

**Genetic and Biochemical Analysis of the Grb10-Interacting
GYF Protein 2 (GIGYF2):**

**A Link Between the Insulin and Insulin-Like Growth Factor Hormonal
Systems and Parkinson's Disease**

By

William G. Tsiaras

B.A., Dartmouth College, 1999

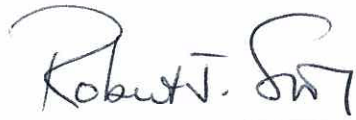
A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in the Program in Molecular Biology, Cell Biology, and
Biochemistry, Division of Biology and Medicine at Brown University

Providence, Rhode Island

May 2010

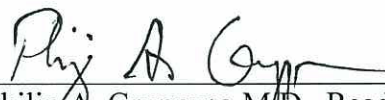
This dissertation by William George Tsiaras is accepted in its present form by
the Division of Biology and Medicine as satisfying the dissertation
requirement for the degree of Doctor of Philosophy.

Date 4/21/08


Robert J. Smith M.D., Advisor

Recommended to the Graduate Council

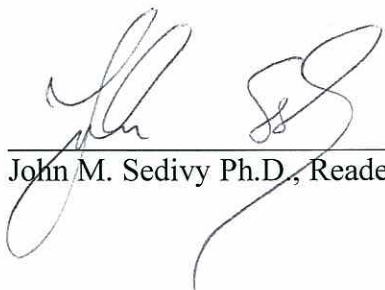
Date 4/21/08


Philip A. Gruppuso M.D., Reader

Date 4/21/08


Daniele Guardavaccaro Ph.D., Reader

Date 4/21/08


John M. Sedivy Ph.D., Reader

Approved by the Graduate Council

Date _____

Sheila Bonde, Dean of the Graduate School

CURRICULUM VITAE

William George Tsiaras

Born: July 12, 1977
Philadelphia, Pennsylvania, USA

Education

- | | |
|--------------|--|
| 2000-Present | Combined MD/PhD Program, Brown University, Providence, RI

Alpert Medical School of Brown University, Providence, RI <ul style="list-style-type: none">▪ MD candidate May 2010
Graduate Program in Molecular Biology, Cell Biology, and Biochemistry, Division of Biology and Medicine, Brown University, Providence, RI <ul style="list-style-type: none">▪ PhD candidate May 2010 |
| 1995-1999 | Dartmouth College, Hanover, NH <ul style="list-style-type: none">▪ BA June 1999, Biochemistry and Molecular Biology |

Honors/Awards

- | | |
|-----------|---|
| 2009 | Member, Gold Humanism Honor Society at the Alpert Medical School of Brown University; honorees selected for excellence in clinical care, leadership, compassion and dedication to service |
| 2002-2008 | MD/PhD Research Fellowship, Division of Biology and Medicine, Brown University |
| 2007 | Poster Award Winner, first place in the Graduate Program in Molecular Biology, Cell Biology and Biochemistry annual poster competition |
| 1999 | Graduated <i>Cum Laude</i> , Dartmouth College |
| 1999 | Academic All-Ivy League, awarded to varsity Ivy League athletes for exceptional academic and athletic performance |

Teaching Experience

2004	Graduate Student Liaison, Harriet W. Sheridan Center for Teaching and Learning, Brown University
2003	Teaching Assistant, Genetics, Brown University
1997-1998	Tutor, Biology, Dartmouth College

Research Experience

2009	Co-Investigator, advised by Martin A. Weinstock MD, PhD, Division of Dermatoepidemiology, Department of Dermatology, Veterans Affairs Medical Center and Alpert Medical School of Brown University, Providence, RI
2009	Co-Investigator, advised by Leslie Robinson-Bostom MD and Gladys H. Telang MD, Division of Dermatopathology, Department of Dermatology, Rhode Island Hospital and Alpert Medical School of Brown University, Providence, RI
2002-2008	Graduate Student, advised by Robert J. Smith MD, Division of Endocrinology, Department of Medicine, Rhode Island Hospital and Alpert Medical School of Brown University, Providence, RI
1999-2000	Research Assistant, laboratory of Benjamin G. Neel MD, PhD, Division of Hematology and Oncology, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA
1998	Research Assistant, laboratory of Michael P. Czech PhD, Program in Molecular Medicine, University of Massachusetts Medical Center, Worcester, MA
1997	Research Assistant, laboratory of William F. Hickey MD, Department of Pathology, Dartmouth-Hitchcock Medical Center, Lebanon, NH
1995, 1996	Research Assistant, laboratory of David M. Berson PhD, Department of Neuroscience, Brown University, Providence, RI

University Committees

2003	MD/PhD Program Admissions Committee, Brown University
------	---

Professional Memberships

2008	Fellow/Student Associate of The Endocrine Society
------	---

Publications

- **Tsiaras WG** and Weinstock MA. (2010) Factors Influencing Vitamin D Status. [Review] Manuscript submitted to Acta Derm-Venereol.
- **Tsiaras WG**, Giovannone B, Smith RJ. (2010) The Parkinson's Disease Protein GIGYF2 Regulates Cyclin D1 and the CUL7 Ubiquitin Ligase Complex. Manuscript in preparation.
- Cleveland-Donovan K, Maile LA, **Tsiaras WG**, Tchkonja T, Kirkland JL, Boney CM. (2010) IGF-I activation of the AKT pathway is impaired in visceral but not subcutaneous human preadipocytes. Manuscript submitted to Endocrinology.
- **Tsiaras WG** and Weinstock MA. (2010) Commentary: Ultraviolet Irradiation and Oral Ingestion as Sources of Optimal Vitamin D. J Am Acad Dermatol. In press.
- Giovannone B, **Tsiaras WG**, de la Monte S, Klysik J, Lautier C, Goldwurm S, Smith RJ. (2009) *GIGYF2* Gene Disruption in Mice Results in Neurodegeneration and Altered Insulin-like Growth Factor Signaling. Hum. Mol. Genet. 18(23): 4629-39. PMID: 19744960.
- Isayama T, O'Brien BJ, Ugalde I, Muller JF, Frenz A, Aurora V, **Tsiaras W**, Berson DM. (2009) Morphology of Ferret (*Mustela putorius furo*) Retinal Ganglion Cells. J. Comp. Neurol. 517(4): 459-80. PMID: 19790267.
- Lautier C, Goldwurm S, Dürr A, Giovannone B, **Tsiaras WG**, Pezzoli G, Brice A, Smith RJ. (2008) Mutations in the *GIGYF2* (*TNRC15*) Gene at the PARK11 Locus in Familial Parkinson Disease. Am. J. Hum. Genet. 82(4): 822-833. PMID: 18358451.
- Lehmann U, Schmitz J, Weissenbach M, Sobota RM, Hortner M, Friederichs K, Behrmann I, **Tsiaras W**, Sasaki A, Schneider-Mergener J, Yoshimura A, Neel BG, Heinrich PC, Schaper F. (2003) SHP2 and SOCS3 Contribute to Tyr-759-Dependent Attenuation of Interleukin-6 Signaling through gp130. J. Biol. Chem. 278(1): 661-671. PMID: 12403768.
- Zhang SQ, **Tsiaras WG**, Araki T, Wen G, Minichiello L, Klein R, Neel BG. (2002) Receptor-Specific Regulation of Phosphatidylinositol 3'-Kinase Activation by the Protein Tyrosine Phosphatase Shp2. Mol. Cell. Biol. 22(12): 4062-4072. PMID: 12024020.
- **Tsiaras W***, Klarlund JK*, Holik JJ, Chawla A, Czech MP. (2000) Distinct Polyphosphoinositide Binding Selectivities for Pleckstrin Homology Domains of GRP1-Like Proteins Based on Diglycine Versus Triglycine Motifs. J. Biol. Chem. 275(42): 32816-32821. PMID: 10913124. (*, These authors contributed equally to this work)

Oral Presentations

- Tsiaras WG, Giovannone B, Karashchuk G, Smith R. Regulation of IGF-I Receptor Signaling and the CUL7 Ubiquitin Ligase by the Parkinson's Disease Protein GIGYF2. The Fourth International Congress of the Growth Hormone Research Society and the Insulin-like Growth Factors Society, 2008
- Tsiaras WG, Giovannone B, Karashchuk G, Smith RJ. Regulation of IGF-I Receptor Signaling and the CUL7 Ubiquitin Ligase Complex by the Parkinson's Disease Associated Protein GIGYF2. The Endocrine Society's 90th Annual Meeting, 2008

Poster Presentations/Abstracts

- Tsiaras WG, Karashchuk G, Giovannone B, Smith RJ. A Molecular Link Between Insulin-like Growth Factor Signaling and Parkinson's Disease. Brown University-Affiliated Hospitals 16th Annual Research Celebration, 2008
- Giovannone B, Tsiaras WG, DeLaMonte S, Lautier C, Klysik J, Smith RJ. Studies on IGF and Insulin Signaling Identify a Gene Involved in Neurodegenerative Disease. The Endocrine Society's 89th Annual Meeting, 2007
- Tsiaras WG and Smith RJ. Interaction Between the Neurodegenerative Disease Linked Protein GIGYF2 and the E3-Ubiquitin Ligase CUL7. Brown University-Affiliated Hospitals 14th Annual Research Celebration, 2006
- Tsiaras WG and Smith RJ. Analysis of GIGYF2 in the Development of Oral and Cervical Squamous Cell Carcinoma. Brown University-Affiliated Hospitals 12th Annual Research Celebration, 2004
- Tsiaras WG and Smith RJ. Analysis of GIGYF2 in the Development of Oral and Cervical Squamous Cell Carcinoma. COBRE Center for Cancer Research Development 2nd Annual Research Symposium, 2004
- Isayama T, O'Brian B, Ugalde I, Frenz A, Aurora V, Tsiaras W, Muler J, Berson D. Morphology of Ferret Retinal Ganglion Cells. Annual Meeting of the Association for Research in Vision and Ophthalmology, Iovs 39(4), 1998

ACKNOWLEDGEMENTS

I would like to thank my thesis advisor and mentor, Dr. Robert Smith for his tremendous guidance and support throughout the years. As an incredibly accomplished and respected physician-scientist, Bob has served as a role model and source of great inspiration for me. GIGYF2 has been an interesting, yet at times, very difficult protein to work on, and I credit Bob's unwaivering vision and level-headedness with carrying us through the many challenges. He had the courage to follow the science as it took us in unique and wonderful directions and for that I am truly grateful.

I would also like to thank my thesis committee, Dr. Philip Gruppuso, Dr. John Sedivy, and Dr. Daniele Guardavaccaro, for their expert opinions, challenging questions, and endless encouragement; with a special thank you to Daniele, for being a seemingly inexhaustible source of advice and reagents, and for his many trips up from New York City to help keep us moving in the right direction. Additionally, I would like to thank faculty, staff, and fellow students in the Molecular Biology, Cell Biology, and Biochemistry Program, for inspiring courses, insightful comments, and their support and friendship. I am so appreciative of the opportunity to have worked with such an incredible group of people.

I thank all the past and present members of the Smith lab for so many years of fun, learning, and adventure. I especially would like to thank Dr. Barbara Giovannone. Without her hard work, technical expertise, and most importantly, her friendship, this work would never have come to fruition.

I am deeply grateful for the love, caring, and support of my family. My parents, Dr. William and Nancy Tsiaras, and my brothers, Matt and Phil, have been there for me

every step of the way, and I can't thank them enough for their advice, encouragement, and home-cooked meals when they were needed most. I also need to thank my parents in law, Roger and Ginny Valkenburgh, for being the two best cheerleaders I know. To my son Luke, a special thanks. You are such a sweet, curious, and intelligent little boy, and fortunately, too young to remember all those nights Daddy spent in the lab away from home. Finally, I owe my deepest, and most heartfelt gratitude to my wife, Sarah. This is as much your accomplishment as it is mine. Words cannot express how thankful I am for your love and patience. Together, with plenty of snacks, we can get through just about anything.

TABLE OF CONTENTS

CHAPTER 1: Introduction	1
Section 1. Insulin and IGF systems:	
<i>Components</i>	2
<i>Signal transduction</i>	4
Section 2. Insulin and IGF-I receptor binding protein Grb10:	
<i>Identification and characterization of Grb10</i>	7
<i>Biological function of Grb10</i>	9
<i>Proposed mechanisms of Grb10 action:</i>	
<i>Receptor turnover</i>	10
<i>Steric effects</i>	12
<i>Adaptor protein function of Grb10</i>	14
Section 3. Grb10-interacting GYF proteins:	
<i>Identification of GIGYFs</i>	15
<i>GIGYF genes: evolutionary conservation and expression</i>	16
<i>GIGYF proteins</i>	18
Thesis objective	23
REFERENCES	24
CHAPTER 2: Mutations in the <i>GIGYF2</i> Gene at the PARK11 Locus in Familial Parkinson's Disease	39
CHAPTER INTRODUCTION	40
ABSTRACT	47
INTRODUCTION	48
MATERIALS AND METHODS	50
RESULTS	53
DISCUSSION	57
ACKNOWLEDGEMENTS	61
REFERENCES	62
FIGURE LEGENDS	70

CHAPTER 3: Partial Loss of the <i>GIGYF2</i> (PARK11 Region) Parkinson's Disease Gene in Mice Results in Motor Dysfunction and Neurodegeneration	80
CHAPTER INTRODUCTION	81
ABSTRACT	85
INTRODUCTION	86
MATERIALS AND METHODS	89
RESULTS	94
DISCUSSION	102
ACKNOWLEDGEMENTS	109
REFERENCES	110
FIGURE LEGENDS	117
 CHAPTER 4: The Parkinson's Disease Protein GIGYF2 Regulates Cyclin D1 and the CUL7 Ubiquitin Ligase Complex	129
INTRODUCTION	131
MATERIALS AND METHODS	134
RESULTS	141
DISCUSSION	151
REFERENCES	158
FIGURE LEGENDS	165
 CHAPTER 5: Discussion	181
OVERVIEW	182
GIGYF2: a link between insulin/IGFs and Parkinson's disease	184
Potential effect of <i>GIGYF2</i> mutations	189
The GIGYF2-CUL7 complex and cyclin D1 degradation	191
A model for shared function of GIGYF2, Grb10 and CUL7	194
REFERENCES	198

LIST OF TABLES

CHAPTER 2.

Table 2.1	Population phenotype descriptions	72
Table 2.2	<i>GIGYF2</i> gene primers used for PCR and sequencing analysis	73
Table 2.3	<i>GIGYF2</i> mutations and gene-sequence variation identified in PD and control populations	74
Table 2.4	Phenotype of patients with <i>GIGYF2</i> mutations	75

CHAPTER 3.

Table 3.1	Perinatal lethality of <i>Gigyf2</i> ^{-/-} mice	121
-----------	--	-----

LIST OF FIGURES

CHAPTER 1.

Fig. 1.1	Signal transduction pathways of ligand activated insulin and IGF-I receptors (MAPK).....	5
Fig. 1.2	Signal transduction pathways of ligand activated insulin and IGF-I receptors (PI3K).....	7
Fig. 1.3	Mechanisms of Grb10-inhibition of ligand activated insulin and IGF-I receptors	13
Fig. 1.4	Grb10-interacting GYF proteins GIGYF1 and GIGYF2.....	18
Fig. 1.5	GIGYF-GYF domain interaction with Grb10 proline-rich region	21

CHAPTER 2.

Fig. 2.1	Genetic map of the PARK11 locus, extending from markers D2S396 to D2S338	76
Fig. 2.2	Chromatograms illustrating <i>GIGYF2</i> gene mutations	77
Fig. 2.3	Alignment of GIGYF2-mutation protein-sequence regions in multiple species.....	78
Fig. 2.4	Pedigrees of patients with <i>GIGYF2</i> missense mutations	79

CHAPTER 3.

Fig. 3.1	Gene-Trap disruption of <i>Gigyf2</i> gene locus.....	122
Fig. 3.2	Phenotype of <i>Gigyf2</i> knockout mice.....	123
Fig. 3.3	<i>Gigyf2</i> expression in knockout mice.....	124
Fig. 3.4	Motor ataxia in 15-month-old <i>Gigyf2</i> heterozygous mice.....	125
Fig. 3.5	Motor neuron degeneration in <i>Gigyf2</i> +/- mice	126
Fig. 3.6	Alpha-synuclein positive neuritic plaques	127
Fig. 3.7	IGF-I stimulated cell signaling in <i>Gigyf2</i> -/- mouse embryonic fibroblasts.....	128

CHAPTER 4.

Fig. 4.1	Loss of GIGYF2 results in decreased cyclin D1 protein levels.....	171
Fig. 4.2	Increased cyclin D1 protein degradation in <i>Gigyf2</i> ^{-/-} MEFs	172
Fig. 4.3	Co-immunoprecipitation of cullin protein CUL7 with GIGYF2.....	173

Fig. 4.4	GIGYF2 forms an endogenous complex with CUL7 and p53	174
Fig. 4.5	GIGYF2 forms a complex with CUL7-PARC-p53 that excludes SKP1 and FBXW8.....	175
Fig. 4.6	Cytoplasmic localization of the GIGYF2-CUL7 complex	176
Fig. 4.7	A C-terminal fragment of GIGYF2 is sufficient for CUL7 interaction	177
Fig. 4.8	GIGYF2 protein is not rapidly degraded by the ubiquitin- proteasome system	178
Fig. 4.9	GIGYF2 C-terminal fragment overexpression disrupts the SKP1-CUL7-FBXW8 complex	179
Fig. 4.10	Overexpression of GIGYF2 increases the level of cyclin D1 protein	180

CHAPTER 1

Introduction

INTRODUCTION

The insulin and insulin-like growth factor hormonal systems mediate numerous biological processes, and their requirement for normal health and survival is evolutionarily conserved across many different species. These two hormonal systems are similar in many ways, yet their key functions are distinct. Insulin is the primary hormonal regulator of carbohydrate, lipid and protein metabolism in mammals. Decreased production and secretion of insulin, and/or resistance to its actions, result in diabetes mellitus, a life-threatening disease that is becoming increasingly more common throughout the world. Insulin-like growth factor (IGF), on the other hand, stimulates cell growth, proliferation, survival, migration and differentiation. The IGF system is a critical mediator of normal organism growth and development, and its dysfunction can result in diseases of excess or insufficient growth. Recently, a great deal of interest has been focused on insulin and IGF actions in the central nervous system (CNS). It is becoming increasingly clear that these hormonal systems are critical mediators of CNS function, and that altered insulin and IGF action contributes to the development of specific neurodegenerative diseases.

Section 1: Insulin and IGF systems

Components

The insulin and IGF hormonal systems are principally composed of three polypeptide ligands (insulin, IGF-I, and IGF-II) and three different receptors (the insulin, IGF-I, and hybrid receptors) (87, 92). The three ligands are each synthesized as single pro-hormones, which are processed in the secretory pathway to yield the mature bioactive

polypeptides (29). Insulin is produced in the β -cells of the endocrine pancreas. The primary function of these cells is to store and secrete insulin in response to specific physiologic stimuli. Glucose is the most important regulator of insulin secretion; however, other circulating metabolites, various hormones, and even neuronal input contribute to the release of insulin from β -cells (50). Circulating levels of IGF-I and IGF-II hormone are predominantly controlled by synthesis and secretion from the liver. The majority of IGF-I produced by the liver is in response to growth hormone stimulation, while IGF-II production appears to be growth hormone independent. IGF-I and IGF-II are also produced locally by a number of different tissues and cell types and have important paracrine and autocrine actions (45). The bioavailability of IGFs is further controlled by six high-affinity IGF-binding proteins, IGFBP-1 through -6. Over 99% of circulating IGFs are bound by IGFBPs, primarily by IGFBP-3, with the primary function of complex formation being to prolong the serum half-life of the IGFs (30).

The vast majority of insulin, IGF-I and IGF-II effects are mediated by insulin and type I insulin-like growth factor cell surface receptors. The type I insulin-like growth factor receptor (IGF-IR) and the insulin receptor (IR) are approximately 70% homologous to each other at the primary amino acid sequence level (2). Each receptor is synthesized as a single pre-propeptide chain, which gets enzymatically cleaved, glycosylated, and dimerized in the endoplasmic reticulum and Golgi apparatus to yield α - and β -subunits. Mature, fully processed receptors consist of two extracellular 125- to 135-kDa α -chains and two transmembrane 90- to 95-kDa β -chains, linked together through multiple disulphide bonds (87, 92). Ligand binding to receptor α -subunits leads to a conformational change resulting in activation of the intrinsic tyrosine kinase domains

in the intracellular β -subunits. This activation leads to *trans*-autophosphorylation, in which the kinase domain transfers the γ -phosphate group of ATP to tyrosyl side chains of the adjacent β -subunit of the same receptor. This process is required for full activation of the receptor tyrosine kinase domains and initiation of subsequent intracellular signaling (11).

The high degree of sequence and structural similarity amongst the components of the insulin and IGF axes allows for considerable crossover between the two hormonal systems. Homology between the IR and the IGF-IR results in single α - β -pairs from each receptor associating with each other to form hybrid receptors (21). This heterodimeric hybrid receptor formation occurs in proportion to the expression level of the individual constituents. Hybrid receptors bind IGFs with an affinity similar to that seen with the homodimeric IGF-IR and to insulin with much lower affinity. They have been isolated from a number of different tissues, but their physiologic significance is unknown (91). There is also considerable homology between the three polypeptide ligands. The structures of specific domains within the IGF-I and IGF-II ligands are similar to those seen in the X-ray crystallographic and nuclear magnetic resonance derived structures of insulin and proinsulin (7, 13, 84). This allows for some degree of binding of insulin and IGFs to non-cognate receptors. The affinities of these interactions are relatively low, but may be physiologically relevant in disease states characterized by high levels of hormone (13).

