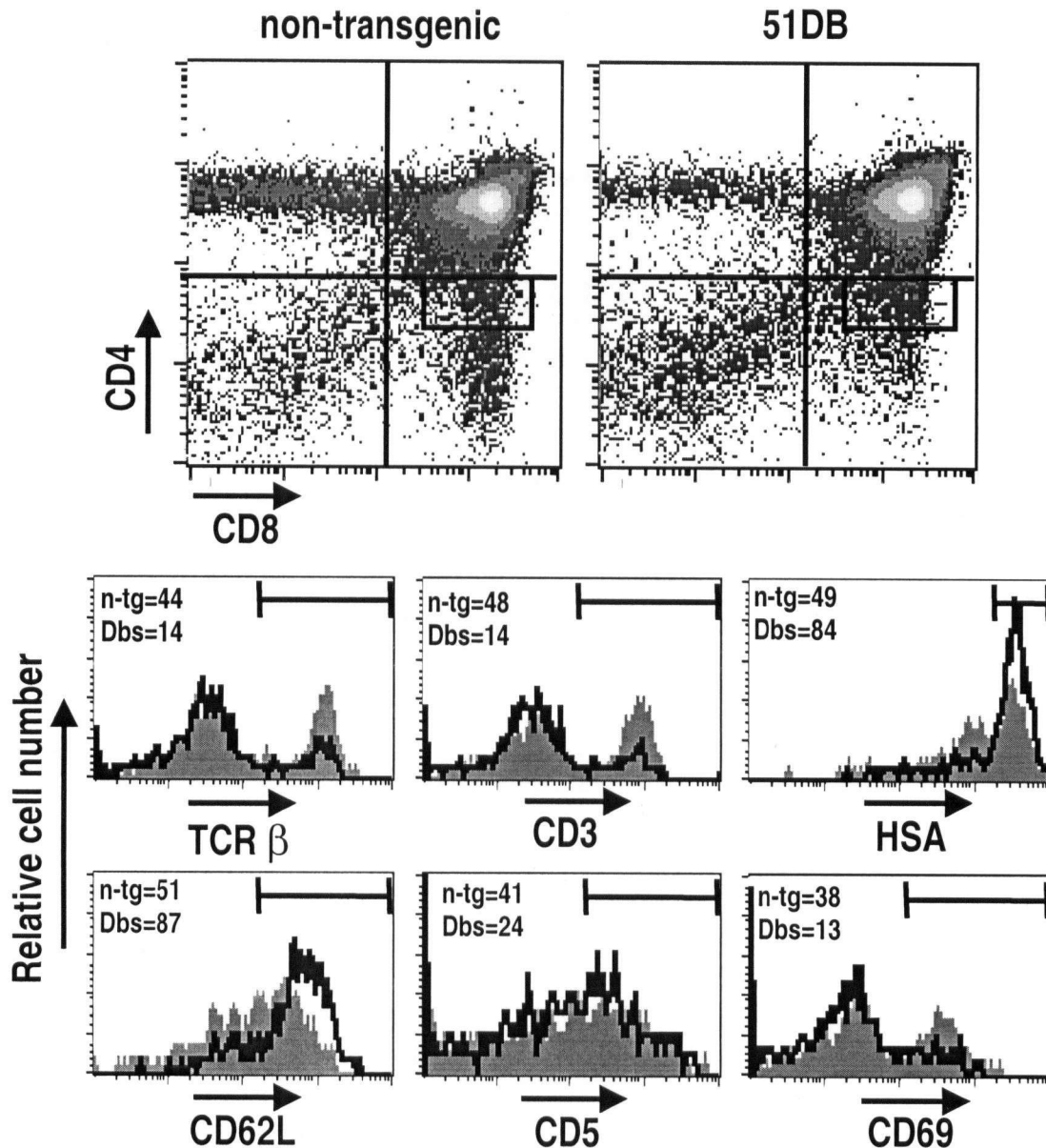
Following maturation from DPs, SP thymocytes can be further subdivided with the more mature cells bearing lower levels of cell surface HSA [342]. While expression of HSA by DN, DP and CD4 SP thymocytes were equivalent to those of normal mice, a higher proportion of transgenic SP thymocytes expressed HSA (**Figure 5.13**). Interestingly, HSA expression levels by the majority of transgenic CD8 SP thymocytes were similar to those of DP thymocytes. TCR signal intensity during development parallels CD5 surface level expression. CD5 expression normally increases after positive selection and has been reported to correlate with TCR signaling intensity [343]. Expression of CD5 by DN, DP and CD4 SP thymocytes were equivalent to those of normal mice, however a lower proportion of CD8 SP thymocytes expressed high levels of CD5 (**Figure 5.13**). CD5 expression by transgenic CD4 SP thymocytes however was elevated. CD62L is up-regulated in the latest stages of SP thymocyte development and is involved in mature thymocyte export to the periphery [344] [345]. DN, DP, CD4 and CD8 SP thymocytes from Dbs transgenic mice express higher levels of CD62L (**Figure 5.13**). During positive selection of DP thymocytes CD69 is transiently expressed and CD4⁺/CD8⁺/CD69⁺ thymocytes have initiated, but not yet completed, positive selection [250]. While expression levels of CD69 by DN, DP and CD4 SP thymocytes are equivalent to those of normal mice, the proportion of CD69⁺ CD8 SP thymocytes is reduced in Dbs transgenic mice (**Figure 5.13**).

CD8 SP thymocytes from 51DB mice that expressed the lowest levels of CD4 were depleted more than CD8 SP thymocytes expressing low levels of CD4. To determine whether the expression patterns of TCR β , CD3, HSA, CD62L, CD5 and CD69 differed between these two populations of CD8 SP thymocytes, CD4^{low}CD8⁺ and CD4⁻CD8⁺ thymocytes were gated and marker expression was measured. Although expression patterns were similar to what was described in **Figure 5.13**, the magnitude of the differences between non-transgenic and transgenic thymocytes were more pronounced in the CD4^{low} CD8 SP population than in the CD4⁻CD8 SP population. Fewer transgenic CD4^{low} CD8 SP thymocytes expressed TCR β , CD3, CD5 and CD69 than transgenic CD4⁻CD8 SP thymocytes (**Figure 5.14**). More transgenic CD4^{low} CD8 SP thymocytes expressed HSA than transgenic CD4⁻CD8 SP thymocytes (**Figure 5.14**). Although expression of CD62L by CD8 SP thymocytes was elevated in transgenic thymocytes, the proportion of CD62L^{high} thymocytes in the

Figure 5.14. Developmental marker expression by $CD4^{low}/CD8^{+}$ and $CD4^{-}/CD8^{+}$ thymocytes.

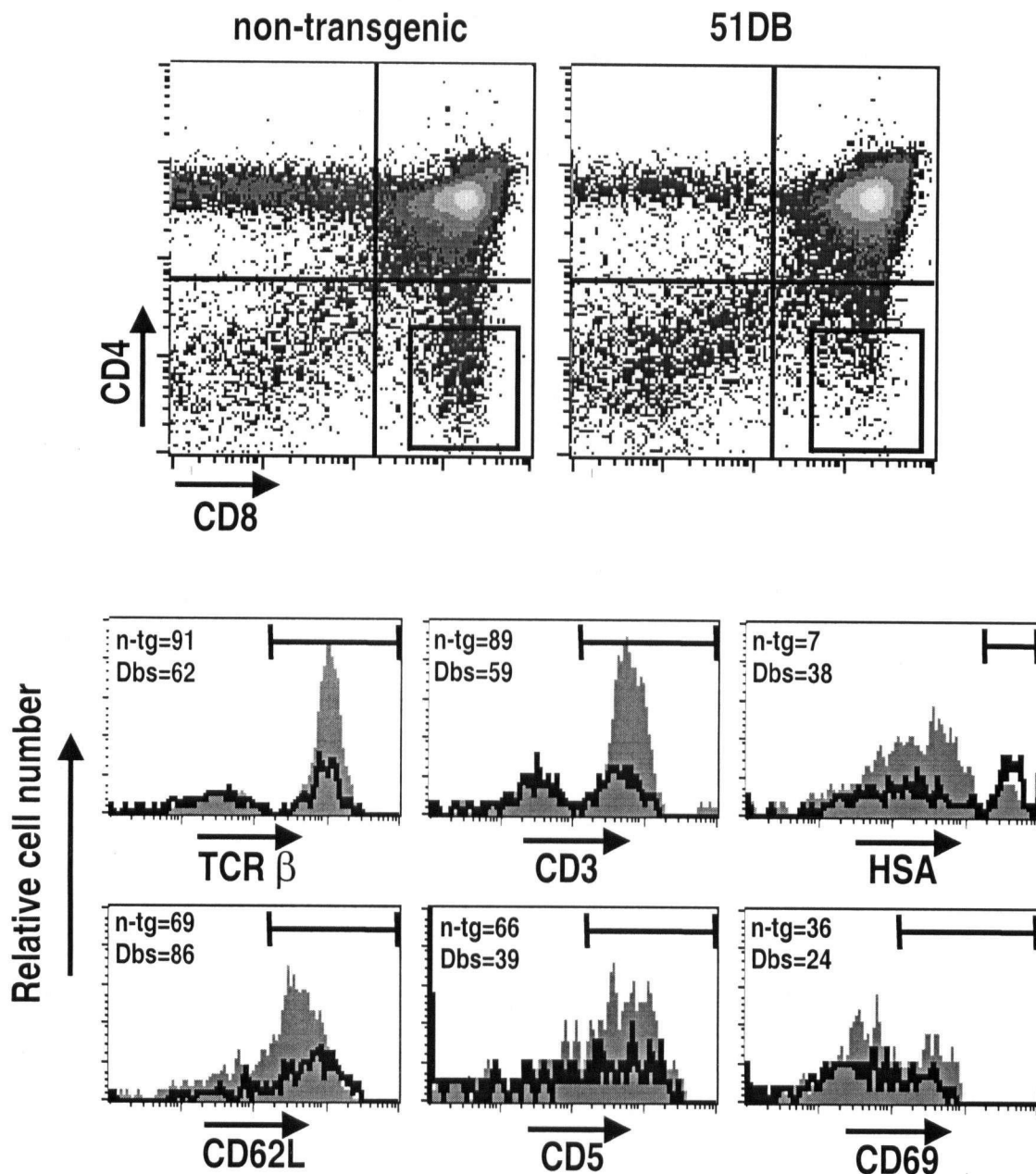
Thymocytes from Dbs transgenic and non-transgenic littermate mice from the 51DB transgenic line were isolated, incubated with antibodies directed against CD4, CD8 and TCR β , CD3, HSA, CD62L, CD5 or CD69 and analyzed by flow cytometry. Histogram overlays indicate fluorescence intensities of non-transgenic (solid gray) and Dbs transgenic (black line) thymocytes within the indicated gated population. Numbers indicate percent of thymocytes within each marker region.

a) $CD4^{low}/CD8^{+}$ thymocyte gate.



$CD4^{low}$ and $CD4^{-}$ $CD8^{+}$ thymocyte populations were equivalent (Figure 5.14). Transgenic $CD8^{+}$ thymocytes that expressed the lowest levels of CD4 more closely resembled normal thymocytes in terms of expression of these developmental markers.

b) CD4/CD8⁺ thymocyte gate.

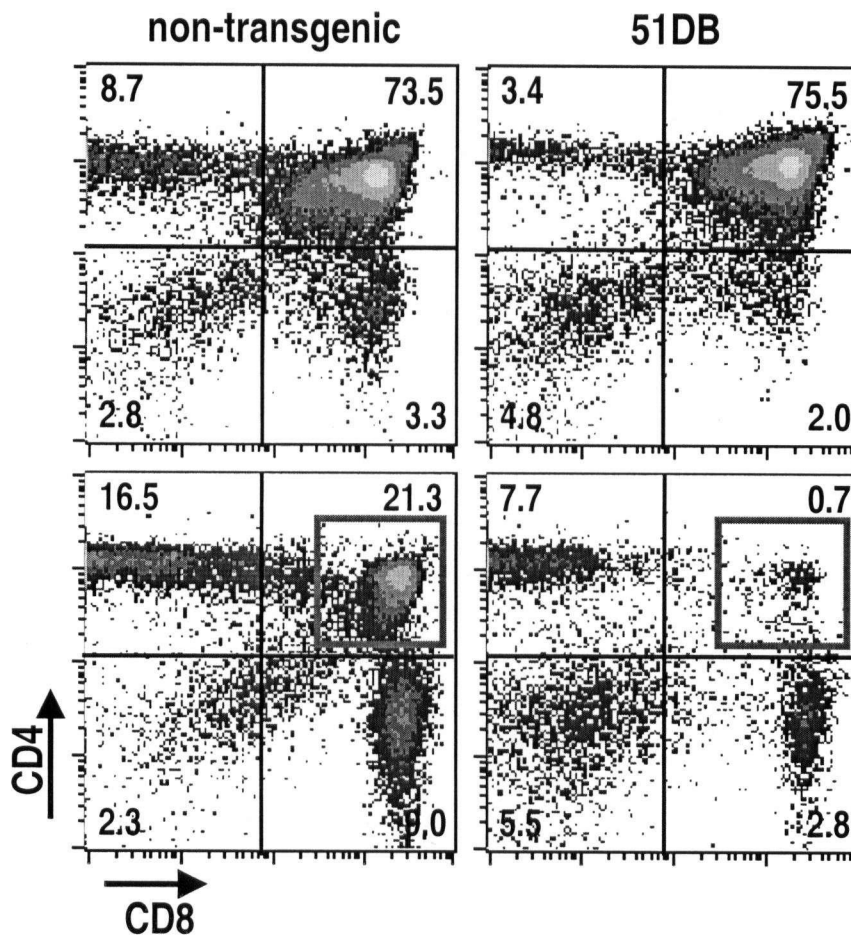


During thymocyte differentiation, thymocytes are positively selected for self-MHC recognition and negatively for recognition of endogenous MHC-presented peptides and most die by apoptosis as a consequence of negative selection. DbS transgenic DP thymocytes expressed increased levels of TCR components including CD3 and TCR β. Immature DP thymocytes are susceptible to a variety of cell death-inducing stimuli, including anti-CD3 cross-linking. To assess whether this form of apoptosis is affected by activated DbS expression, monoclonal antibodies with specificity for the TCR associated CD3 complex were intra-peritoneally injected into mice and thymocytes were analyzed at 48 hours. Cross-linking of surface CD3ε on DbS transgenic thymocytes resulted in a reduction in the total number

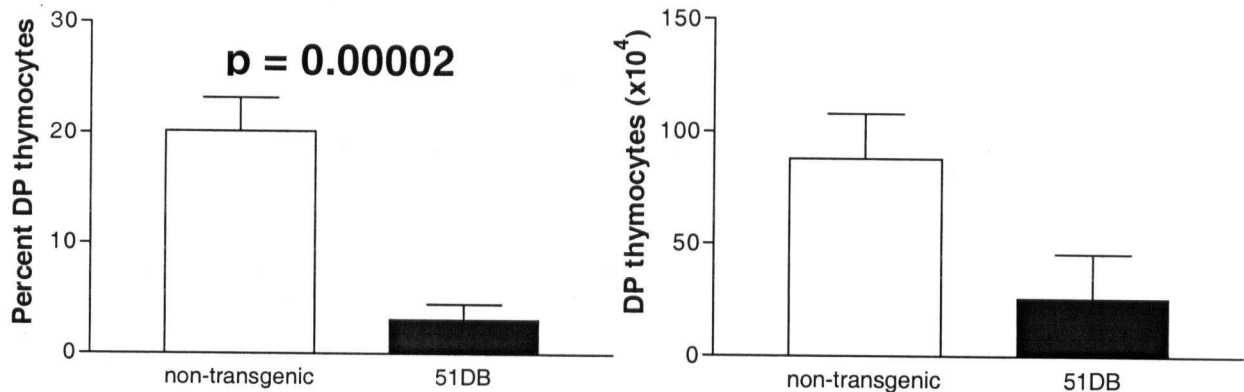
of thymocytes (**Figure 5.15**). Analysis of thymocyte CD4 and CD8 expression revealed that the proportion and numbers of DP thymocytes were reduced in 51DB thymuses 48 hours after injection of anti-CD3 (**Figure 5.15**). Activated Dbs expression thus increased the severity of anti-CD3-mediated DP thymocyte apoptosis.

Figure 5.15. Anti-CD3-mediated DP thymocyte apoptosis.

a) Flow cytometric analysis of thymocytes from the 51DB transgenic line. Non-transgenic or transgenic littermate mice were injected with either PBS (control) or anti-CD3 and thymocytes were isolated 48 hours later, incubated with antibodies directed against CD4 and CD8 and analyzed by flow cytometry. Quadrant numbers indicate the proportions of total thymocytes at 48 hours.

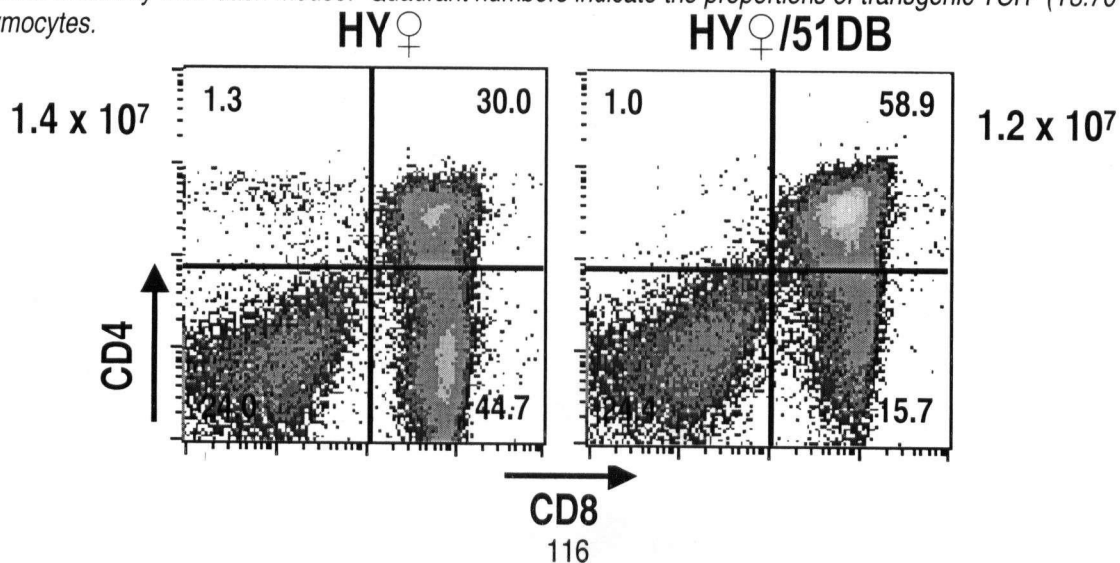


b) DP thymocyte calculations 48 hours after anti-CD3 injection. Percent total DP thymocytes are indicated on the left. Total DP thymocytes are indicated on the right. The number indicates the probability value associated with a paired t-test.



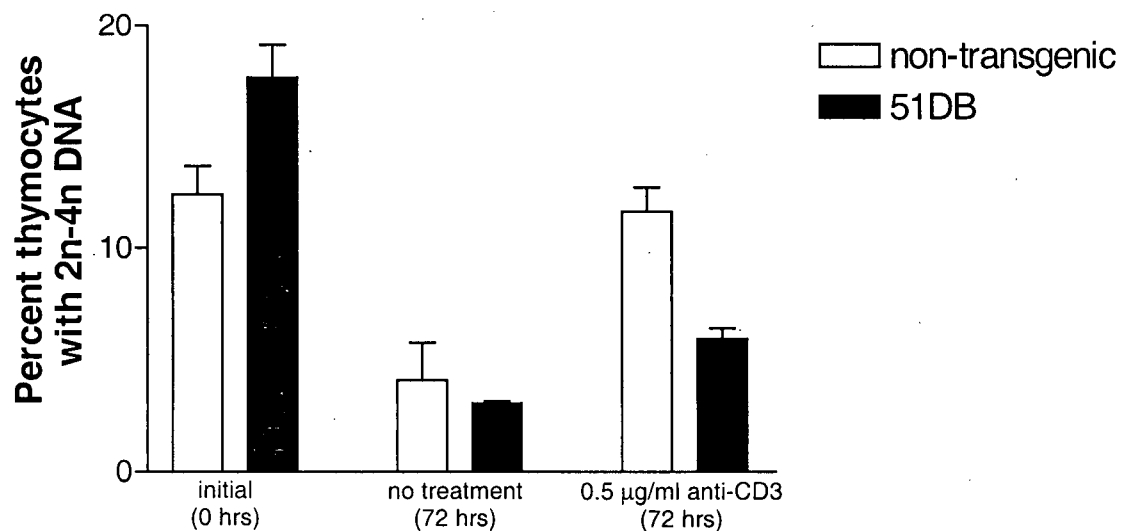
Reduced numbers of SP thymocytes in Dbs transgenic mice may also result from a reduced frequency of positive selection. In order to test the hypothesis that activated Dbs expression impairs positive selection, Dbs transgenic mice were crossed with TCR-HY transgenic mice. HY mice express a transgenic alpha beta T cell receptor (TCR) that recognizes a male specific antigen (HY) in the context of MHC Class I (H-2D^b) [346] [347]. Expression of the transgene in female mice normally results in a large fraction of CD8⁺ T cells with T cell receptors for HY antigen, while male mice are severely depleted of mature thymocytes. If activated Dbs expression impairs thymocyte positive selection, we would expect female offspring from these crosses to display reduced numbers of CD8 SP thymocytes. Female HY/Dbs doubly transgenic mice (HY ♀ /51DB) displayed reduced numbers of CD8 SP thymocytes (**Figure 5.16**). Activated Dbs expression therefore impairs positive selection, at least in the context of this positively selecting TCR transgenic system.

Figure 5.16. Transgenic TCR HY crosses. Six week old Dbs transgenic and TCR-HY mice were crossed and thymocytes from female offspring were incubated with antibodies directed against CD4, CD8 and an antibody specific for the TCR transgene (T3.70) and analyzed by flow cytometry. Exponential numbers indicate total thymus cellularity from each mouse. Quadrant numbers indicate the proportions of transgenic TCR⁺ (T3.70⁺) thymocytes.



To determine the effect of activated Dbs expression on the cell cycle status of thymocytes in the presence or absence of anti-CD3 in culture, DNA content was measured after 72 hours. A lower proportion of Dbs transgenic thymocytes contained $>2n$ DNA in the presence of high anti-CD3 concentrations (**Figure 5.17**). These results suggest that proliferative responses to anti-CD3 are impaired in transgenic thymocytes.

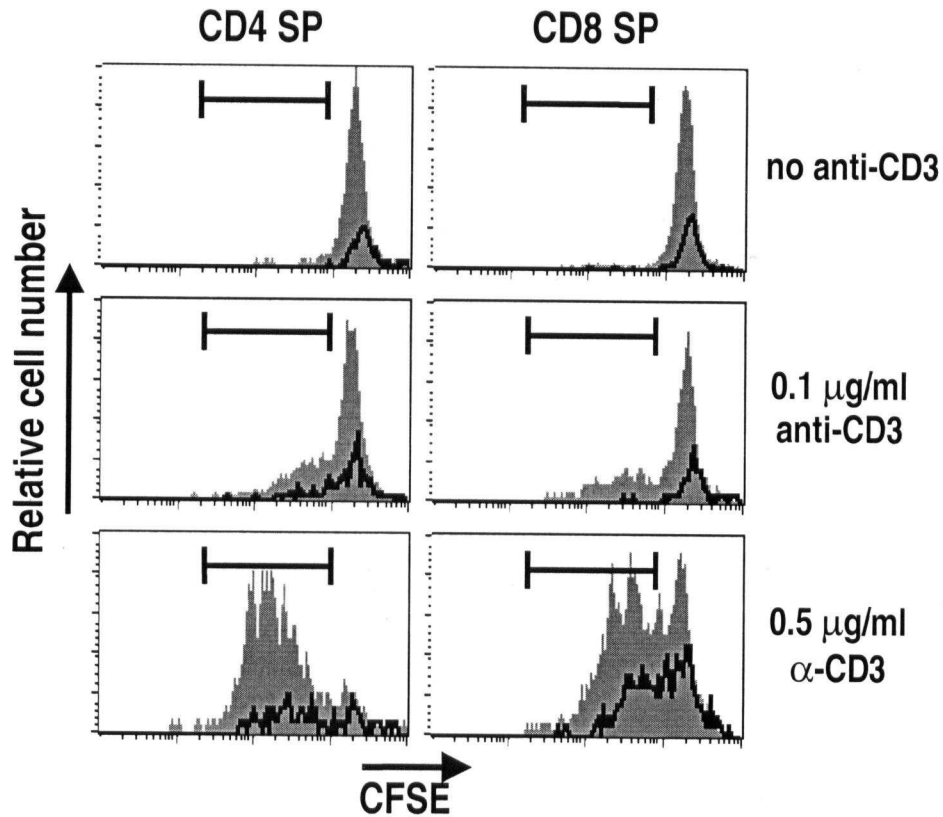
Figure 5.17. Cell cycle analysis of cultured thymocytes. Thymocytes from non-transgenic and Dbs transgenic mice from the 51DB line were isolated and cultured in serum-supplemented medium either with or without anti-CD3. 72 hours later, cultured thymocytes were isolated, fixed, incubated with propidium iodide and analyzed by flow cytometry. Bars represent the percent of $2n$ - $4n$ DNA-containing thymocytes initially (0 hours) and after 72 hours in culture with or without anti-CD3.



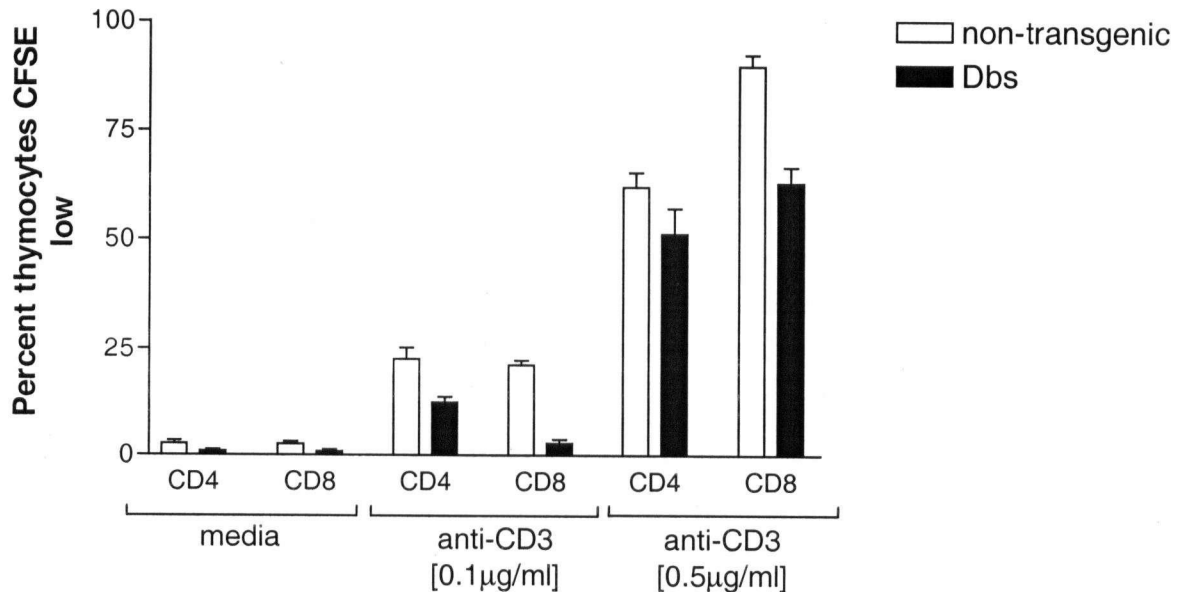
Carboxyfluorescein succinimidyl ester (CFSE) labeling prior to the initiation of cultures was also used to directly assess the proliferative history of the thymocyte subsets in the presence or absence of anti-CD3 during culture. In the absence of anti-CD3 there was no proliferation of CD4 SP or CD8 SP thymocytes from either non-transgenic or Dbs transgenic mice after 72 hours (**Figure 5.18**). In the presence of low and high anti-CD3 concentrations, the Dbs transgene caused reductions in proliferation of both CD4 and CD8 SP thymocytes (**Figures 5.18**). The Dbs transgene therefore impairs the proliferative responses of Dbs SP thymocytes in culture in the presence of TCR-mediated signaling.

Figure 5.18. CFSE analysis of cultured thymocytes. Thymocytes from non-transgenic and Dbs transgenic mice from the 51DB line were isolated, incubated with CFSE and cultured in serum-supplemented medium either with or without anti-CD3. 72 hours later, cultured thymocytes were isolated, incubated with antibodies directed against CD4 or CD8 and analyzed by flow cytometry.

a) Histogram overlays represent CFSE fluorescence at 72 hours in serum supplemented medium with or without anti-CD3 (non-transgenic = solid gray; Dbs transgenic = black line).

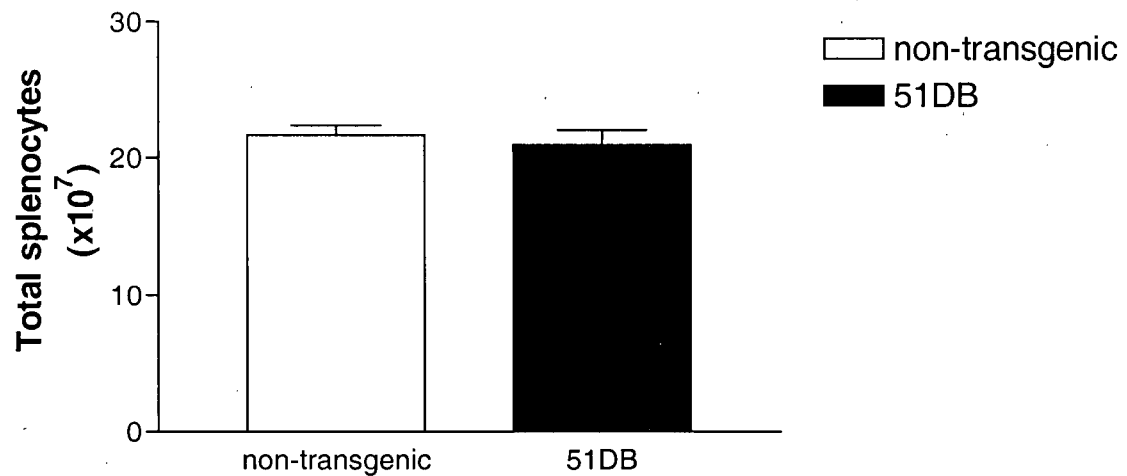


b) Bars represent the percent CFSE low (indicated by the marker region above) thymocytes after 72 hours in culture with or without anti-CD3.



SP thymocytes are present at reduced frequencies in Dbs transgenic thymuses. To determine the effect of activated Dbs expression on splenic cellularity, total spleen cells from 51DB mice were isolated and counted. Total numbers of splenocytes from 51DB transgenic mice were equivalent to those of non-transgenic controls (**Figure 5.19**).

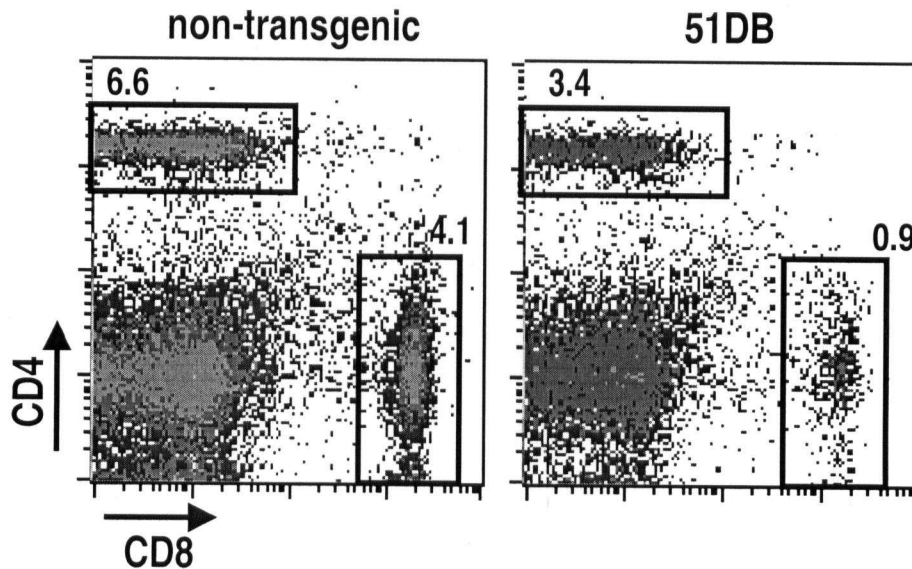
Figure 5.19. Spleen cell counts. Total cells from non-transgenic and Dbs transgenic mice from the 51DB line were counted.



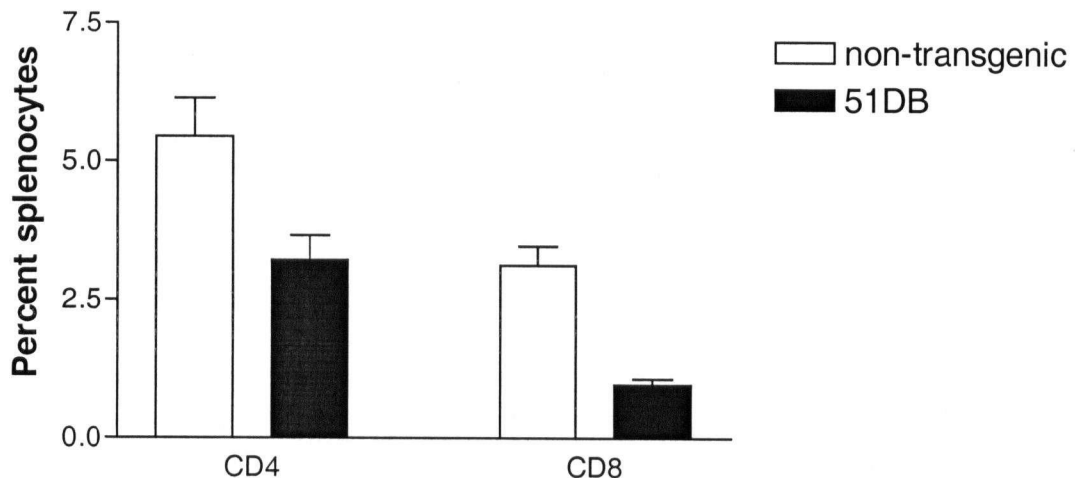
To determine the effect of activated Dbs expression on mature T cells, splenocytes were incubated with antibodies directed against CD4 and CD8 and analyzed by flow cytometry. CD4 and CD8 T cells were present at about half the normal numbers in Dbs transgenic mice (**Figure 5.20**).

Figure 5.20. Flow cytometric analysis of spleen cells.

a) Spleen cells were isolated, incubated with antibodies directed against CD4 and CD8 and analyzed by flow cytometry. Numbers indicate the percent total CD4⁺ and CD8⁺ T cells within spleens from non-transgenic and Dbs transgenic littermates from the 51DB line.



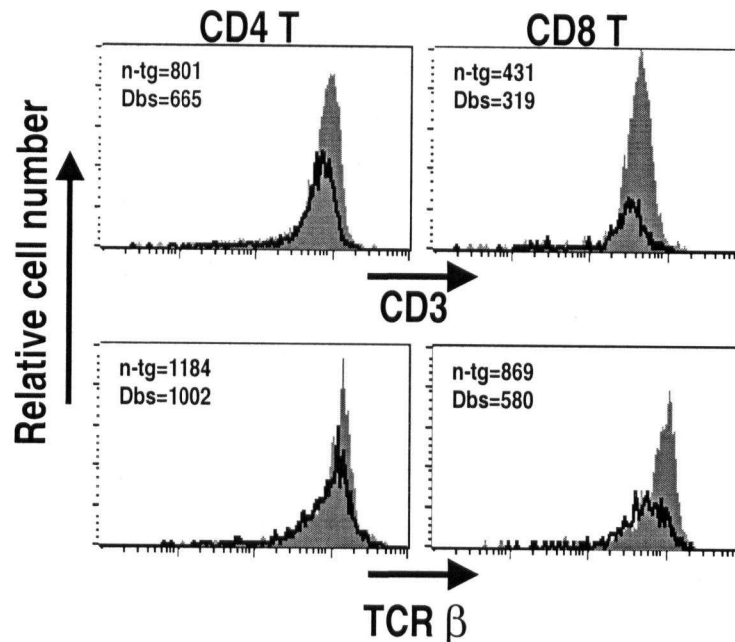
b) Bars represent the percent total CD4⁺ and CD8⁺ T cells within spleens from multiple non-transgenic and Dbs transgenic littermates from the 51DB line.



Expression levels of TCR β and CD3 by 51DB SP thymocytes were reduced slightly (**Figures 5.13** and **5.14**). To determine the effect of activated Dbs on TCR component expression by splenic T cells, expression of TCR β and CD3 were measured on CD4 and CD8 T cells from 51DB and non-transgenic

control mice. As indicated by MFI values, expression of CD3 and TCR β were reduced on 51DB CD4 and CD8 splenic T cells (**Figure 5.21**). Activated Dbs expression thus impairs the generation of normal numbers of mature SP thymocytes and mature peripheral T cells.

Figure 5.21. Flow cytometric analysis of splenic T cells. Spleen cells were isolated, incubated with antibodies directed against CD4 and CD8 and either CD3 ϵ or TCR β and analyzed by flow cytometry. Histogram overlays indicate fluorescence intensity of non-transgenic (solid gray) and Dbs transgenic (black line) CD4⁺ and CD8⁺ T cells from the 51DB transgenic line. Numbers represent the mean fluorescence intensity of the histogram peaks.



5.3 Discussion

Several members of the Rho family of small GTPases, including Rho, Rac1 and Cdc42, are involved in signaling during thymocyte and T cell development [272] [275] [296] [291] [278] (refer to section 1.5). Expression of an activated form of Dbs, a positive regulator of RhoA and Cdc42, also has a dramatic impact on T cell development. Notably, Dbs transgenic mice display increased numbers of DN thymocytes and reduced numbers of SP thymocytes. Although the Dbs transgene is expressed within the thymus from these mice, the relative expression levels within each of the thymocyte subsets is not known. DN thymocytes from Dbs transgenic mice accumulate at the latest stages of thymocyte development (DN3 and DN4), but not in the earliest stages. Assuming that the transgene is expressed in DN thymocytes from Dbs transgenic mice, then increased numbers of DN3 and DN4, but not DN1 or DN2 thymocytes, may simply reflect the expression profile of the transgene. Activated Dbs transgene expression is under the control of a *TCR β* promoter and endogenous *TCR β* gene expression begins during the DN2/DN3 stages (CD44^{low}/-/CD25⁺) [348]. Thus if transgene expression resembles that of the endogenous *TCR β* gene, then the Dbs transgene may not be expressed in the earlier stages of thymocyte development. This may explain why the accumulation is restricted to the later stages of thymocyte development in transgenic mice. It is also possible that the immunoglobulin μ enhancer extends transgene expression to an earlier stage of development. Detailed expression analysis of the transgene will be useful in further interpretation of the DN thymocyte phenotype displayed by Dbs transgenic mice.

Interestingly, transgenic mice expressing activated mutants of either RhoA or Cdc42 display similar increases in numbers of DN thymocytes [278] [296]. Dbs-mediated activation of Rho and/or Cdc42 may lead to increased numbers of DN thymocytes in these transgenic mice. In principle, elevated numbers of DN thymocytes in RhoA, Cdc42 and Dbs transgenic thymuses may result from an increased proliferative and/or survival capacity of these cells. It is known that inactivating Rho impairs DN thymocyte proliferation and survival and that activated Cdc42 enhances DN thymocyte proliferation [272] [275] [349] [296]. Cell cycle analysis suggested that Dbs transgenic thymocytes were cycling more rapidly. Subsequent BrdU incorporation experiments revealed that Dbs transgenic DN thymocytes are proliferating more rapidly than non-transgenic controls. Interestingly, DP and SP thymocytes from Dbs transgenic mice were also proliferating more rapidly; thus increased proliferation was not restricted to the DN subset. As an activator of Cdc42 and Rho, activated Dbs may promote DN thymocyte proliferation. It is not known whether Dbs transgenic DN thymocytes display an

enhanced survival capacity. The relative contribution of Cdc42 and/or Rho activation to the DN phenotype downstream of Dbs also remains to be determined. Furthermore, it is important to note that although Dbs has been shown to activate Cdc42 and RhoA, but not Rac1, other members of this family of GTPases have not been tested. There are at least thirteen other members of the Rho family of GTPases and some of them are related in terms of sequence similarity; thus it is possible that Dbs is acting on other members, in addition to RhoA and Cdc42.