Signal transduction

Ligand induced activation of IGF-IR or IR intracellular kinase domains results in phosphorylation of tyrosyl residues in proximal molecules such as the insulin receptor

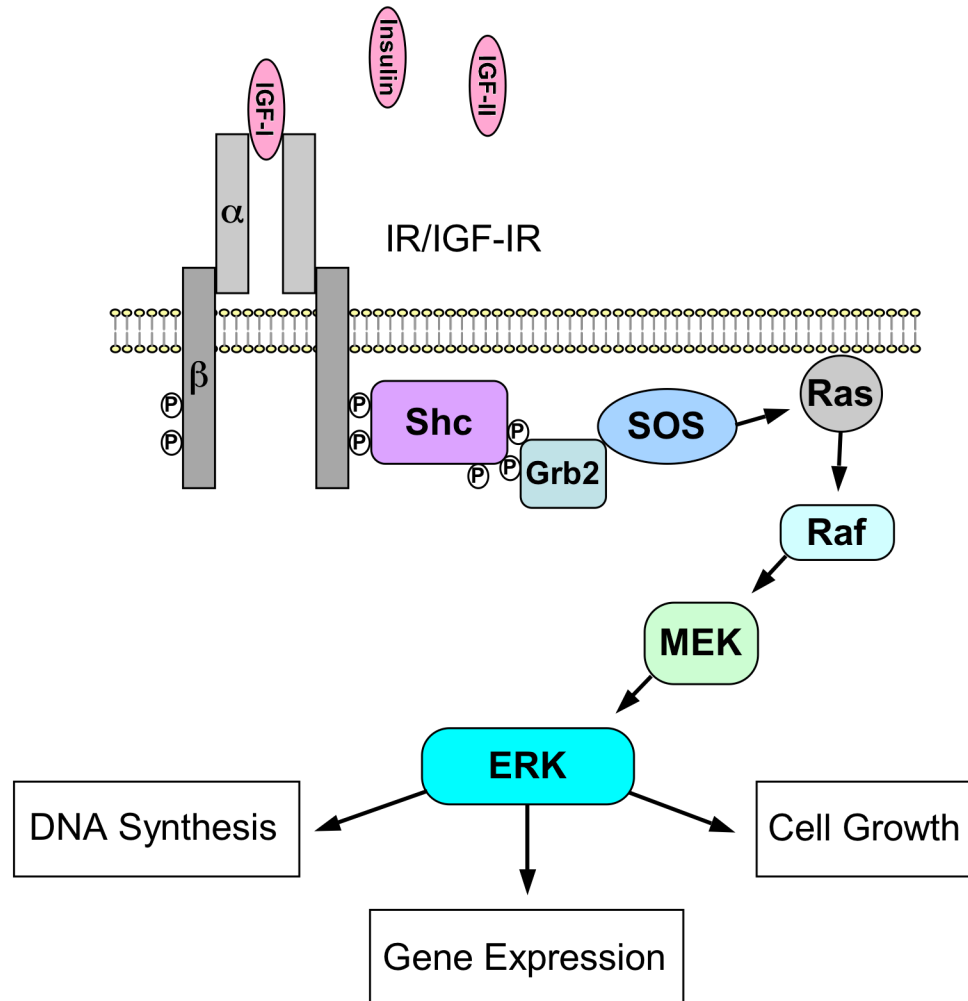


Figure 1. Signal transduction pathways of ligand activated insulin and IGF-I receptors. A schematic representation of the mitogen activated protein kinase (MAPK) signaling pathway and biological outcomes.

substrates (IRS) 1-4 and Src homology/collagen (Shc) proteins. Once phosphorylated, these receptor substrate molecules recruit and form complexes with key signaling molecules like the growth factor receptor bound protein Grb2, and the p85 regulatory subunit of the phosphatidyl inositol 3-kinase (PI3K) (19, 42, 86, 99). Coupling of activated receptors to Grb2 and p85 results in activation of two important signaling pathways, the mitogen activated protein kinase (MAPK) pathway (Fig. 1) and the PI3K

pathway (Fig. 2). These two pathways are the primary intracellular mediators of insulin and IGF actions (6, 54).

In the MAPK pathway, Grb2 association with phosphorylated IRS or Shc molecules results in recruitment of the guanine nucleotide exchange factor SOS. Recruitment of SOS to the plasma membrane leads to activation of the small membrane associated GTPase Ras. Activated Ras engages a complex network of effector molecules many of which directly alter transcription factor activity resulting in induction of cellular proliferation. In most cell systems, the dominant Ras signaling pathway is the Ras/Raf/MEK/ERK serine/threonine kinase cascade. Once activated, ERK undergoes nuclear translocation where it phosphorylates and stabilizes the highly pleiotropic transcription factor c-Myc, as well as c-Jun, c-Fos and the Ets-domain transcription factor Elk-1, resulting in significant changes in gene expression (43, 59, 87).

The lipid kinase PI3K binds to tyrosine phosphorylated IRS molecules through its p85 regulatory subunit. PI3K can also be recruited to the membrane by activated Ras molecules. At the membrane, PI3K phosphorylates phosphatidyl inositol-4,5-bisphosphate lipids at the 3' position on the inositol head group. This generates local, high concentrations of phosphatidyl inositol-3,4,5-trisphosphate (PIP3), which recruits and activates proteins containing pleckstrin homology (PH) domains (44). The PH domain-containing serine/threonine kinase Akt is recruited to PIP3 rich membranes where it then becomes activated following phosphorylation by the phosphatidyl inositide-dependent kinase PDK1 (59). Activated Akt mediates a wide range of insulin/IGF actions including mitogenesis, inhibition of apoptosis, protein synthesis, glucose transport

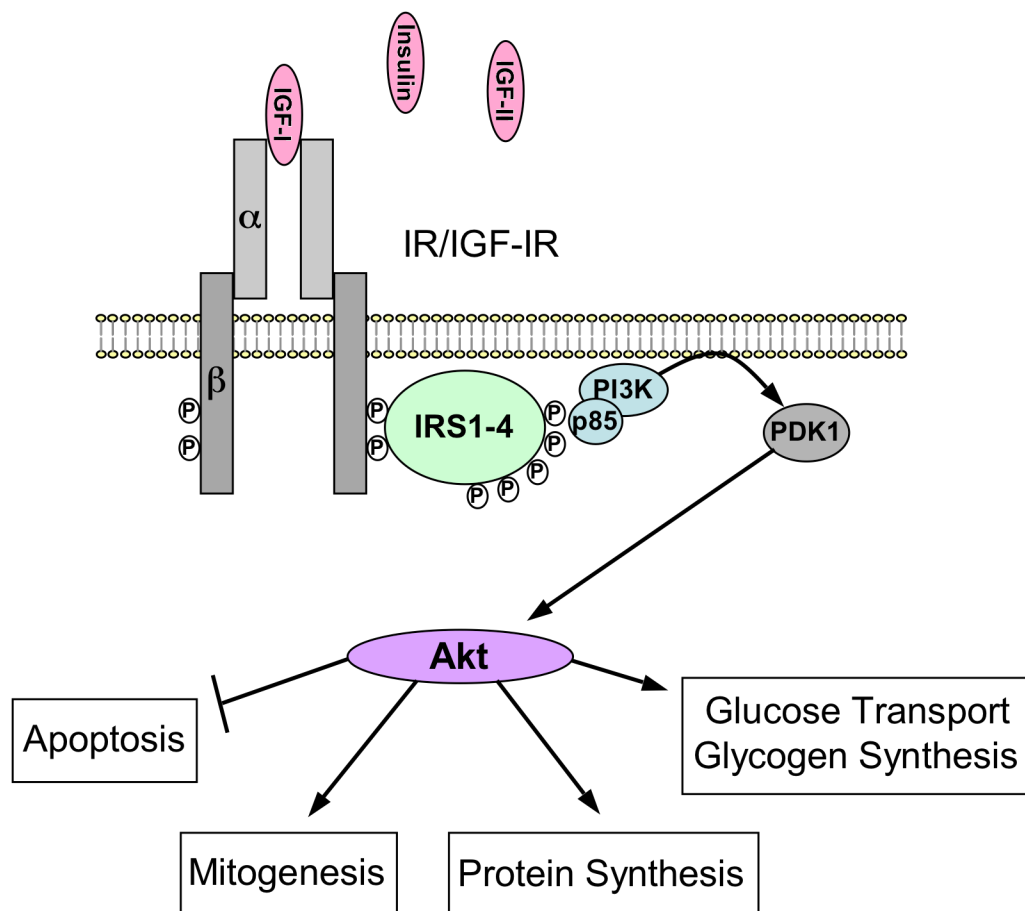


Figure 2. Signal transduction pathways of ligand activated insulin and IGF-I receptors. A schematic representation of the phosphatidylinositol 3-kinase (PI3K) signaling pathway and biological outcomes.

and glycogen synthesis. Akt can also translocate to the nucleus where it plays an important role in the regulation of a number of different transcription factors (92, 105).

Section 2: Insulin and IGF-I receptor binding protein Grb10

Identification and characterization of Grb10

The major IR/IGF-IR signal transduction pathways involve recruitment of receptor substrates to initiate signaling. There are, however, a number of proteins that

interact directly with activated receptors, which are not substrates for receptor tyrosine kinase activity. Perhaps the best studied of these is the Grb10 adaptor protein. Grb10 belongs to the Grb7/10/14 family of structurally related receptor binding proteins (20, 34, 38, 53, 67, 76). An additional member of this family is the *Caenorhabditis elegans* protein Mig-10, which shares high sequence homology with some, but not all, portions of the Grb7/10/14 proteins (58). This family is characterized by the presence of an N-terminal proline-rich region, a Ras-association (RA)-like domain, a PH domain, a C-terminal Src-homology region 2 (SH2) domain, and a conserved region between the PH and SH2 domains (BPS). This structural topology is conserved in all the family members with the exception of Mig-10, which lacks C-terminal BPS and SH2 domains (56).

Grb10 was initially identified in the NIH-3T3 mouse cell line as a binding partner for the epidermal growth factor (EGF) receptor (78). It was subsequently identified in yeast two-hybrid screens of both mouse and human cDNA libraries based on its ability to interact with insulin and IGF-I receptors (34, 57). There are now several reports indicating Grb10 interaction with a number of receptor tyrosine kinases including the Ret, platelet-derived growth factor, vascular endothelial growth factor, fibroblast growth factor, c-kit/SCF, and Eph-related receptors (8, 26, 41, 79, 94, 103). A total of 4 human and 3 mouse isoforms of Grb10 have been discovered. Each of these variants is produced by alternative splicing of a single *Grb10* gene transcript resulting, primarily, in differential sequence in the N-terminal and PH domains. Grb10 is widely expressed in mouse and human embryonic and adult tissues. High expression of Grb10 protein and mRNA are seen in the testis, skeletal muscle, pancreas and brain, with intermediate expression found in placenta, heart, kidney, liver, lung, spleen, prostate, ovary, small

intestine, colon and adipose tissues (reviewed in (56)). Expression of mouse and human isoforms of Grb10 is tissue-specific, although multiple isoforms of mouse Grb10 appear to be co-expressed in differentiated adipocytes (15). Analysis of Grb10 cellular localization indicates that all Grb10 isoforms are localized diffusely in the cytoplasm and become transiently recruited to the plasma membrane upon receptor activation (15, 20). Immunocytochemistry and subcellular fractionation studies also reveal an association between Grb10 and mitochondria. The significance of mitochondrial localization is not entirely clear, but there is some indication that it may facilitate Grb10 regulation of apoptosis (72).

Biological function of Grb10

The functional significance of Grb10 interaction with EGF and other related tyrosine kinase receptors has not been clearly established. There is, however, a substantial amount of evidence at cellular and organismal levels supporting a role for Grb10 in the regulation of insulin and IGF-I receptor activity. Overexpression of Grb10 fragments and full-length protein results in both enhancement and inhibition of IR and IGF-IR signaling and downstream effects (12, 57, 65, 68, 69, 76, 96, 103, 106, 107). These contradictory results have been attributed to the use of different cell lines, Grb10 isoforms, and Grb10 expression levels. It is also likely that overexpressed Grb10 can both augment and interfere with the function of endogenous Grb10, complicating the interpretation of these results. Recently, a clearer picture of Grb10 function in IR/IGF-IR activity has emerged from studies in which levels of endogenous Grb10 were reduced either by gene disruption or RNA interference. In mice, *Grb10* gene disruption by gene-trap insertion results in significant placental and embryonic overgrowth. At birth, these

mice are approximately 30% larger than wild type control animals, implicating Grb10 as a potent suppressor of somatic growth (9). It has also been shown that *Grb10* gene disruption results in enhanced insulin-stimulated Akt and ERK activation in skeletal muscle and fat and increased systemic insulin sensitivity (104). Consistent with this, at the cellular level, reduction of endogenous Grb10 by small interfering RNAs results in increased IGF-I-mediated phosphorylation of IRS, Akt and ERK, and insulin-stimulated phosphorylation of ERK, Shc, and Akt (18, 52). Together these findings suggest that Grb10 plays an important role in negatively regulating IGF and insulin effects *in vivo*.

Grb10 has also been genetically linked to human growth and metabolic disorders. A single nucleotide mutation in the *Grb10* gene, resulting in a proline to serine substitution at codon 95, was detected in two unrelated patients with Silver-Russell syndrome (110). This disorder is characterized by prenatal and postnatal growth retardation accompanied by dysmorphic features, such as triangular facies and fifth-finger clinodactyly (80, 85, 93). Grb10 has also been linked to type 2 diabetes mellitus. There is significant association between the human Grb10 single nucleotide polymorphism (SNP) rs4947710 and type 2 diabetes in Caucasian subjects from Italy, and between the Grb10 SNP rs2237457 and type 2 diabetes in an Old Order Amish population (14, 83). The results of these genetic studies are consistent with the effects of Grb10 gene disruption in mice and suggest that Grb10 plays a physiologically important role in insulin and IGF actions in humans.

Proposed mechanisms of Grb10 action: 1) Receptor turnover

There are two primary mechanisms by which Grb10 negatively regulates insulin and IGF-I receptors (Fig. 3). First, Grb10 stimulates internalization and degradation of

both insulin and IGF-I receptors, resulting in a decrease in receptor signaling capacity. Following ligand binding, activated insulin and IGF-I receptors are recruited to clathrin coated pits and rapidly endocytosed from the surface of the plasma membrane. Receptor-containing vesicles fuse with early endosomes where the acidic interior of these structures facilitates ligand dissociation resulting in receptor inactivation. Insulin and IGF ligands are rapidly degraded by endosomal specific proteases, preventing reactivation of the receptors. Internalized receptors are then recycled back to the plasma membrane or targeted for degradation by lysosome or proteasome dependent mechanisms (60, 108). Covalent attachment of the protein ubiquitin to activated receptors promotes receptor internalization and degradation. Ubiquitin is a small, highly conserved polypeptide that is covalently ligated to target proteins through a sequence of reactions involving a ubiquitin activating enzyme (E1), a ubiquitin carrier or conjugating enzyme (E2), and a ubiquitin ligase (E3). Ubiquitin-modification has a wide range of effects on protein activity, localization and degradation (36). Mono- or multiubiquitylation (monoubiquitylation at multiple sites) of receptor tyrosine kinases increases receptor affinity for lysosomal sorting machinery (32, 33, 37, 89). Attachment of single or multiple polymeric ubiquitin chains to receptors, a process referred to as polyubiquitylation, targets receptors for proteasome dependent degradation (90).

It has recently been observed that Grb10 overexpression results in decreased levels of the insulin receptor. Likewise, reduction of endogenous Grb10 levels by RNA interference leads to an increase in steady state levels of the receptor. Grb10 does not appear to affect IR mRNA levels, but rather promotes insulin stimulated ubiquitylation and degradation of the receptor through a proteasome dependent mechanism (82). It has

also been demonstrated that mouse Grb10 α can interact with the Neural precursor cell-expressed developmentally down-regulated 4 (Nedd4) protein (66). Nedd4 is an E3 ubiquitin ligase. It utilizes a homologous to the E6-AP carboxyl-terminus (HECT) domain to catalyze the transfer of ubiquitin to target substrates (39). Grb10-Nedd4 association does not result in ubiquitylation of Grb10; rather it serves to recruit Nedd4 to ligand activated IGF-I receptors. This results in Nedd4-dependent multiubiquitylation of the receptor, leading to increased receptor internalization and lysosomal degradation. There is also evidence suggesting that Grb10 promotes proteasome dependent degradation of the IGF-IR. The involvement of Nedd4-ubiquitylation of the receptor in this process has not been clearly established (62, 100).

2) Steric effects

Grb10 is rapidly recruited to activated insulin and IGF-I receptors following hormone stimulation. It has been demonstrated, that Grb10 interacts directly with specific phosphotyrosines in the activation loop of the IR/IGF-IR kinase domain, and remains tightly associated with the receptor for over 20 minutes following hormone stimulation (16, 27, 35, 95). Grb10 also has a propensity for oligomerization, and both dimeric and tetrameric forms of Grb10 have been detected in solution (17). The SH2 domain of Grb10 readily forms homodimers, and the amino acid residues involved in this process appear to be distinct from those responsible for phosphotyrosine binding (95). This implies that Grb10 may associate with the IR and IGF-IR as a large homo-dimeric or –tetrameric complex. The formation of this complex has been proposed to physically interfere with the access of substrate proteins to the receptor. In support of this, it was recently shown that Grb10 inhibition of insulin stimulated PI3K activity occurs through

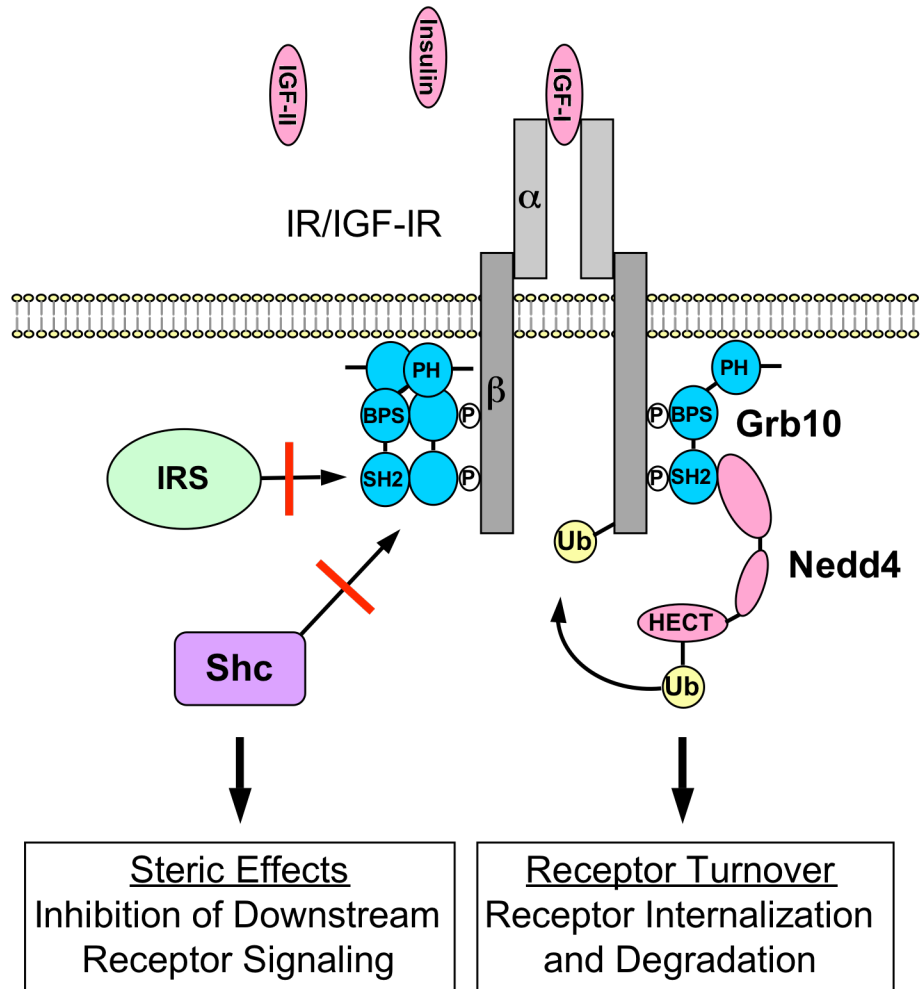


Figure 3. Mechanisms of Grb10-inhibition of ligand activated insulin and IGF-I receptors. Schematic representation of the steric effects due to Grb10 oligomer binding, preventing access of proximal signaling molecules IRS and Shc to the receptor (left); and Grb10 recruitment of Nedd4 ubiquitin (Ub) ligase, resulting in receptor turnover (right).

steric hindrance of IRS1 and IRS2 access to the IR (106). This mechanism is further supported by the observation that reduction of endogenous Grb10 levels by RNA interference, results in increased phosphatase-dependent dephosphorylation of the IGF-IR (18). Thus, Grb10-receptor association prevents proximal receptor substrates, as well as

phosphatases, and potentially additional receptor binding proteins, from directly interacting with the activated receptor.

Adaptor protein function of Grb10

Grb10 may also modulate receptor function by acting as an adaptor protein to recruit additional regulatory factors to the receptor. Adaptor proteins typically lack intrinsic enzymatic activity. Instead these proteins contain multiple protein-protein interaction domains and regulate signal transduction through the formation of protein complexes. Grb10 has the characteristic structure of an adaptor protein, with five potential protein-protein interaction motifs spread across the molecule. At the C-terminal end of the protein, Grb10 contains a BPS domain and an SH2 domain. The SH2 domain of Grb10 is approximately 100 amino acids in length and is defined by its sequence homology to a portion of the non-receptor tyrosine kinase Src. SH2 domains mediate interaction with phosphorylated tyrosine motifs. This domain is found in a wide variety of signal transduction proteins and plays a critical role in the recruitment of these proteins to sites of active signaling (88). Situated just N-terminal to the SH2 domain is the roughly 80 amino acid BPS domain. This protein domain is unique to the Grb7/10/14 family of proteins. It is classified as intrinsically unstructured although it may become transiently structured upon receptor interaction (35, 63, 64). The SH2 domain of Grb10, in combination with the BPS domain, mediates Grb10 interaction with tyrosine phosphorylated IR. Grb10 interaction with the IGF-IR appears to be primarily dependent on the BPS domain, with the SH2 domain playing more of a minor role in IGF-IR association.

Interaction between the C-terminal domains of Grb10 and insulin and IGF-I receptors leaves the N-terminus of the molecule free for additional protein-protein interactions. Human Grb10 isoforms contain two conserved proline-rich stretches, also referred to as proline-rich boxes, at their N-termini. Mouse Grb10 isoforms share these two proline-rich boxes and contain a 3rd highly conserved N-terminal proline-rich box (27, 38). A number of protein-protein interaction domains recognize and bind specifically to proline-rich motifs, like those found in Grb10, to facilitate protein complex formation (1, 111). Despite this, relatively few N-terminal Grb10 interacting proteins have been identified. The proline interacting, Src-homology region 3 (SH3) domain of the proto-oncogene c-Abl shows a capacity for N-terminal Grb10 interaction *in vitro* (20). *In vivo* association and potential biological significance of this interaction have not been determined. Recently, the proline-rich region of mouse Grb10 was found to interact with two novel proteins designated Grb10-interacting GYF protein 1 (GIGYF1) and GIGYF2 (27). The characterization and Grb10 binding capacity of these proteins will be addressed in the following section with particular emphasis on the GIGYF2 protein, as it is the primary focus of this dissertation.

Section 3: Grb10-interacting GYF proteins

Identification of GIGYFs

To identify additional factors that may participate in Grb10 regulation of the insulin and IGF-I receptors, Giovannone et al. (2003) performed a yeast two-hybrid cDNA library screen with the N-terminal proline-rich region of mouse Grb10 δ . A total of 15 independent Grb10-interactive clones were identified from 2 different mouse

cDNA libraries. Five of the clones consisted of sequence fragments of the two novel proteins GIGYF1 and GIGYF2. Analysis of full-length nucleotide and amino acid sequence revealed that GIGYF1 and GIGYF2 are homologous proteins. They have an overall amino acid sequence similarity of approximately 40% but show a higher degree homology within specific domains. Interaction between GIGYF1 and Grb10 was confirmed by co-immunoprecipitation studies in mouse fibroblasts. Full-length GIGYF1 protein associates with Grb10 in serum depleted cells. IGF-I stimulation of these cells results in increased Grb10-GIGYF1 interaction, and rapid recruitment of both proteins to the activated IGF-IR. GIGYF1 transiently interacts with the IGF-IR and then dissociates from the Grb10-receptor complex. The fragment of GIGYF1 that was identified by yeast two-hybrid cloning, when overexpressed, results in an increase in IGF-IR activation and downstream signaling (27). Whether this GIGYF1 fragment is augmenting endogenous GIGYF1 function or acting in a dominant-negative manner is not known. While the mechanism of action has not been established, these results indicate that the GIGYF proteins may participate in Grb10-mediated regulation of the IGF-IR.