The acquisition of CD25 expression by CD25⁺/CD44⁺ (DN1) thymocytes is accompanied by pre-TCR-mediated proliferation. The pre-TCR, composed of the pre-T α surrogate chain and the β chain is expressed following successful TCR β gene rearrangements (reviewed in [240] [350]). Rag2^{-/-} mice do not rearrange TCR β genes and do not express a pre-TCR; consequently, these mice display a block at the DN3 stage of thymocyte development. Expression of an activated mutant of Rac1 over-rides this developmental block and restores CD4⁺CD8⁺ differentiation in Rag2^{-/-} mice [291]. Activated Rho expression, however is unable to restore DP differentiation in the absence of the Rag2 [278]. Increased activity of certain Rho family GTPases, but not others is thus sufficient to mediate differentiation signals downstream of the pre-TCR. The role of Cdc42 in DN thymocyte differentiation is not known. The Dbs transgene causes increased proliferation and accumulation of DN3 and DN4 thymocytes and, as an activator of multiple Rho family GTPases, may also provide signals important in differentiation of DN thymocytes to DP thymocytes. In order to determine whether the Dbs transgene augments proliferative signals mediated by the pre-TCR, Dbs transgenic mice were crossed with Rag2^{-/-} mice. In Dbs/Rag2^{-/-} mice, the Dbs transgene was unable to substitute for pre-TCR signals required for DN differentiation and failed to over-ride the developmental block in the Rag2^{-/-} genetic background. Moreover, these results indicate that activation of Cdc42 or Rho via activated Dbs expression is unable to drive DN differentiation to DP thymocytes. Although these findings do not exclude a possible role for endogenous Dbs in DN thymocyte development, it is clear that activated Dbs cannot fully compensate for pre-TCR signals required for differentiation of DN thymocytes. It is important to note that expression levels of the transgene in DN thymocytes from Dbs/Rag2^{-/-} mice have also not been determined. Total numbers of DN3 Dbs/Rag2^{-/-} thymocytes, however were increased relative to Rag2^{-/-} DN3 thymocytes, indicating that Dbs-mediated DN thymocyte accumulation still occurs in the absence of functional pre-TCR. Together these results indicate that DN thymocyte proliferation in Dbs transgenic mice occurs independently of pre-TCR signals.

Dbs transgenic mice also display reduced numbers of SP thymocytes and mature peripheral T cells. The reduced cell numbers observed in Dbs transgenic mice could result from decreased production or increased destruction. Developing T cells undergo a rigorous selection process in the thymus and normally, very few cells are chosen to mature. Thymocytes are faced with three choices in the thymus: death by neglect, death by negative selection and survival by positive selection. All three fates are determined by the TCR. Apoptosis is tightly regulated during the development of thymocytes. Moreover, TCR ligation is a minimal requirement for negative selection of DP thymocytes and the avidity of TCR for self-Ag/MHC appears to determine the fate of these immature thymocytes. Failure to produce a TCR or production of one with strong reactivity toward self-peptide/MHC complexes results in programmed cell death. Positive selection results from intermediary TCR signals between these two extremes. The mechanism by which the TCR distinguishes between minimal and maximal binding to self-peptide ligands is unclear. There is considerable evidence supporting a role for TCR affinity in this discrimination; an avidity model proposes that positive selection is the result of low avidity thymocyte interactions, whereas high avidity interactions elicit negative selection [351] [352] [353] [354] [355] [356] [357] [358] (reviewed in [246] [359] [360] [361]).

Interestingly, Dbs transgenic thymocytes express higher levels of CD3 and TCR β , two components of the TCR. Many factors, including proximal TCR signaling molecules, the Jnk and Erk pathways, the PLC- γ pathway, transcription factors and accessory surface molecules, are known to influence thymocyte selection either by acting in parallel to or downstream of the TCR (reviewed in [246]). Notably, factors that affect TCR surface expression levels can also have an impact on selection events in the thymus. One consequence of receptor internalization is the attenuation of further signaling [362]. Inhibition of thymocyte TCR internalization enhances TCR signaling and, as a result, thymocyte negative selection [363]. Altered activity of some Rho family GTPase members can also influence expression of TCR components. Transgenic mice expressing activated Rac1 display elevated TCR expression levels and enhanced negative selection [291]. Thymocytes from activated Rho transgenic mice, however do not display defects in TCR expression levels or signals involved in negative selection; thus altered TCR expression is mediated by some Rho family GTPases, but not others. Reduced numbers of SP thymocytes and mature peripheral T cells displayed by Dbs transgenic mice may be the result of enhanced TCR signaling leading to more pronounced negative selection. The hypersensitivity of Dbs transgenic DP thymocytes to anti-CD3-mediated apoptosis supports this model. The defects displayed by these mice, however are unlikely to be a result of Rac1 activation, since Dbs

is unable to activate Rac1 *in vitro*. Although it is possible that increased TCR expression and hypersensitivity to anti-CD3-mediated apoptosis in Dbs transgenic mice is mediated by Cdc42, it remains to be determined whether activation of this GTPase is actually involved.

Activated Dbs transgene expression increases expression of the TCR. Increased TCR expression may lower the signaling threshold for thymocytes that would normally be negatively selected, thus effectively reducing the pool of thymocytes available for positive selection and differentiation to SP thymocytes. Overall, enhanced TCR signaling and the increased incidence of thymocyte apoptosis impairs the production of SP thymocytes. Enhanced Rho family GTPase activity is known to influence thymocyte apoptosis. Transgenic mice expressing activated Cdc42, for example display an increased incidence of Fas-independent apoptosis in the thymus [296]. Moreover transgenic mice expressing wild type and activated Rac2 also exhibit reduced numbers of SP thymocytes and an increased incidence of thymocyte apoptosis [364]. Although not actually demonstrated in either of these reports, Cdc42 or Rac2 activation may also influence thymocyte selection events via altered TCR signaling. The observation that thymocyte apoptosis in the thymuses of Cdc42 transgenic mice is Fas-independent, points to a defect in thymocyte selection. Thus it is possible that activated Dbs-mediated activation of Cdc42 is at least partly responsible for the restricted production of SP thymocytes in Dbs transgenic mice. Although thymocyte hypersensitivity to apoptosis induced by MHC-TCR interactions is likely to cause the reduced numbers of SP thymocytes in Dbs transgenic mice, this still needs to be demonstrated. An early hallmark of apoptotic cells is the translocation of phosphatidylserine from the inside to the outside of the plasma membrane, which can be readily detected by Annexin V binding [365]. The incidence of apoptosis in Dbs transgenic thymuses has not been determined.

The actin cytoskeleton is involved in sustaining TCR signal transduction and Vav1, another Dbl family member, enhances cytoskeletal reorganization and TCR clustering [366] [367] [368]. Dbs, as a regulator of the actin cytoskeletal changes, may influence TCR expression and signaling via the actin cytoskeleton. The mechanism by which activated Dbs increases TCR levels remains to be determined.

There are at least two candidates likely to be involved in apoptosis of DP thymocytes downstream of the TCR. Transgenic mice expressing activated Rac2 or Cdc42 both displayed increased Jnk kinase activity in addition to increased thymocyte apoptosis [296] [364]. It is reasonable to hypothesize that a Jnk signaling pathway may be involved in apoptosis of thymocytes in these mice. Interestingly, Dbs

has been shown to activate Jnk in fibroblast cell lines, presumably via Rho and/or Cdc42 activation [111]. As an activator of Cdc42, activated Dbs may enhance Jnk-mediated apoptotic signals in DP thymocytes, thus contributing to their death. Interestingly, NF- κ B is required for anti-CD3-mediated apoptosis of DP thymocytes [320]. Transgenic mice that express a super-inhibitory mutant form of inhibitor κ B- α are resistant to anti-CD3-mediated apoptosis *in vivo*, indicating that NF- κ B is required for TCR-mediated DP thymocyte apoptosis [369]. Dbs has also been shown to activate NF- κ B in NIH 3T3 fibroblasts [111]. Increased NF- κ B activity via activated Dbs transgene expression may further contribute to the sensitivity of Dbs transgenic DP thymocytes to CD3-mediated apoptosis. Although these are potential apoptotic signaling mediators, the actual contributions of these factors to the thymocyte defects displayed by Dbs transgenic mice are not known.

Cdc42 triggers distinct apoptotic pathways in thymocytes and peripheral T cells; thymocyte apoptosis in activated Cdc42 transgenic mice was independent of Fas, whereas peripheral spleen and lymph node T cell apoptosis was Fas-dependent [296]. Although it is clear that activated Dbs expression influences TCR signaling and sensitivity to CD3-mediated signals and that this is likely to affect negative selection in thymuses from these mice, it is assumed that the reduced numbers of peripheral T cells are simply a reflection of impaired thymocyte selection events. Cdc42 activation via transgenic Dbs may also lead to distinct defects in mechanisms of peripheral T cell apoptosis. Fas-dependency on peripheral T cell apoptosis has not been examined.

Impaired positive selection may also lead to fewer SP thymocytes. TCR HY transgenic mice with the Dbs transgene displayed fewer CD8 SP thymocytes indicating that Dbs expression impairs positive selection. Thus, increased TCR expression in Dbs transgenic mice may reduce the frequency of positive selection by inducing apoptosis in cells that would normally be positively selected. In Dbs transgenic mice, strong TCR signaling, negative selection and impaired positive selection may all contribute to an overall reduction in SP thymocytes. Once again, the relative contribution of different Rho family GTPases to the thymocyte defects observed in Dbs transgenic mice remains to be determined. Although it is known that enhanced Rho activity does not influence thymocyte negative selection, activated RhoA augments thymocyte positive selection in the TCR-HY transgenic system [278]. It is therefore unlikely that activated Dbs-mediated RhoA activation is involved in this process. Understanding the contributions of different Rho family GTPases downstream of Dbs in thymocyte selection events will be an interesting area of future research.

Interestingly, DP and SP thymocytes, in addition to DNs, proliferated more rapidly *in vivo* when compared to non-transgenic controls. Unlike DN thymocytes, however increased proliferation of DP and SP thymocytes did not lead to accumulation. Instead, thymocyte numbers within these subsets were reduced in transgenic mice. Dbs was isolated based on its ability to transform NIH 3T3 fibroblasts and was subsequently shown to stimulate transcription from a cyclin D1 reporter construct [132] [111]. Thus, as with other components of Rho signaling pathways, Dbs may mediate cell cycle progression via induction of cyclins. Interestingly, altered activity of cell cycle components can influence thymocyte proliferation. For example, mice deficient in the cyclin-dependent kinase inhibitor p16^{INK4a} display thymic hyperplasia [370]. As a potential activator of cyclin D1, activated Dbs may promote DP and SP thymocyte turnover. Whether enhanced proliferation of DP and SP thymocytes is a direct effect of activated Dbs expression or a secondary effect of altered positive and negative selection remains to be determined.

Attempts were made to further characterize the SP thymocytes from Dbs transgenic mice and analyses revealed some additional unusual features. In terms of expression of TCR β , CD3 ϵ , HSA and CD69, CD4 SP thymocytes from Dbs transgenic mice appeared normal, albeit at reduced frequency. CD62L expression levels by this subset, as well as DN, DP and CD8 SP thymocytes, however were elevated. The significance of increased CD62L expression is not known. Interestingly, CD5 expression was increased on CD4 SP thymocytes. CD5 is a negative regulator of TCR signaling and increased CD5 expression by CD4 SP thymocytes is one potential explanation for the impaired proliferative response of this thymocyte subset [247].

Unlike CD4 SP thymocytes, CD8 SP thymocytes differed more in terms of expression of TCR β , CD3 ϵ , HSA, CD69 and CD5. TCR-dependent maturational events define the earliest stage of positive selection; these events include up-regulation of TCR β , CD3 ϵ , bcl-2, CD69 and CD5 and down-regulation of HSA (reviewed in [244]). With the exception of CD62L, CD8 SP thymocytes appeared less mature in terms of expression of TCR β , CD3 ϵ , CD69, CD5, bcl-2 and HSA. Activated Dbs expression appears to differentially influence the SP thymocyte subsets that develop in these mice. Although both CD4 and CD8 SP thymocytes are present at reduced frequencies in Dbs transgenic mice, CD4 SP thymocytes appear normal and CD8 SP thymocytes appear less mature. The last stage of SP thymocyte development prior to emigration to the periphery is characterized by a proliferative

expansion phase [371] [372]. The apparent 'immature' SP thymocyte population from Dbs transgenic mice may be less responsive to TCR stimulation, which could explain their impaired proliferative responses to anti-CD3 in culture. Most of the cells that would normally respond to TCR stimulation when placed in culture may not be present in thymuses from Dbs transgenic mice. The differential effect of activated Dbs expression on SP thymocyte subsets is interesting, although not understood; future experiments are required to further characterize development of these thymocyte subsets.

The significance of increased CD62L (L-selectin) expression also needs to be further addressed. The selectins are a family of vascular adhesion molecules that possess close structural and functional relationships and whose primary role is to promote rolling behaviour of leucocytes along endothelium prior to firm adhesion and subsequent emigration (reviewed in [373]). CD62L is up-regulated in the latest stages of SP thymocyte development and is involved in mature thymocyte emigration to the periphery [344] [345]. Although not required for thymocyte emigration from the thymus, CD62L-deficient mice display defects in lymphocyte homing to lymphoid tissues and sites of inflammation [373]. Interestingly, CD62L expression levels may underpin the migration of T cells to specific peripheral lymphoid organs. While CD62L^{high} thymic emigrants preferentially migrate to the lymph nodes, CD62L^{low} thymic emigrants migrate to the spleen [374] [375]. Thus altered expression of CD62L has the potential to influence peripheral lymphocyte migration patterns. The effect of activated Dbs expression on thymocyte emigration to the periphery has not been addressed.

Considerable evidence indicates that Rho GTPase family members can influence cell adhesion via CD62L as well as other molecules. Neutrophils from Rac2^{-/-} mice, for example display impaired CD62L-mediated adhesion to GlyCAM-1, the CD62L ligand [376]. It is also known that RhoA can regulate cell adhesion in lymphocytes [265]. Thymocytes express the integrin $\alpha_4\beta_1$ which acts as the main receptor for the extracellular matrix protein fibronectin in these cells [377] [378]. Thymocytes from activated RhoA and Rac1 transgenic mice adhere more to fibronectin than control thymocytes [278] [379]. Also, cells expressing the Cdc42 and Rac1 GAP ARHGAP9 display impaired adhesion to fibronectin [380]. As a regulator of Rho and Cdc42, Dbs may influence thymocyte adhesion to fibronectin. The effect of activated Dbs expression on lymphocyte adhesion to GlyCAM-1 and fibronectin has not been determined and would be an interesting area of future research.

Chapter 6 – Conclusion

Members of the $G\alpha_{12}$ and Rho GTPase families coordinate a wide range of cellular processes and deregulated GTPase activity can adversely affect cells. While studies involving GTPases have provided many insights into their involvement in an array of cellular processes, most have utilized constitutively active or inactive mutants. Consequently, the roles of GTPase regulators have often been overlooked. Balanced regulation of GTPase activity involves several types of proteins and is critical in coordinating normal cellular responses. GTPase regulatory proteins including GPCRs, GAPs and GEFs are diverse and influence the activities of one or several types of GTPases. Studies involving GTPase regulators are important for several reasons. Regulators stimulate either exchange of GTP for GDP or catalyze hydrolysis of GTP, depending upon whether they are positive or negative GTPase regulators. Altering expression levels of any of these regulators influences the rate of GDP/GTP exchange or hydrolysis but does not render the GTPase constitutively active or inactive in terms of the type of nucleotide bound. This is an important point; although imbalanced, the GTPase cycle is maintained upon over-expression of GTPase regulators. One weakness associated with the use of GTPase mutants is the inability of these proteins to undergo complete GDP/GTP exchange or hydrolysis cycles. Altering the GTPase cycling rates through the use of GTPase regulators is one way of overcoming this problem. GTPase regulators themselves are also subject to an array of regulatory constraints. The mechanisms by which these regulators are activated are often quite complex and can include, for example, phosphorylation, protein-protein interactions and sub-cellular relocalization events, as is the case with Vav1. The view that GTPases are simply 'on' or 'off', is too simple; GTPase activity is much more complex and depends on not only the presence of regulators, but the sub-cellular context as well as the presence and activities of an array of other interacting proteins. Thus characterizing regulators of GTPases is important in understanding both the normal cellular functions of GTPases and the mechanisms by which GTPase signals lead to abnormal cellular responses.

Gain of function studies are important in characterizing regulators of GTPases as well as the cellular responses they coordinate. As with loss of function approaches, gain of function studies involving GTPase regulators provide insights into the potential roles of these proteins in cellular processes. This thesis addressed the contributions of several GTPase regulators, G2A, PAR-1, Lsc and Dbs, in fibroblast growth control as well as lymphocyte development and function. Activation of $G\alpha_{12}$, $G\alpha_{13}$ and Rho are required for transformation via G2A and PAR-1. These $G\alpha$ proteins are unique in that they are activated upstream of Rho. $G\alpha$ -mediated activation of GEFs and ultimately, the smaller molecular

weight GTPases, appears to be an important mechanism of linking divergent GTPases downstream of GPCR activation.

In an attempt to implicate $G\alpha_{12}$ and $G\alpha_{13}$ in lymphocyte development, transgenic mice expressing LscRGS, a negative regulator or GAP for these GTPases, were generated. LscRGS expression did not overtly affect lymphocyte development. The results presented in Chapter 4 highlight the limitations of a transgenic expression approach of a GAP in characterization of these $G\alpha$ proteins. If transgenic mice had displayed a lymphocyte developmental defect, it would have been reasonable to conclude that altered $G\alpha_{12}$ or $G\alpha_{13}$ are important in lymphocyte development. Unfortunately within the measurement parameters chosen, I was unable to demonstrate any effect of LscRGS expression on lymphocyte development of transgenic mice. These results are difficult to interpret. Several factors may have contributed to the apparent normal phenotype of lymphocytes from LscRGS transgenic mice, including insufficient LscRGS expression levels in lymphocytes, negative regulatory influences on LscRGS or a complete lack of $G\alpha_{12}$ or $G\alpha_{13}$ involvement in lymphocyte development. While all of these are possible explanations for the normal phenotype displayed by these transgenic mice, I am very reluctant to believe that these $G\alpha$ proteins are not involved in some aspect of lymphocyte signaling. It is quite likely that some lymphocyte defect is awaiting discovery in LscRGS transgenic mice.

To determine the effect of increased GEF expression on lymphocyte development, transgenic mice expressing an activated form of Dbs in lymphocytes were generated and analyzed. Expression of activated Dbs in lymphocytes promoted the accumulation of early thymocytes and restricted the production of mature thymocytes in transgenic mice. Although results from these studies indicate a potential role for Dbs in thymocyte development, forced expression of activated Dbs does not enable me to conclude that this GEF is actually involved in normal lymphocyte development. More simply, these analyses demonstrate what Dbs is capable of doing, not necessarily what Dbs is actually doing in lymphocytes. This is an inherent limitation of all gain of function experiments. The next logical step would be to generate a loss of function model of Dbs and observe the effects in lymphocytes. Generating and analyzing Dbs^{-/-} mice would be an interesting avenue for future research.

Awareness of the limitations associated with gain of function studies is important in interpretation of experimental results. Results presented in this thesis have nevertheless provided insights into the contributions of GTPase regulators in signal transduction and regulation of cellular growth control, differentiation and transformation.

References

1. Tao, W., D. Pennica, L. Xu, R.F. Kalejta and A.J. Levine, *Wrch-1, a novel member of the Rho gene family that is regulated by Wnt-1*. Genes Dev, 2001. **15**(14): p. 1796-807.
2. Vignal, E., M. De Toledo, F. Comunale, A. Ladopoulou, C. Gauthier-Rouviere, A. Blangy and P. Fort, *Characterization of TCL, a new GTPase of the rho family related to TC10 and Ccdc42*. J Biol Chem, 2000. **275**(46): p. 36457-64.
3. Han, J.S., J.H. Kim, J.G. Kim, J.B. Park, D.Y. Noh and K.H. Lee, *Molecular cloning and sequencing of rat Cdc42 GTPase cDNA*. Exp Mol Med, 2000. **32**(3): p. 115-9.
4. Hall, A., *Rho GTPases and the actin cytoskeleton*. Science, 1998. **279**(5350): p. 509-14.
5. Mackay, D.J. and A. Hall, *Rho GTPases*. J Biol Chem, 1998. **273**(33): p. 20685-8.
6. Aspenstrom, P., *The Rho GTPases have multiple effects on the actin cytoskeleton*. Exp Cell Res, 1999. **246**(1): p. 20-5.
7. Bishop, A.L. and A. Hall, *Rho GTPases and their effector proteins*. Biochem J, 2000. **348 Pt 2**: p. 241-55.
8. Rittinger, K., P.A. Walker, J.F. Eccleston, K. Nurmahomed, D. Owen, E. Laue, S.J. Gamblin and S.J. Smerdon, *Crystal structure of a small G protein in complex with the GTPase-activating protein rhoGAP*. Nature, 1997. **388**(6643): p. 693-7.
9. Li, R. and Y. Zheng, *Residues of the Rho family GTPases Rho and Cdc42 that specify sensitivity to Dbl-like guanine nucleotide exchange factors*. J Biol Chem, 1997. **272**(8): p. 4671-9.
10. Mott, H.R., D. Owen, D. Niellispach, P.N. Lowe, E. Manser, L. Lim and E.D. Laue, *Structure of the small G protein Cdc42 bound to the GTPase-binding domain of ACK*. Nature, 1999. **399**(6734): p. 384-8.
11. Worthylake, D.K., K.L. Rossman and J. Sondek, *Crystal structure of Rac1 in complex with the guanine nucleotide exchange region of Tiam1*. Nature, 2000. **408**(6813): p. 682-8.
12. Morreale, A., M. Venkatesan, H.R. Mott, D. Owen, D. Niellispach, P.N. Lowe and E.D. Laue, *Structure of Cdc42 bound to the GTPase binding domain of PAK*. Nat Struct Biol, 2000. **7**(5): p. 384-8.
13. Rossman, K.L., D.K. Worthylake, J.T. Snyder, D.P. Siderovski, S.L. Campbell and J. Sondek, *A crystallographic view of interactions between Dbs and Cdc42: PH domain-assisted guanine nucleotide exchange*. Embo J, 2002. **21**(6): p. 1315-26.
14. Klockow, B., M.R. Ahmadian, C. Block and A. Wittinghofer, *Oncogenic insertional mutations in the P-loop of Ras are overactive in MAP kinase signaling*. Oncogene, 2000. **19**(47): p. 5367-76.
15. Freeman, J.L., A. Abo and J.D. Lambeth, *Rac "insert region" is a novel effector region that is implicated in the activation of NADPH oxidase, but not PAK65*. J Biol Chem, 1996. **271**(33): p. 19794-801.
16. Wu, W.J., D.A. Leonard, A.C. R and D. Manor, *Interaction between Cdc42Hs and RhoGDI is mediated through the Rho insert region*. J Biol Chem, 1997. **272**(42): p. 26153-8.
17. Hoffman, G.R., N. Nassar and R.A. Cerione, *Structure of the Rho family GTP-binding protein Cdc42 in complex with the multifunctional regulator RhoGDI*. Cell, 2000. **100**(3): p. 345-56.

18. Cherfilis, J. and P. Chardin, *GEFs: structural basis for their activation of small GTP-binding proteins*. Trends Biochem Sci, 1999. **24**(8): p. 306-11.
19. Lin, R., R.A. Cerione and D. Manor, *Specific contributions of the small GTPases Rho, Rac, and Cdc42 to Dbl transformation*. J Biol Chem, 1999. **274**(33): p. 23633-41.
20. Feig, L.A. and G.M. Cooper, *Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP*. Mol Cell Biol, 1988. **8**(8): p. 3235-43.
21. Feig, L.A., *Tools of the trade: use of dominant-inhibitory mutants of Ras-family GTPases*. Nat Cell Biol, 1999. **1**(2): p. E25-7.
22. Quilliam, L.A., K. Kato, K.M. Rabun, M.M. Hisaka, S.Y. Huff, S. Campbell-Burk and C.J. Der, *Identification of residues critical for Ras(17N) growth-inhibitory phenotype and for Ras interaction with guanine nucleotide exchange factors*. Mol Cell Biol, 1994. **14**(2): p. 1113-21.
23. Sekine, A., M. Fujiwara and S. Narumiya, *Asparagine residue in the rho gene product is the modification site for botulinum ADP-ribosyltransferase*. J Biol Chem, 1989. **264**(15): p. 8602-5.
24. Just, I., C. Mohr, G. Schallehn, L. Menard, J.R. Didsbury, J. Vandekerckhove, J. van Damme and K. Aktories, *Purification and characterization of an ADP-ribosyltransferase produced by Clostridium limosum*. J Biol Chem, 1992. **267**(15): p. 10274-80.
25. Chardin, P., P. Boquet, P. Madaule, M.R. Popoff, E.J. Rubin and D.M. Gill, *The mammalian G protein rhoC is ADP-ribosylated by Clostridium botulinum exoenzyme C3 and affects actin microfilaments in Vero cells*. Embo J, 1989. **8**(4): p. 1087-92.
26. Mohr, C., G. Koch, I. Just and K. Aktories, *ADP-ribosylation by Clostridium botulinum C3 exoenzyme increases steady-state GTPase activities of recombinant rhoA and rhoB proteins*. FEBS Lett, 1992. **297**(1-2): p. 95-9.
27. Van Aelst, L. and C. D'Souza-Schorey, *Rho GTPases and signaling networks*. Genes Dev, 1997. **11**(18): p. 2295-322.
28. Kaibuchi, K., S. Kuroda and M. Amano, *Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells*. Annu Rev Biochem, 1999. **68**: p. 459-86.
29. Chimini, G. and P. Chavrier, *Function of Rho family proteins in actin dynamics during phagocytosis and engulfment*. Nat Cell Biol, 2000. **2**(10): p. E191-6.
30. Evers, E.E., G.C. Zondag, A. Malliri, L.S. Price, J.P. ten Klooster, R.A. van der Kammen and J.G. Collard, *Rho family proteins in cell adhesion and cell migration*. Eur J Cancer, 2000. **36**(10): p. 1269-74.
31. Ridley, A.J., *Rho family proteins: coordinating cell responses*. Trends Cell Biol, 2001. **11**(12): p. 471-7.
32. Ridley, A.J. and A. Hall, *The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors*. Cell, 1992. **70**(3): p. 389-99.
33. Ridley, A.J., H.F. Paterson, C.L. Johnston, D. Diekmann and A. Hall, *The small GTP-binding protein rac regulates growth factor-induced membrane ruffling*. Cell, 1992. **70**(3): p. 401-10.
34. Kozma, R., S. Ahmed, A. Best and L. Lim, *The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts*. Mol Cell Biol, 1995. **15**(4): p. 1942-52.

35. Nobes, C.D. and A. Hall, *Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia.* Cell, 1995. **81**(1): p. 53-62.
36. Sander, E.E., J.P. ten Klooster, S. van Delft, R.A. van der Kammen and J.G. Collard, *Rac downregulates Rho activity: reciprocal balance between both GTPases determines cellular morphology and migratory behavior.* J Cell Biol, 1999. **147**(5): p. 1009-22.
37. Nobes, C.D., I. Lauritzen, M.G. Mattei, S. Paris, A. Hall and P. Chardin, *A new member of the Rho family, Rnd1, promotes disassembly of actin filament structures and loss of cell adhesion.* J Cell Biol, 1998. **141**(1): p. 187-97.
38. Avraham, H. and R.A. Weinberg, *Characterization and expression of the human rhoH12 gene product.* Mol Cell Biol, 1989. **9**(5): p. 2058-66.
39. Prendergast, G.C., R. Khosravi-Far, P.A. Solski, H. Kurzawa, P.F. Lebowitz and C.J. Der, *Critical role of Rho in cell transformation by oncogenic Ras.* Oncogene, 1995. **10**(12): p. 2289-96.
40. Perona, R., P. Esteve, B. Jimenez, R.P. Ballesteros, S. Ramon y Cajal and J.C. Lacal, *Tumorigenic activity of rho genes from Aplysia californica.* Oncogene, 1993. **8**(5): p. 1285-92.
41. Roux, P., C. Gauthier-Rouviere, S. Doucet-Brutin and P. Fort, *The small GTPases Cdc42Hs, Rac1 and RhoG delineate Raf-independent pathways that cooperate to transform NIH3T3 cells.* Curr Biol, 1997. **7**(9): p. 629-37.
42. Qiu, R.G., J. Chen, D. Kirn, F. McCormick and M. Symons, *An essential role for Rac in Ras transformation.* Nature, 1995. **374**(6521): p. 457-9.
43. van Leeuwen, F.N., R.A. van der Kammen, G.G. Habets and J.G. Collard, *Oncogenic activity of Tiam1 and Rac1 in NIH3T3 cells.* Oncogene, 1995. **11**(11): p. 2215-21.
44. Westwick, J.K., Q.T. Lambert, G.J. Clark, M. Symons, L. Van Aelst, R.G. Pestell and C.J. Der, *Rac regulation of transformation, gene expression, and actin organization by multiple, PAK-independent pathways.* Mol Cell Biol, 1997. **17**(3): p. 1324-35.
45. Lin, R., S. Bagrodia, R. Cerione and D. Manor, *A novel Cdc42Hs mutant induces cellular transformation.* Curr Biol, 1997. **7**(10): p. 794-7.
46. Hernandez-Alcoceba, R., L. del Peso and J.C. Lacal, *The Ras family of GTPases in cancer cell invasion.* Cell Mol Life Sci, 2000. **57**(1): p. 65-76.
47. Fritz, G., I. Just and B. Kaina, *Rho GTPases are over-expressed in human tumors.* Int J Cancer, 1999. **81**(5): p. 682-7.
48. Suwa, H., G. Ohshio, T. Imamura, G. Watanabe, S. Arai, M. Imamura, S. Narumiya, H. Hiai and M. Fukumoto, *Overexpression of the rhoC gene correlates with progression of ductal adenocarcinoma of the pancreas.* Br J Cancer, 1998. **77**(1): p. 147-52.
49. Jordan, P., R. Brazao, M.G. Boavida, C. Gespach and E. Chastre, *Cloning of a novel human Rac1b splice variant with increased expression in colorectal tumors.* Oncogene, 1999. **18**(48): p. 6835-9.
50. Clark, E.A., T.R. Golub, E.S. Lander and R.O. Hynes, *Genomic analysis of metastasis reveals an essential role for RhoC.* Nature, 2000. **406**(6795): p. 532-5.
51. Dallery, E., S. Galiegue-Zouitina, M. Collyn-d'Hooghe, S. Quief, C. Denis, M.P. Hildebrand, D. Lantoine, C. Dewindt, H. Tilly, C. Bastard and et al., *TTF, a gene encoding a novel small G*

- protein, fuses to the lymphoma-associated LAZ3 gene by t(3;4) chromosomal translocation. *Oncogene*, 1995. **10**(11): p. 2171-8.
52. Preudhomme, C., C. Roumier, M.P. Hildebrand, E. Dallery-Prudhomme, D. Lantoine, J.L. Lai, A. Daudignon, C. Adenis, F. Bauters, P. Fenaux, J.P. Kerckaert and S. Galiegue-Zouitina, *Nonrandom 4p13 rearrangements of the RhoH/TTF gene, encoding a GTP-binding protein, in non-Hodgkin's lymphoma and multiple myeloma*. *Oncogene*, 2000. **19**(16): p. 2023-32.
 53. Neudauer, C.L., G. Joberty, N. Tatsis and I.G. Macara, *Distinct cellular effects and interactions of the Rho-family GTPase TC10*. *Curr Biol*, 1998. **8**(21): p. 1151-60.
 54. Guasch, R.M., P. Scambler, G.E. Jones and A.J. Ridley, *RhoE regulates actin cytoskeleton organization and cell migration*. *Mol Cell Biol*, 1998. **18**(8): p. 4761-71.
 55. Tsubakimoto, K., K. Matsumoto, H. Abe, J. Ishii, M. Amano, K. Kaibuchi and T. Endo, *Small GTPase RhoD suppresses cell migration and cytokinesis*. *Oncogene*, 1999. **18**(15): p. 2431-40.
 56. Tapon, N. and A. Hall, *Rho, Rac and Cdc42 GTPases regulate the organization of the actin cytoskeleton*. *Curr Opin Cell Biol*, 1997. **9**(1): p. 86-92.
 57. Ihara, K., S. Muraguchi, M. Kato, T. Shimizu, M. Shirakawa, S. Kuroda, K. Kaibuchi and T. Hakoshima, *Crystal structure of human RhoA in a dominantly active form complexed with a GTP analogue*. *J Biol Chem*, 1998. **273**(16): p. 9656-66.
 58. Wei, Y., Y. Zhang, U. Derewenda, X. Liu, W. Minor, R.K. Nakamoto, A.V. Somlyo, A.P. Somlyo and Z.S. Derewenda, *Crystal structure of RhoA-GDP and its functional implications*. *Nat Struct Biol*, 1997. **4**(9): p. 699-703.
 59. Ishizaki, T., M. Naito, K. Fujisawa, M. Maekawa, N. Watanabe, Y. Saito and S. Narumiya, *p160ROCK, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions*. *FEBS Lett*, 1997. **404**(2-3): p. 118-24.
 60. Amano, M., Y. Fukata and K. Kaibuchi, *Regulation and functions of Rho-associated kinase*. *Exp Cell Res*, 2000. **261**(1): p. 44-51.
 61. Madaule, P., M. Eda, N. Watanabe, K. Fujisawa, T. Matsuoka, H. Bito, T. Ishizaki and S. Narumiya, *Role of citron kinase as a target of the small GTPase Rho in cytokinesis*. *Nature*, 1998. **394**(6692): p. 491-4.
 62. Madaule, P., T. Furuyashiki, M. Eda, H. Bito, T. Ishizaki and S. Narumiya, *Citron, a Rho target that affects contractility during cytokinesis*. *Microsc Res Tech*, 2000. **49**(2): p. 123-6.
 63. Teramoto, H., O.A. Coso, H. Miyata, T. Igishi, T. Miki and J.S. Gutkind, *Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c-Jun N-terminal kinase/stress-activated protein kinase pathway. A role for mixed lineage kinase 3/protein-tyrosine kinase 1, a novel member of the mixed lineage kinase family*. *J Biol Chem*, 1996. **271**(44): p. 27225-8.
 64. Bock, B.C., P.O. Vacratsis, E. Qamirani and K.A. Gallo, *Cdc42-induced activation of the mixed-lineage kinase SPRK in vivo. Requirement of the Cdc42/Rac interactive binding motif and changes in phosphorylation*. *J Biol Chem*, 2000. **275**(19): p. 14231-41.
 65. Manser, E. and L. Lim, *Roles of PAK family kinases*. *Prog Mol Subcell Biol*, 1999. **22**: p. 115-33.
 66. Bagrodia, S. and R.A. Cerione, *Pak to the future*. *Trends Cell Biol*, 1999. **9**(9): p. 350-5.
 67. Manser, E., T. Leung, H. Salihuddin, L. Tan and L. Lim, *A non-receptor tyrosine kinase that inhibits the GTPase activity of p21cdc42*. *Nature*, 1993. **363**(6427): p. 364-7.

68. Tolias, K.F., L.C. Cantley and C.L. Carpenter, *Rho family GTPases bind to phosphoinositide kinases*. J Biol Chem, 1995. **270**(30): p. 17656-9.
69. Bokoch, G.M., C.J. Vlahos, Y. Wang, U.G. Knaus and A.E. Traynor-Kaplan, *Rac GTPase interacts specifically with phosphatidylinositol 3-kinase*. Biochem J, 1996. **315**(Pt 3): p. 775-9.
70. Palazzo, A.F., T.A. Cook, A.S. Alberts and G.G. Gundersen, *mDia mediates Rho-regulated formation and orientation of stable microtubules*. Nat Cell Biol, 2001. **3**(8): p. 723-9.
71. Reid, T., T. Furuyashiki, T. Ishizaki, G. Watanabe, N. Watanabe, K. Fujisawa, N. Morii, P. Madaule and S. Narumiya, *Rhotekin, a new putative target for Rho bearing homology to a serine/threonine kinase, PKN, and rhotillin in the rho-binding domain*. J Biol Chem, 1996. **271**(23): p. 13556-60.
72. Watanabe, N., P. Madaule, T. Reid, T. Ishizaki, G. Watanabe, A. Kakizuka, Y. Saito, K. Nakao, B.M. Jockusch and S. Narumiya, *p140mDia, a mammalian homolog of Drosophila diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin*. Embo J, 1997. **16**(11): p. 3044-56.
73. Symons, M., J.M. Derry, B. Karlak, S. Jiang, V. Lemahieu, F. McCormick, U. Francke and A. Abo, *Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization*. Cell, 1996. **84**(5): p. 723-34.
74. Snapper, S.B. and F.S. Rosen, *The Wiskott-Aldrich syndrome protein (WASP): roles in signaling and cytoskeletal organization*. Annu Rev Immunol, 1999. **17**: p. 905-29.
75. O'Sullivan, E., C. Kinnon and P. Brickell, *Wiskott-Aldrich syndrome protein, WASP*. Int J Biochem Cell Biol, 1999. **31**(3-4): p. 383-7.
76. Abo, A., E. Pick, A. Hall, N. Totty, C.G. Teahan and A.W. Segal, *Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1*. Nature, 1991. **353**(6345): p. 668-70.
77. Nisimoto, Y., J.L. Freeman, S.A. Motalebi, M. Hirshberg and J.D. Lambeth, *Rac binding to p67(phox). Structural basis for interactions of the Rac1 effector region and insert region with components of the respiratory burst oxidase*. J Biol Chem, 1997. **272**(30): p. 18834-41.
78. Tu, H. and M. Wigler, *Genetic evidence for Pak1 autoinhibition and its release by Cdc42*. Mol Cell Biol, 1999. **19**(1): p. 602-11.
79. Amano, M., K. Chihara, N. Nakamura, T. Kaneko, Y. Matsuura and K. Kaibuchi, *The COOH terminus of Rho-kinase negatively regulates rho-kinase activity*. J Biol Chem, 1999. **274**(45): p. 32418-24.
80. Matsui, T., M. Amano, T. Yamamoto, K. Chihara, M. Nakafuku, M. Ito, T. Nakano, K. Okawa, A. Iwamatsu and K. Kaibuchi, *Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho*. Embo J, 1996. **15**(9): p. 2208-16.
81. Watanabe, G., Y. Saito, P. Madaule, T. Ishizaki, K. Fujisawa, N. Morii, H. Mukai, Y. Ono, A. Kakizuka and S. Narumiya, *Protein kinase N (PKN) and PKN-related protein rhotillin as targets of small GTPase Rho*. Science, 1996. **271**(5249): p. 645-8.
82. Ramesh, N., I.M. Anton, N. Martinez-Quiles and R.S. Geha, *Waltzing with WASP*. Trends Cell Biol, 1999. **9**(1): p. 15-9.
83. Gallego, M.D., M. Santamaria, J. Pena and I.J. Molina, *Defective actin reorganization and polymerization of Wiskott-Aldrich T cells in response to CD3-mediated stimulation*. Blood, 1997. **90**(8): p. 3089-97.