GIGYF genes: evolutionary conservation and expression

The gene for mouse GIGYF1 maps to chromosome 5. It is a relatively small gene with 24 exons extending over just 7000 base pairs of genomic sequence. The orthologous human gene maps to chromosome 7q22 and encodes a protein that is over 90% identical in amino acid sequence to the mouse protein. Both human and mouse *GIGYF1* genes were originally designated *PERQ1* in recognition of the high abundance of proline, glutamic acid, arginine and glutamine found in the proteins they encode (27, 28, 109). Genes encoding GIGYF2 are found on chromosome 1 in mice and on

chromosome 2q37.1 in humans. In contrast to the *GIGYF1* gene, both the mouse and human *GIGYF2* genes are quite large. They consist of 27 exons, interspersed with large introns, extending over 90,000 and 160,000 bases of the mouse and human genomes, respectively. *GIGYF2* genes were originally designated *TNRC15* (Trinucleotide Repeat Containing 15) due to the presence of long stretches of glutamines encoded by CAG trinucleotide repeats. This is a characteristic of both *GIGYF1* and *GIGYF2*, with a number of glutamine repeats, from 2-12 residues in length, found in the central and C-terminal portions of the proteins (27, 40). Both *GIGYF1* and *GIGYF2* genes appear to be highly conserved in the euteleostomi clade of vertebrates, which includes all mammals, birds, amphibians, reptiles and most fish. Single genes, encoding proteins with similarities to both *GIGYF1* and *GIGYF2*, are present in *Drosophila* (*D. melanogaster* CG11148, FlyBase) and *Caenorhabditis* (*C. elegans* R10D12.14, WormBase) species. Thus, it is likely that *GIGYF1* and *GIGYF2* are paralogs, arising from the duplication of a common ancestral gene at some point in evolution.

In the mouse, *Gigyf1* and *Gigyf2* genes appear to be co-expressed in a number of different tissues. Northern blot analysis indicates expression of both transcripts in heart, brain, lung, liver, testis, and kidney tissue. *GIGYF1* mRNA is also found in the spleen. Low expression of both genes is seen in mouse skeletal muscle (27). In humans, both *GIGYF1* and *GIGYF2* are widely expressed, with particular enrichment in the brain. Based on analysis of expressed sequence tags (ESTs), a high level of expression is predicted for both genes with *GIGYF1* expressed at 1.6 times the average gene and *GIGYF2* expressed at 4.4 times the average gene (97).

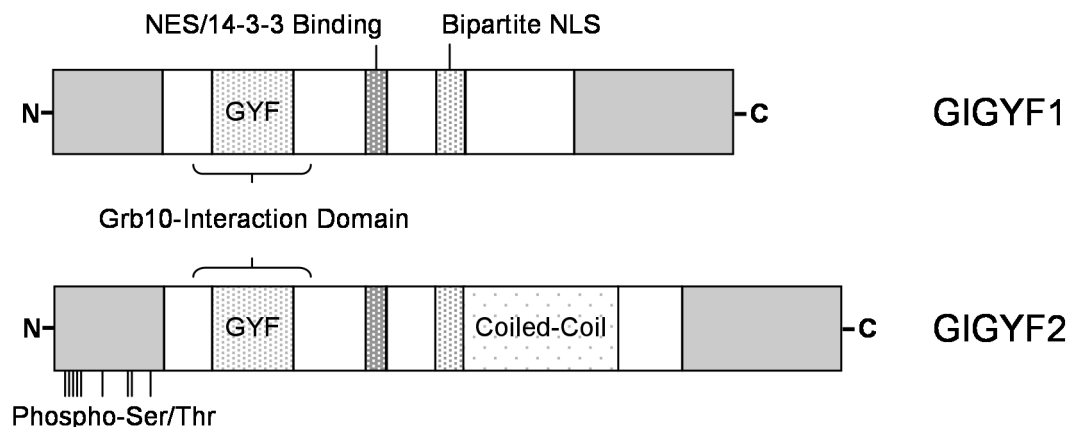


Figure 4. Grb10-interacting GYF proteins GIGYF1 and GIGYF2. Diagram showing domain topology of GIGYF proteins. Regions of high amino acid sequence homology in N- and C-termini are indicated by gray shading. Structural motifs shared by GIGYF1 and GIGYF2 are labeled. GIGYF2-specific coiled-coil domain and phosphorylated serine/threonine sites are also shown. NES = nuclear export signal and NLS = nuclear localization signal.

GIGYF proteins

GIGYF1 and GIGYF2 proteins (Fig. 4) contain large sections of high sequence homology (>70%) at both the N- and C-termini. Both proteins contain a predicted bipartite nuclear localization signal and candidate binding sites for protein phosphatase 1 (sequence: RVPF), 14-3-3 (RSXpSXP), and phospholipase C γ (V(F)DDY) (10, 27). GIGYF2 contains a 200 amino acid section immediately C-terminal to the bipartite nuclear localization signal that is not found in GIGYF1 (human GIGYF2 residues 723-923). This entire region is predicted to form a long coiled-coil domain containing alternating clusters of negative (29.4% glutamic acid) and positive amino acids (7% lysine and 21.9% arginine), as well as a high number of glutamine (17.4%) and hydrophobic residues (14.9%) (5). Functional significance of these predicted sites has not been determined. A comprehensive proteomic analysis of 14-3-3-binding proteins

did, however, detect GIGYF2 in association with 14-3-3 in mitotic HeLa cells (human cervix epithelial adenocarcinoma) (61). 14-3-3 proteins have been shown to play an important role in regulating the subcellular localization of their binding partners. This typically occurs when 14-3-3 proteins bind near a nuclear export or nuclear import signal, physically blocking access to these sites (reviewed in (71)). It is interesting to note that the potential 14-3-3 binding site in GIGYF2 lies within a predicted CRM1-dependent nuclear export signal (human GIGYF2 residues 673-687) (81). This presents the intriguing possibility that GIGYF2 subcellular localization is regulated by cell cycle dependent 14-3-3 binding.

Several proteomic studies have identified GIGYF2 as a serine/threonine phosphorylated protein. All of the phosphorylated residues are located in the first ~400 amino acids of GIGYF2 (3, 4, 70, 77, 98, 102). A number of these studies identified a cluster of 6 phosphorylated serine/threonine residues over a stretch of 12 amino acids (human GIGYF2 residues 19-30) (3, 4, 70, 77, 98). It is expected that this degree of phosphorylation in such a concentrated region would create a focus of negative charge at the N-terminus of GIGYF2. How this might translate into effects on GIGYF2 function is not known. Two groups identified phospho-GIGYF2 in mouse post-synaptic density and synaptosomal preparations suggesting a role for GIGYF2 in these critical neuronal structures (70, 98). Finally, three of the phosphorylated residues identified show evidence of growth factor regulation. Phosphorylation of serine 26, serine 30 and serine 158, was found to increase following EGF stimulation of HeLa cells. Peak phosphorylation at residues 26 and 30 was seen at 5 minutes and at residue 158 at 10 minutes following addition of EGF (77).

Analysis of GIGYF sequences in multiple species reveals the presence of an evolutionarily conserved domain of approximately 70 amino acids in the N-terminal half of each protein (human GIGYF1 residues 472-537 and human GIGYF2 residues 531-596). This domain is over 80% identical between GIGYF1 and GIGYF2 and 60% identical between GIGYF2 and the *D. melanogaster* GIGYF ortholog. Contained within this region is a consensus GYF domain (27). The GYF domain is a proline-rich sequence recognition motif that was first identified in the T-lymphocyte CD2-binding protein 2 (CD2BP2) (22, 75). The signature motif of the GYF domain includes the glycyl-tyrosyl-phenylalanyl tripeptide contained within the sequence W-X-Y-X₆₋₁₁-GPF-X₄-M-X₂-W-X₃-GYF. This sequence adopts a bulge-helix-bulge topology and utilizes stacked aromatic amino acids for recognition of paired proline residues. GYF domains appear to be present in all eukaryotic genomes. However, the number of GYF domain-containing proteins within each species is relatively small. In mammals, there are only three GYF domain-containing proteins, CD2BP2, GIGYF1 and GIGYF2 (23, 24, 31, 49, 75).

Based on sequence homology, the GYF domains of the GIGYF proteins are classified as members of the SMY2 subtype (48). The prototypical member of this family is the *Saccharomyces cerevisiae* protein SMY2, which was initially cloned as a suppressor of mutations in the yeast myosin MYO2 (55). Proteomic analysis of the GIGYF2-GYF domain revealed that its preferred recognition motif is prolyl-prolyl-glycyl- Φ (PPG Φ), with Φ standing for hydrophobic residues. This appears to be the conserved recognition motif for all GYF domains of the SMY2 subtype. A search for candidate GIGYF2-GYF domain targets, using yeast two-hybrid and *in vitro* proteomic approaches, identified a number of proteins involved in mRNA processing (46, 48).

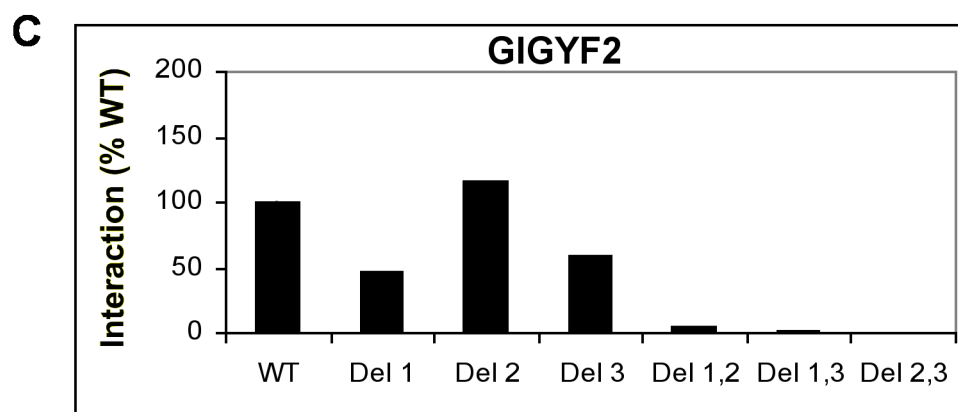
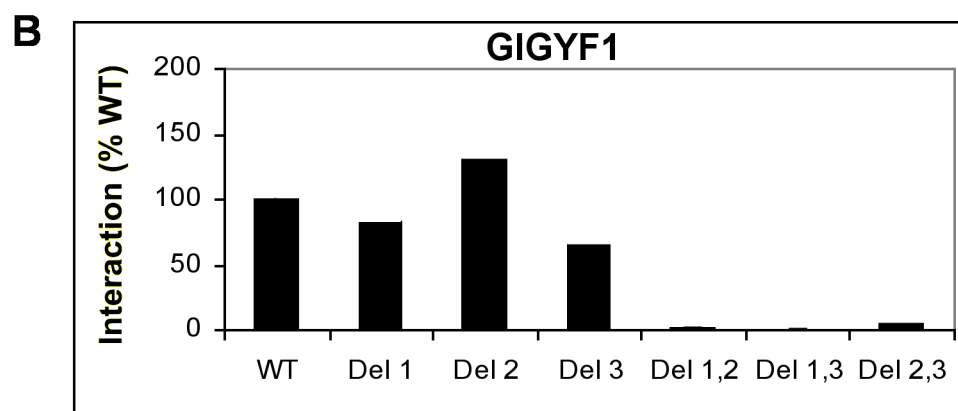
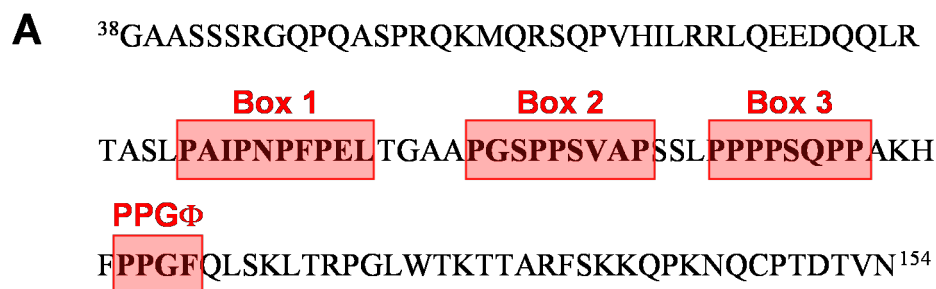


Figure 5. GIGYF-GYF domain interaction with Grb10 proline-rich region. Shown in (A), the sequence of mouse Grb10 δ proline-rich region (amino acids 38-154) with location of proline-rich boxes 1-3 and GYF domain consensus recognition motif (PPG Φ) indicated. Relative strength of interaction with wild type (WT) and various proline-rich box deletions of Grb10 is shown for GIGYF1-GYF domain in panel (B) and GIGYF2-GYF domain in panel (C). Adapted from: Giovannone et al., (2003) (27).

Several GYF domain-containing proteins, including SMY2 and CD2BP2, have now been functionally linked to the regulation of splicing, transport and translation of mRNA (25, 47, 51, 73, 74, 101). The extent to which GIGYF proteins are involved in these processes has not been examined.

The GYF domains of mouse GIGYF1 and GIGYF2 were found to be necessary and sufficient for interaction with the N-terminal proline-rich region of Grb10. In addition to this, a recent yeast two-hybrid analysis revealed that GIGYF1 and GIGYF2-GYF domains interact specifically with Grb10 and not with the N-terminal proline-rich regions of Grb7 or Grb14 (18). The three proline-rich boxes identified in mouse Grb10 do not contain the PPG Φ sequence predicted to interact with the GIGYF2-GYF domain. Immediately adjacent to the 3rd proline-rich box there is a PPGF motif that fits the consensus recognition sequence. Whether or not this motif contributes to GIGYF-Grb10 interaction is not known. Deletion analysis, however, reveal that removal of any two of the three established Grb10 proline-rich boxes results in complete loss of GIGYF-Grb10 interaction. Furthermore, excision of proline-rich box 3 for GIGYF1, and box 1 for GIGYF2, significantly decreases Grb10 binding to GIGYF-GYF domains (Fig. 5) (27). These results suggest a role for individual boxes in GIGYF1 or GIGYF2-specific binding, and a clear requirement for at least two of the three established proline-rich boxes for GIGYF-Grb10 interaction.

Thesis objective

GIGYF1 and GIGYF2 are evolutionarily conserved proteins that contain a number of interesting structural motifs, and are highly expressed in multiple tissues. Giovannone et al. (2003) have established a role for GIGYF1 in Grb10-regulation of insulin and IGF-I receptors. Although it is structurally homologous, the function of GIGYF2 is not known. Insulin and IGF-I hormonal systems are required for human growth, development and metabolism. Precise regulation of these systems by intracellular binding partners like Grb10 and GIGYF1 is critical. As a physiologic regulator of insulin and IGF action, Grb10 has emerged as a promising drug target for the treatment of diseases ranging from diabetes to cancer. For this type of treatment strategy to succeed, a detailed understanding of Grb10 and interacting proteins that may participate in its actions is required. Thus, the primary objective of this thesis is to examine the biological significance and molecular function of the GIGYF2 protein. In the following data chapters, three distinct, yet complementary approaches will be used to address this objective. Chapter two describes analysis of the GIGYF2 gene in human disease, with the objective of linking GIGYF2 to specific pathophysiologic processes. In chapter three, GIGYF2's role in growth and development, and participation in IGF-I receptor action, will be assessed following gene disruption in the mouse. In the final data chapter, the molecular function of GIGYF2 will be addressed through characterization of GIGYF2 deficient cells, and analysis of GIGYF2 interacting proteins.

REFERENCES

1. **Ball, L. J., R. Kuhne, J. Schneider-Mergener, and H. Oschkinat.** 2005.
Recognition of Proline-Rich Motifs by Protein-Protein-Interaction Domains.
Angew Chem Int Ed Engl **44**:2852-2869.
2. **Baserga, R., F. Peruzzi, and K. Reiss.** 2003. The IGF-1 receptor in cancer
biology. *Int J Cancer* **107**:873-877.
3. **Beausoleil, S. A., M. Jedrychowski, D. Schwartz, J. E. Elias, J. Villen, J. Li, M.
A. Cohn, L. C. Cantley, and S. P. Gygi.** 2004. Large-scale characterization of
HeLa cell nuclear phosphoproteins. *Proc Natl Acad Sci U S A* **101**:12130-12135.
4. **Beausoleil, S. A., J. Villen, S. A. Gerber, J. Rush, and S. P. Gygi.** 2006. A
probability-based approach for high-throughput protein phosphorylation analysis
and site localization. *Nat Biotechnol* **24**:1285-1292.
5. **Berger, B., D. B. Wilson, E. Wolf, T. Tonchev, M. Milla, and P. S. Kim.** 1995.
Predicting coiled coils by use of pairwise residue correlations. *Proc Natl Acad Sci
U S A* **92**:8259-8263.
6. **Bevan, P.** 2001. Insulin signalling. *J Cell Sci* **114**:1429-1430.
7. **Brzozowski, A. M., E. J. Dodson, G. G. Dodson, G. N. Murshudov, C. Verma,
J. P. Turkenburg, F. M. de Bree, and Z. Dauter.** 2002. Structural origins of the
functional divergence of human insulin-like growth factor-I and insulin.
Biochemistry **41**:9389-9397.
8. **Cailliau, K., V. Le Marcis, V. Bereziat, D. Perdereau, B. Cariou, J. P. Vilain,
A. F. Burnol, and E. Browaeys-Poly.** 2003. Inhibition of FGF receptor signalling

- in *Xenopus* oocytes: differential effect of Grb7, Grb10 and Grb14. *FEBS Lett* **548**:43-48.
9. **Charalambous, M., F. M. Smith, W. R. Bennett, T. E. Crew, F. Mackenzie, and A. Ward.** 2003. Disruption of the imprinted Grb10 gene leads to disproportionate overgrowth by an Igf2-independent mechanism. *Proc Natl Acad Sci U S A* **100**:8292-8297.
 10. **Cokol, M., R. Nair, and B. Rost.** 2000. Finding nuclear localization signals. *EMBO Rep* **1**:411-415.
 11. **De Meyts, P., and J. Whittaker.** 2002. Structural biology of insulin and IGF1 receptors: implications for drug design. *Nat Rev Drug Discov* **1**:769-783.
 12. **Deng, Y., S. Bhattacharya, O. R. Swamy, R. Tandon, Y. Wang, R. Janda, and H. Riedel.** 2003. Growth factor receptor-binding protein 10 (Grb10) as a partner of phosphatidylinositol 3-kinase in metabolic insulin action. *J Biol Chem* **278**:39311-39322.
 13. **Denley, A., L. J. Cosgrove, G. W. Booker, J. C. Wallace, and B. E. Forbes.** 2005. Molecular interactions of the IGF system. *Cytokine Growth Factor Rev* **16**:421-439.
 14. **Di Paola, R., E. Ciociola, W. Boonyasrisawat, D. Nolan, J. Duffy, G. Miscio, C. Cisternino, G. Fini, V. Tassi, A. Doria, and V. Trischitta.** 2006. Association of hGrb10 genetic variations with type 2 diabetes in Caucasian subjects. *Diabetes Care* **29**:1181-1183.
 15. **Dong, L. Q., H. Du, S. G. Porter, L. F. J. Kolakowski, A. V. Lee, L. J. Mandarino, J. Fan, D. Yee, and F. Liu.** 1997. Cloning, chromosome localization,

- expression, and characterization of an Src homology 2 and pleckstrin homology domain-containing insulin receptor binding protein hGrb10gamma. *J Biol Chem* **272**:29104-29112.
16. **Dong, L. Q., S. Farris, J. Christal, and F. Liu.** 1997. Site-directed mutagenesis and yeast two-hybrid studies of the insulin and insulin-like growth factor-1 receptors: the Src homology-2 domain-containing protein hGrb10 binds to the autophosphorylated tyrosine residues in the kinase domain of the insulin receptor. *Mol Endocrinol* **11**:1757-1765.
 17. **Dong, L. Q., S. Porter, D. Hu, and F. Liu.** 1998. Inhibition of hGrb10 binding to the insulin receptor by functional domain-mediated oligomerization. *J Biol Chem* **273**:17720-17725.
 18. **Dufresne, A. M., and R. J. Smith.** 2005. The adapter protein GRB10 is an endogenous negative regulator of insulin-like growth factor signaling. *Endocrinology* **146**:4399-4409.
 19. **Dupont, J., S. E. Dunn, J. C. Barrett, and D. LeRoith.** 2003. Microarray analysis and identification of novel molecules involved in insulin-like growth factor-1 receptor signaling and gene expression. *Recent Prog Horm Res* **58**:325-342.
 20. **Frantz, J. D., S. Giorgetti-Peraldi, E. A. Ottinger, and S. E. Shoelson.** 1997. Human GRB-IRbeta/GRB10. Splice variants of an insulin and growth factor receptor-binding protein with PH and SH2 domains. *J Biol Chem* **272**:2659-2667.
 21. **Frattali, A. L., J. L. Treadway, and J. E. Pessin.** 1992. Insulin/IGF-1 hybrid receptors: implications for the dominant-negative phenotype in syndromes of insulin resistance. *J Cell Biochem* **48**:43-50.

22. **Freund, C., V. Dotsch, K. Nishizawa, E. L. Reinherz, and G. Wagner.** 1999.
The GYF domain is a novel structural fold that is involved in lymphoid signaling through proline-rich sequences. *Nat Struct Biol* **6**:656-660.
23. **Freund, C., R. Kuhne, S. Park, K. Thiemke, E. L. Reinherz, and G. Wagner.** 2003. Structural investigations of a GYF domain covalently linked to a proline-rich peptide. *J Biomol NMR* **27**:143-149.
24. **Freund, C., R. Kuhne, H. Yang, S. Park, E. L. Reinherz, and G. Wagner.** 2002. Dynamic interaction of CD2 with the GYF and the SH3 domain of compartmentalized effector molecules. *EMBO J* **21**:5985-5995.
25. **Georgiev, A., M. Sjostrom, and A. Wieslander.** 2007. Binding specificities of the GYF domains from two *Saccharomyces cerevisiae* paralogs. *Protein Eng Des Sel* **20**:443-452.
26. **Giorgetti-Peraldi, S., J. Murdaca, J. C. Mas, and E. Van Obberghen.** 2001. The adapter protein, Grb10, is a positive regulator of vascular endothelial growth factor signaling. *Oncogene* **20**:3959-3968.
27. **Giovannone, B., E. Lee, L. Laviola, F. Giorgino, K. A. Cleveland, and R. J. Smith.** 2003. Two novel proteins that are linked to insulin-like growth factor (IGF-I) receptors by the Grb10 adapter and modulate IGF-I signaling. *J Biol Chem* **278**:31564-31573.
28. **Glockner, G., S. Scherer, R. Schattevoy, A. Borigt, J. Weber, L. C. Tsui, and A. Rosenthal.** 1998. Large-scale sequencing of two regions in human chromosome 7q22: analysis of 650 kb of genomic sequence around the EPO and CUTL1 loci reveals 17 genes. *Genome Res* **8**:1060-1073.

29. **Goodman, H. M.** 1994. Basic medical endocrinology. **Raven Press series in physiology**:xv, 332 p.
30. **Grimberg, A., and P. Cohen.** 2000. Role of insulin-like growth factors and their binding proteins in growth control and carcinogenesis. *J Cell Physiol* **183**:1-9.
31. **Gu, W., M. Kofler, I. Antes, C. Freund, and V. Helms.** 2005. Alternative binding modes of proline-rich peptides binding to the GYF domain. *Biochemistry* **44**:6404-6415.
32. **Haglund, K., P. P. Di Fiore, and I. Dikic.** 2003. Distinct monoubiquitin signals in receptor endocytosis. *Trends Biochem Sci* **28**:598-603.
33. **Haglund, K., S. Sigismund, S. Polo, I. Szymkiewicz, P. P. Di Fiore, and I. Dikic.** 2003. Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. *Nat Cell Biol* **5**:461-466.
34. **Hansen, H., U. Svensson, J. Zhu, L. Laviola, F. Giorgino, G. Wolf, R. J. Smith, and H. Riedel.** 1996. Interaction between the Grb10 SH2 domain and the insulin receptor carboxyl terminus. *J Biol Chem* **271**:8882-8886.
35. **He, W., D. W. Rose, J. M. Olefsky, and T. A. Gustafson.** 1998. Grb10 interacts differentially with the insulin receptor, insulin-like growth factor I receptor, and epidermal growth factor receptor via the Grb10 Src homology 2 (SH2) domain and a second novel domain located between the pleckstrin homology and SH2 domains. *J Biol Chem* **273**:6860-6867.
36. **Hershko, A., and A. Ciechanover.** 1998. The ubiquitin system. *Annu Rev Biochem* **67**:425-479.

37. **Hicke, L.** 2001. A new ticket for entry into budding vesicles-ubiquitin. *Cell* **106**:527-530.
38. **Holt, L. J., and K. Siddle.** 2005. Grb10 and Grb14: enigmatic regulators of insulin action--and more? *Biochem J* **388**:393-406.
39. **Huibregtse, J. M., M. Scheffner, S. Beaudenon, and P. M. Howley.** 1995. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proc Natl Acad Sci U S A* **92**:2563-2567.
40. **Ishikawa, K., T. Nagase, M. Suyama, N. Miyajima, A. Tanaka, H. Kotani, N. Nomura, and O. Ohara.** 1998. Prediction of the coding sequences of unidentified human genes. X. The complete sequences of 100 new cDNA clones from brain which can code for large proteins in vitro. *DNA Res* **5**:169-176.
41. **Jahn, T., P. Seipel, S. Urschel, C. Peschel, and J. Duyster.** 2002. Role for the adaptor protein Grb10 in the activation of Akt. *Mol Cell Biol* **22**:979-991.
42. **Kanzaki, M., and J. E. Pessin.** 2001. Signal integration and the specificity of insulin action. *Cell Biochem Biophys* **35**:191-209.
43. **Katz, M., I. Amit, and Y. Yarden.** 2007. Regulation of MAPKs by growth factors and receptor tyrosine kinases. *Biochim Biophys Acta* **1773**:1161-1176.
44. **Khan, A. H., and J. E. Pessin.** 2002. Insulin regulation of glucose uptake: a complex interplay of intracellular signalling pathways. *Diabetologia* **45**:1475-1483.
45. **Khandwala, H. M., I. E. McCutcheon, A. Flyvbjerg, and K. E. Friend.** 2000. The effects of insulin-like growth factors on tumorigenesis and neoplastic growth. *Endocr Rev* **21**:215-244.

46. **Kofler, M., K. Heuer, T. Zech, and C. Freund.** 2004. Recognition sequences for the GYF domain reveal a possible spliceosomal function of CD2BP2. *J Biol Chem* **279**:28292-28297.
47. **Kofler, M., K. Motzny, M. Beyermann, and C. Freund.** 2005. Novel interaction partners of the CD2BP2-GYF domain. *J Biol Chem* **280**:33397-33402.
48. **Kofler, M., K. Motzny, and C. Freund.** 2005. GYF domain proteomics reveals interaction sites in known and novel target proteins. *Mol Cell Proteomics* **4**:1797-1811.
49. **Kofler, M. M., and C. Freund.** 2006. The GYF domain. *FEBS J* **273**:245-256.
50. **Kulkarni, R. N.** 2004. The islet beta-cell. *Int J Biochem Cell Biol* **36**:365-371.
51. **Laggerbauer, B., S. Liu, E. Makarov, H. P. Vornlocher, O. Makarova, D. Ingelfinger, T. Achsel, and R. Luhrmann.** 2005. The human U5 snRNP 52K protein (CD2BP2) interacts with U5-102K (hPrp6), a U4/U6.U5 tri-snRNP bridging protein, but dissociates upon tri-snRNP formation. *RNA* **11**:598-608.
52. **Langlais, P., L. Q. Dong, F. J. Ramos, D. Hu, Y. Li, M. J. Quon, and F. Liu.** 2004. Negative regulation of insulin-stimulated mitogen-activated protein kinase signaling by Grb10. *Mol Endocrinol* **18**:350-358.
53. **Laviola, L., F. Giorgino, J. C. Chow, J. A. Baquero, H. Hansen, J. Ooi, J. Zhu, H. Riedel, and R. J. Smith.** 1997. The adapter protein Grb10 associates preferentially with the insulin receptor as compared with the IGF-I receptor in mouse fibroblasts. *J Clin Invest* **99**:830-837.
54. **LeRoith, D., and C. T. J. Roberts.** 2003. The insulin-like growth factor system and cancer. *Cancer Lett* **195**:127-137.

55. **Lillie, S. H., and S. S. Brown.** 1992. Suppression of a myosin defect by a kinesin-related gene. *Nature* **356**:358-361.
56. **Lim, M. A., H. Riedel, and F. Liu.** 2004. Grb10: more than a simple adaptor protein. *Front Biosci* **9**:387-403.
57. **Liu, F., and R. A. Roth.** 1995. Grb-IR: a SH2-domain-containing protein that binds to the insulin receptor and inhibits its function. *Proc Natl Acad Sci U S A* **92**:10287-10291.
58. **Manser, J., C. Roonprapunt, and B. Margolis.** 1997. *C. elegans* cell migration gene mig-10 shares similarities with a family of SH2 domain proteins and acts cell nonautonomously in excretory canal development. *Dev Biol* **184**:150-164.
59. **Massague, J.** 2004. G1 cell-cycle control and cancer. *Nature* **432**:298-306.
60. **McPherson, P. S., B. K. Kay, and N. K. Hussain.** 2001. Signaling on the endocytic pathway. *Traffic* **2**:375-384.
61. **Meek, S. E., W. S. Lane, and H. Piwnica-Worms.** 2004. Comprehensive proteomic analysis of interphase and mitotic 14-3-3-binding proteins. *J Biol Chem* **279**:32046-32054.
62. **Monami, G., V. Emiliozzi, and A. Morrione.** 2008. Grb10/Nedd4-mediated multiubiquitination of the insulin-like growth factor receptor regulates receptor internalization. *J Cell Physiol*
63. **Moncoq, K., I. Broutin, C. T. Craescu, P. Vachette, A. Ducruix, and D. Durand.** 2004. SAXS study of the PIR domain from the Grb14 molecular adaptor: a natively unfolded protein with a transient structure primer? *Biophys J* **87**:4056-4064.

64. **Moncoq, K., I. Broutin, V. Larue, D. Perdereau, K. Cailliau, E. Browaeys-Poly, A. F. Burnol, and A. Ducruix.** 2003. The PIR domain of Grb14 is an intrinsically unstructured protein: implication in insulin signaling. *FEBS Lett* **554**:240-246.
65. **Morrione, A.** 2003. Grb10 adapter protein as regulator of insulin-like growth factor receptor signaling. *J Cell Physiol* **197**:307-311.
66. **Morrione, A., P. Plant, B. Valentinis, O. Staub, S. Kumar, D. Rotin, and R. Baserga.** 1999. mGrb10 interacts with Nedd4. *J Biol Chem* **274**:24094-24099.
67. **Morrione, A., B. Valentinis, S. Li, J. Y. Ooi, B. Margolis, and R. Baserga.** 1996. Grb10: A new substrate of the insulin-like growth factor I receptor. *Cancer Res* **56**:3165-3167.
68. **Morrione, A., B. Valentinis, M. Resnicoff, S. Xu, and R. Baserga.** 1997. The role of mGrb10alpha in insulin-like growth factor I-mediated growth. *J Biol Chem* **272**:26382-26387.
69. **Mounier, C., L. Lavoie, V. Dumas, K. Mohammad-Ali, J. Wu, A. Nantel, J. J. Bergeron, D. Y. Thomas, and B. I. Posner.** 2001. Specific inhibition by hGRB10zeta of insulin-induced glycogen synthase activation: evidence for a novel signaling pathway. *Mol Cell Endocrinol* **173**:15-27.
70. **Munton, R. P., R. Tweedie-Cullen, M. Livingstone-Zatchej, F. Weinandy, M. Waidelich, D. Longo, P. Gehrig, F. Potthast, D. Rutishauser, B. Gerrits, C. Panse, R. Schlapbach, and I. M. Mansuy.** 2007. Qualitative and quantitative analyses of protein phosphorylation in naive and stimulated mouse synaptosomal preparations. *Mol Cell Proteomics* **6**:283-293.

71. **Muslin, A. J., and H. Xing.** 2000. 14-3-3 proteins: regulation of subcellular localization by molecular interference. *Cell Signal* **12**:703-709.
72. **Nantel, A., M. Huber, and D. Y. Thomas.** 1999. Localization of endogenous Grb10 to the mitochondria and its interaction with the mitochondrial-associated Raf-1 pool. *J Biol Chem* **274**:35719-35724.
73. **Naranda, T., M. Kainuma, S. E. MacMillan, and J. W. Hershey.** 1997. The 39-kilodalton subunit of eukaryotic translation initiation factor 3 is essential for the complex's integrity and for cell viability in *Saccharomyces cerevisiae*. *Mol Cell Biol* **17**:145-153.
74. **Nielsen, T. K., S. Liu, R. Luhrmann, and R. Ficner.** 2007. Structural basis for the bifunctionality of the U5 snRNP 52K protein (CD2BP2). *J Mol Biol* **369**:902-908.
75. **Nishizawa, K., C. Freund, J. Li, G. Wagner, and E. L. Reinherz.** 1998. Identification of a proline-binding motif regulating CD2-triggered T lymphocyte activation. *Proc Natl Acad Sci U S A* **95**:14897-14902.
76. **O'Neill, T. J., D. W. Rose, T. S. Pillay, K. Hotta, J. M. Olefsky, and T. A. Gustafson.** 1996. Interaction of a GRB-IR splice variant (a human GRB10 homolog) with the insulin and insulin-like growth factor I receptors. Evidence for a role in mitogenic signaling. *J Biol Chem* **271**:22506-22513.
77. **Olsen, J. V., B. Blagoev, F. Gnad, B. Macek, C. Kumar, P. Mortensen, and M. Mann.** 2006. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* **127**:635-648.

78. **Ooi, J., V. Yajnik, D. Immanuel, M. Gordon, J. J. Moskow, A. M. Buchberg, and B. Margolis.** 1995. The cloning of Grb10 reveals a new family of SH2 domain proteins. *Oncogene* **10**:1621-1630.
79. **Pandey, A., H. Duan, P. P. Di Fiore, and V. M. Dixit.** 1995. The Ret receptor protein tyrosine kinase associates with the SH2-containing adapter protein Grb10. *J Biol Chem* **270**:21461-21463.
80. **Price, S. M., R. Stanhope, C. Garrett, M. A. Preece, and R. C. Trembath.** 1999. The spectrum of Silver-Russell syndrome: a clinical and molecular genetic study and new diagnostic criteria. *J Med Genet* **36**:837-842.
81. **Puntervoll, P., R. Linding, C. Gemund, S. Chabanis-Davidson, M. Mattingsdal, S. Cameron, D. M. Martin, G. Ausiello, B. Brannetti, A. Costantini, F. Ferre, V. Maselli, A. Via, G. Cesareni, F. Diella, G. Superti-Furga, L. Wyrwicz, C. Ramu, C. McGuigan, R. Gudavalli, I. Letunic, P. Bork, L. Rychlewski, B. Kuster, M. Helmer-Citterich, W. N. Hunter, R. Aasland, and T. J. Gibson.** 2003. ELM server: A new resource for investigating short functional sites in modular eukaryotic proteins. *Nucleic Acids Res* **31**:3625-3630.
82. **Ramos, F. J., P. R. Langlais, D. Hu, L. Q. Dong, and F. Liu.** 2006. Grb10 mediates insulin-stimulated degradation of the insulin receptor: a mechanism of negative regulation. *Am J Physiol Endocrinol Metab* **290**:E1262-6.
83. **Rampersaud, E., C. M. Damcott, M. Fu, H. Shen, P. McArdle, X. Shi, J. Shelton, J. Yin, Y. P. Chang, S. H. Ott, L. Zhang, Y. Zhao, B. D. Mitchell, J. O'Connell, and A. R. Shuldiner.** 2007. Identification of novel candidate genes for type 2 diabetes from a genome-wide association scan in the Old Order Amish:

evidence for replication from diabetes-related quantitative traits and from independent populations. *Diabetes* **56**:3053-3062.

84. **Rinderknecht, E., and R. E. Humbel.** 1978. The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *J Biol Chem* **253**:2769-2776.
85. **RUSSELL, A.** 1954. A syndrome of intra-uterine dwarfism recognizable at birth with cranio-facial dysostosis, disproportionately short arms, and other anomalies (5 examples). *Proc R Soc Med* **47**:1040-1044.
86. **Saltiel, A. R., and J. E. Pessin.** 2002. Insulin signaling pathways in time and space. *Trends Cell Biol* **12**:65-71.
87. **Samani, A. A., S. Yakar, D. LeRoith, and P. Brodt.** 2007. The role of the IGF system in cancer growth and metastasis: overview and recent insights. *Endocr Rev* **28**:20-47.
88. **Schlessinger, J., and M. A. Lemmon.** 2003. SH2 and PTB domains in tyrosine kinase signaling. *Sci STKE* **2003**:RE12.
89. **Shih, S. C., K. E. Sloper-Mould, and L. Hicke.** 2000. Monoubiquitin carries a novel internalization signal that is appended to activated receptors. *EMBO J* **19**:187-198.
90. **Shtiegman, K., and Y. Yarden.** 2003. The role of ubiquitylation in signaling by growth factors: implications to cancer. *Semin Cancer Biol* **13**:29-40.
91. **Siddle, K., M. A. Soos, C. E. Field, and B. T. Nave.** 1994. Hybrid and atypical insulin/insulin-like growth factor I receptors. *Horm Res* **41 Suppl 2**:56-64; discussion 65.

92. **Siddle, K., B. Urso, C. A. Niesler, D. L. Cope, L. Molina, K. H. Surinya, and M. A. Soos.** 2001. Specificity in ligand binding and intracellular signalling by insulin and insulin-like growth factor receptors. *Biochem Soc Trans* **29**:513-525.
93. **SILVER, H. K., W. KIYASU, J. GEORGE, and W. C. DEAMER.** 1953. Syndrome of congenital hemihypertrophy, shortness of stature, and elevated urinary gonadotropins. *Pediatrics* **12**:368-376.
94. **Stein, E., D. P. Cerretti, and T. O. Daniel.** 1996. Ligand activation of ELK receptor tyrosine kinase promotes its association with Grb10 and Grb2 in vascular endothelial cells. *J Biol Chem* **271**:23588-23593.
95. **Stein, E. G., R. Ghirlando, and S. R. Hubbard.** 2003. Structural basis for dimerization of the Grb10 Src homology 2 domain. Implications for ligand specificity. *J Biol Chem* **278**:13257-13264.
96. **Stein, E. G., T. A. Gustafson, and S. R. Hubbard.** 2001. The BPS domain of Grb10 inhibits the catalytic activity of the insulin and IGF1 receptors. *FEBS Lett* **493**:106-111.
97. **Thierry-Mieg, D., and J. Thierry-Mieg.** 2006. AceView: a comprehensive cDNA-supported gene and transcripts annotation. *Genome Biol* **7 Suppl 1**:S12.1-14.
98. **Trinidad, J. C., C. G. Specht, A. Thalhammer, R. Schoepfer, and A. L. Burlingame.** 2006. Comprehensive identification of phosphorylation sites in postsynaptic density preparations. *Mol Cell Proteomics* **5**:914-922.
99. **Van Obberghen, E., V. Baron, L. Delahaye, B. Emanuelli, N. Filippa, S. Giorgetti-Peraldi, P. Lebrun, I. Mothe-Satney, P. Peraldi, S. Rocchi, D. Sawka-**

- Verhelle, S. Tartare-Deckert, and J. Giudicelli.** 2001. Surfing the insulin signaling web. *Eur J Clin Invest* **31**:966-977.
100. **Vecchione, A., A. Marchese, P. Henry, D. Rotin, and A. Morrione.** 2003. The Grb10/Nedd4 complex regulates ligand-induced ubiquitination and stability of the insulin-like growth factor I receptor. *Mol Cell Biol* **23**:3363-3372.
101. **Verlhac, M. H., R. H. Chen, P. Hanachi, J. W. Hershey, and R. Derynck.** 1997. Identification of partners of TIF34, a component of the yeast eIF3 complex, required for cell proliferation and translation initiation. *EMBO J* **16**:6812-6822.
102. **Villen, J., S. A. Beausoleil, S. A. Gerber, and S. P. Gygi.** 2007. Large-scale phosphorylation analysis of mouse liver. *Proc Natl Acad Sci U S A* **104**:1488-1493.
103. **Wang, J., H. Dai, N. Yousaf, M. Moussaif, Y. Deng, A. Boufelliga, O. R. Swamy, M. E. Leone, and H. Riedel.** 1999. Grb10, a positive, stimulatory signaling adapter in platelet-derived growth factor BB-, insulin-like growth factor I-, and insulin-mediated mitogenesis. *Mol Cell Biol* **19**:6217-6228.
104. **Wang, L., B. Balas, C. Y. Christ-Roberts, R. Y. Kim, F. J. Ramos, C. K. Kikani, C. Li, C. Deng, S. Reyna, N. Musi, L. Q. Dong, R. A. DeFronzo, and F. Liu.** 2007. Peripheral disruption of the Grb10 gene enhances insulin signaling and sensitivity in vivo. *Mol Cell Biol* **27**:6497-6505.
105. **White, M. F.** 2003. Insulin signaling in health and disease. *Science* **302**:1710-1711.
106. **Wick, K. R., E. D. Werner, P. Langlais, F. J. Ramos, L. Q. Dong, S. E. Shoelson, and F. Liu.** 2003. Grb10 inhibits insulin-stimulated insulin receptor substrate (IRS)-phosphatidylinositol 3-kinase/Akt signaling pathway by disrupting

- the association of IRS-1/IRS-2 with the insulin receptor. *J Biol Chem* **278**:8460-8467.
107. **Wick, M. J., L. Q. Dong, D. Hu, P. Langlais, and F. Liu.** 2001. Insulin receptor-mediated p62dok tyrosine phosphorylation at residues 362 and 398 plays distinct roles for binding GTPase-activating protein and Nck and is essential for inhibiting insulin-stimulated activation of Ras and Akt. *J Biol Chem* **276**:42843-42850.
 108. **Wiley, H. S., and P. M. Burke.** 2001. Regulation of receptor tyrosine kinase signaling by endocytic trafficking. *Traffic* **2**:12-18.
 109. **Wilson, M. D., C. Riemer, D. W. Martindale, P. Schnupf, A. P. Boright, T. L. Cheung, D. M. Hardy, S. Schwartz, S. W. Scherer, L. C. Tsui, W. Miller, and B. F. Koop.** 2001. Comparative analysis of the gene-dense ACHE/TFR2 region on human chromosome 7q22 with the orthologous region on mouse chromosome 5. *Nucleic Acids Res* **29**:1352-1365.
 110. **Yoshihashi, H., K. Maeyama, R. Kosaki, T. Ogata, M. Tsukahara, Y. Goto, J. Hata, N. Matsuo, R. J. Smith, and K. Kosaki.** 2000. Imprinting of human GRB10 and its mutations in two patients with Russell-Silver syndrome. *Am J Hum Genet* **67**:476-482.
 111. **Zarrinpar, A., R. P. Bhattacharyya, and W. A. Lim.** 2003. The structure and function of proline recognition domains. *Sci STKE* **2003**:RE8.

CHAPTER 2

Mutations in the *GIGYF2* Gene at the PARK11 Locus in Familial Parkinson's Disease

CHAPTER INTRODUCTION

Grb10-interacting GYF domain proteins 1 and 2 (GIGYF1/2) were originally identified by yeast two-hybrid cloning with the Grb10 adapter protein (2). Grb10 functions as a negative regulator of insulin receptor and insulin-like growth factor (IGF-I) receptor signaling. Disruption of the *Grb10* gene in mice results in increased insulin sensitivity and fetal and placental overgrowth indicating that Grb10 plays an important role in insulin and IGF-I system physiology (1, 11, 13). Grb10 has several protein-protein interaction domains and may modulate insulin and IGF-I receptor function through the recruitment of other proteins to receptor signaling complexes. Consistent with this hypothesis, a GYF domain fragment of the GIGYF1 protein becomes transiently linked to the activated IGF-I receptor through association with an N-terminal proline-rich region of Grb10. Overexpression of this Grb10 binding fragment of GIGYF1 results in significant augmentation of IGF-I receptor signaling, suggesting a potential regulatory role for the GIGYF1 protein in IGF-I action (2).

GIGYF1 and GIGYF2 proteins share an overall amino acid sequence homology of approximately 40% but the GYF domains of the two proteins are over 80% identical. Despite this sequence conservation, the GYF domain of GIGYF2 fails to show Grb10 interaction capacity in co-immunoprecipitation studies. When overexpressed, this GYF domain fragment of GIGYF2 localizes strongly to the cell nucleus and therefore, may be sequestered away from Grb10-IGF-I receptor complexes at the plasma membrane (Tsiaras, unpublished and (4)). Complicating matters further, overexpression of full-length GIGYF2 protein results in significant inclusion body formation and cytotoxicity

preventing adequate assessment of Grb10 interaction (2). These technical issues have made it difficult to determine the extent of GIGYF2 involvement in Grb10/IGF-I receptor activity and severely impeded analysis of GIGYF2 function.

As an alternative approach to studying GIGYF2, we examined available human genetic and functional genomic data, with the objective of linking GIGYF2 to specific pathophysiologic processes. The *GIGYF2* gene maps to chromosome 2 at position 2q37.1 and covers approximately 163 kilobases on the direct strand (National Center for Biotechnology Information human genome build 36, March 2006). GIGYF2 mRNA is encoded by 27 exons and is expressed at varying levels in a number of different tissues. One tissue with particularly high expression of GIGYF2 is the human brain (3, 12). GIGYF2 transcripts were initially identified in screens of adult and fetal brain cDNA libraries for clones containing CAG repeats (7). Expansion of DNA regions containing CAG and other trinucleotide repeats is a type of genetic mutation found in a number of neurodegenerative disorders. Interestingly, GIGYF2 mRNA was also isolated from human spinal cord in a screen for genes containing unstable trinucleotide repeat regions as candidates for disorders of motor function (5). Analysis of genome-wide disease linkage studies revealed that the q36-37 region of chromosome 2 shows significant association with Parkinson's disease (PD) a relatively common progressive neurodegenerative disorder (6, 8-10). This region of chromosome 2 has been designated PARK11 to reflect its association with PD. The PARK11 locus is an approximately 18 cM region of 2q36-37, containing 73 potential candidate genes. The highest degree of linkage within this locus was obtained for dinucleotide repeat microsatellite marker D2S206 (logarithm of the odds score, LOD = 5.1). Mapping of the D2S206 sequence

(GenBank accession: Z17274) revealed that it resides within intron 21 of the *GIGYF2* gene.

Together, the genetic and genomic data suggested that *GIGYF2* may play a role in neurodegeneration, and more specifically, in the development of PD. In this chapter, we investigated the potential involvement of the *GIGYF2* gene in PD through genetic analysis of a European population with familial PD. The 27 coding exons of *GIGYF2* were sequenced in patients with PD and corresponding controls. This resulted in the identification of 7 different missense mutations resulting in single amino acid substitutions in the *GIGYF2* sequence. *GIGYF2* mutations were found in close to 5% of the PD patients and not in controls. These data indicate that mutations in the *GIGYF2* gene are a frequent cause of familial PD. Furthermore, this represents the first link between a potential insulin/IGF-I regulatory protein and PD. The results of this study are presented here in the form of a manuscript, which has been published in the American Journal of Human Genetics (doi:10.1016/j.ajhg.2008.01.015)¹. Corinne Lautier is the primary author of the work and performed the genetic screening of the familial PD population. I performed the initial search of available disease linkage data resulting in the identification of chromosome 2q36-37 association with PD, and I mapped the D2S206 marker to intron 21 of the *GIGYF2* gene. I also participated in, and made substantial contributions to all aspects of experimental design, data analysis, and manuscript preparation.