84. Ren, X.D. and M.A. Schwartz, *Determination of GTP loading on Rho*. Methods Enzymol, 2000. **325**: p. 264-72.
85. Olson, M.F., A. Ashworth and A. Hall, *An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1*. Science, 1995. **269**(5228): p. 1270-2.
86. Welsh, C.F., K. Roovers, J. Villanueva, Y. Liu, M.A. Schwartz and R.K. Assoian, *Timing of cyclin D1 expression within G1 phase is controlled by Rho*. Nat Cell Biol, 2001. **3**(11): p. 950-7.
87. Mettouchi, A., S. Klein, W. Guo, M. Lopez-Lago, E. Lemichez, J.K. Westwick and F.G. Giancotti, *Integrin-specific activation of Rac controls progression through the G(1) phase of the cell cycle*. Mol Cell, 2001. **8**(1): p. 115-27.
88. Gjoerup, O., J. Lukas, J. Bartek and B.M. Willumsen, *Rac and Cdc42 are potent stimulators of E2F-dependent transcription capable of promoting retinoblastoma susceptibility gene product hyperphosphorylation*. J Biol Chem, 1998. **273**(30): p. 18812-8.
89. Hirai, A., S. Nakamura, Y. Noguchi, T. Yasuda, M. Kitagawa, I. Tatsuno, T. Oeda, K. Tahara, T. Terano, S. Narumiya, L.D. Kohn and Y. Saito, *Geranylgeranylated rho small GTPase(s) are essential for the degradation of p27Kip1 and facilitate the progression from G1 to S phase in growth-stimulated rat FRTL-5 cells*. J Biol Chem, 1997. **272**(1): p. 13-6.
90. Weber, J.D., W. Hu, S.C. Jefcoat, Jr., D.M. Raben and J.J. Baldassare, *Ras-stimulated extracellular signal-related kinase 1 and RhoA activities coordinate platelet-derived growth factor-induced G1 progression through the independent regulation of cyclin D1 and p27*. J Biol Chem, 1997. **272**(52): p. 32966-71.
91. Hu, W., C.J. Bellone and J.J. Baldassare, *RhoA stimulates p27(Kip) degradation through its regulation of cyclin E/CDK2 activity*. J Biol Chem, 1999. **274**(6): p. 3396-401.
92. Coso, O.A., M. Chiariello, J.C. Yu, H. Teramoto, P. Crespo, N. Xu, T. Miki and J.S. Gutkind, *The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway*. Cell, 1995. **81**(7): p. 1137-46.
93. Minden, A., A. Lin, F.X. Claret, A. Abo and M. Karin, *Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs*. Cell, 1995. **81**(7): p. 1147-57.
94. Teramoto, H., P. Crespo, O.A. Coso, T. Igishi, N. Xu and J.S. Gutkind, *The small GTP-binding protein rho activates c-Jun.N-terminal kinases/stress-activated protein kinases in human kidney 293T cells. Evidence for a Pak-independent signaling pathway*. J Biol Chem, 1996. **271**(42): p. 25731-4.
95. Marinissen, M.J., M. Chiariello and J.S. Gutkind, *Regulation of gene expression by the small GTPase Rho through the ERK6 (p38 gamma) MAP kinase pathway*. Genes Dev, 2001. **15**(5): p. 535-53.
96. Norman, C., M. Runswick, R. Pollock and R. Treisman, *Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-fos serum response element*. Cell, 1988. **55**(6): p. 989-1003.
97. Shaw, P.E., H. Schroter and A. Nordheim, *The ability of a ternary complex to form over the serum response element correlates with serum inducibility of the human c-fos promoter*. Cell, 1989. **56**(4): p. 563-72.
98. Treisman, R., *Ternary complex factors: growth factor regulated transcriptional activators*. Curr Opin Genet Dev, 1994. **4**(1): p. 96-101.

99. Price, M.A., C. Hill and R. Treisman, *Integration of growth factor signals at the c-fos serum response element*. Philos Trans R Soc Lond B Biol Sci, 1996. **351**(1339): p. 551-9.
100. Hill, C.S., J. Wynne and R. Treisman, *The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF*. Cell, 1995. **81**(7): p. 1159-70.
101. Hill, C.S. and R. Treisman, *Differential activation of c-fos promoter elements by serum, lysophosphatidic acid, G proteins and polypeptide growth factors*. Embo J, 1995. **14**(20): p. 5037-47.
102. Murphy, G.A., P.A. Solski, S.A. Jillian, P. Perez de la Ossa, P. D'Eustachio, C.J. Der and M.G. Rush, *Cellular functions of TC10, a Rho family GTPase: regulation of morphology, signal transduction and cell growth*. Oncogene, 1999. **18**(26): p. 3831-45.
103. Reif, K., C.D. Nobes, G. Thomas, A. Hall and D.A. Cantrell, *Phosphatidylinositol 3-kinase signals activate a selective subset of Rac/Rho-dependent effector pathways*. Curr Biol, 1996. **6**(11): p. 1445-55.
104. Wang, Y., M. Falasca, J. Schlessinger, S. Malstrom, P. Tschlis, J. Settleman, W. Hu, B. Lim and R. Prywes, *Activation of the c-fos serum response element by phosphatidyl inositol 3-kinase and rho pathways in HeLa cells*. Cell Growth Differ, 1998. **9**(7): p. 513-22.
105. Poser, S., S. Impey, K. Trinh, Z. Xia and D.R. Storm, *SRF-dependent gene expression is required for PI3-kinase-regulated cell proliferation*. Embo J, 2000. **19**(18): p. 4955-66.
106. Sotiropoulos, A., D. Gineitis, J. Copeland and R. Treisman, *Signal-regulated activation of serum response factor is mediated by changes in actin dynamics*. Cell, 1999. **98**(2): p. 159-69.
107. Arber, S., F.A. Barbayannis, H. Hanser, C. Schneider, C.A. Stanyon, O. Bernard and P. Caroni, *Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase*. Nature, 1998. **393**(6687): p. 805-9.
108. Perona, R., S. Montaner, L. Saniger, I. Sanchez-Perez, R. Bravo and J.C. Lacal, *Activation of the nuclear factor-kappaB by Rho, CDC42, and Rac-1 proteins*. Genes Dev, 1997. **11**(4): p. 463-75.
109. Zhang, S., J. Han, M.A. Sells, J. Chernoff, U.G. Knaus, R.J. Ulevitch and G.M. Bokoch, *Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1*. J Biol Chem, 1995. **270**(41): p. 23934-6.
110. Montaner, S., R. Perona, L. Saniger and J.C. Lacal, *Multiple signalling pathways lead to the activation of the nuclear factor kappaB by the Rho family of GTPases*. J Biol Chem, 1998. **273**(21): p. 12779-85.
111. Whitehead, I.P., Q.T. Lambert, J.A. Glaven, K. Abe, K.L. Rossman, G.M. Mahon, J.M. Trzaskos, R. Kay, S.L. Campbell and C.J. Der, *Dependence of Dbl and Dbs transformation on MEK and NF-kappaB activation*. Mol Cell Biol, 1999. **19**(11): p. 7759-70.
112. Montaner, S., R. Perona, L. Saniger and J.C. Lacal, *Activation of serum response factor by RhoA is mediated by the nuclear factor-kappaB and C/EBP transcription factors*. J Biol Chem, 1999. **274**(13): p. 8506-15.
113. Frost, J.A., J.L. Swantek, S. Stippec, M.J. Yin, R. Gaynor and M.H. Cobb, *Stimulation of NFkappa B activity by multiple signaling pathways requires PAK1*. J Biol Chem, 2000. **275**(26): p. 19693-9.

114. Charvet, C., P. Auberger, S. Tartare-Deckert, A. Bernard and M. Deckert, *Vav1 couples T cell receptor to serum response factor-dependent transcription via a MEK-dependent pathway*. J Biol Chem, 2002. **271**: p. 21.
115. Bar-Sagi, D. and A. Hall, *Ras and Rho GTPases: a family reunion*. Cell, 2000. **103**(2): p. 227-38.
116. Khosravi-Far, R., P.A. Solski, G.J. Clark, M.S. Kinch and C.J. Der, *Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation*. Mol Cell Biol, 1995. **15**(11): p. 6443-53.
117. Qiu, R.G., J. Chen, F. McCormick and M. Symons, *A role for Rho in Ras transformation*. Proc Natl Acad Sci U S A, 1995. **92**(25): p. 11781-5.
118. Qiu, R.G., A. Abo, F. McCormick and M. Symons, *Cdc42 regulates anchorage-independent growth and is necessary for Ras transformation*. Mol Cell Biol, 1997. **17**(6): p. 3449-58.
119. Olson, M.F., H.F. Paterson and C.J. Marshall, *Signals from Ras and Rho GTPases interact to regulate expression of p21Waf1/Cip1*. Nature, 1998. **394**(6690): p. 295-9.
120. Vidal, A., S.S. Millard, J.P. Miller and A. Koff, *Rho activity can alter the translation of p27 mRNA and is important for RasV12 induced transformation in a manner dependent on p27 status*. J Biol Chem, 2002. **277**: p. 1.
121. Eva, A. and S.A. Aaronson, *Isolation of a new human oncogene from a diffuse B-cell lymphoma*. Nature, 1985. **316**(6025): p. 273-5.
122. Srivastava, S.K., R.H. Wheelock, S.A. Aaronson and A. Eva, *Identification of the protein encoded by the human diffuse B-cell lymphoma (db1) oncogene*. Proc Natl Acad Sci U S A, 1986. **83**(23): p. 8868-72.
123. Hart, M.J., A. Eva, T. Evans, S.A. Aaronson and R.A. Cerione, *Catalysis of guanine nucleotide exchange on the CDC42Hs protein by the db1 oncogene product*. Nature, 1991. **354**(6351): p. 311-4.
124. Yaku, H., T. Sasaki and Y. Takai, *The Dbl oncogene product as a GDP/GTP exchange protein for the Rho family: its properties in comparison with those of Smg GDS*. Biochem Biophys Res Commun, 1994. **198**(2): p. 811-7.
125. Venter, J.C., M.D. Adams, E.W. Myers, P.W. Li, R.J. Mural, G.G. Sutton, H.O. Smith, M. Yandell, C.A. Evans, R.A. Holt, J.D. Gocayne, P. Amanatides, R.M. Ballew, D.H. Huson, J.R. Wortman, Q. Zhang, C.D. Kodira, X.H. Zheng, L. Chen, M. Skupski, G. Subramanian, P.D. Thomas, J. Zhang, G.L. Gabor Miklos, C. Nelson, S. Broder, A.G. Clark, J. Nadeau, V.A. McKusick, N. Zinder, A.J. Levine, R.J. Roberts, M. Simon, C. Slayman, M. Hunkapiller, R. Bolanos, A. Delcher, I. Dew, D. Fasulo, M. Flanigan, L. Florea, A. Halpern, S. Hannenhalli, S. Kravitz, S. Levy, C. Mobarry, K. Reinert, K. Remington, J. Abu-Threideh, E. Beasley, K. Biddick, V. Bonazzi, R. Brandon, M. Cargill, I. Chandramouliswaran, R. Charlab, K. Chaturvedi, Z. Deng, V. Di Francesco, P. Dunn, K. Eilbeck, C. Evangelista, A.E. Gabrielian, W. Gan, W. Ge, F. Gong, Z. Gu, P. Guan, T.J. Heiman, M.E. Higgins, R.R. Ji, Z. Ke, K.A. Ketchum, Z. Lai, Y. Lei, Z. Li, J. Li, Y. Liang, X. Lin, F. Lu, G.V. Merkulov, N. Milshina, H.M. Moore, A.K. Naik, V.A. Narayan, B. Neelam, D. Nusskern, D.B. Rusch, S. Salzberg, W. Shao, B. Shue, J. Sun, Z. Wang, A. Wang, X. Wang, J. Wang, M. Wei, R. Wides, C. Xiao, C. Yan, A. Yao, J. Ye, M. Zhan, W. Zhang, H. Zhang, Q. Zhao, L. Zheng, F. Zhong, W. Zhong, S. Zhu, S. Zhao, D. Gilbert, S. Baumhueter, G. Spier, C. Carter, A. Cravchik, T. Woodage, F. Ali, H. An, A. Awe, D. Baldwin, H. Baden, M. Barnstead, I. Barrow, K. Beeson, D. Busam, A. Carver, A. Center, M.L. Cheng, L. Curry, S.

- Danaher, L. Davenport, R. Desilets, S. Dietz, K. Dodson, L. Doup, S. Ferriera, N. Garg, A. Gluecksmann, B. Hart, J. Haynes, C. Haynes, C. Heiner, S. Hladun, D. Hostin, J. Houck, T. Howland, C. Ibegwam, J. Johnson, F. Kalush, L. Kline, S. Koduru, A. Love, F. Mann, D. May, S. McCawley, T. McIntosh, I. McMullen, M. Moy, L. Moy, B. Murphy, K. Nelson, C. Pfannkoch, E. Pratts, V. Puri, H. Qureshi, M. Reardon, R. Rodriguez, Y.H. Rogers, D. Romblad, B. Ruhfel, R. Scott, C. Sitter, M. Smallwood, E. Stewart, R. Strong, E. Suh, R. Thomas, N.N. Tint, S. Tse, C. Vech, G. Wang, J. Wetter, S. Williams, M. Williams, S. Windsor, E. Winn-Deen, K. Wolfe, J. Zaveri, K. Zaveri, J.F. Abril, R. Guigo, M.J. Campbell, K.V. Sjolander, B. Karlak, A. Kejariwal, H. Mi, B. Lazareva, T. Hatton, A. Narechania, K. Diemer, A. Muruganujan, N. Guo, S. Sato, V. Bafna, S. Istrail, R. Lippert, R. Schwartz, B. Walenz, S. Yooseph, D. Allen, A. Basu, J. Baxendale, L. Blick, M. Caminha, J. Carnes-Stine, P. Caulk, Y.H. Chiang, M. Coyne, C. Dahlke, A. Mays, M. Dombroski, M. Donnelly, D. Ely, S. Esparham, C. Fosler, H. Gire, S. Glanowski, K. Glasser, A. Glodek, M. Gorokhov, K. Graham, B. Gropman, M. Harris, J. Heil, S. Henderson, J. Hoover, D. Jennings, C. Jordan, J. Jordan, J. Kasha, L. Kagan, C. Kraft, A. Levitsky, M. Lewis, X. Liu, J. Lopez, D. Ma, W. Majoros, J. McDaniel, S. Murphy, M. Newman, T. Nguyen, N. Nguyen, M. Nodell, S. Pan, J. Peck, M. Peterson, W. Rowe, R. Sanders, J. Scott, M. Simpson, T. Smith, A. Sprague, T. Stockwell, R. Turner, E. Venter, M. Wang, M. Wen, D. Wu, M. Wu, A. Xia, A. Zandieh and X. Zhu, *The sequence of the human genome*. Science, 2001. **291**(5507): p. 1304-51.
126. Glaven, J.A., I.P. Whitehead, T. Nomanbhoy, R. Kay and R.A. Cerione, *Lfc and Lsc oncoproteins represent two new guanine nucleotide exchange factors for the Rho GTP-binding protein*. J Biol Chem, 1996. **271**(44): p. 27374-81.
 127. Hart, M.J., A. Eva, D. Zangrilli, S.A. Aaronson, T. Evans, R.A. Cerione and Y. Zheng, *Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the dbl oncogene product*. J Biol Chem, 1994. **269**(1): p. 62-5.
 128. Whitehead, I.P., R. Khosravi-Far, H. Kirk, G. Trigo-Gonzalez, C.J. Der and R. Kay, *Expression cloning of lsc, a novel oncogene with structural similarities to the Dbl family of guanine nucleotide exchange factors*. J Biol Chem, 1996. **271**(31): p. 18643-50.
 129. Hart, M.J., S. Sharma, N. elMasry, R.G. Qiu, P. McCabe, P. Polakis and G. Bollag, *Identification of a novel guanine nucleotide exchange factor for the Rho GTPase*. J Biol Chem, 1996. **271**(41): p. 25452-8.
 130. Hart, M.J., X. Jiang, T. Kozasa, W. Roscoe, W.D. Singer, A.G. Gilman, P.C. Sternweis and G. Bollag, *Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by Galpha13*. Science, 1998. **280**(5372): p. 2112-4.
 131. Kozasa, T., X. Jiang, M.J. Hart, P.M. Sternweis, W.D. Singer, A.G. Gilman, G. Bollag and P.C. Sternweis, *p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13*. Science, 1998. **280**(5372): p. 2109-11.
 132. Whitehead, I., H. Kirk and R. Kay, *Retroviral transduction and oncogenic selection of a cDNA encoding Dbs, a homolog of the Dbl guanine nucleotide exchange factor*. Oncogene, 1995. **10**(4): p. 713-21.
 133. Habets, G.G., E.H. Scholtes, D. Zuydgeest, R.A. van der Kammen, J.C. Stam, A. Berns and J.G. Collard, *Identification of an invasion-inducing gene, Tiam-1, that encodes a protein with homology to GDP-GTP exchangers for Rho-like proteins*. Cell, 1994. **77**(4): p. 537-49.
 134. Reuther, G.W., Q.T. Lambert, M.A. Booden, K. Wennerberg, B. Becknell, G. Marcucci, J. Sondek, M.A. Caligiuri and C.J. Der, *Leukemia-associated Rho guanine nucleotide exchange*

- factor, a *Dbl* family protein found mutated in leukemia, causes transformation by activation of *RhoA*. *J Biol Chem*, 2001. **276**(29): p. 27145-51.
135. Fukuhara, S., H. Chikumi and J.S. Gutkind, *Leukemia-associated Rho guanine nucleotide exchange factor (LARG) links heterotrimeric G proteins of the G(12) family to Rho*. *FEBS Lett*, 2000. **485**(2-3): p. 183-8.
 136. Fukuhara, S., C. Murga, M. Zohar, T. Igishi and J.S. Gutkind, *A novel PDZ domain containing guanine nucleotide exchange factor links heterotrimeric G proteins to Rho*. *J Biol Chem*, 1999. **274**(9): p. 5868-79.
 137. Toksoz, D. and D.A. Williams, *Novel human oncogene lbc detected by transfection with distinct homology regions to signal transduction products*. *Oncogene*, 1994. **9**(2): p. 621-8.
 138. Chan, A.M., E.S. McGovern, G. Catalano, T.P. Fleming and T. Miki, *Expression cDNA cloning of a novel oncogene with sequence similarity to regulators of small GTP-binding proteins*. *Oncogene*, 1994. **9**(4): p. 1057-63.
 139. Bustelo, X.R., K.L. Suen, K. Leftheris, C.A. Meyers and M. Barbacid, *Vav cooperates with Ras to transform rodent fibroblasts but is not a Ras GDP/GTP exchange factor*. *Oncogene*, 1994. **9**(8): p. 2405-13.
 140. Khosravi-Far, R., M.A. White, J.K. Westwick, P.A. Soliski, M. Chrzanowska-Wodnicka, L. Van Aelst, M.H. Wigler and C.J. Der, *Oncogenic Ras activation of Raf/mitogen-activated protein kinase- independent pathways is sufficient to cause tumorigenic transformation*. *Mol Cell Biol*, 1996. **16**(7): p. 3923-33.
 141. Katzav, S., D. Martin-Zanca and M. Barbacid, *vav, a novel human oncogene derived from a locus ubiquitously expressed in hematopoietic cells*. *Embo J*, 1989. **8**(8): p. 2283-90.
 142. Zheng, Y., M.F. Olson, A. Hall, R.A. Cerione and D. Toksoz, *Direct involvement of the small GTP-binding protein Rho in lbc oncogene function*. *J Biol Chem*, 1995. **270**(16): p. 9031-4.
 143. Lorenzi, M.V., P. Castagnino, Q. Chen, Y. Hori and T. Miki, *Distinct expression patterns and transforming properties of multiple isoforms of Ost, an exchange factor for RhoA and Cdc42*. *Oncogene*, 1999. **18**(33): p. 4742-55.
 144. Ron, D., M. Zannini, M. Lewis, R.B. Wickner, L.T. Hunt, G. Graziani, S.R. Tronick, S.A. Aaronson and A. Eva, *A region of proto-dbl essential for its transforming activity shows sequence similarity to a yeast cell cycle gene, CDC24, and the human breakpoint cluster gene, bcr*. *New Biol*, 1991. **3**(4): p. 372-9.
 145. Zhu, K., B. Debreceni, R. Li and Y. Zheng, *Identification of Rho GTPase-dependent sites in the Dbl homology domain of oncogenic Dbl that are required for transformation*. *J Biol Chem*, 2000. **275**(34): p. 25993-6001.
 146. Glaven, J.A., I. Whitehead, S. Bagrodia, R. Kay and R.A. Cerione, *The Dbl-related protein, Lfc, localizes to microtubules and mediates the activation of Rac signaling pathways in cells*. *J Biol Chem*, 1999. **274**(4): p. 2279-85.
 147. Zheng, Y., D.J. Fischer, M.F. Santos, G. Tigyi, N.G. Pasteris, J.L. Gorski and Y. Xu, *The faciogenital dysplasia gene product FGD1 functions as a Cdc42Hs- specific guanine-nucleotide exchange factor*. *J Biol Chem*, 1996. **271**(52): p. 33169-72.
 148. Michiels, F., G.G. Habets, J.C. Stam, R.A. van der Kammen and J.G. Collard, *A role for Rac in Tiam1-induced membrane ruffling and invasion*. *Nature*, 1995. **375**(6529): p. 338-40.