¹ In accordance with the copyright policy of the American Journal of Human Genetics, the manuscript presented here is not the final published version. However, this version is identical in scientific content to the accepted, published form.

REFERENCES

1. **Charalambous, M., F. M. Smith, W. R. Bennett, T. E. Crew, F. Mackenzie, and A. Ward.** 2003. Disruption of the imprinted Grb10 gene leads to disproportionate overgrowth by an Igf2-independent mechanism. *Proc Natl Acad Sci U S A* **100**:8292-8297.
2. **Giovannone, B., E. Lee, L. Laviola, F. Giorgino, K. A. Cleveland, and R. J. Smith.** 2003. Two novel proteins that are linked to insulin-like growth factor (IGF-I) receptors by the Grb10 adapter and modulate IGF-I signaling. *J Biol Chem* **278**:31564-31573.
3. **Ishikawa, K., T. Nagase, M. Suyama, N. Miyajima, A. Tanaka, H. Kotani, N. Nomura, and O. Ohara.** 1998. Prediction of the coding sequences of unidentified human genes. X. The complete sequences of 100 new cDNA clones from brain which can code for large proteins in vitro. *DNA Res* **5**:169-176.
4. **Kofler, M., K. Motzny, and C. Freund.** 2005. GYF domain proteomics reveals interaction sites in known and novel target proteins. *Mol Cell Proteomics* **4**:1797-1811.
5. **Malaspina, A., N. Kaushik, and J. de Belleruche.** 2001. A survey of trinucleotide/tandem repeat-containing transcripts (TNRTs) isolated from human spinal cord to identify genes containing unstable DNA regions as candidates for disorders of motor function. *Brain Res Bull* **56**:299-306.
6. **Maraganore, D. M., M. de Andrade, T. G. Lesnick, K. J. Strain, M. J. Farrer, W. A. Rocca, P. V. Pant, K. A. Frazer, D. R. Cox, and D. G. Ballinger.** 2005.

- High-resolution whole-genome association study of Parkinson disease. *Am J Hum Genet* **77**:685-693.
7. **Margolis, R. L., M. R. Abraham, S. B. Gatchell, S. H. Li, A. S. Kidwai, T. S. Breschel, O. C. Stine, C. Callahan, M. G. McInnis, and C. A. Ross.** 1997. cDNAs with long CAG trinucleotide repeats from human brain. *Hum Genet* **100**:114-122.
 8. **Pankratz, N., W. C. Nichols, S. K. Uniacke, C. Halter, J. Murrell, A. Rudolph, C. W. Shults, P. M. Conneally, and T. Foroud.** 2003. Genome-wide linkage analysis and evidence of gene-by-gene interactions in a sample of 362 multiplex Parkinson disease families. *Hum Mol Genet* **12**:2599-2608.
 9. **Pankratz, N., W. C. Nichols, S. K. Uniacke, C. Halter, A. Rudolph, C. Shults, P. M. Conneally, and T. Foroud.** 2002. Genome screen to identify susceptibility genes for Parkinson disease in a sample without parkin mutations. *Am J Hum Genet* **71**:124-135.
 10. **Pankratz, N., W. C. Nichols, S. K. Uniacke, C. Halter, A. Rudolph, C. Shults, P. M. Conneally, and T. Foroud.** 2003. Significant linkage of Parkinson disease to chromosome 2q36-37. *Am J Hum Genet* **72**:1053-1057.
 11. **Smith, F. M., L. J. Holt, A. S. Garfield, M. Charalambous, F. Koumanov, M. Perry, R. Bazzani, S. A. Sheardown, B. D. Hegarty, R. J. Lyons, G. J. Cooney, R. J. Daly, and A. Ward.** 2007. Mice with a disruption of the imprinted Grb10 gene exhibit altered body composition, glucose homeostasis, and insulin signaling during postnatal life. *Mol Cell Biol* **27**:5871-5886.

12. **Thierry-Mieg, D., and J. Thierry-Mieg.** 2006. AceView: a comprehensive cDNA-supported gene and transcripts annotation. *Genome Biol* **7 Suppl 1**:S12.1-14.
13. **Wang, L., B. Balas, C. Y. Christ-Roberts, R. Y. Kim, F. J. Ramos, C. K. Kikani, C. Li, C. Deng, S. Reyna, N. Musi, L. Q. Dong, R. A. DeFronzo, and F. Liu.** 2007. Peripheral disruption of the Grb10 gene enhances insulin signaling and sensitivity in vivo. *Mol Cell Biol* **27**:6497-6505.

Title: Mutations in the *GIGYF2* (*TNRC15*) gene at the PARK11 locus in familial
Parkinson's disease

Corinne Lautier, Stefano Goldwurm, Alexandra Dürr, Barbara Giovannone,
William G. Tsias, Gianni Pezzoli, Alexis Brice, Robert J. Smith

From Division of Endocrinology, Rhode Island Hospital, Alpert Medical School of
Brown University, Providence, RI, 02903, USA (C.L.; B.G.; W.G.T.; R.J.S.), INSERM,
U679 and Pierre and Marie Curie-Paris6 University, UMR S679, Pitié-Salpêtrière
Hospital, Paris, 75013, France (C.L.; A.D.; A.B.), AP-HP, Pitié-Salpêtrière Hospital,
Department of Genetics and Cytogenetics, Paris, 75013, France (C.L.; A.D.; A.B.), and
Parkinson Institute, Istituti Clinici di Perfezionamento, Milan, 20126, Italy (S.G.; G.P.)

Address for correspondence and reprints: Dr. Robert J. Smith, Division of
Endocrinology, Alpert Medical School of Brown University, Rhode Island Hospital, One
Hoppin Street, Suite 200, Providence, RI, 02903, USA. E-mail: rsmith4@lifelife.org
Phone: 1-401-444-3420

Fax: 1-401-444-4921

ABSTRACT

The genetic basis for association of the PARK11 region of chromosome 2 with familial Parkinson's disease (PD) is unknown. This study examined the *GIGYF2* (Grb10-Interacting GYF Protein-2) (*TNRC15*) gene, which contains the PARK11 microsatellite marker with the highest linkage score (D2S206, LOD 5.14). The 27 coding exons of the *GIGYF2* gene were sequenced in 123 Italian and 126 French Caucasian patients with familial PD, plus 131 Italian and 96 French controls. A total of 7 different *GIGYF2* missense mutations resulting in single amino acid substitutions were present in 12 unrelated PD index patients (4.8%) and not in controls. Three amino acid insertions or deletions were found in four other index patients and absent in controls. These 10 sequence changes were absent from a further 91 controls based on specific exon sequencing. In four families with amino acid substitutions in which at least one other PD case was available, the *GIGYF2* mutations (Asn56Ser, Thr112Ala, and Asp606Glu) segregated with PD. There were, however, two unaffected carriers in one family, suggesting age-dependent or incomplete penetrance. One index case (PD onset age 33) inherited a *GIGYF2* mutation (Ile278Val) from her affected father (PD onset age 66) and a previously described PD-linked mutation in the *LRRK2* gene (Ile1371Val) from her affected mother (PD onset age 61). The earlier onset and severe clinical course in the index patient suggests additive effects of the *GIGYF2* and *LRRK2* mutations. These data strongly support *GIGYF2* as a PARK11 gene with a causal role in familial PD.

INTRODUCTION

Parkinson's disease (PD [MIM 168600]) is a neurodegenerative disorder affecting 1-2 % of the population above age 60.¹ The cause of dopaminergic neuronal loss in the nigro-striatal pathway and consequent bradykinesia, resting tremor, muscular rigidity, and postural instability that characterize PD is largely unknown. Although only 10-30% of PD is familial,² there is strong interest in identifying genes that contribute to familial PD, since these genes are expected to define mechanisms and new therapeutic approaches that apply to more common sporadic forms of the disease.

Family-based whole genome linkage scans have identified 13 chromosomal regions (PARK1 to PARK13) that show linkage to PD.³⁻¹⁴ Candidate gene analysis at these genomic loci linked to PD has identified eight causative genes in PD (*SNCA* [MIM 163890], *PARK2* [*Parkin*] [MIM 602544], *UCHL1* [MIM 191342], *PINK1* [MIM 608309], *PARK7* [*DJI*] [MIM 602533], *LRRK2* [MIM 609007], *ATP13A2* [MIM 610513] and *HTRA2* [MIM 606441]).^{12,15-26} The mechanisms through which these genes contribute to the development of PD have not yet been clearly established. Autosomal dominant transmission is associated with mutations in the *SNCA*, *LRRK2* and, probably, *UCHL1* and *HTRA2* genes, whereas the 4 other genes are autosomal recessive. These genes only account for a small proportion of PD cases, except in specific clinical subgroups or populations. This is the case for the G2019S mutation in the *LRRK2* gene found in up to 18% of Ashkenazi Jews and 39% of North African Arabs with PD, but in only 1 to 3% of their corresponding controls.^{27,28}

The PARK11 locus on chromosome 2q36-37 was initially identified by whole genome linkage analysis in a population of PD patients with at least one first-degree affected relative.^{8,29,30} This linkage region was not confirmed in another PD population.³¹ However, an earlier association analysis,³² which represents a more powerful method for identifying risk factor genes,³³ also revealed significant association between markers in the PARK11 region and PD.

The PARK11 locus corresponds to an 18 cM interval between microsatellite markers D2S396 and D2S338, with LOD scores of 2.9 and 2.4 respectively^{8,29,30} that contains 73 potential candidate genes (Fig. 1). The PARK11 microsatellite marker D2S206, which has the highest LOD score of 5.14, is contained within intron 21 of a 27 exon, 1299 amino acid encoding gene that has been alternatively designated *GIGYF2* (Grb10-Interacting GYF Protein-2)³⁴ or *TNRC15* (Trinucleotide Repeat Containing 15). We previously identified GIGYF2 by yeast two-hybrid screening as one of two homologous proteins, GIGYF1 (or PERQ1) and GIGYF2, interacting through their GYF domain with the Grb10 adapter protein³⁴. Grb10 and the GIGYF proteins are of interest for their potential involvement in insulin-like growth factor (IGF) and insulin signaling.³⁴⁻³⁹ Since the IGFs and insulin have important effects in the central nervous system^{40,41} and are potentially associated with PD,⁴²⁻⁴⁵ we investigated the involvement of the *GIGYF2* (*TNRC15*) gene in PD.

MATERIALS AND METHODS

Study Population

Index cases with familial Parkinson's disease (PD) (n=123) defined by the presence of at least one first-degree affected relative (parent, child or sibling) and healthy controls (n=131) were obtained from an Italian DNA bank assembled by the Parkinson Institute - Istituti Clinici di Perfezionamento, Milan, Italy (Table 1). DNA from a second set of patients with familial PD compatible with dominant transmission (n=126) and controls (n=96) was obtained from the French Parkinson Disease Genetics Study Group (Paris, France) (Table 1). The clinical diagnosis of PD in both populations was established according to widely accepted criteria.^{46,47} This required the presence of bradykinesia and at least one of the following: resting tremor, rigidity and postural instability; a positive response to dopaminergic therapy; the absence of atypical features or other causes of parkinsonism. Control DNAs were from unrelated individuals from the same populations. All controls were free of symptoms suggestive of PD, ≥ 45 years old, and with a negative family history for movement disorders at least in their first-degree relatives. The project was approved by the local ethical authorities, and written informed consent was obtained from all subjects.

The study population was analyzed for the *LRRK2* G2019S mutation. Six of the 123 Italian patients with familial PD (4.8%) carry the G2019S mutation, which was absent from the 131 Italian controls.⁴⁸ In the French population, only 2 of the 126 familial PD patients (1.6%) and 1 of the 96 controls (1.0%) carried the G2019S mutation.^{27,49}

GIGYF2 Gene Sequencing and Mutation Analysis

Genomic primer design for all exons was performed using Vector NTI-Suite 6.0 (InforMax, Invitrogen). Each exon and at least 50 bp of flanking intronic sequence was PCR amplified from genomic DNA with the primer pairs listed in Table 2. PCR amplification was carried out using Taq DNA polymerase (FastStart Taq, 5U/ μ l, Roche) and 25 pmol of forward and reverse primers. PCR was performed in 50 μ l volumes with conditions of 95°C for 2 min followed by 35 cycles of 95°C for 30 sec, annealing temperature for 30 sec (58°C for exons 1,3,4,6,8-10,13-15,17,18,21,23-27; 53°C for exons 2,22; 47°C for exons 5,19; 59°C for exon 7; 56°C for exons 11,12,16; and 61°C for exon 20), and 68°C for 1 min, with a final 68°C hold for 5 min. Exons 9 and 10 plus the intervening intron were small enough to be amplified in a single PCR reaction. The PCR products were purified using Montage PCR₉₆ Plate vacuum filtration (Millipore) and sequenced with either the forward or reverse primer designed for the PCR amplification, except for several with a sequencing primer indicated by “seq” after the primer name in Table 2. Sequencing products were analyzed on an ABI 3730xl or ABI 3700 sequencer using Sequence Analysis 3.3 software (Applied Biosystems). All 27 coding exons of the *GIGYF2* gene were sequenced in the 249 familial PD cases and 227 controls. All missense mutations, deletions, and insertions were subcloned for confirmation. Purified PCR amplification products were vector ligated using the TOPO-TA cloning sequencing kit (Invitrogen). At least six recombinant colonies were selected at random for each variation and sequenced.

After the completion of these analyses, the 8 exons found to contain mutations (exons 2, 4, 8, 9, 11, 14, 25, and 26) were PCR-amplified and sequenced in 91 additional DNA samples obtained from the French Parkinson Disease Genetics Study Group.

Statistical Analysis

Data are presented as mean $\pm$ standard deviation. A database including genetic and phenotypic data for each PD patient and control was constructed in Statview 5.1 (SAS Institute, Cary, NC), and all statistical analyses were performed with this program. Allelic frequency (q) of *GIGYF2* gene variation was expressed as relative frequency in $2n$ chromosomes (with n the number of subjects). Statistical significance was determined by Chi square testing ($P < 0.05$). Hardy-Weinberg equilibrium was considered for Hardy-Weinberg Chi square test < 3.84 .

RESULTS

GIGYF2 was screened for mutations as a candidate gene at the PARK11 locus by complete sequencing of all 27 coding exons in the index cases of families with PD from Caucasian populations (n=249). This included 123 unrelated Italian PD patients and 126 unrelated French PD patients with at least one affected first-degree relative. For comparison sequencing of the 27 coding exons of *GIGYF2* also was performed in 131 Italian and 96 French unrelated controls. The general characteristics of PD index cases and controls are shown in Table 1. Although most patients developed PD after age 45 (n=167), there were also cases with early (n=78) and juvenile onset (n=4). Most patients had only one affected first-degree relative.

We identified 7 different heterozygous amino acid changes in the *GIGYF2* gene in 12 unrelated index cases with PD ($P_{mut}X$, Table 3). None of these 7 *GIGYF2* mutations found in PD patients were observed in 227 controls. They consist of seven single nucleotide changes resulting in amino acid substitutions in exon 2 (Asn56Ser), exon 4 (Thr112Ala), exon 8 (Ile278Val), exon 9 (Ser335Thr), exon 11 (Asn457Thr), exon 14 (Asp606Glu) and exon 26 (Val1242Ile) (Table 3, Fig. 2). The Asn56Ser substitution was found in four unrelated PD patients (one Italian and three French), the Asn457Thr substitution was present in three unrelated PD patients (two Italian and one French). All other amino acid substitutions were observed only in a single PD patient. In contrast, sequencing all coding exons in 227 unrelated control individuals detected a single 71 year old healthy Italian woman without a family history of PD, with an amino acid substitution in exon 25 (His1171Arg, Ctrl_{mut}-1, Table 3, Fig. 2), which was not found in the PD population or other control subjects. The eight exons shown to contain mutations

in the PD patients and the single control (exons 2, 4, 8, 9, 11, 14, 25, 26) then were sequenced in a further 91 control DNA samples from the French Parkinson Disease Genetics Study Group. In this set of additional controls, none of the identified mutations and no new variations were found.

Most of the seven single amino acid substitutions identified in *GIGYF2* in patients represent conservative amino acid sequence changes, but alignment of the human *GIGYF2* protein sequence with 12 other species demonstrates they occur within highly conserved amino acid blocks and involve residues that are highly although not absolutely conserved across species (Fig. 3). In addition, these amino acid substitutions are significantly more frequent in PD patients (12 of 249) than in controls (1 of 227) ($P < 0.003$).

Among the 12 PD probands with *GIGYF2* mutations, 8 had at least one parent affected, one had both parents affected, and 3 had a single affected sib (Fig. 4). PD was present by medical history across three successive generations in 3 of the families and across two generations in 5 of the families (Fig. 4). Among the probands of families with *GIGYF2* missense mutations, there were a total of 6 females and 6 males with the diagnosis of PD, and the occurrence of PD in 2 or 3 successive generations in most families is consistent with autosomal dominant inheritance. DNA samples were available from at least one affected relative in 4 families. In family 068-004, PD was present in three successive generations, and the affected mother of the male proband was shown to have the same Asp606Glu mutation (Fig. 4). The proband was diagnosed with PD at age 42, and his mother at age 83. In family 057-009, the male index case inherited the Asn56Ser mutation from his affected mother. The age of PD onset was 41 in the proband

and 73 in the mother. Among eight siblings of the proband, three were genotyped. Two of these, a dizygotic female twin and a brother, bear the same mutation but do not have clinically apparent PD. Their ages at the time of most recent medical history were 61 and 73 years. Follow-up will be required to determine whether or not they ultimately develop PD. Another unaffected brother, age 67, does not carry the Asn56Ser mutation. In family 28-V-0056 the male index case had an affected brother, and both carried the Thr112Ala mutation. Although both brothers had onset at 43, neither their mother who died at age 72 nor their father at 52 had known PD (Fig. 4).

In the Italian family 1034-D-0385, the female index case inherited the Ile278Val candidate mutation from her father. Sequence analysis of a second PD-associated gene, LRRK2, in this family revealed the previously described LRRK2 Ile1371Val mutation in the index case and her mother.⁵⁰ The father had PD onset at age 66, the mother at age 61, and the index case bearing the two different gene mutations had PD onset at the much earlier age of 31 years. She has had progressive, severe PD with marked fluctuations in clinical course in spite of initial response to L-dopa therapy. At 50 years of age, she currently is partially compensated on intraduodenal L-dopa infusions.

Three other nucleotide variations were found in 4 index cases with PD and none of the controls. They consisted of a deletion of eight (Del LPQQQQQQ 1209-1216) and four codons (Del PPQQ 1221-1224), and an insertion of two codons (Ins QQ 1217) in exon 25 (Table 3). The Del LPQQQQQQ 1209-1216 deletion was detected in two unrelated French PD patients. The InsQQ1217 insertion was associated with the Asn457Thr missense mutation in a patient with onset at age 43. These deletion and insertion sequences are in a region of Gln (Q) and Pro (P) repeats in exon 25 that is not

conserved across species. Furthermore, four other insertions and deletions have been observed in this region, with similar frequencies in PD patients and controls. One was previously described in dbSNP (rs10555297, Table 3) and three are new (Variant-1-2-3, Table 3). We therefore speculate that the rare deletion and insertion sequences identified in PD patients may represent less frequent variants without functional significance.

In addition, we found one amino acid substitution already described in dbSNP (rs2289912, Table 3) as well as 10 non-synonymous SNPs, including four previously described in dbSNP (SNP-rsxxxx, Table 3) and six new SNPs (SNP-X, Table 3). All these variants appear to be polymorphisms based on their similar frequencies in PD cases and controls.

DISCUSSION

Our study has examined *GIGYF2* (*TNRC15*) as a candidate gene for PARK11. This was accomplished by complete sequencing of all 27 coding exons of the *GIGYF2* gene in two different populations of Caucasian patients with familial PD and in population-matched controls (123 Italian PD patients and 131 controls, and 126 French PD patients and 96 controls). We found 7 different missense mutations in the *GIGYF2* gene in 12 index patients with PD from Italy (n=7) and France (n=5). The following evidence supports the pathogenic role of these amino acid changes: i) they are not found in geographically matched controls (a single amino acid change was detected in only 1 of 127 controls) ($P < 0.003$); ii) the 7 PD missense mutations (Asn56Ser, Thr112Ala, Ile278Val, Ser335Thr, Asn457Thr, Asp606Glu, and Val1242Ile) and the control mutation (His171Arg) were absent from an additional 91 controls; iii) they involve amino acids that are relatively conserved across 12 non-human species; iv) when available affected relatives were sampled, they also carried the same missense mutation (except for one of the parents in the case with bilineal transmission of PD). These data provide strong support for a role of mutations in the *GIGYF2* gene as a frequent cause of familial PD, at least in Italy and France, and a major contributing factor at the PARK11 locus. The other sequence variants found in patients are probably not causative, because they affect a non-conserved region containing highly polymorphic repeats rich in Q and P residues. They are more likely rare polymorphisms.

The 12 index cases with PD and *GIGYF2* missense mutations include 6 women and 6 men with a mean age at onset of 48.7 ± 10.2 years (range 33-68). The clinical

features of these patients did not differ from those of typical idiopathic PD (Table 4).

Unilateral tremor and bradykinesia appeared at onset in most of the mutation carriers, all of whom responded to L-dopa and developed dyskinesia and/or motor fluctuations as the disease progressed.

The *GIGYF2* missense mutations are all found in the heterozygous state compatible with autosomal dominant transmission. This inheritance pattern is also supported by the observation of parent-child transmission in 11 of the 12 pedigrees. However, age-dependent penetrance or reduced penetrance is likely. In family 20-V-0056, the parents of the two affected sibs died without being diagnosed with PD and, in family 757-028, two sibs with the mutation are still healthy even though they are older than their affected brother. It will be important to evaluate precisely the penetrance of *GIGYF2* mutations and also determine their frequency in idiopathic PD.

It has been suggested that interactions between multiple genetic factors may have a role in the development of PD.⁵² In the first report on the PARK11 locus, the LOD score of 5.14 for the entire study population was noted to decrease to 4.12 when 11 of 65 families with known *Parkin* mutations were excluded.³⁰ In the present study, we provide further evidence for gene interaction involving the PARK11 locus in an individual with co-occurrence of Ile278Val *GIGYF2* and Ile1371Val *LRRK2* mutations, who had much earlier onset of PD than either parent with single gene mutations. Since *GIGYF2* and *LRRK2* mutations each have been found in approximately 5 and 6% of familial PD patients, respectively, it should be possible to identify additional patients with both mutations to further test their potential interaction. Although the functional role of the *LRRK2* protein is not known, it contains a mixed function kinase-like sequence, and the

most common *LRRK2* mutations (G2019S and Arg1441Cys) enhance the kinase activity.⁵³ Since GIGYF2 initially was identified through its binding to the Grb10 adapter protein,³⁴ and Grb10 is thought to function by linking proteins to tyrosine kinases,³⁶ it will be important in future studies to determine whether GIGYF2 interacts with the *LRRK2* kinase.

The function of the GIGYF2 protein presently is unknown. Consistent with the development of a neurodegenerative disease in humans with GIGYF2 mutations, GIGYF2 mRNA is strongly expressed in multiple regions of the central nervous system (Novartis Gene Expression Atlas, <http://expression.gnf.org>). Together with the homologous GIGYF1 protein, GIGYF2 was cloned from a mouse cDNA expression library by yeast 2-hybrid screening with an N-terminal fragment of the Grb10 adapter protein.³⁴ The Grb10 N-terminus contains a proline-rich region, and both GIGYF proteins contain a GYF motif, which had previously been shown to bind proline-rich sequences.⁵⁴ Using the yeast 2-hybrid method, the GYF domains of GIGYF1 and GIGYF2 were confirmed to interact with the Grb10 proline-rich region (Giovannone et al., 2003). The GYF domain is named for a Gly-Tyr-Phe triad and forms a bulge-helix-bulge structure important for binding to proline sequences.⁵⁴ Based on its flanking amino acid residues, the GYF domain of GIGYF2 can be classified as a member of the SMY2 sub-type.⁵⁵ There is evidence that this class of GYF domains has a preferred pro-rich binding motif that is present in several proteins with roles in mRNA splicing. GYF domain-containing proteins in general are hypothesized to function in mRNA splicing and/or other aspects of mRNA processing, such as nuclear export.

The interaction between the GIGYF proteins and the Grb10 adapter protein evident in the yeast 2-hybrid system has been confirmed in mammalian cells expressing endogenous levels of Grb10 and a myc-tagged GYF domain-containing fragment of the GIGYF1 protein.³⁴ Further studies demonstrated that this fragment of GIGYF1 was recruited to activated IGF-I receptors, presumably via the Grb10 adapter. Of interest, overexpression of the GIGYF1 fragment resulted in augmented IGF-I receptor signaling. Similar studies with full-length GIGYF1 or GIGYF2 have not been possible, since the full-length proteins form toxic inclusion bodies in cells when overexpressed, presumably as a consequence of protein self-aggregation.³⁴ In future experiments at GIGYF2 cellular concentrations below the threshold for inclusion body formation, it will be important to determine whether the association of GIGYF2 with PD involves its interaction with Grb10 and the IGF and/or insulin signaling pathways. It also will be important to determine whether the mutations that we have identified lead to the development of PD by altering GIGYF2 interactions with Grb10 and the IGF/insulin pathways or other functions of the GIGYF2 protein.

ACKNOWLEDGEMENTS

We thank the patients and their relatives for their contribution. The Italian population DNA samples were from the "Human genetic bank of patients affected by Parkinson disease and parkinsonisms" of the Parkinson Institute - Istituti Clinici di Perfezionamento. This DNA bank is supported by the Italian Telethon Foundation (grant n°: GTF04007). We also thank the French Parkinson Disease Genetics Study Group (Y. Agid, A.-M. Bonnet, M. Borg, A. Brice, E. Broussolle, Ph. Damier, A. Destée, A. Dürr, F. Durif, S. Lesage, E. Lohmann, P. Pollak, O. Rascol, F. Tison, C. Tranchant, M. Verin, F. Viallet, and M. Vidailhet) and the DNA and cell bank of the Institut Fédératif des Neurosciences (IFR), 070, CHU Pitié-Salpêtrière, AP-HP, Paris, France for sample preparation. We express gratitude to V. Bonifati and A. Di Fonzo for sharing their LRRK2 data on family 1034-D-0385, and to S. de la Monte and J. Klysik for their contributions to the study project evolution. This work was supported by grants from the Fondation Fertilité et Stérilité, France (C. Lautier), the Association Française des Femmes Diplômées des Universités, France (C. Lautier), the Association France Parkinson, France (A. Brice, C. Lautier), the Agence Nationale de la Recherche (ANR grant n°: ANR-05-NEUR-019; A. Brice), the Hallett Center for Diabetes and Endocrinology, USA (R.J. Smith), and the National Institutes of Health, USA (NIH grant n°: DK43038; R.J. Smith).

REFERENCES

1. Lang AE, Lozano AM (1998) Parkinson's disease. First of two parts. *N Engl J Med* 339:1044-1053
2. Payami H, Zarepari S (1998) Genetic epidemiology of Parkinson's disease. *J Geriatr Psychiatry Neurol* 11:98-106
3. Funayama M, Hasegawa K, Kowa H, Saito M, Tsuji S, Obata F (2002) A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2-q13.1. *Ann Neurol* 51:296-301
4. Gasser T, Muller-Myhsok B, Wszolek ZK, Oehlmann R, Calne DB, Bonifati V, Bereznai B, Fabrizio E, Vieregge P, Horstmann RD (1998) A susceptibility locus for Parkinson's disease maps to chromosome 2p13. *Nat Genet* 18:262-265
5. Hicks AA, Petursson H, Jonsson T, Stefansson H, Johannsdottir HS, Sainz J, Frigge ML, Kong A, Gulcher JR, Stefansson K, Sveinbjornsdottir S (2002) A susceptibility gene for late-onset idiopathic Parkinson's disease. *Ann Neurol* 52:549-555
6. Liu Y, Fallon L, Lashuel HA, Liu Z, Lansbury PTJ (2002) The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility. *Cell* 111:209-218
7. Matsumine H, Saito M, Shimoda-Matsubayashi S, Tanaka H, Ishikawa A, Nakagawa-Hattori Y, Yokochi M, Kobayashi T, Igarashi S, Takano H, Sanpei K, Koike R, Mori H, Kondo T, Mizutani Y, Schaffer AA, Yamamura Y, Nakamura S, Kuzuhara S, Tsuji S, Mizuno Y (1997) Localization of a gene for an autosomal recessive form of juvenile Parkinsonism to chromosome 6q25.2-27. *Am J Hum Genet* 60:588-596

8. Pankratz N, Nichols WC, Uniacke SK, Halter C, Rudolph A, Shults C, Conneally PM, Foroud T (2002) Genome screen to identify susceptibility genes for Parkinson disease in a sample without parkin mutations. *Am J Hum Genet* 71:124-135
9. Polymeropoulos MH, Higgins JJ, Golbe LI, Johnson WG, Ide SE, Di Iorio G, Sanges G, Stenroos ES, Pho LT, Schaffer AA, Lazzarini AM, Nussbaum RL, Duvoisin RC (1996) Mapping of a gene for Parkinson's disease to chromosome 4q21-q23. *Science* 274:1197-1199
10. Schultheis PJ, Hagen TT, O'Toole KK, Tachibana A, Burke CR, McGill DL, Okunade GW, Shull GE (2004) Characterization of the P5 subfamily of P-type transport ATPases in mice. *Biochem Biophys Res Commun* 323:731-738
11. Scott WK, Nance MA, Watts RL, Hubble JP, Koller WC, Lyons K, Pahwa R, et al. (2001) Complete genomic screen in Parkinson disease: evidence for multiple genes. *JAMA* 286:2239-2244
12. Strauss KM, Martins LM, Plun-Favreau H, Marx FP, Kautzmann S, Berg D, Gasser T, Wszolek Z, Muller T, Bornemann A, Wolburg H, Downward J, Riess O, Schulz JB, Kruger R (2005) Loss of function mutations in the gene encoding Omi/HtrA2 in Parkinson's disease. *Hum Mol Genet* 14:2099-2111
13. Valente EM, Bentivoglio AR, Dixon PH, Ferraris A, Ialongo T, Frontali M, Albanese A, Wood NW (2001) Localization of a novel locus for autosomal recessive early-onset parkinsonism, PARK6, on human chromosome 1p35-p36. *Am J Hum Genet* 68:895-900
14. Van Duijn CM, Dekker MC, Bonifati V, Galjaard RJ, Houwing-Duistermaat JJ, Snijders PJ, Testers L, Breedveld GJ, Horstink M, Sandkuijl LA, van Swieten JC,

- Oostra BA, Heutink P (2001) Park7, a novel locus for autosomal recessive early-onset parkinsonism, on chromosome 1p36. *Am J Hum Genet* 69:629-634
15. Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, Dekker MC, Squitieri F, Ibanez P, Joosse M, van Dongen JW, Vanacore N, van Swieten JC, Brice A, Meco G, van Duijn CM, Oostra BA, Heutink P (2003) Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science* 299:256-259
 16. Brice A (2005) Genetics of Parkinson's disease: LRRK2 on the rise. *Brain* 128:2760-2762
 17. Hedrich K, Kann M, Lanthaler AJ, Dalski A, Eskelson C, Landt O, Schwinger E, Vieregge P, Lang AE, Breakefield XO, Ozelius LJ, Pramstaller PP, Klein C (2001) The importance of gene dosage studies: mutational analysis of the parkin gene in early-onset parkinsonism. *Hum Mol Genet* 10:1649-1656
 18. Ibanez P, Bonnet AM, Debarges B, Lohmann E, Tison F, Pollak P, Agid Y, Durr A, Brice A (2004) Causal relation between alpha-synuclein gene duplication and familial Parkinson's disease. *Lancet* 364:1169-1171
 19. Ibanez P, De Michele G, Bonifati V, Lohmann E, Thobois S, Pollak P, Agid Y, Heutink P, Durr A, Brice A (2003) Screening for DJ-1 mutations in early onset autosomal recessive parkinsonism. *Neurology* 61:1429-1431
 20. Ibanez P, Lesage S, Lohmann E, Thobois S, De Michele G, Borg M, Agid Y, Durr A, Brice A (2006) Mutational analysis of the PINK1 gene in early-onset parkinsonism in Europe and North Africa. *Brain* 129:686-694

21. Lesage S, Lohmann E, Tison F, Durif F, Durr A, Brice A (2007) Rare heterozygous parkin variants in French early-onset Parkinson's disease patients and controls. *J Med Genet* Aug 31 [Epub ahead of print]
22. Lincoln S, Vaughan J, Wood N, Baker M, Adamson J, Gwinn-Hardy K, Lynch T, Hardy J, Farrer M (1999) Low frequency of pathogenic mutations in the ubiquitin carboxy-terminal hydrolase gene in familial Parkinson's disease. *Neuroreport* 10:427-429
23. Paisan-Ruiz C, Lang AE, Kawarai T, Sato C, Salehi-Rad S, Fisman GK, Al-Khairallah T, St George-Hyslop P, Singleton A, Rogaeva E (2005) LRRK2 gene in Parkinson disease: mutation analysis and case control association study. *Neurology* 65:696-700
24. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 276:2045-2047
25. Ramirez A, Heimbach A, Grundemann J, Stiller B, Hampshire D, Cid LP, Goebel I, Mubaidin AF, Wriekat AL, Roeper J, Al-Din A, Hillmer AM, Karsak M, Liss B, Woods CG, Behrens MI, Kubisch C (2006) Hereditary parkinsonism with dementia is caused by mutations in ATP13A2, encoding a lysosomal type 5 P-type ATPase. *Nat Genet* 38:1184-1191
26. Valente EM, Abou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, Ali Z, Del Turco D, Bentivoglio AR, Healy DG, Albanese A, Nussbaum R, Gonzalez-

- Maldonado R, Deller T, Salvi S, Cortelli P, Gilks WP, Latchman DS, Harvey RJ, Dallapiccola B, Auburger G, Wood NW (2004) Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* 304:1158-1160
27. Lesage S, Durr A, Tazir M, Lohmann E, Leutenegger AL, Janin S, Pollak P, Brice A (2006) LRRK2 G2019S as a cause of Parkinson's disease in North African Arabs. *N Engl J Med* 354:422-423
 28. Ozelius LJ, Senthil G, Saunders-Pullman R, Ohmann E, Deligtisch A, Tagliati M, Hunt AL, Klein C, Henick B, Hailpern SM, Lipton RB, Soto-Valencia J, Risch N, Bressman SB (2006) LRRK2 G2019S as a cause of Parkinson's disease in Ashkenazi Jews. *N Engl J Med* 354:424-425
 29. Pankratz N, Nichols WC, Uniacke SK, Halter C, Murrell J, Rudolph A, Shults CW, Conneally PM, Foroud T (2003) Genome-wide linkage analysis and evidence of gene-by-gene interactions in a sample of 362 multiplex Parkinson disease families. *Hum Mol Genet* 12:2599-2608
 30. Pankratz N, Nichols WC, Uniacke SK, Halter C, Rudolph A, Shults C, Conneally PM, Foroud T (2003) Significant linkage of Parkinson disease to chromosome 2q36-37. *Am J Hum Genet* 72:1053-1057
 31. Prestel J, Sharma M, Leitner P, Zimprich A, Vaughan JR, Durr A, Bonifati V, De Michele G, Hanagasi HA, Farrer M, Hofer A, Asmus F, Volpe G, Meo G, Brice A, Wood NW, Muller-Myhsok B, Gasser T (2005) PARK11 is not linked with Parkinson's disease in European families. *Eur J Hum Genet* 13:193-197

32. Maraganore DM, de Andrade M, Lesnick TG, Strain KJ, Farrer MJ, Rocca WA, Pant PV, Frazer KA, Cox DR, Ballinger DG (2005) High-resolution whole-genome association study of Parkinson disease. *Am J Hum Genet* 77:685-693
33. Risch N, Merikangas K (1996) The future of genetic studies of complex human diseases. *Science* 273:1516-1517
34. Giovannone B, Lee E, Laviola L, Giorgino F, Cleveland KA, Smith RJ (2003) Two novel proteins that are linked to insulin-like growth factor (IGF-I) receptors by the Grb10 adapter and modulate IGF-I signaling. *J Biol Chem* 278:31564-31573
35. Dufresne AM, Smith RJ (2005) The adapter protein GRB10 is an endogenous negative regulator of insulin-like growth factor signaling. *Endocrinology* 146:4399-4409
36. Holt LJ, Siddle K (2005) Grb10 and Grb14: enigmatic regulators of insulin action--and more? *Biochem J* 388:393-406
37. Langlais P, Dong LQ, Ramos FJ, Hu D, Li Y, Quon MJ, Liu F (2004) Negative regulation of insulin-stimulated mitogen-activated protein kinase signaling by Grb10. *Mol Endocrinol* 18:350-358
38. Laviola L, Giorgino F, Chow JC, Baquero JA, Hansen H, Ooi J, Zhu J, Riedel H, Smith RJ (1997) The adapter protein Grb10 associates preferentially with the insulin receptor as compared with the IGF-I receptor in mouse fibroblasts. *J Clin Invest* 99:830-837
39. Mori K, Giovannone B, Smith RJ (2005) Distinct Grb10 domain requirements for effects on glucose uptake and insulin signaling. *Mol Cell Endocrinol* 230:39-50

40. Russo VC, Gluckman PD, Feldman EL, Werther GA (2005) The insulin-like growth factor system and its pleiotropic functions in brain. *Endocr Rev* 26:916-943
41. Schulingkamp RJ, Pagano TC, Hung D, Raffa RB (2000) Insulin receptors and insulin action in the brain: review and clinical implications. *Neurosci Biobehav Rev* 24:855-872
42. Craft S, Watson GS (2004) Insulin and neurodegenerative disease: shared and specific mechanisms. *Lancet Neurol* 3:169-178
43. Hu G, Jousilahti P, Bidel S, Antikainen R, Tuomilehto J (2007) Type 2 diabetes and the risk of Parkinson's disease. *Diabetes Care* 30:842-847
44. Offen D, Shtail B, Hadad D, Weizman A, Melamed E, Gil-Ad I (2001) Protective effect of insulin-like-growth-factor-1 against dopamine-induced neurotoxicity in human and rodent neuronal cultures: possible implications for Parkinson's disease. *Neurosci Lett* 316:129-132
45. Takahashi M, Yamada T, Tooyama I, Moroo I, Kimura H, Yamamoto T, Okada H (1996) Insulin receptor mRNA in the substantia nigra in Parkinson's disease. *Neurosci Lett* 204:201-204
46. Hughes AJ, Daniel SE, Kilford L, Lees AJ (1992) Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. *J Neurol Neurosurg Psychiatry* 55:181-184
47. Hughes AJ, Daniel SE, Lees AJ (2001) Improved accuracy of clinical diagnosis of Lewy body Parkinson's disease. *Neurology* 57:1497-1499
48. Goldwurm S, Di Fonzo A, Simons EJ, Rohe CF, Zini M, Canesi M, Tesei S, et al. (2005) The G6055A (G2019S) mutation in LRRK2 is frequent in both early and

late onset Parkinson's disease and originates from a common ancestor. *J Med Genet* 42:e65

49. Lesage S, Ibanez P, Lohmann E, Pollak P, Tison F, Tazir M, Leutenegger AL, Guimaraes J, Bonnet AM, Agid Y, Durr A, Brice A (2005) G2019S LRRK2 mutation in French and North African families with Parkinson's disease. *Ann Neurol* 58:784-787
50. Di Fonzo A, Tassorelli C, De Mari M, Chien HF, Ferreira J, Rohe CF, Riboldazzi G, et al. (2006) Comprehensive analysis of the LRRK2 gene in sixty families with Parkinson's disease. *Eur J Hum Genet* 14:322-331
51. Fahn S, Elton, RL, and the UPDRS Development Committee (1987) Unified Parkinson's Disease Rating Scale. In: Fahn S, Marsden CD, Calne D, Goldstein M (eds) *Recent developments in Parkinson's disease*. Vol. 2. Macmillan Healthcare Information, Florham Park, pp 153–163
52. Gasser T (2005) Genetics of Parkinson's disease. *Curr Opin Neurol* 18:363-369
53. West AB, Moore DJ, Biskup S, Bugayenko A, Smith WW, Ross CA, Dawson VL, Dawson TM (2005) Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity. *Proc Natl Acad Sci U S A* 102:16842-16847
54. Freund C, Dotsch V, Nishizawa K, Reinherz EL, Wagner G (1999) The GYF domain is a novel structural fold that is involved in lymphoid signaling through proline-rich sequences. *Nat Struct Biol* 6:656-660
55. Kofler MM, Freund C (2006) The GYF domain. *FEBS J* 273:245-256

FIGURE LEGENDS

Figure 1. Genetic map of the PARK11 locus, extending from markers D2S396 to D2S338. Microsatellite markers enclosed within boxes have been significantly linked to Parkinson's disease.²⁹ Dots indicate markers localized within the respective genes. Gene mapping data are from the National Center for Biotechnology Information (NCBI) website.

Figure 2. Chromatograms illustrating *GIGYF2* gene mutations. All show analysis by forward sequencing, except for the exon 4 (Thr112Ala) mutation, which was sequenced in reverse, and insertion (Ins QQ 1217) and deletions (Del LPQQQQQQ 1209-1216 and Del PPQQ 1221-1224), for which individual alleles were sequenced after subcloning.

Figure 3. Alignment of *GIGYF2* mutation protein sequence regions in multiple species. Overall identity of available amino acid sequences of various species with *Homo sapiens* (gi 42476299) is: *Pan troglodytes* (gi 114583902) 99%, *Macaca mulatta* (gi 109101490) 99%, *Canis familiaris* (gi 73993973) 97%, *Bos taurus* (gi 156120403) 95%, *Equus caballus* (gi 149711637) 95%, *Rattus norvegicus* (gi 109495408) 94%, *Mus musculus* (gi 34365797) 93%, *Gallus gallus* (gi 118094814) 86%, *Monodelphis domestica* (gi 126314299) 86%, *Danio rerio* (gi 71834468) 56%, *Xenopus laevis* (gi 148223517) 56%, and *Ornithorhynchus anatinus* (gi 149599305) 39%. The dotted lines correspond to sequence regions not yet available or sequence regions of known sequence but non-homology.

Figure 4. Pedigrees of patients with *GIGYF2* missense mutations. Arrows indicate the index cases. Patients with PD are in black, with current age or age at death indicated if known. Age of onset is the number adjacent to PD. Numbers under other family members correspond to the best available estimate of current age or the age at death based on information provided by the index cases. Asterisks indicate individuals from whom DNA was obtained. When information on mutation segregation was available, the genotypes are indicated in the box below the pedigree.

Table 1. Population phenotype descriptions. The L-dopa response was considered positive when at least 30% improvement was observed based on clinical assessment.

	Italian population		French population	
	PD n=123	Control n=131	PD n=126	Control n=96
Parkinson age of onset, Control age of study entry (Mean $\pm$ SD)	53 $\pm$ 12	69 $\pm$ 9	48 $\pm$ 12	64 $\pm$ 9
Young onset $\leq$ 21 years	0		4	
Early onset $\leq$ 45 years	28		50	
Late onset $>$ 45 years	95		72	
At least one first-degree relative affected (%)	100		100	
Only father affected (%)	25.6		33.9	
Only mother affected (%)	24.0		32.2	
Both parents affected (%)	0.8		0.9	
Only siblings affected (%)	44.6		1.7	
Only progeny affected (%)	0		6.0	
At least one parent and one sibling affected (%)	5.0		18.3	
At least one parent and one progeny affected (%)	0		4.4	
At least one parent, one sibling and one progeny affected (%)	0		1.7	
At least one sibling and one progeny affected (%)	0		0.9	
Consanguinity (%)	8	ND	0	ND
Sex ratio (F/M)	54/69	75/56	63/63	61/35
Resting tremor (%)	80	0	78.3	0
Bradykinesia (%)	99	0	93.0	0
Rigidity (%)	78	0	88.9	0
Dementia (%)	5.9	0	11.7	0
Disease duration (mean year $\pm$ SD)	14.0 $\pm$ 6.4	0	10.2 $\pm$ 9.4	0
L-Dopa response (%)	98	0	94	0
L-Dopa treatment duration (mean year $\pm$ SD)	11.5 $\pm$ 6.6	0	8.25 $\pm$ 6.25	0

Table 2. *GIGYF2* gene primers used for PCR and sequencing analysis.