149. Nagata, K., M. Driessens, N. Lamarche, J.L. Gorski and A. Hall, *Activation of G1 progression, JNK mitogen-activated protein kinase, and actin filament assembly by the exchange factor FGD1*. J Biol Chem, 1998. **273**(25): p. 15453-7.
150. Westwick, J.K., R.J. Lee, Q.T. Lambert, M. Symons, R.G. Pestell, C.J. Der and I.P. Whitehead, *Transforming potential of Dbl family proteins correlates with transcription from the cyclin D1 promoter but not with activation of Jun NH2-terminal kinase, p38/Mpk2, serum response factor, or c-Jun*. J Biol Chem, 1998. **273**(27): p. 16739-47.
151. Mao, J., H. Yuan, W. Xie and D. Wu, *Guanine nucleotide exchange factor GEF115 specifically mediates activation of Rho and serum response factor by the G protein alpha subunit Galpha13*. Proc Natl Acad Sci U S A, 1998. **95**(22): p. 12973-6.
152. Jin, S. and J.H. Exton, *Activation of RhoA by association of Galpha(13) with Dbl*. Biochem Biophys Res Commun, 2000. **277**(3): p. 718-21.
153. Bustelo, X.R., *Regulatory and signaling properties of the Vav family*. Mol Cell Biol, 2000. **20**(5): p. 1461-77.
154. Tatsumoto, T., X. Xie, R. Blumenthal, I. Okamoto and T. Miki, *Human ECT2 is an exchange factor for Rho GTPases, phosphorylated in G2/M phases, and involved in cytokinesis*. J Cell Biol, 1999. **147**(5): p. 921-8.
155. Cerione, R.A. and Y. Zheng, *The Dbl family of oncogenes*. Curr Opin Cell Biol, 1996. **8**(2): p. 216-22.
156. Whitehead, I.P., S. Campbell, K.L. Rossman and C.J. Der, *Dbl family proteins*. Biochim Biophys Acta, 1997. **1332**(1): p. F1-23.
157. Zheng, Y., *Dbl family guanine nucleotide exchange factors*. Trends Biochem Sci, 2001. **26**(12): p. 724-32.
158. Zheng, Y., D. Zangrilli, R.A. Cerione and A. Eva, *The pleckstrin homology domain mediates transformation by oncogenic dbl through specific intracellular targeting*. J Biol Chem, 1996. **271**(32): p. 19017-20.
159. Olson, M.F., P. Sterpetti, K. Nagata, D. Toksoz and A. Hall, *Distinct roles for DH and PH domains in the Lbc oncogene*. Oncogene, 1997. **15**(23): p. 2827-31.
160. Whitehead, I., H. Kirk, C. Tognon, G. Trigo-Gonzalez and R. Kay, *Expression cloning of lfc, a novel oncogene with structural similarities to guanine nucleotide exchange factors and to the regulatory region of protein kinase C*. J Biol Chem, 1995. **270**(31): p. 18388-95.
161. Lemmon, M.A., K.M. Ferguson and C.S. Abrams, *Pleckstrin homology domains and the cytoskeleton*. FEBS Lett, 2002. **513**(1): p. 71-6.
162. Russo, C., Y. Gao, P. Mancini, C. Vanni, M. Porotto, M. Falasca, M.R. Torrisi, Y. Zheng and A. Eva, *Modulation of oncogenic DBL activity by phosphoinositol phosphate binding to pleckstrin homology domain*. J Biol Chem, 2001. **276**(22): p. 19524-31.
163. Liu, X., H. Wang, M. Eberstadt, A. Schnuchel, E.T. Olejniczak, R.P. Meadows, J.M. Schkeryantz, D.A. Janowick, J.E. Harlan, E.A. Harris, D.E. Staunton and S.W. Fesik, *NMR structure and mutagenesis of the N-terminal Dbl homology domain of the nucleotide exchange factor Trio*. Cell, 1998. **95**(2): p. 269-77.
164. Zhu, K., B. Debreceni, F. Bi and Y. Zheng, *Oligomerization of DH domain is essential for Dbl-induced transformation*. Mol Cell Biol, 2001. **21**(2): p. 425-37.

165. Bi, F., B. Debreceni, K. Zhu, B. Salani, A. Eva and Y. Zheng, *Autoinhibition mechanism of proto-Dbl*. Mol Cell Biol, 2001. **21**(5): p. 1463-74.
166. Aghazadeh, B., W.E. Lowry, X.Y. Huang and M.K. Rosen, *Structural basis for relief of autoinhibition of the Dbl homology domain of proto-oncogene Vav by tyrosine phosphorylation*. Cell, 2000. **102**(5): p. 625-33.
167. Han, J., K. Luby-Phelps, B. Das, X. Shu, Y. Xia, R.D. Mosteller, U.M. Krishna, J.R. Falck, M.A. White and D. Broek, *Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav*. Science, 1998. **279**(5350): p. 558-60.
168. Nimnual, A.S., B.A. Yatsula and D. Bar-Sagi, *Coupling of Ras and Rac guanosine triphosphatases through the Ras exchanger Sos*. Science, 1998. **279**(5350): p. 560-3.
169. Wells, C.D., S. Gutowski, G. Bollag and P.C. Sternweis, *Identification of potential mechanisms for regulation of p115 RhoGEF through analysis of endogenous and mutant forms of the exchange factor*. J Biol Chem, 2001. **276**(31): p. 28897-905.
170. Rumenapp, U., A. Blomquist, G. Schworer, H. Schablowski, A. Psoma and K.H. Jakobs, *Rho-specific binding and guanine nucleotide exchange catalysis by KIAA0380, a dbl family member*. FEBS Lett, 1999. **459**(3): p. 313-8.
171. Aravind, L., A.F. Neuwald and C.P. Ponting, *Sec14p-like domains in NF1 and Dbl-like proteins indicate lipid regulation of Ras and Rho signaling*. Curr Biol, 1999. **9**(6): p. R195-7.
172. Horii, Y., J.F. Beeler, K. Sakaguchi, M. Tachibana and T. Miki, *A novel oncogene, ost, encodes a guanine nucleotide exchange factor that potentially links Rho and Rac signaling pathways*. Embo J, 1994. **13**(20): p. 4776-86.
173. Olson, M.F., *Guanine nucleotide exchange factors for the Rho GTPases: a role in human disease?* J Mol Med, 1996. **74**(10): p. 563-71.
174. McWhirter, J.R. and J.Y. Wang, *Activation of tyrosinase kinase and microfilament-binding functions of c-abl by bcr sequences in bcr/abl fusion proteins*. Mol Cell Biol, 1991. **11**(3): p. 1553-65.
175. McWhirter, J.R. and J.Y. Wang, *Effect of Bcr sequences on the cellular function of the Bcr-Abl oncoprotein*. Oncogene, 1997. **15**(14): p. 1625-34.
176. Kin, Y., G. Li, M. Shibuya and Y. Maru, *The Dbl homology domain of BCR is not a simple spacer in P210BCR-ABL of the Philadelphia chromosome*. J Biol Chem, 2001. **276**(42): p. 39462-8.
177. Kourlas, P.J., M.P. Strout, B. Becknell, M.L. Veronese, C.M. Croce, K.S. Theil, R. Krahe, T. Ruutu, S. Knuutila, C.D. Bloomfield and M.A. Caligiuri, *Identification of a gene at 11q23 encoding a guanine nucleotide exchange factor: evidence for its fusion with MLL in acute myeloid leukemia*. Proc Natl Acad Sci U S A, 2000. **97**(5): p. 2145-50.
178. Pasteris, N.G., A. Cadle, L.J. Logie, M.E. Porteous, C.E. Schwartz, R.E. Stevenson, T.W. Glover, R.S. Wilroy and J.L. Gorski, *Isolation and characterization of the faciogenital dysplasia (Aarskog- Scott syndrome) gene: a putative Rho/Rac guanine nucleotide exchange factor*. Cell, 1994. **79**(4): p. 669-78.
179. Schoneberg, T., G. Schultz and T. Gudermann, *Structural basis of G protein-coupled receptor function*. Mol Cell Endocrinol, 1999. **151**(1-2): p. 181-93.
180. Hur, E.M. and K.T. Kim, *G protein-coupled receptor signalling and cross-talk. Achieving rapidity and specificity*. Cell Signal, 2002. **14**(5): p. 397-405.

181. Offermanns, S., *In vivo functions of heterotrimeric G-proteins: studies in G α -deficient mice.* Oncogene, 2001. **20**(13): p. 1635-42.
182. Radhika, V. and N. Dhanasekaran, *Transforming G proteins.* Oncogene, 2001. **20**(13): p. 1607-14.
183. Clapham, D.E. and E.J. Neer, *G protein beta gamma subunits.* Annu Rev Pharmacol Toxicol, 1997. **37**: p. 167-203.
184. Sondek, J., A. Bohm, D.G. Lambright, H.E. Hamm and P.B. Sigler, *Crystal structure of a G-protein beta gamma dimer at 2.1 Å resolution.* Nature, 1996. **379**(6563): p. 369-74.
185. Ross, E.M. and T.M. Wilkie, *GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins.* Annu Rev Biochem, 2000. **69**: p. 795-827.
186. Faurobert, E. and J.B. Hurley, *The core domain of a new retina specific RGS protein stimulates the GTPase activity of transducin in vitro.* Proc Natl Acad Sci U S A, 1997. **94**(7): p. 2945-50.
187. Popov, S., K. Yu, T. Kozasa and T.M. Wilkie, *The regulators of G protein signaling (RGS) domains of RGS4, RGS10, and GAIP retain GTPase activating protein activity in vitro.* Proc Natl Acad Sci U S A, 1997. **94**(14): p. 7216-20.
188. Mukhopadhyay, S. and E.M. Ross, *Rapid GTP binding and hydrolysis by G(q) promoted by receptor and GTPase-activating proteins.* Proc Natl Acad Sci U S A, 1999. **96**(17): p. 9539-44.
189. Rasenick, M.M., M. Watanabe, M.B. Lazarevic, S. Hattar and H.E. Hamm, *Synthetic peptides as probes for G protein function. Carboxyl-terminal G α s peptides mimic Gs and evoke high affinity agonist binding to beta-adrenergic receptors.* J Biol Chem, 1994. **269**(34): p. 21519-25.
190. Akhter, S.A., L.M. Luttrell, H.A. Rockman, G. Iaccarino, R.J. Lefkowitz and W.J. Koch, *Targeting the receptor-Gq interface to inhibit in vivo pressure overload myocardial hypertrophy.* Science, 1998. **280**(5363): p. 574-7.
191. Gilchrist, A., M. Bunemann, A. Li, M.M. Hosey and H.E. Hamm, *A dominant-negative strategy for studying roles of G proteins in vivo.* J Biol Chem, 1999. **274**(10): p. 6610-6.
192. Spada, A. and L. Vallar, *G-protein oncogenes in acromegaly.* Horm Res, 1992. **38**(1-2): p. 90-3.
193. Whitehead, I.P., I.E. Zohn and C.J. Der, *Rho GTPase-dependent transformation by G protein-coupled receptors.* Oncogene, 2001. **20**(13): p. 1547-55.
194. Young, D., G. Waitches, C. Birchmeier, O. Fasano and M. Wigler, *Isolation and characterization of a new cellular oncogene encoding a protein with multiple potential transmembrane domains.* Cell, 1986. **45**(5): p. 711-9.
195. Gutkind, J.S., E.A. Novotny, M.R. Brann and K.C. Robbins, *Muscarinic acetylcholine receptor subtypes as agonist-dependent oncogenes.* Proc Natl Acad Sci U S A, 1991. **88**(11): p. 4703-7.
196. Julius, D., T.J. Livelli, T.M. Jessell and R. Axel, *Ectopic expression of the serotonin 1c receptor and the triggering of malignant transformation.* Science, 1989. **244**(4908): p. 1057-62.
197. Allen, L.F., R.J. Lefkowitz, M.G. Caron and S. Cotecchia, *G-protein-coupled receptor genes as protooncogenes: constitutively activating mutation of the α 1B-adrenergic receptor enhances mitogenesis and tumorigenicity.* Proc Natl Acad Sci U S A, 1991. **88**(24): p. 11354-8.
198. Parma, J., L. Duprez, J. Van Sande, P. Cochaux, C. Gervy, J. Mockel, J. Dumont and G. Vassart, *Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenomas.* Nature, 1993. **365**(6447): p. 649-51.

199. Du Villard, J.A., R. Wicker, P. Crespo, D. Russo, S. Filetti, J.S. Gutkind, A. Sarasin and H.G. Suarez, *Role of the cAMP and MAPK pathways in the transformation of mouse 3T3 fibroblasts by a TSHR gene constitutively activated by point mutation*. *Oncogene*, 2000. **19**(42): p. 4896-905.
200. Cuttitta, F., D.N. Carney, J. Mulshine, T.W. Moody, J. Fedorko, A. Fischler and J.D. Minna, *Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer*. *Nature*, 1985. **316**(6031): p. 823-6.
201. Zohn, I.E., M. Klinger, X. Karp, H. Kirk, M. Symons, M. Chrzanowska-Wodnicka, C.J. Der and R.J. Kay, *G2A is an oncogenic G protein-coupled receptor*. *Oncogene*, 2000. **19**(34): p. 3866-77.
202. Martin, C.B., G.M. Mahon, M.B. Klinger, R.J. Kay, M. Symons, C.J. Der and I.P. Whitehead, *The thrombin receptor, PAR-1, causes transformation by activation of Rho-mediated signaling pathways*. *Oncogene*, 2001. **20**(16): p. 1953-63.
203. Macfarlane, S.R., M.J. Seatter, T. Kanke, G.D. Hunter and R. Plevin, *Proteinase-activated receptors*. *Pharmacol Rev*, 2001. **53**(2): p. 245-82.
204. Van Obberghen-Schilling, E., J.C. Chambard, S. Paris, G. L'Allemain and J. Pouyssegur, *alpha-Thrombin-induced early mitogenic signalling events and G0 to S- phase transition of fibroblasts require continual external stimulation*. *Embo J*, 1985. **4**(11): p. 2927-32.
205. Even-Ram, S., B. Uziely, P. Cohen, S. Grisaru-Granovsky, M. Maoz, Y. Ginzburg, R. Reich, I. Vlodavsky and R. Bar-Shavit, *Thrombin receptor overexpression in malignant and physiological invasion processes*. *Nat Med*, 1998. **4**(8): p. 909-14.
206. Henrikson, K.P., S.L. Salazar, J.W. Fenton, 2nd and B.T. Pentecost, *Role of thrombin receptor in breast cancer invasiveness*. *Br J Cancer*, 1999. **79**(3-4): p. 401-6.
207. Lyons, J., C.A. Landis, G. Harsh, L. Vallar, K. Grunewald, H. Feichtinger, Q.Y. Duh, O.H. Clark, E. Kawasaki, H.R. Bourne and et al., *Two G protein oncogenes in human endocrine tumors*. *Science*, 1990. **249**(4969): p. 655-9.
208. Vallar, L., *Oncogenic role of heterotrimeric G proteins*. *Cancer Surv*, 1996. **27**: p. 325-38.
209. Ham, J., M. Ivan, D. Wynford-Thomas and M.F. Scanlon, *GH3 cells expressing constitutively active Gs alpha (Q227L) show enhanced hormone secretion and proliferation*. *Mol Cell Endocrinol*, 1997. **127**(1): p. 41-7.
210. Gupta, S.K., C. Gallego, G.L. Johnson and L.E. Heasley, *MAP kinase is constitutively activated in gip2 and src transformed rat 1a fibroblasts*. *J Biol Chem*, 1992. **267**(12): p. 7987-90.
211. Winitz, S., M. Russell, N.X. Qian, A. Gardner, L. Dwyer and G.L. Johnson, *Involvement of Ras and Raf in the Gi-coupled acetylcholine muscarinic m2 receptor activation of mitogen-activated protein (MAP) kinase kinase and MAP kinase*. *J Biol Chem*, 1993. **268**(26): p. 19196-9.
212. Gupta, S.K., C. Gallego, J.M. Lowndes, C.M. Pleiman, C. Sable, B.J. Eisfelder and G.L. Johnson, *Analysis of the fibroblast transformation potential of GTPase-deficient gip2 oncogenes*. *Mol Cell Biol*, 1992. **12**(1): p. 190-7.
213. Lowndes, J.M., S.K. Gupta, S. Osawa and G.L. Johnson, *GTPase-deficient G alpha i2 oncogene gip2 inhibits adenylylcyclase and attenuates receptor-stimulated phospholipase A2 activity*. *J Biol Chem*, 1991. **266**(22): p. 14193-7.
214. Wong, Y.H., A. Federman, A.M. Pace, I. Zachary, T. Evans, J. Pouyssegur and H.R. Bourne, *Mutant alpha subunits of Gi2 inhibit cyclic AMP accumulation*. *Nature*, 1991. **351**(6321): p. 63-5.

215. Kalinec, G., A.J. Nazarali, S. Hermouet, N. Xu and J.S. Gutkind, *Mutated alpha subunit of the Gq protein induces malignant transformation in NIH 3T3 cells*. *Mol Cell Biol*, 1992. **12**(10): p. 4687-93.
216. De Vivo, M., J. Chen, J. Codina and R. Iyengar, *Enhanced phospholipase C stimulation and transformation in NIH-3T3 cells expressing Q209LGq-alpha-subunits*. *J Biol Chem*, 1992. **267**(26): p. 18263-6.
217. Chan, A.M., T.P. Fleming, E.S. McGovern, M. Chedid, T. Miki and S.A. Aaronson, *Expression cDNA cloning of a transforming gene encoding the wild-type G alpha 12 gene product*. *Mol Cell Biol*, 1993. **13**(2): p. 762-8.
218. Gutkind, J.S., *Cell growth control by G protein-coupled receptors: from signal transduction to signal integration*. *Oncogene*, 1998. **17**(11 Reviews): p. 1331-42.
219. Buhl, A.M., N.L. Johnson, N. Dhanasekaran and G.L. Johnson, *G alpha 12 and G alpha 13 stimulate Rho-dependent stress fiber formation and focal adhesion assembly*. *J Biol Chem*, 1995. **270**(42): p. 24631-4.
220. Collins, L.R., W.A. Ricketts, J.M. Olefsky and J.H. Brown, *The G12 coupled thrombin receptor stimulates mitogenesis through the Shc SH2 domain*. *Oncogene*, 1997. **15**(5): p. 595-600.
221. Prasad, M.V., J.M. Dermott, L.E. Heasley, G.L. Johnson and N. Dhanasekaran, *Activation of Jun kinase/stress-activated protein kinase by GTPase- deficient mutants of G alpha 12 and G alpha 13*. *J Biol Chem*, 1995. **270**(31): p. 18655-9.
222. Voyno-Yasenetskaya, T.A., M.P. Faure, N.G. Ahn and H.R. Bourne, *Galpha12 and Galpha13 regulate extracellular signal-regulated kinase and c-Jun kinase pathways by different mechanisms in COS-7 cells*. *J Biol Chem*, 1996. **271**(35): p. 21081-7.
223. Collins, L.R., A. Minden, M. Karin and J.H. Brown, *Galpha12 stimulates c-Jun NH2-terminal kinase through the small G proteins Ras and Rac*. *J Biol Chem*, 1996. **271**(29): p. 17349-53.
224. Mitsui, H., N. Takuwa, K. Kurokawa, J.H. Exton and Y. Takuwa, *Dependence of activated Galpha12-induced G1 to S phase cell cycle progression on both Ras/mitogen-activated protein kinase and Ras/Rac1/Jun N-terminal kinase cascades in NIH3T3 fibroblasts*. *J Biol Chem*, 1997. **272**(8): p. 4904-10.
225. Dermott, J.M., M.R. Reddy, D. Onesime, E.P. Reddy and N. Dhanasekaran, *Oncogenic mutant of Galpha12 stimulates cell proliferation through cyclooxygenase-2 signaling pathway*. *Oncogene*, 1999. **18**(51): p. 7185-9.
226. Needham, L.K. and E. Rozengurt, *Galpha12 and Galpha13 stimulate Rho-dependent tyrosine phosphorylation of focal adhesion kinase, paxillin, and p130 Crk-associated substrate*. *J Biol Chem*, 1998. **273**(23): p. 14626-32.
227. Shi, C.S., S. Sinnarajah, H. Cho, T. Kozasa and J.H. Kehrl, *G13alpha-mediated PYK2 activation. PYK2 is a mediator of G13alpha - induced serum response element-dependent transcription*. *J Biol Chem*, 2000. **275**(32): p. 24470-6.
228. Fromm, C., O.A. Coso, S. Montaner, N. Xu and J.S. Gutkind, *The small GTP-binding protein Rho links G protein-coupled receptors and Galpha12 to the serum response element and to cellular transformation*. *Proc Natl Acad Sci U S A*, 1997. **94**(19): p. 10098-103.
229. Vara Prasad, M.V., S.K. Shore and N. Dhanasekaran, *Activated mutant of G alpha 13 induces Egr-1, c-fos, and transformation in NIH 3T3 cells*. *Oncogene*, 1994. **9**(8): p. 2425-9.