Primer Code	Sequence	PCR	Sequencing	Primer Size (bp)	PCR Size (bp)
GG2-E1F	GTCTGTTCTACCTCTCTCACT	PCR		22	485 (E1F-E1R)
GG2-E1F seq	ACAGGTTTCTTCACATA		SEQ	17	
GG2-E1R	GTTTTCAGCGAGGCACATAA	PCR	SEQ	20	
GG2-E2F	CTGATACTTTGAGATAG	PCR	SEQ	17	643 (E2F-E2R)
GG2-E2R	GCTCCTCTGTTGGCTCTTT	PCR	SEQ	20	
GG2-E3F	TGCTAAGGACACATACAGTTGC	PCR	SEQ	22	735(E3F-E3R)
GG2-E3R	TGATTTTCTCCCTCCCCAA	PCR	SEQ	20	
GG2-E4F	GTGTTATTAGATGGTTGCT	PCR	SEQ	20	728 (E4F-E4R)
GG2-E4R	AAGTTCTCTATCCTCCTCCT	PCR	SEQ	20	
GG2-E5F	ATTTCATATTCAACTGT	PCR		18	910 (E5F-E5R)
GG2-E5F seq	TTTATGGTGGTGAATGTGTGC		SEQ	21	
GG2-E5R	CAAAAACACCTCTAATGCTCA	PCR		21	
GG2-E5R seq	GATGTTCAATACTTA		SEQ	17	
GG2-E6F	TAGAAAAATGGGAAAGTGAGTGTC	PCR	SEQ	24	807(E6F-E6R)
GG2-E6R	TGGCATTATTTTCTCCTCAG	PCR		20	
GG2-E6R seq	CTGATACAAAAGCAAAGAT		SEQ	19	
GG2-E7F	GGCGTTGTGTGTGGATGAGG	PCR	SEQ	23	625 (E7F-E7R)
GG2-E7R	CTTCCCTTAITTCAGTCC	PCR	SEQ	19	
GG2-E8F	CTTTAGAGTATGTTGCCTGG	PCR	SEQ	20	662 (E8F-E8R)
GG2-E8R	TCTTATCGGGTCTTTGGC	PCR	SEQ	19	
GG2-E9-10F	TTAGAGGATGCTGAAGAAG	PCR	SEQ	19	678 (E9-10F - E9-10R)
GG2-E9-10R	GCATAAGCAGAAGCCTAACT	PCR	SEQ	20	
GG2-E11F	GTAGTTAGCCTTCACAGA	PCR	SEQ	18	296 (E11F-E11R)
GG2-E11R	TAAGAGGAGACAAAAAGAAAT	PCR	SEQ	20	
GG2-E12F	ATACCATTTAGAGTTGACCA	PCR		20	873 (E12F-E12R)
GG2-E12F seq	TGGCATTGTTGGGAGAAGTTAC		SEQ	22	
GG2-E12R	GGGATGCTCAACTGGTATGT	PCR	SEQ	20	
GG2-E13F	TTGACAGATGCCACCTCG	PCR	SEQ	18	321 (E13F-E13R)
GG2-E13R	GTGTTCCGTGACTAATA	PCR	SEQ	18	
GG2-E14F	GATGCTGAGTTTTTATTTC	PCR	SEQ	19	325 (E14F-E14R)
GG2-E14R	AGGTGAGACTTGAGAGCAGA	PCR	SEQ	20	
GG2-E15F	CTGAATAGACCATTTCG	PCR		18	557 (E15F-E15R)
GG2-E15F seq	TTTCTAATAATGTCCTGGG		SEQ	21	
GG2-E15R	CGCTTATCTCCACCTGAAAT	PCR	SEQ	20	
GG2-E16F	CAATAACCTGGGAGAACCTGC	PCR		21	443 (E16F-E16R)
GG2-E16F seq	GCAATGACAAGACAAG		SEQ	18	
GG2-E16R	ATAAAAGAGATGCTGACT	PCR	SEQ	18	
GG2-E17F	GCTCATTTAGGCACAGGGA	PCR	SEQ	19	634 (E17F-E17R)
GG2-E17R	CGGGCTTTTGGCTGCTACG	PCR		20	
GG2-E17R seq	AGCAATGTTATGTCCTG		SEQ	18	
GG2-E18F	GAGTGTCTCTGCTTGGTT	PCR	SEQ	20	239 (E18F-E18R)
GG2-E18R	GGTCAGAGTTCAGATACA	PCR	SEQ	20	
GG2-E19F	ATACTTTTACTCCAGAT	PCR	SEQ	18	538 (E19F-E19R)
GG2-E19R	TGAGCAAAAACAGAAAGAT	PCR	SEQ	20	
GG2-E20F	GCTTTCTGTCTATCTCTAT	PCR	SEQ	20	516 (E20F-E20R)
GG2-E20R	CTGGGTGATGGGAGTGAGAC	PCR	SEQ	20	
GG2-E21F	TGGAGTTGAGAGAAATGC	PCR	SEQ	18	308 (E21F-E21R)
GG2-E21R	GTAACCACTAACCCATAAT	PCR	SEQ	19	
GG2-E22F	TATCTAAAAACAGGAATC	PCR	SEQ	18	329 (E22F-E22R)
GG2-E22R	TAAAAAGATAAGAAGACAGT	PCR	SEQ	20	
GG2-E23F	CGCCAGCAGAGGGAGTTGAT	PCR		20	550 (E23F-E23R)
GG2-E23F seq	AGTATTATCTTTCTGAC		SEQ	18	
GG2-E23R	CATAGGTGTGGTCATTTTG	PCR	SEQ	19	
GG2-E24F	CATCCATCCAGATTGCCA	PCR	SEQ	19	305 (E24F-E24R)
GG2-E24R	GTCTGTCTGTCTATCCAC	PCR	SEQ	20	
GG2-E25F	ATGTGGTATGGATGTTGTGATTAT	PCR		24	616 (E25F-E25R)
GG2-E25F seq	TTACCAAGTGTCTGTCAATTA		SEQ	20	
GG2-E25R	TGGGAGATACACAAAGGT	PCR		18	
GG2-E25R seq	CATTTCTGGGTACTCA		SEQ	18	
GG2-E26F	CTGAGTGGGATGTCTTGGTCTA	PCR		22	395 (E26F-E26R)
GG2-E26F seq	AGGGTGAGCCAAGTCTGAATCTGC		SEQ	24	
GG2-E26R	GCACTCTCCAACACTCA	PCR	SEQ	18	
GG2-E27F	ACAGACTTACATTCCAGC	PCR	SEQ	18	246 (E27F-E27R)
GG2-E27R	AAGAGTAAATGGTGAGTAAGTGT	PCR	SEQ	23	

Table 3. *GIGYF2* mutations and gene sequence variation identified in PD and control populations.

Exon	Variation Reference	Nucleotide Change	Amino Acid Change	Distance (bp)	Major Alleles	Allele Frequency (q)		
						PD 498 Chr.	Control 454 Chr.	p Value
GIGYF2 Mutations								
Ex2	PD _{mut} -1	c.167A → G	Asn56Ser (AAC-AGC)	16776	A	0.008	0.000	0.056
Ex4	PD _{mut} -2	c.334A → G	Thr112Ala (ACA-GCA)	25326	A	0.002	0.000	0.339
Ex8	PD _{mut} -3	c.832A → G	Ile278Val (ATA-GTA)	59866	A	0.002	0.000	0.339
Ex9	PD _{mut} -4	c.1003T → A	Ser335Thr (TCT-ACT)	60129	T	0.002	0.000	0.339
Ex11	PD _{mut} -5	c.1370A → C	Asn457Thr (AAT-ACT)	63886	A	0.006	0.000	0.098
Ex14	PD _{mut} -6	c.1818C → G	Asp606Glu (GAC-GAG)	78790	C	0.002	0.000	0.339
Ex25	PD _{mut} -7	c.3625_3648del24	Del LPQQQQQQ 1209-1216	116588		0.004	0.000	0.184
Ex25	PD _{mut} -8	c.3648_3649insCAACAG	Ins QQ 1217	116611		0.002	0.000	0.357
Ex25	PD _{mut} -9	c.3661_3672del12	Del PPQQ 1221-1224	116624		0.002	0.000	0.357
Ex26	PD _{mut} -10	c.3724G → A	Val1242Ile (GTA-ATA)	119378	G	0.002	0.000	0.339
Ex25	Ctrl _{mut} -1	C.3512A → G	His1171Arg (CAT-CGT)	116475	A	0.000	0.002	0.286
GIGYF2 Variants								
Ex11	Variant-rs2289912	c.1378C → A	Pro469Thr (CCT-ACT)	63894	C	0.018	0.020	0.843
Ex25	Variant-1	c.3613_3633del21	Del QQQQLPQ 1205-1211	116576		0.056	0.057	0.924
Ex25	Variant-rs10555297	c.3630_3632del3	Del Q 1210	116593	DelACA	0.464	0.457	0.843
Ex25	Variant-2	c.3633_3634insCAG	Ins Q 1212	116596		0.016	0.021	0.555
Ex25	Variant-3	c.3673_3684del12	Del PPQQ 1225-1228	116636		0.018	0.007	0.144
GIGYF2 SNPs								
Ex2	SNP-1	c.69T → C	Ser23 (AGT-AGC)	16678	T	0.002	0.002	0.948
Ex8	SNP-2	c.828G → C	Gly276 (GGG-GGC)	59862	G	0.002	0.002	0.948
Ex9	SNP-3	c.1047T → C	Asp349 (GAT-GAC)	60173	T	0.002	0.000	0.407
Ex12	SNP-rs2305138	c.1554G → A	Glu518 (GAG-GAA)	65187	G	0.054	0.035	0.159
Ex13	SNP-4	c.1716G → T	Ala572 (GCG-GCT)	75626	G	0.006	0.000	0.150
Ex21	SNP-5	c.2835G → T	Ser945 (TCG-TCT)	108989	G	0.004	0.000	0.241
Ex22	SNP-rs3816334	c.2940G → A	Gln980 (CAG-CAA)	113169	G	0.281	0.319	0.198
Ex25	SNP-rs7563724	c.3630A → G	Pro1210 (CCA-CCG)	116593	A	0.014	0.021	0.445
Ex25	SNP-rs12328151	c.3651G → A	Pro1217 (CCG-CCA)	116614	G	0.227	0.225	0.948
Ex27	SNP-6	c.3855G → A	Ser1285 (TCG-TCA)	125899	G	0.006	0.009	0.615

Table 4. Phenotype of patients with GIGYF2 mutations.

Patient Number	677-0-0092	057-009	329-013	757-028	28-V-0056	1034-D-0385	283-Z-0103	102-C-0274	489-C-0532	397-011	068-004	848-Z-0079
GIGYF2 Mutation	Asn56Ser				Thr112Asp	Ile278Val	Ser335Thr	Asn457Thr			Asp606Glu	Val1242Ile
Sex (M/F)	M	M	F	M	M	F	F	F	F	M	M	F
Origin (ITA/FR)	ITA	FR	FR	FR	ITA	ITA	ITA	ITA	ITA	FR	FR	ITA
Age of Onset (yr)	64	41	48	49	43	33	59	50	44	43	42	68
Disease Duration at Examination (yr)	7	9	10	15	14	19	15	25	8	25	15	17
Resting Tremor	—	ND	+	—	+	+	+	+	+	+	+	+
Bradykinesia	+	ND	+	+	+	+	+	+	+	+	+	+
Rigidity	+	ND	+	+	+	+	+	+	+	+	+	+
Dementia	—	ND	—	—	—	—	—	—	—	—	—	—
L-Dopa Response	+	ND	+	+	+	+	+	+	+	+	+	+
L-Dopa Duration (yr)	7	ND	8	10	10	15	13	21	3	25	7	17
UPDRS (ON)	14	ND	ND	9	19	24	22	26	13	35	9	18
UPDRS (OFF)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	16	37
H & Y (ON)	2.5	ND	ND	1.5	2.5	3	3	3	2	2.5	2.5	2.5
H & Y (OFF)	ND	ND	ND	ND	ND	4	3	ND	ND	ND	ND	3

(ND) not determined, (–) phenotype absent, (+) phenotype present, (UPDRS) Unified Parkinson's Disease Rating Scale,⁵¹ (H & Y) Modified Hoehn and Yahr staging⁵¹

Figure 1

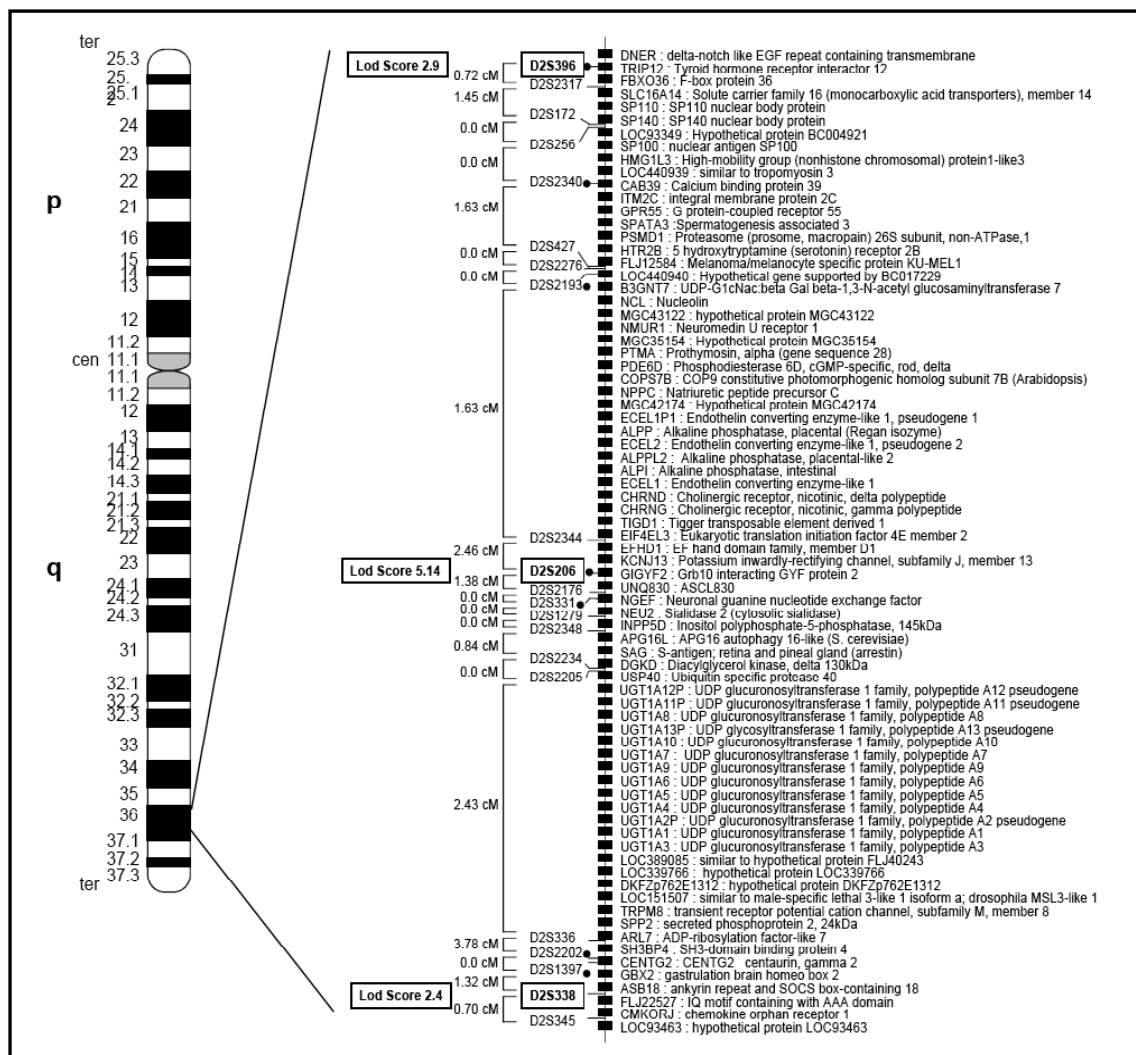


Figure 2

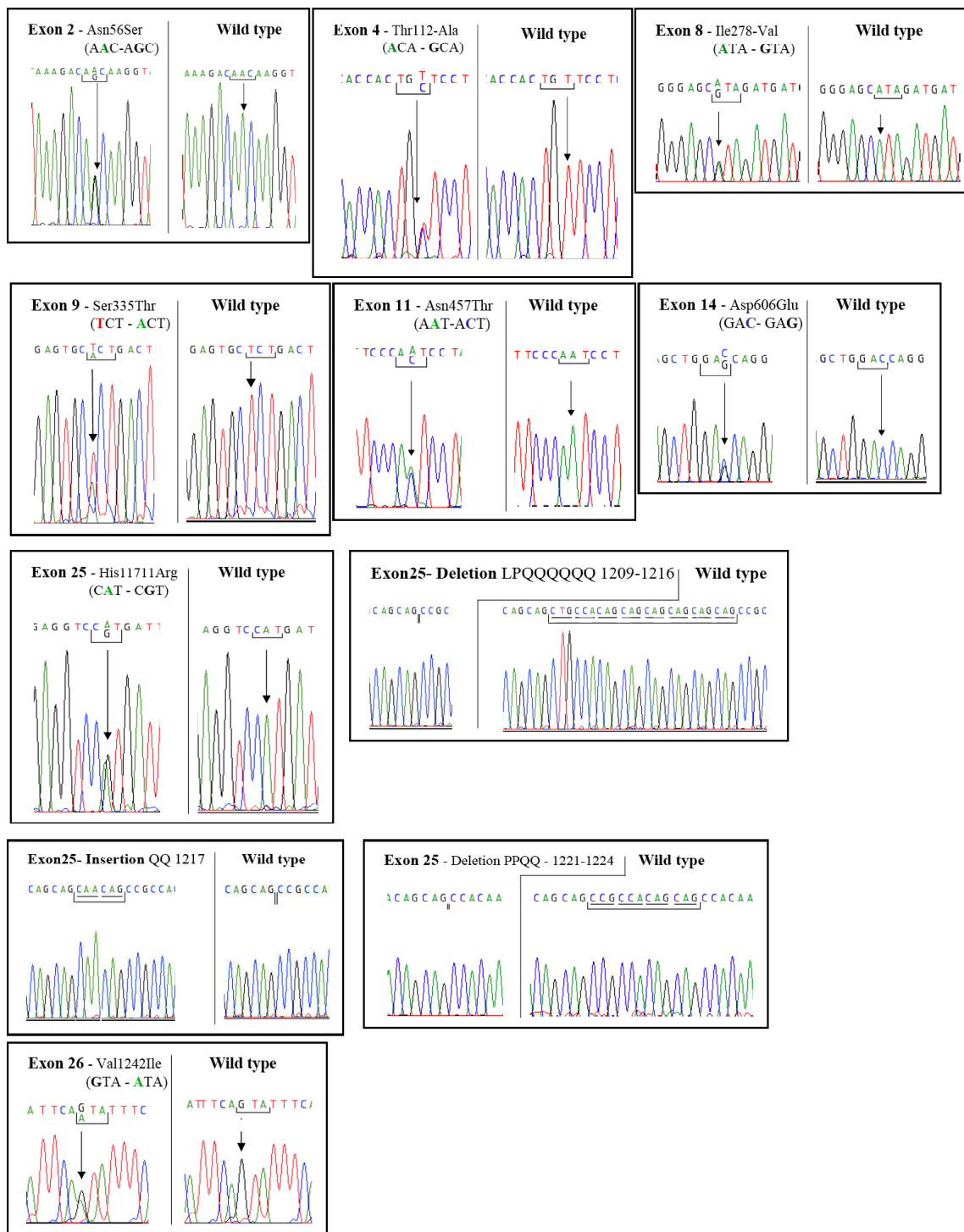
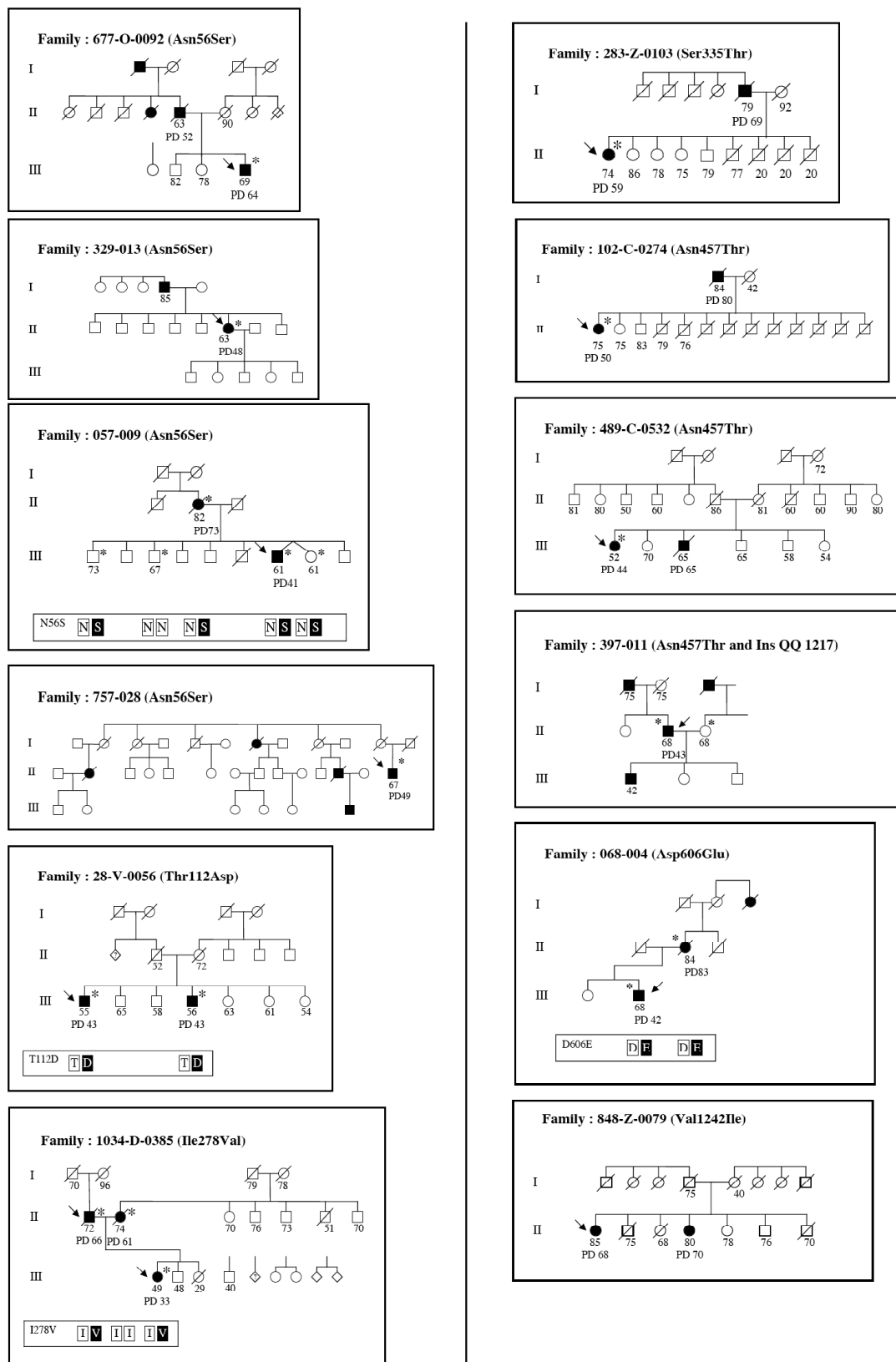


Figure 3

	Asn56Ser	Thr112Asp	Ile278Val	Ser335Thr	Asn457Thr	Asp606Glu	His1171Arg	Val1242Ile
Homo sapiens	LFLKDNKIPS	GGGTVVGAPR	SGSGIDDDRD	GEECSDSEGS	PPVNPNSPTL	PHMGELDQER	ESPYEVHDIYI	LHSVFQTN
Pan troglodytes	LFLKDNKIPS	GGGTVVGAPR	SGSGIDDDRD	GEECSDSEGS	PPVNPNSPTL	PHMGELDQER	ESPYEVHDIYI	LHSVFQTN
Macaca mullata	LFLKDNKIPS	GGGTVVGAPR	SGSGIDDDRD	GEECSDSEGS	PPVNPNSPTL	PHMGELDQER	ESPYEVHDIYI	LHSVFQTN
Canis familiaris	LFLKDNKIPS	GGGTVVGAPR	SGSGIDDDRD	GEECSDSESS	PPVNPNSPAL	PHMGELDQER	ESPYEVHDIYI	LHSVFQTN
Bos taurus	LFLKDNKIPS	GGGTVVGAPR	SGSGIDDDRD	GEECSDSEGS	PPVPSPSPAL	PHMGELDQER	ESPYEVHDIYI	LHSVFQTN
Equus caballus	LFLKDNKIPS	GGGTVVGAPR	SGSGIDDDRD	GEECSDSESS	PPVPSPSPAL	PHMGELDQER	ESPYEVHDIYI	LHSVFQTN
Rattus norvegicus	LFLKDYKIPF	GGGTVVGAPR	SGSGIDDDRD	GEERSDSDSS	PPLPTPSAS	PHMGELDQER	ESPYEVHDIYT	LHSVFQTN
Mus musculus	LFLKDYKIPF	GGGTVVGAPR	SGSGIDDDRD	GEERSDSDSS	PPVPTPSAS	PHMGELDOER	ESPYEVHDIYT	LHSVFQTN
Gallus gallus	LFLKDNKIPS	GG-TVVGAPR	SGSGMDDRD	GEERSDSEGS	PPVNSNPAL	PHLGELDQER	ESPYEVHDIYI	LHSVFQTN
Monodelphis domestica	LFLKDNKIPS	GGGTAVGAPR	SGSGIDDDRD	GEERSDSEGS	PPVASAGPAL	PHLGELDQER	ESPYEVHDIYI	LHSVFQTN
Danio rerio	LYVKDNMIPV	GGGTAVGAPR	SGSGAEERD	-----	-----	PILGDADQER	DSPYEVHDIYV	-----
Xenopus laevis	LFLKDNKVPS	GGGTVVGAPR	SGSGAEERD	GADLSDSDGS	-----	PRLGDLDPER	ESPYDVHDIYI	-----
Ornithorhynchus anatinus	LFLKDNKIPS	GGGTVVGAPR	-----	-----	-----	-----	ESPYEVHDIYI	LHSVFQTN

Figure 4



CHAPTER 3

Partial Loss of the *GIGYF2* (PARK11 Region) Parkinson's Disease Gene in Mice Results in Motor Dysfunction and Neurodegeneration

CHAPTER INTRODUCTION

GIGYF2 is one of two homologous GYF domain-containing proteins that may play a role in Grb10-mediated regulation of insulin receptor (IR) and insulin-like growth factor receptor (IGF-IR) signaling. In chapter 2, we used a genetic approach to assess the potential biological significance of the GIGYF2 protein. This revealed significant linkage of the *GIGYF2* gene region (PARK11 locus) to familial Parkinson's disease (PD). Sequencing of the *GIGYF2* gene in a European population with familial PD identified mutations in *GIGYF2* in close to 5% of affected individuals (1). These data suggest that mutation of the *GIGYF2* gene is a potentially frequent cause of familial PD.