230. Gohla, A., R. Harhammer and G. Schultz, *The G-protein G13 but not G12 mediates signaling from lysophosphatidic acid receptor via epidermal growth factor receptor to Rho*. J Biol Chem, 1998. **273**(8): p. 4653-9.
231. Gohla, A., S. Offermanns, T.M. Wilkie and G. Schultz, *Differential involvement of Galpha12 and Galpha13 in receptor-mediated stress fiber formation*. J Biol Chem, 1999. **274**(25): p. 17901-7.
232. Mao, J., W. Xie, H. Yuan, M.I. Simon, H. Mano and D. Wu, *Tec/Bmx non-receptor tyrosine kinases are involved in regulation of Rho and serum response factor by Galpha12/13*. Embo J, 1998. **17**(19): p. 5638-46.
233. Fleming, I.N., C.M. Elliott, J.G. Collard and J.H. Exton, *Lysophosphatidic acid induces threonine phosphorylation of Tiam1 in Swiss 3T3 fibroblasts via activation of protein kinase C*. J Biol Chem, 1997. **272**(52): p. 33105-10.
234. Zohn, I.E., M. Symons, M. Chrzanowska-Wodnicka, J.K. Westwick and C.J. Der, *Mas oncogene signaling and transformation require the small GTP-binding protein Rac*. Mol Cell Biol, 1998. **18**(3): p. 1225-35.
235. Kabarowski, J.H., J.D. Feramisco, L.Q. Le, J.L. Gu, S.W. Luoh, M.I. Simon and O.N. Witte, *Direct genetic demonstration of G alpha 13 coupling to the orphan G protein-coupled receptor G2A leading to RhoA-dependent actin rearrangement*. Proc Natl Acad Sci U S A, 2000. **97**(22): p. 12109-14.
236. Shortman, K. and L. Wu, *Early T lymphocyte progenitors*. Annu Rev Immunol, 1996. **14**: p. 29-47.
237. Killeen, N., B.A. Irving, S. Pippig and K. Ziegler, *Signaling checkpoints during the development of T lymphocytes*. Curr Opin Immunol, 1998. **10**(3): p. 360-7.
238. Zuniga-Pflucker, J.C. and M.J. Lenardo, *Regulation of thymocyte development from immature progenitors*. Curr Opin Immunol, 1996. **8**(2): p. 215-24.
239. Godfrey, D.I. and A. Zlotnik, *Control points in early T-cell development*. Immunol Today, 1993. **14**(11): p. 547-53.
240. von Boehmer, H. and H.J. Fehling, *Structure and function of the pre-T cell receptor*. Annu Rev Immunol, 1997. **15**: p. 433-52.
241. Mombaerts, P., A.R. Clarke, M.A. Rudnicki, J. Iacomini, S. Itohara, J.J. Lafaille, L. Wang, Y. Ichikawa, R. Jaenisch, M.L. Hooper and et al., *Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages*. Nature, 1992. **360**(6401): p. 225-31.
242. Fehling, H.J., A. Krotkova, C. Saint-Ruf and H. von Boehmer, *Crucial role of the pre-T-cell receptor alpha gene in development of alpha beta but not gamma delta T cells*. Nature, 1995. **375**(6534): p. 795-8.
243. Fehling, H.J. and H. von Boehmer, *Early alpha beta T cell development in the thymus of normal and genetically altered mice*. Curr Opin Immunol, 1997. **9**(2): p. 263-75.
244. Gidos, C.J., *Positive selection of CD4+ and CD8+ T cells*. Curr Opin Immunol, 1996. **8**(2): p. 225-32.
245. Saito, T. and N. Watanabe, *Positive and negative thymocyte selection*. Crit Rev Immunol, 1998. **18**(4): p. 359-70.
246. Sebzda, E., S. Mariathasan, T. Ohteki, R. Jones, M.F. Bachmann and P.S. Ohashi, *Selection of the T cell repertoire*. Annu Rev Immunol, 1999. **17**: p. 829-74.

247. Azzam, H.S., J.B. DeJarnette, K. Huang, R. Emmons, C.S. Park, C.L. Sommers, D. El-Khoury, E.W. Shores and P.E. Love, *Fine tuning of TCR signaling by CD5*. J Immunol, 2001. **166**(9): p. 5464-72.
248. Kishimoto, H. and J. Sprent, *The thymus and negative selection*. Immunol Res, 2000. **21**(2-3): p. 315-23.
249. Kisielow, P. and A. Miazek, *Positive selection of T cells: rescue from programmed cell death and differentiation require continual engagement of the T cell receptor*. J Exp Med, 1995. **181**(6): p. 1975-84.
250. Hare, K.J., E.J. Jenkinson and G. Anderson, *CD69 expression discriminates MHC-dependent and -independent stages of thymocyte positive selection*. J Immunol, 1999. **162**(7): p. 3978-83.
251. Swat, W., M. Dessing, H. von Boehmer and P. Kisielow, *CD69 expression during selection and maturation of CD4+8+ thymocytes*. Eur J Immunol, 1993. **23**(3): p. 739-46.
252. Nakayama, T., D.J. Kasprowicz, M. Yamashita, L.A. Schubert, G. Gillard, M. Kimura, A. Didierlaurent, H. Koseki and S.F. Ziegler, *The generation of mature, single-positive thymocytes in vivo is dysregulated by CD69 blockade or overexpression*. J Immunol, 2002. **168**(1): p. 87-94.
253. Hough, M.R., F. Takei, R.K. Humphries and R. Kay, *Defective development of thymocytes overexpressing the costimulatory molecule, heat-stable antigen*. J Exp Med, 1994. **179**(1): p. 177-84.
254. Tanchot, C., M.M. Rosado, F. Agenes, A.A. Freitas and B. Rocha, *Lymphocyte homeostasis*. Semin Immunol, 1997. **9**(6): p. 331-7.
255. LeBien, T.W., *B-cell lymphopoiesis in mouse and man*. Curr Opin Immunol, 1998. **10**(2): p. 188-95.
256. Patrick, C.W., Jr., T.W. Smith, L.V. McIntire and H.S. Juneja, *Cellular interactions among marrow stromal and normal/neoplastic pre-B- and B-lymphoblastic cells*. Leuk Lymphoma, 1996. **22**(3-4): p. 205-19.
257. Peschon, J.J., P.J. Morrissey, K.H. Grabstein, F.J. Ramsdell, E. Maraskovsky, B.C. Gliniak, L.S. Park, S.F. Ziegler, D.E. Williams, C.B. Ware and et al., *Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice*. J Exp Med, 1994. **180**(5): p. 1955-60.
258. Hardy, R.R., C.E. Carmack, S.A. Shinton, J.D. Kemp and K. Hayakawa, *Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow*. J Exp Med, 1991. **173**(5): p. 1213-25.
259. Healy, J.I. and C.C. Goodnow, *Positive versus negative signaling by lymphocyte antigen receptors*. Annu Rev Immunol, 1998. **16**: p. 645-70.
260. Campbell, K.S., *Signal transduction from the B cell antigen-receptor*. Curr Opin Immunol, 1999. **11**(3): p. 256-64.
261. Reif, K. and D.A. Cantrell, *Networking Rho family GTPases in lymphocytes*. Immunity, 1998. **8**(4): p. 395-401.
262. Aktories, K., *Rho proteins: targets for bacterial toxins*. Trends Microbiol, 1997. **5**(7): p. 282-8.
263. Lang, P., L. Guizani, I. Vitte-Mony, R. Stancou, O. Dorseuil, G. Gacon and J. Bertoglio, *ADP-ribosylation of the ras-related, GTP-binding protein RhoA inhibits lymphocyte-mediated cytotoxicity*. J Biol Chem, 1992. **267**(17): p. 11677-80.

264. Lang, P. and J. Bertoglio, *Inhibition of lymphocyte-mediated cytotoxicity by Clostridium botulinum C3 transferase*. Methods Enzymol, 1995. **256**: p. 320-7.
265. Laudanna, C., J.J. Campbell and E.C. Butcher, *Role of Rho in chemoattractant-activated leukocyte adhesion through integrins*. Science, 1996. **271**(5251): p. 981-3.
266. Adamson, P., S. Etienne, P.O. Couraud, V. Calder and J. Greenwood, *Lymphocyte migration through brain endothelial cell monolayers involves signaling through endothelial ICAM-1 via a rho-dependent pathway*. J Immunol, 1999. **162**(5): p. 2964-73.
267. Vicente-Manzanares, M., J.R. Cabrero, M. Rey, M. Perez-Martinez, A. Ursa, K. Itoh and F. Sanchez-Madrid, *A role for the Rho-p160 Rho coiled-coil kinase axis in the chemokine stromal cell-derived factor-1alpha-induced lymphocyte actomyosin and microtubular organization and chemotaxis*. J Immunol, 2002. **168**(1): p. 400-10.
268. Woodside, D.G., D.K. Wooten and B.W. McIntyre, *Adenosine diphosphate (ADP)-ribosylation of the guanosine triphosphatase (GTPase) rho in resting peripheral blood human T lymphocytes results in pseudopodial extension and the inhibition of T cell activation*. J Exp Med, 1998. **188**(7): p. 1211-21.
269. Angkachatchai, V. and T.H. Finkel, *ADP-ribosylation of rho by C3 ribosyltransferase inhibits IL-2 production and sustained calcium influx in activated T cells*. J Immunol, 1999. **163**(7): p. 3819-25.
270. Borroto, A., D. Gil, P. Delgado, M. Vicente-Manzanares, A. Alcover, F. Sanchez-Madrid and B. Alarcon, *Rho regulates T cell receptor ITAM-induced lymphocyte spreading in an integrin-independent manner*. Eur J Immunol, 2000. **30**(12): p. 3403-10.
271. Kobayashi, M., E. Azuma, M. Ido, M. Hirayama, Q. Jiang, S. Iwamoto, T. Kumamoto, H. Yamamoto, M. Sakurai and Y. Komada, *A pivotal role of Rho GTPase in the regulation of morphology and function of dendritic cells*. J Immunol, 2001. **167**(7): p. 3585-91.
272. Henning, S.W., R. Galandini, A. Hall and D.A. Cantrell, *The GTPase Rho has a critical regulatory role in thymus development*. Embo J, 1997. **16**(9): p. 2397-407.
273. Akashi, K., M. Kondo, U. von Freeden-Jeffry, R. Murray and I.L. Weissman, *Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice*. Cell, 1997. **89**(7): p. 1033-41.
274. Maraskovsky, E., L.A. O'Reilly, M. Teepe, L.M. Corcoran, J.J. Peschon and A. Strasser, *Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor- deficient mice but not in mutant rag-1-/- mice*. Cell, 1997. **89**(7): p. 1011-9.
275. Galandini, R., S.W. Henning and D.A. Cantrell, *Different functions of the GTPase Rho in prothymocytes and late pre-T cells*. Immunity, 1997. **7**(1): p. 163-74.
276. Cleverley, S.C., P.S. Costello, S.W. Henning and D.A. Cantrell, *Loss of Rho function in the thymus is accompanied by the development of thymic lymphoma*. Oncogene, 2000. **19**(1): p. 13-20.
277. Cleverley, S., S. Henning and D. Cantrell, *Inhibition of Rho at different stages of thymocyte development gives different perspectives on Rho function*. Curr Biol, 1999. **9**(12): p. 657-60.
278. Corre, I., M. Gomez, S. Vielkind and D.A. Cantrell, *Analysis of thymocyte development reveals that the GTPase RhoA is a positive regulator of T cell receptor responses in vivo*. J Exp Med, 2001. **194**(7): p. 903-14.
279. Henning, S.W. and D.A. Cantrell, *GTPases in antigen receptor signalling*. Curr Opin Immunol, 1998. **10**(3): p. 322-9.

280. Genot, E., S. Cleverley, S. Henning and D. Cantrell, *Multiple p21ras effector pathways regulate nuclear factor of activated T cells*. *Embo J*, 1996. **15**(15): p. 3923-33.
281. Turner, H. and D.A. Cantrell, *Distinct Ras effector pathways are involved in Fc epsilon R1 regulation of the transcriptional activity of Elk-1 and NFAT in mast cells*. *J Exp Med*, 1997. **185**(1): p. 43-53.
282. Woodrow, M., N.A. Clipstone and D. Cantrell, *p21ras and calcineurin synergize to regulate the nuclear factor of activated T cells*. *J Exp Med*, 1993. **178**(5): p. 1517-22.
283. Bustelo, X.R. and M. Barbacid, *Tyrosine phosphorylation of the vav proto-oncogene product in activated B cells*. *Science*, 1992. **256**(5060): p. 1196-9.
284. Turner, M., P.J. Mee, A.E. Walters, M.E. Quinn, A.L. Mellor, R. Zamoyska and V.L. Tybulewicz, *A requirement for the Rho-family GTP exchange factor Vav in positive and negative selection of thymocytes*. *Immunity*, 1997. **7**(4): p. 451-60.
285. Crespo, P., K.E. Schuebel, A.A. Ostrom, J.S. Gutkind and X.R. Bustelo, *Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product*. *Nature*, 1997. **385**(6612): p. 169-72.
286. Fischer, K.D., A. Zmudzinas, S. Gardner, M. Barbacid, A. Bernstein and C. Guidos, *Defective T-cell receptor signalling and positive selection of Vav- deficient CD4+ CD8+ thymocytes*. *Nature*, 1995. **374**(6521): p. 474-7.
287. Zhang, R., F.W. Alt, L. Davidson, S.H. Orkin and W. Swat, *Defective signalling through the T- and B-cell antigen receptors in lymphoid cells lacking the vav proto-oncogene*. *Nature*, 1995. **374**(6521): p. 470-3.
288. Penninger, J.M., K.D. Fischer, T. Sasaki, I. Kozieradzki, J. Le, K. Tedford, K. Bachmaier, P.S. Ohashi and M.F. Bachmann, *The oncogene product Vav is a crucial regulator of primary cytotoxic T cell responses but has no apparent role in CD28-mediated co-stimulation*. *Eur J Immunol*, 1999. **29**(5): p. 1709-18.
289. Yu, H., D. Leitenberg, B. Li and R.A. Flavell, *Deficiency of small GTPase Rac2 affects T cell activation*. *J Exp Med*, 2001. **194**(7): p. 915-26.
290. Croker, B.A., D.M. Tarlinton, L.A. Cluse, A.J. Tuxen, A. Light, F.C. Yang, D.A. Williams and A.W. Roberts, *The Rac2 guanosine triphosphatase regulates B lymphocyte antigen receptor responses and chemotaxis and is required for establishment of B-1a and marginal zone B lymphocytes*. *J Immunol*, 2002. **168**(7): p. 3376-86.
291. Gomez, M., V. Tybulewicz and D.A. Cantrell, *Control of pre-T cell proliferation and differentiation by the GTPase Rac-1*. *Nat Immunol*, 2000. **1**(4): p. 348-52.
292. Stowers, L., D. Yelon, L.J. Berg and J. Chant, *Regulation of the polarization of T cells toward antigen-presenting cells by Ras-related GTPase CDC42*. *Proc Natl Acad Sci U S A*, 1995. **92**(11): p. 5027-31.
293. Kolluri, R., K.F. Tolias, C.L. Carpenter, F.S. Rosen and T. Kirchhausen, *Direct interaction of the Wiskott-Aldrich syndrome protein with the GTPase Cdc42*. *Proc Natl Acad Sci U S A*, 1996. **93**(11): p. 5615-8.
294. Kirchhausen, T. and F.S. Rosen, *Disease mechanism: unravelling Wiskott-Aldrich syndrome*. *Curr Biol*, 1996. **6**(6): p. 676-8.
295. Devriendt, K., A.S. Kim, G. Mathijs, S.G. Frints, M. Schwartz, J.J. Van Den Oord, G.E. Verhoef, M.A. Boogaerts, J.P. Fryns, D. You, M.K. Rosen and P. Vandenberghe, *Constitutively activating*

- mutation in WASP causes X-linked severe congenital neutropenia. *Nat Genet*, 2001. **27**(3): p. 313-7.
296. Na, S., B. Li, I.S. Grewal, H. Enslen, R.J. Davis, J.H. Hanke and R.A. Flavell, *Expression of activated CDC42 induces T cell apoptosis in thymus and peripheral lymph organs via different pathways*. *Oncogene*, 1999. **18**(56): p. 7966-74.
 297. Zaffran, Y., O. Destaing, A. Roux, S. Ory, T. Nheu, P. Jurdic, C. Rabourdin-Combe and A.L. Astier, *CD46/CD3 costimulation induces morphological changes of human T cells and activation of Vav, Rac, and extracellular signal-regulated kinase mitogen-activated protein kinase*. *J Immunol*, 2001. **167**(12): p. 6780-5.
 298. Avraham, A., S. Jung, Y. Samuels, R. Seger and Y. Ben-Neriah, *Co-stimulation-dependent activation of a JNK-kinase in T lymphocytes*. *Eur J Immunol*, 1998. **28**(8): p. 2320-30.
 299. Chang, J.H., J.C. Pratt, S. Sawasdikosol, R. Kapeller and S.J. Burakoff, *The small GTP-binding protein Rho potentiates AP-1 transcription in T cells*. *Mol Cell Biol*, 1998. **18**(9): p. 4986-93.
 300. Hehner, S.P., T.G. Hofmann, A. Ushmorov, O. Dienz, I. Wing-Lan Leung, N. Lassam, C. Scheidereit, W. Droge and M.L. Schmitz, *Mixed-lineage kinase 3 delivers CD3/CD28-derived signals into the I κ B kinase complex*. *Mol Cell Biol*, 2000. **20**(7): p. 2556-68.
 301. Chatah, N.E. and C.S. Abrams, *G-protein-coupled receptor activation induces the membrane translocation and activation of phosphatidylinositol-4-phosphate 5- kinase I alpha by a Rac- and Rho-dependent pathway*. *J Biol Chem*, 2001. **276**(36): p. 34059-65.
 302. Glassford, J., M. Holman, L. Banerji, E. Clayton, G.G. Klaus, M. Turner and E.W. Lam, *Vav is required for cyclin D2 induction and proliferation of mouse B lymphocytes activated via the antigen Receptor*. *J Biol Chem*, 2001. **276**(44): p. 41040-8.
 303. Baggiolini, M., *Chemokines and leukocyte traffic*. *Nature*, 1998. **392**(6676): p. 565-8.
 304. Hornquist, C.E., X. Lu, P.M. Rogers-Fani, U. Rudolph, S. Shappell, L. Birnbaumer and G.R. Harriman, *G(alpha)i2-deficient mice with colitis exhibit a local increase in memory CD4+ T cells and proinflammatory Th1-type cytokines*. *J Immunol*, 1997. **158**(3): p. 1068-77.
 305. Amatruda, T.T., 3rd, D.A. Steele, V.Z. Slepak and M.I. Simon, *G alpha 16, a G protein alpha subunit specifically expressed in hematopoietic cells*. *Proc Natl Acad Sci U S A*, 1991. **88**(13): p. 5587-91.
 306. Lippert, E., K. Baltensperger, Y. Jacques and S. Hermouet, *G alpha 16 protein expression is up- and down-regulated following T-cell activation: disruption of this regulation impairs activation-induced cell responses*. *FEBS Lett*, 1997. **417**(3): p. 292-6.
 307. Davignon, I., M.D. Catalina, D. Smith, J. Montgomery, J. Swantek, J. Croy, M. Siegelman and T.M. Wilkie, *Normal hematopoiesis and inflammatory responses despite discrete signaling defects in Galpha15 knockout mice*. *Mol Cell Biol*, 2000. **20**(3): p. 797-804.
 308. Strathmann, M.P. and M.I. Simon, *G alpha 12 and G alpha 13 subunits define a fourth class of G protein alpha subunits*. *Proc Natl Acad Sci U S A*, 1991. **88**(13): p. 5582-6.
 309. Offermanns, S., V. Mancino, J.P. Revel and M.I. Simon, *Vascular system defects and impaired cell chemokinesis as a result of Galpha13 deficiency*. *Science*, 1997. **275**(5299): p. 533-6.
 310. Moratz, C., V.H. Kang, K.M. Druey, C.S. Shi, A. Scheschonka, P.M. Murphy, T. Kozasa and J.H. Kehrl, *Regulator of G protein signaling 1 (RGS1) markedly impairs Gi alpha signaling responses of B lymphocytes*. *J Immunol*, 2000. **164**(4): p. 1829-38.

311. Reif, K. and J.G. Cyster, *RGS molecule expression in murine B lymphocytes and ability to down-regulate chemotaxis to lymphoid chemokines*. J Immunol, 2000. **164**(9): p. 4720-9.
312. Girkontaite, I., K. Missy, V. Sakik, A. Harenberg, K. Tedford, T. Potzel, K. Pfeffer and K.D. Fischer, *Lsc is required for marginal zone B cells, regulation of lymphocyte motility and immune responses*. Nat Immunol, 2001. **2**(9): p. 855-62.
313. Weng, Z., A.C. Fluckiger, S. Nisitani, M.I. Wahl, L.Q. Le, C.A. Hunter, A.A. Fernal, M.M. Le Beau and O.N. Witte, *A DNA damage and stress inducible G protein-coupled receptor blocks cells in G2/M*. Proc Natl Acad Sci U S A, 1998. **95**(21): p. 12334-9.
314. Le, L.Q., J.H. Kabarowski, Z. Weng, A.B. Satterthwaite, E.T. Harvill, E.R. Jensen, J.F. Miller and O.N. Witte, *Mice lacking the orphan G protein-coupled receptor G2A develop a late-onset autoimmune syndrome*. Immunity, 2001. **14**(5): p. 561-71.
315. Voyno-Yasenetskaya, T.A., A.M. Pace and H.R. Bourne, *Mutant alpha subunits of G12 and G13 proteins induce neoplastic transformation of Rat-1 fibroblasts*. Oncogene, 1994. **9**(9): p. 2559-65.
316. Engler-Blum, G., M. Meier, J. Frank and G.A. Muller, *Reduction of background problems in nonradioactive northern and Southern blot analyses enables higher sensitivity than 32P-based hybridizations*. Anal Biochem, 1993. **210**(2): p. 235-44.
317. Pear, W.S., G.P. Nolan, M.L. Scott and D. Baltimore, *Production of high-titer helper-free retroviruses by transient transfection*. Proc Natl Acad Sci U S A, 1993. **90**(18): p. 8392-6.
318. Normont, A.M., L.Y. Bogatzki, B.N. Gantner and M.J. Bevan, *Murine CCR9, a chemokine receptor for thymus-expressed chemokine that is up-regulated following pre-TCR signaling*. J Immunol, 2000. **164**(2): p. 639-48.
319. Chaffin, K.E., C.R. Beals, T.M. Wilkie, K.A. Forbush, M.I. Simon and R.M. Perlmutter, *Dissection of thymocyte signaling pathways by in vivo expression of pertussis toxin ADP-ribosyltransferase*. Embo J, 1990. **9**(12): p. 3821-9.
320. Hettmann, T., J. DiDonato, M. Karin and J.M. Leiden, *An essential role for nuclear factor kappaB in promoting double positive thymocyte apoptosis*. J Exp Med, 1999. **189**(1): p. 145-58.
321. Shinkai, Y., G. Rathbun, K.P. Lam, E.M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A.M. Stall and et al., *RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement*. Cell, 1992. **68**(5): p. 855-67.
322. Gilchrist, A., M.R. Mazzoni, B. Dineen, A. Dice, J. Linden, W.R. Proctor, C.R. Lupica, T.V. Dunwiddie and H.E. Hamm, *Antagonists of the receptor-G protein interface block Gi-coupled signal transduction*. J Biol Chem, 1998. **273**(24): p. 14912-9.
323. Xu, N., L. Bradley, I. Ambudkar and J.S. Gutkind, *A mutant alpha subunit of G12 potentiates the eicosanoid pathway and is highly oncogenic in NIH 3T3 cells*. Proc Natl Acad Sci U S A, 1993. **90**(14): p. 6741-5.
324. Xu, N., T. Voyno-Yasenetskaya and J.S. Gutkind, *Potent transforming activity of the G13 alpha subunit defines a novel family of oncogenes*. Biochem Biophys Res Commun, 1994. **201**(2): p. 603-9.
325. Zachary, I., S.B. Masters and H.R. Bourne, *Increased mitogenic responsiveness of Swiss 3T3 cells expressing constitutively active Gs alpha*. Biochem Biophys Res Commun, 1990. **168**(3): p. 1184-93.

326. Pace, A.M., Y.H. Wong and H.R. Bourne, *A mutant alpha subunit of Gi2 induces neoplastic transformation of Rat-1 cells*. Proc Natl Acad Sci U S A, 1991. **88**(16): p. 7031-5.
327. Offermanns, S. and M.I. Simon, *Genetic analysis of mammalian G-protein signalling*. Oncogene, 1998. **17**(11 Reviews): p. 1375-81.
328. Zohn, I.M., S.L. Campbell, R. Khosravi-Far, K.L. Rossman and C.J. Der, *Rho family proteins and Ras transformation: the RHOad less traveled gets congested*. Oncogene, 1998. **17**(11 Reviews): p. 1415-38.
329. Kabarowski, J.H., K. Zhu, L.Q. Le, O.N. Witte and Y. Xu, *Lysophosphatidylcholine as a ligand for the immunoregulatory receptor G2A*. Science, 2001. **293**(5530): p. 702-5.
330. van Corven, E.J., P.L. Hordijk, R.H. Medema, J.L. Bos and W.H. Moolenaar, *Pertussis toxin-sensitive activation of p21ras by G protein-coupled receptor agonists in fibroblasts*. Proc Natl Acad Sci U S A, 1993. **90**(4): p. 1257-61.
331. Seasholtz, T.M., M. Majumdar and J.H. Brown, *Rho as a mediator of G protein-coupled receptor signaling*. Mol Pharmacol, 1999. **55**(6): p. 949-56.
332. Macey, M.G., G.L. Howells, S.R. Stone and A.C. Newland, *Analysis of the platelet-type thrombin receptor in 20 cases of large granular lymphocyte proliferations*. Leukemia, 1996. **10**(4): p. 687-92.
333. Moser, B. and P. Loetscher, *Lymphocyte traffic control by chemokines*. Nat Immunol, 2001. **2**(2): p. 123-8.
334. Luther, S.A. and J.G. Cyster, *Chemokines as regulators of T cell differentiation*. Nat Immunol, 2001. **2**(2): p. 102-7.
335. Sanchez-Madrid, F. and M.A. del Pozo, *Leukocyte polarization in cell migration and immune interactions*. Embo J, 1999. **18**(3): p. 501-11.
336. Wang, J., A. Ducret, Y. Tu, T. Kozasa, R. Aebersold and E.M. Ross, *RGSZ1, a Gz-selective RGS protein in brain. Structure, membrane association, regulation by G α phaz phosphorylation, and relationship to a Gz gtpase-activating protein subfamily*. J Biol Chem, 1998. **273**(40): p. 26014-25.
337. Niu, J., K. Druey, A. Scheschonka, A. Davis, E. Reed, V. Kolenko, R. Bodnar, T. Voyno-Yasenetskaya, X. Du, J. Kehrl and N.O. Dulin, *RGS3 interacts with 14-3-3 via the N-terminal region distinct from RGS domain*. Biochem J, 2002. **1**.
338. Ishii, M., A. Inanobe and Y. Kurachi, *PIP3 inhibition of RGS protein and its reversal by Ca²⁺/calmodulin mediate voltage-dependent control of the G protein cycle in a cardiac K⁺ channel*. Proc Natl Acad Sci U S A, 2002. **99**(7): p. 4325-30.
339. Moore, N.C., G. Anderson, G.T. Williams, J.J. Owen and E.J. Jenkinson, *Developmental regulation of bcl-2 expression in the thymus*. Immunology, 1994. **81**(1): p. 115-9.
340. Siegel, R.M., M. Katsumata, T. Miyashita, D.C. Louie, M.I. Greene and J.C. Reed, *Inhibition of thymocyte apoptosis and negative antigenic selection in bcl-2 transgenic mice*. Proc Natl Acad Sci U S A, 1992. **89**(15): p. 7003-7.
341. Strasser, A., A.W. Harris, H. von Boehmer and S. Cory, *Positive and negative selection of T cells in T-cell receptor transgenic mice expressing a bcl-2 transgene*. Proc Natl Acad Sci U S A, 1994. **91**(4): p. 1376-80.

342. Marodon, G. and B. Rocha, *Generation of mature T cell populations in the thymus: CD4 or CD8 down-regulation occurs at different stages of thymocyte differentiation*. Eur J Immunol, 1994. **24**(1): p. 196-204.
343. Azzam, H.S., A. Grinberg, K. Lui, H. Shen, E.W. Shores and P.E. Love, *CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity*. J Exp Med, 1998. **188**(12): p. 2301-11.
344. Gallatin, W.M., I.L. Weissman and E.C. Butcher, *A cell-surface molecule involved in organ-specific homing of lymphocytes*. Nature, 1983. **304**(5921): p. 30-4.
345. Shortman, K., A. Wilson, W. Van Ewijk and R. Scollay, *Phenotype and localization of thymocytes expressing the homing receptor-associated antigen MEL-14: arguments for the view that most mature thymocytes are located in the medulla*. J Immunol, 1987. **138**(2): p. 342-51.
346. Kisielow, P., H.S. Teh, H. Bluthmann and H. von Boehmer, *Positive selection of antigen-specific T cells in thymus by restricting MHC molecules*. Nature, 1988. **335**(6192): p. 730-3.
347. Teh, H.S., P. Kisielow, B. Scott, H. Kishi, Y. Uematsu, H. Bluthmann and H. von Boehmer, *Thymic major histocompatibility complex antigens and the alpha beta T-cell receptor determine the CD4/CD8 phenotype of T cells*. Nature, 1988. **335**(6187): p. 229-33.
348. Godfrey, D.I., J. Kennedy, P. Mombaerts, S. Tonegawa and A. Zlotnik, *Onset of TCR-beta gene rearrangement and role of TCR-beta expression during CD3-CD4-CD8- thymocyte differentiation*. J Immunol, 1994. **152**(10): p. 4783-92.
349. Costello, P.S., S.C. Cleverley, R. Galandriani, S.W. Henning and D.A. Cantrell, *The GTPase rho controls a p53-dependent survival checkpoint during thymopoiesis*. J Exp Med, 2000. **192**(1): p. 77-85.
350. Moroy, T. and H. Karsunky, *Regulation of pre-T-cell development*. Cell Mol Life Sci, 2000. **57**(6): p. 957-75.
351. Alam, S.M., P.J. Travers, J.L. Wung, W. Nasholds, S. Redpath, S.C. Jameson and N.R. Gascoigne, *T-cell-receptor affinity and thymocyte positive selection*. Nature, 1996. **381**(6583): p. 616-20.
352. Hogquist, K.A., S.C. Jameson, W.R. Heath, J.L. Howard, M.J. Bevan and F.R. Carbone, *T cell receptor antagonist peptides induce positive selection*. Cell, 1994. **76**(1): p. 17-27.
353. Jameson, S.C., K.A. Hogquist and M.J. Bevan, *Specificity and flexibility in thymic selection*. Nature, 1994. **369**(6483): p. 750-2.
354. Delaney, J.R., Y. Sykulev, H.N. Eisen and S. Tonegawa, *Differences in the level of expression of class I major histocompatibility complex proteins on thymic epithelial and dendritic cells influence the decision of immature thymocytes between positive and negative selection*. Proc Natl Acad Sci U S A, 1998. **95**(9): p. 5235-40.
355. Sebzda, E., T.M. Kundig, C.T. Thomson, K. Aoki, S.Y. Mak, J.P. Mayer, T. Zamborelli, S.G. Nathanson and P.S. Ohashi, *Mature T cell reactivity altered by peptide agonist that induces positive selection*. J Exp Med, 1996. **183**(3): p. 1093-104.
356. Cook, J.R., E.M. Wormstall, T. Hornell, J. Russell, J.M. Connolly and T.H. Hansen, *Quantitation of the cell surface level of Ld resulting in positive versus negative selection of the 2C transgenic T cell receptor in vivo*. Immunity, 1997. **7**(2): p. 233-41.

357. Fukui, Y., T. Ishimoto, M. Utsuyama, T. Gytoku, T. Koga, K. Nakao, K. Hirokawa, M. Katsuki and T. Sasazuki, *Positive and negative CD4+ thymocyte selection by a single MHC class II/peptide ligand affected by its expression level in the thymus*. Immunity, 1997. **6**(4): p. 401-10.
358. Sebзда, E., V.A. Wallace, J. Mayer, R.S. Yeung, T.W. Mak and P.S. Ohashi, *Positive and negative thymocyte selection induced by different concentrations of a single peptide*. Science, 1994. **263**(5153): p. 1615-8.
359. Ashton-Rickardt, P.G. and S. Tonegawa, *A differential-avidity model for T-cell selection*. Immunol Today, 1994. **15**(8): p. 362-6.
360. Williams, O., Y. Tanaka, R. Tarazona and D. Kioussis, *The agonist-antagonist balance in positive selection*. Immunol Today, 1997. **18**(3): p. 121-6.
361. Sprent, J., D. Lo, E.K. Gao and Y. Ron, *T cell selection in the thymus*. Immunol Rev, 1988. **101**: p. 173-90.
362. Valitutti, S., S. Muller, M. Dessing and A. Lanzavecchia, *Signal extinction and T cell repolarization in T helper cell-antigen-presenting cell conjugates*. Eur J Immunol, 1996. **26**(9): p. 2012-6.
363. Andre, P., J. Boretto, A.O. Hueber, A. Regnier-Vigouroux, J.P. Gorvel, P. Ferrier and P. Chavrier, *A dominant-negative mutant of the Rab5 GTPase enhances T cell signaling by interfering with TCR down-modulation in transgenic mice*. J Immunol, 1997. **159**(11): p. 5253-63.
364. Lores, P., L. Morin, R. Luna and G. Gacon, *Enhanced apoptosis in the thymus of transgenic mice expressing constitutively activated forms of human Rac2GTPase*. Oncogene, 1997. **15**(5): p. 601-5.
365. Martin, S.J., C.P. Reutelingsperger, A.J. McGahon, J.A. Rader, R.C. van Schie, D.M. LaFace and D.R. Green, *Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl*. J Exp Med, 1995. **182**(5): p. 1545-56.
366. Valitutti, S., M. Dessing, K. Aktories, H. Gallati and A. Lanzavecchia, *Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton*. J Exp Med, 1995. **181**(2): p. 577-84.
367. Fischer, K.D., Y.Y. Kong, H. Nishina, K. Tedford, L.E. Marengere, I. Kozieradzki, T. Sasaki, M. Starr, G. Chan, S. Gardener, M.P. Nghiem, D. Bouchard, M. Barbacid, A. Bernstein and J.M. Penninger, *Vav is a regulator of cytoskeletal reorganization mediated by the T-cell receptor*. Curr Biol, 1998. **8**(10): p. 554-62.
368. Holsinger, L.J., I.A. Graef, W. Swat, T. Chi, D.M. Bautista, L. Davidson, R.S. Lewis, F.W. Alt and G.R. Crabtree, *Defects in actin-cap formation in Vav-deficient mice implicate an actin requirement for lymphocyte signal transduction*. Curr Biol, 1998. **8**(10): p. 563-72.
369. Hettmann, T. and J.M. Leiden, *NF-kappa B is required for the positive selection of CD8+ thymocytes*. J Immunol, 2000. **165**(9): p. 5004-10.
370. Sharpless, N.E., N. Bardeesy, K.H. Lee, D. Carrasco, D.H. Castrillon, A.J. Aguirre, E.A. Wu, J.W. Horner and R.A. DePinho, *Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis*. Nature, 2001. **413**(6851): p. 86-91.
371. Penit, C. and F. Vasseur, *Expansion of mature thymocyte subsets before emigration to the periphery*. J Immunol, 1997. **159**(10): p. 4848-56.

372. Le Campion, A., F. Vasseur and C. Penit, *Regulation and kinetics of premigrant thymocyte expansion*. Eur J Immunol, 2000. **30**(3): p. 738-46.
373. Arbones, M.L., D.C. Ord, K. Ley, H. Ratech, C. Maynard-Curry, G. Otten, D.J. Capon and T.F. Tedder, *Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice*. Immunity, 1994. **1**(4): p. 247-60.
374. Steeber, D.A., N.E. Green, S. Sato and T.F. Tedder, *Humoral immune responses in L-selectin-deficient mice*. J Immunol, 1996. **157**(11): p. 4899-907.
375. Holder, J.E., W.G. Kimpton, E.A. Washington and R.N. Cahill, *L-selectin expression on thymic emigrants defines two distinct tissue- migration pathways*. Immunology, 1999. **98**(3): p. 422-6.
376. Roberts, A.W., C. Kim, L. Zhen, J.B. Lowe, R. Kapur, B. Petryniak, A. Spaetti, J.D. Pollock, J.B. Borneo, G.B. Bradford, S.J. Atkinson, M.C. Dinauer and D.A. Williams, *Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense*. Immunity, 1999. **10**(2): p. 183-96.
377. Salomon, D.R., C.F. Mojcik, A.C. Chang, S. Wadsworth, D.H. Adams, J.E. Coligan and E.M. Shevach, *Constitutive activation of integrin alpha 4 beta 1 defines a unique stage of human thymocyte development*. J Exp Med, 1994. **179**(5): p. 1573-84.
378. Crisa, L., V. Cirulli, M.H. Ellisman, J.K. Ishii, M.J. Elises and D.R. Salomon, *Cell adhesion and migration are regulated at distinct stages of thymic T cell development: the roles of fibronectin, VLA4, and VLA5*. J Exp Med, 1996. **184**(1): p. 215-28.
379. Gomez, M., D. Kioussis and D.A. Cantrell, *The GTPase Rac-1 controls cell fate in the thymus by diverting thymocytes from positive to negative selection*. Immunity, 2001. **15**(5): p. 703-13.
380. Furukawa, Y., T. Kawasoe, Y. Daigo, T. Nishiwaki, H. Ishiguro, M. Takahashi, J. Kitayama and Y. Nakamura, *Isolation of a novel human gene, ARHGAP9, encoding a rho-GTPase activating protein*. Biochem Biophys Res Commun, 2001. **284**(3): p. 643-9.