Linkage of GIGYF2 to a human neurodegenerative disease indicates that this protein plays an important physiologic role in the central nervous system. Although significant, these results do not elucidate potential actions of GIGYF2 in other tissues, nor do they address the role GIGYF2 plays in IR/IGF-IR regulation. GIGYF2 is highly expressed in a number of different tissues, and it appears to be expressed throughout development, from the blastocyst stage into adulthood (2, 3). This suggests a potentially fundamental role for GIGYF2 in cellular function, organism growth and development.

In this chapter, we sought to address some of these questions, and further investigate the causal role of GIGYF2 in neurodegeneration through disruption of the mouse *Gigyf2* gene. Analysis of *Gigyf2* null mice revealed significant perinatal lethality indicating a critical function for GIGYF2 in facilitating survival of the organism beyond the first day of life. *Gigyf2* heterozygous mice appear to develop normally and survive into adulthood. When assessed after 12 months of age, a significant percentage of

Gigyf2^{+/-} animals exhibit motor ataxia. The brain and spinal cord of *Gigyf2*^{+/-} mice show histologic evidence of diffuse neuronal degeneration, intracytoplasmic inclusions and α -synuclein positive neuritic plaques. In addition to animal based studies, IGF-IR function was assessed in embryonic fibroblasts derived from *Gigyf2*^{-/-} mice. Compared to littermate matched wild type controls, GIGYF2 deficient cells show decreased IGF-I stimulated IGF-IR tyrosine phosphorylation, increased ERK1/2 activation and a trend towards increased Akt activation. Together these results support a causal role for GIGYF2 in the development or progression of human PD and provide functional evidence of GIGYF2 involvement in IGF-IR regulation.

The results are presented here in the form of a manuscript. Barbara Giovannone produced the *Gigyf2* gene disrupted mice, maintained the colony and generated data for the manuscript. I assisted in mouse colony husbandry and maintenance, and participated in generating much of the experimental data. I am solely responsible for the data in a portion of figure 1 and all of the data in figure 7. In addition, I contributed to all aspects of experimental design, and data analysis, and assisted in writing and preparation of the manuscript.

REFERENCES

1. **Lautier, C., S. Goldwurm, A. Durr, B. Giovannone, W. G. Tsiaras, G. Pezzoli, A. Brice, and R. J. Smith.** 2008. Mutations in the GIGYF2 (TNRC15) Gene at the PARK11 Locus in Familial Parkinson Disease. *Am J Hum Genet*
doi:10.1016/j.ajhg.2008.01.015
2. **Thierry-Mieg, D., and J. Thierry-Mieg.** 2006. AceView: a comprehensive cDNA-supported gene and transcripts annotation. *Genome Biol* **7 Suppl 1**:S12.1-14.
3. **Van Hoof, D., R. Passier, D. Ward-Van Oostwaard, M. W. Pinkse, A. J. Heck, C. L. Mummery, and J. Krijgsveld.** 2006. A quest for human and mouse embryonic stem cell-specific proteins. *Mol Cell Proteomics* **5**:1261-1273.

Title: Partial Loss of the *GIGYF2* (PARK11 Region) Parkinson's Disease Gene in Mice Results in Motor Dysfunction and Neurodegeneration

Barbara Giovannone,¹ William Tsiaras,¹ Suzanne de la Monte,² Jan Klysik,³ Corinne Lautier,^{1,4} Stefano Goldwurm,⁵ and Robert J. Smith^{1*}

¹Division of Endocrinology, Rhode Island Hospital, Warren Alpert Medical School of Brown University, One Hoppin Street, Suite 200, Providence, RI 02903. ²Liver Research Center, Rhode Island Hospital, Warren Alpert Medical School of Brown University, Providence, RI 02903. ³Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, RI. ⁴INSERM U679, Neurology and Experimental Therapeutics, Centre Hospitalier Universitaire Pitie-Salpetriere, Paris, France. ⁵Parkinson Institute, Istituti Clinici di Perfezionamento, Milan, Italy

* To whom correspondence should be addressed. E-mail: rsmith4@lifespan.org

ABSTRACT

Mutations in the *GIGYF2* gene recently were described in approximately 5% of familial Parkinson's disease patients. *GIGYF2* was initially identified through its interaction with Grb10, an adapter protein that binds activated IGF-I and insulin receptors. This study investigated mice with *GIGYF2* gene disruption. *GIGYF2* null mice have early postnatal lethality. Heterozygous *GIGYF2* +/- mice develop normally, but exhibit motor ataxia starting at 12 months of age. The motor impairments are associated with degeneration and rare intracytoplasmic Lewy body-like inclusions in lumbar spinal anterior horns, and α -synuclein positive neuritic plaques in brainstem and cerebellum. Cultured embryo fibroblasts from *GIGYF2* null mice exhibit decreased IGF-I stimulation of IGF-I receptor tyrosine phosphorylation and augmented ERK1/2 phosphorylation. These findings support a causal role for *GIGYF2* in human Parkinson's disease and likely loss of function from the identified *GIGYF2* mutations. *GIGYF2* represents the first Parkinson's disease gene with a functional link to IGF pathway signaling.

INTRODUCTION

Parkinson's disease is the second most common human neurodegenerative disorder, affecting 1 to 2 percent of the population above age 60 and an estimated 5 percent of people above age 85 (1). Its characteristic clinical features of bradykinesia, resting tremor, rigidity, and postural instability (2,3) result from the loss of dopaminergic neurons in the substantia nigra and other regions of the ventral midbrain (4). Neuronal loss often extends beyond these sites to other regions of the central nervous system, and this may explain the frequent occurrence of non-motor system symptoms in Parkinson's disease, including dementia, sleep disturbances, and depression (5). The neurodegeneration typically is progressive, resulting in substantial morbidity and mortality. Current therapies for Parkinson's disease, e.g., treatment with the dopamine precursor L-Dopa, are targeted to the relief of symptoms and do not prevent or reverse neuronal loss. A better understanding of the underlying molecular basis of the disorder can be expected to define strategies for more effective prevention and treatment.

An estimated 10 to 30 percent of Parkinson's disease occurs in families with multiple affected individuals, and family history is second only to age as a predisposing factor (6). It is thought that knowledge of the genes responsible for familial Parkinson's is likely to define molecular pathways with important roles not only in inherited forms of the disease, but also in the more common, sporadic forms. A total of 13 genes thus far have been associated with Parkinson's disease. Among the extensively studied genes, only *LRRK2* has been shown to contribute to a substantial portion of familial Parkinson's disease. The prevalence of *LRRK2* mutations varies markedly in different populations,

but there overall appear to be coding sequence changing mutations in approximately 5 percent of patients with familial Parkinson's and 1 to 2 percent of patients with sporadic disease (7). Mutations in five other genes have been demonstrated in familial Parkinson's disease in multiple populations or families (*SNCA*, *PARK2* [*Parkin*], *PINK1*, *PARK7* [*DJI*], and *ATP13A2*), but these occur at very low frequency and, thus, appear to be only rare causes of Parkinson's disease (8-12). Another five genes have been identified thus far only in single families (*UCHL1*, *Omi/HtrA2*, *Synphylin-1*, *NR4A2/Nurr1*, and *POLG*), and their relationship to Parkinson's disease will require confirmation (13-17). The most recently identified Parkinson's associated gene, *GIGYF2* (Grb10-Interacting GYF Protein 2), was reported by our group to have mutations in 4.8 percent of affected patients in distinct Italian and French familial Parkinson's disease populations (18). *GIGYF2* thus represents, together with *LRRK2*, one of two relatively common genetic factors in familial Parkinson's disease. The *GIGYF2* gene is located within a Parkinson's disease linkage region on chromosome 2q37 designated PARK11 (*GIGYF2* microsatellite D2S206 LOD 5.14) (19) and thus may explain the previously described linkage of this locus to Parkinson's disease.

GIGYF2 was initially identified in our laboratory, together with a homologous protein designated *GIGYF1*, through yeast two-hybrid screening for proteins that bind to the Grb10 adapter protein (20). Grb10 interacts with intracellular domains of activated insulin and IGF-I receptors and negatively regulates insulin and IGF signaling (21,22). As a protein that may be complexed to receptors via the adapter function of Grb10, *GIGYF2* is of interest as a potential mediator or regulator of hormone signaling. This may have relevance to Parkinson's disease, given that the IGFs and insulin have

important actions in the central nervous system and potential association with Parkinson's disease (23-27). The goal of the present study was to use a gene disruption strategy in mice to further investigate the causal role of the GIGYF2 protein in neurodegeneration and Parkinson's disease.

MATERIALS AND METHODS

Generation of KO mice

Gigyf2 gene-trap 129Ola ES cells (XH076) were obtained from Bay Genomics (UC San Francisco), expanded, and injected into C57BL/6-Tyr^{c-Brd} blastocysts. The injected blastocysts were transferred into the uteri of pseudopregnant foster mothers to generate chimeras. The male chimeras were mated with C57BL/6-Tyr^{c-Brd} females, and the *Gigyf2* line was subsequently maintained on a mixed C57BL/6-Tyr^{c-Brd}/129Ola background. All breeding and animal procedures were carried out according to institutional regulations of the Rhode Island Hospital animal facility (IACUC protocol #0221-06).

5'-RACE PCR performed originally by Bay Genomics indicated that the gene-trap integration site was between exons 4 and 5 of the *Gigyf2* gene (an approximately 5kb region). Several primers covering intron 4 were used independently with 6 primers in the trapping vector to amplify the flanking sequences of the integration site. The PCR products were subcloned into the TA vector (Invitrogen, Carlsbad, CA), and the precise integration site was defined by DNA sequencing. Animals were genotyped by PCR using primers that amplified a 290 bp fragment from the disrupted intron 4 of the *Gigyf2* gene (P1-F: 5'-GGGTGCCAAACTCAGTCCATTC-3' and GT2-R: 5'-CGTGTCTCTACAACACACTCCAACC-3') and a 639 bp fragment from the wild-type allele (P2-F: 5'-AAGCAGGGCTGGAGGTAGTC-3' and P2-R: 5'-TTCTCCCTCTGCCCTACATTC-3').

Northern blotting

Total RNA was isolated from primary cultured mouse embryonic fibroblasts (MEFs) using TRI Reagent (Molecular Research Center, Cincinnati, OH), and 30 µg of RNA was resolved on 1% agarose gels under denaturing conditions. After transfer to nitrocellulose, blots were hybridized with one of two probes labeled with [³²P] deoxy-CTP by random priming (Multiple DNA Labeling Kit, Amersham, GE Healthcare-Life Science, Piscataway, NJ). Probe A was generated by amplifying a 450 bp fragment from GIGYF2 cDNA using primers spanning exons 1 to 4 (NT-F: 5'-TTTGGGCCTGAATGGCTCCGTGC-3' and NT-R: 5'-CTTCTTGAAGCTGGAGATCCTCG-3'), thus matching sequence 5' from the gene-trap insertion. Probe B, corresponding to a region downstream from the gene-trap vector, was obtained by HindIII digestion of GIGYF2 cDNA (bp 1738-2769, exons 13-26). Blots were washed twice with 2X SSC-0.05% SDS (40 min each) and once with 0.1X SSC-0.1% SDS, and specific mRNA bands were identified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The GIGYF1 probe for northern blotting was generated by restriction digestion of mouse GIGYF1 cDNA using BamHI-XhoI, generating a 450bp fragment at the N-terminus.

Immunoblotting

For total protein lysates, MEFs derived from *Gigyf2* ^{+/+} and ^{-/-} mice or whole embryos from timed matings, were lysed in a 1% NP-40 buffer (1% Nonidet P-40, 10% glycerol, 137mM NaCl, 1mM MgCl₂, 1mM CaCl₂, 20mM Tris-HCl at pH 7.6, 2mM EDTA, 10mM sodium pyrophosphate, 10mM NaF, 2mM Na₂VO₄, 2mM PMSF, and 8mg/ml leupeptin). For IGF-I stimulation experiments, *Gigyf2* ^{+/+} and ^{-/-} MEFs were

cultured for 6 hours in serum-free Dulbecco's modified Eagle's medium (4.5 g/L glucose) supplemented with 0.5% bovine serum albumin and 25 mM HEPES. Cells were incubated with 10^{-7} M IGF-I (GroPep, Adelaide, Australia) for the indicated duration at 37°C prior to lysis in 1% NP-40 buffer. All lysates were cleared by centrifugation, protein concentrations were determined by Bradford assay, and 50-60 µg protein aliquots were subjected to SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting. Membranes were blocked at room temperature for 1 hour with 5% BSA in Tris-buffered saline-Tween 20 followed by incubation overnight at 4°C with primary antibody. After washing, membranes were incubated with appropriate secondary antibodies conjugated to horseradish peroxidase (Jackson Immuno Research, West Grove, PA). Immunoblots were developed by Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Boston, MA), and digital image acquisition was performed on a FluorChem Imaging System (Alpha Innotech, San Leandro, CA). The following antibodies were used for immunoblotting: peptide-affinity purified anti-GIGYF2 and anti-GIGYF1 polyclonal antibodies, anti-ERK1 (Transduction Laboratories), anti-Akt (Cell Signaling Technology, Beverly, MA), anti-IGF-I receptor (kindly provided by Dr. Kenneth Siddle, Cambridge, UK), and anti-β-actin (C4; Santa Cruz Biotechnology, Santa Cruz, CA); phosphorylation was measured using anti-ACTIVE MAPK (Promega, Madison, WI), anti-phospho-Akt (Thr308) (Cell Signaling Technology), and anti-phospho-IGF-I receptor (pY¹¹⁵⁸-specific) (BioSource, Camarillo, CA).

Histology and Immunohistochemistry

Brain and spinal cord from 12-month-old *Gigylf2* $+/+$ and $+/-$ mouse littermates were fixed overnight in 4% paraformaldehyde/PBS at 4°C, dehydrated with increasing concentrations of ethanol, embedded in paraffin, and sectioned sagittally (7-10 μ m). Luxol Fast Blue/ Hematoxylin & Eosin staining was performed using standard methods. Adjacent sections were rehydrated, treated with Avidin-Biotin blocking solution (Vector Laboratories, Burlingame, CA), and incubated overnight at 4°C in a humidified chamber with sheep anti- α -synuclein antibody (Chemicon, Temecula, CA). Primary antibody detection was performed with biotinylated goat anti-sheep IgG and Vector Elite ABC Reagent (Vector Laboratories). Sections were counterstained with Hematoxylin.

β -Gal staining in whole-mount embryos

Day 14.5 embryos from timed mating were obtained by Caesarean section, washed in PBS, fixed in fresh 2% paraformaldehyde/PBS for 1 hour at 4°C, hemisected, fixed for an additional hour, and then placed in 18% sucrose overnight at 4°C. Brains from adult *Gigylf2* $-/-$ mice were also treated in a similar manner. After removal of sucrose by blotting, the specimens were equilibrated in OCT (Tissuetech, EMS, Hatfield, PA), positioned in a plastic base mold filled with OCT, and frozen. Sagittal sections (10 μ m) were prepared, adhered to slides, washed in PBS, and incubated for 24 hours at 30°C in the dark in fresh X-Gal solution (1mg/ml X-Gal, 35mM potassium ferrocyanide, 35mM potassium ferricyanide, 2mM MgCl₂, 0.02% NP-40 in PBS). After washing in double-distilled water, the sections were counterstained for 2 min with Vector Fast Red (Vector Laboratories), dehydrated and mounted under coverslips.

Motor function studies

The Rota-Rod system (like the Ugo Basile apparatus) was built to measure animal balance and coordination. A 3.1 cm diameter rotating rod was adapted by the addition of a soft rubber cover (made from a bike tire inner tube) (Brooks SP 2004). Mice were tested at 12-15 months of age. Each animal was housed in a single cage and all experiments were performed during the light phase. Animals were first conditioned on a stationary rod for 1 minute and during this time any animal that fell was placed back on the rod. The next day, animals were conditioned at a constant speed of 5rpm for 90 sec. If an animal fell during this time they were placed back on the rod until the end of the conditioning time (Jiang C 2005). After conditioning, animals were subjected to 1 trial per day at 5rpm for 4 days, for a total of 4 trials. The length of time each mouse remained on the rod was recorded.

Statistical Analysis

Statistical analyses were conducted using the Sigma Stat 3.0 and Sigma Plot software (Systat Software, San Jose, CA). One-tailed or two-tailed t tests, and the Mann-Whitney rank sum test were performed as indicated to evaluate the significance of differences between means. Results with $p < 0.05$ were considered to be statistically significant.

RESULTS

***GIGYF2* Gene Disruption**

A gene trap strategy was used to generate a mouse line with inactivation of the *Gigyf2* gene. For this purpose, embryonic stem cells were obtained from Bay Genomics (<http://baygenomics.ucsf.edu>) that contained a gene trap construct composed of a splice acceptor, a beta-geo cassette, and a polyadenylation sequence inserted into the proximal coding region of the mouse *Gigyf2* gene. We mapped the exact insertion point of the construct within the 5 kb intron between *Gigyf2* exons 4 and 5 using intronic and gene trap PCR primer pairs (Fig 1A). This predicted a truncated transcript with deletion of 23 of the total 27 exons of *Gigyf2*. Genotyping of mouse colony progeny obtained from crosses of parental mice heterozygous for the gene trap insert using some of the same PCR primer sets demonstrated E17.5 embryos with each of the three expected genotypes (Fig. 1B). Northern blotting analysis of total RNA extracts from primary mouse embryonic fibroblast (MEF) cultures derived from E12.5 embryos using a probe corresponding to the first 4 exons of *Gigyf2* is shown in Figure 1C. A single 7 kb *GIGYF2* mRNA band was observed in the wild-type *+/+* MEFs, a smaller 6 kb band corresponding to the expected size for the truncated gene trap-containing transcript in the homozygous *GIGYF2* *-/-* MEFs, and both bands in MEFs from heterozygous *+/-* progeny. Similar data were obtained using total RNA extracts from whole E17.5 mouse embryos (data not shown). Northern blotting with a *Gigyf2* cDNA probe corresponding to sequence downstream from the gene trap insert demonstrated a single band in the wild type *+/+* MEFs, a lower intensity single band of the same size in heterozygous *+/-* MEFs,

and no hybridizing band in the $-/-$ MEFs (Fig. 1D). This confirmed the absence of transcribed full-length mRNA sequence downstream from the gene trap. The failure to detect smaller bands on these blots further indicated that bypassing of the gene trap by alternative splicing or alternative start codons was not occurring. Northern blotting with a probe specific for the homologous *Gigyf1* gene (20) demonstrated a single band of equal intensity in mice of all three genotypes (Fig. 1E), indicating a lack of upregulation of *Gigyf1* gene transcription in response to loss of *Gigyf2* expression.

Immunoblotting of total cell lysates with an affinity-purified GIGYF2 anti-peptide antibody confirmed the presence of a GIGYF2 band at the expected 180 kD size of the full-length protein in wild type $+/+$ MEFs (Fig. 1E). The GIGYF2 protein was undetectable in $-/-$ MEFs, and a 180 kD band of approximately 50% lower intensity compared to the control cells was present in the $+/-$ heterozygotes. A similar partial decrease of GIGYF2 protein in $+/-$ heterozygotes and absence of expression in $-/-$ animals was demonstrated by immunoblotting of extracts from whole E14.5 embryos (Fig. 1F). By contrast, immunoblotting of whole embryo (not shown) and MEF extracts with GIGYF1 antibody identified a dominant band of the expected 150 kD size for the full-length GIGYF1 protein, which was present at approximately equal intensity in all three genotypes (Fig. 1G). Thus, consistent with the Northern blotting data, there is a partial (approximately 50 percent) decrease in abundance of the GIGYF2 protein in $+/-$ heterozygotes and a complete absence of detectable GIGYF2 protein in the $-/-$ homozygotes. The homologous GIGYF1 protein is expressed and not changed in abundance in control vs. GIGYF2-deficient mice.

Effects of *Gigyf2* Gene Disruption on Embryonic and Postnatal Survival

Analysis of genotype frequency in late gestation (E17.5-18.5) embryos from heterozygous matings demonstrated the predicted Mendelian genotype distribution, consistent with the generation and survival of heterozygous and homozygous *Gigyf2* gene-disrupted mice equivalent to the wild type (Table 1). However, we observed that a greater than expected number of newborn animals from the heterozygous matings did not survive beyond the first day. When both surviving and non-surviving progeny were collected during the first postnatal day and analyzed, the genotype frequencies of the newborns were close to the Mendelian ratio observed for the late gestation embryos (Table 1). Progeny deaths were limited almost entirely to the first postnatal day and specifically occurred in the *Gigyf2* null (-/-) mice. The modest (25%) decrease in the number of *Gigyf2* -/- compared to wild type +/+ newborn mice likely reflected the failure to recover some non-surviving -/- animals as a consequence of postmortem maternal cannibalism. At the time of weaning (day P21), genotyping revealed the predicted 2:1 ratio of *Gigyf2* +/- to +/+ animals, but a marked decrease in the *Gigyf2* null (-/-) genotype, consistent with approximately 85% lethality of the *Gigyf2* null mice during the first postnatal day (Table 1). The surviving *Gigyf2* -/- mice and also the +/- heterozygotes were indistinguishable from the wild type animals in growth curves and adult size and weight, and there were no genotype-related differences in fertility among the surviving animals.

Examination of fetal animals of all three genotypes in late gestation (E17.5), revealed no differences among the wild type and *Gigyf2* heterozygous and homozygous null mice in gross appearance, linear growth, or body weight (Fig. 2A-C). By contrast,

crown-rump length and body weight became significantly lower in *Gigyf2* null (-/-) animals examined during the first postnatal day (Fig. 2D-F). This correlated with a marked decrease in size of the gastric milk spot in the *Gigyf2* null mice (Fig 2G), indicating a failure of the *Gigyf2* null mice to feed. The rapid loss of weight and less marked decrease in crown-rump length likely were secondary to dehydration, and the magnitude of the feeding deficit appeared adequate to explain the observed mortality rate. The mean body weight and milk spot size of animals heterozygous for *Gigyf2* gene disruption were lower than wild type controls, but the differences were not statistically significant (Fig. 2F-G). Although not confirmed with the number of animals studied, this suggested a possible intermediate phenotype with partial loss of the GIGYF2 protein. The newborn *Gigyf2* null mice had normal motility based on timed self-righting capacity, and detailed anatomic and histologic examination of P1 mice showed no abnormalities in nasal, oropharyngeal, or gastrointestinal structures (not shown). The *Gigyf2* null mice also were not cyanotic and had no detectable pulmonary or cardiac abnormalities. These findings overall are consistent with abnormal feeding secondary to tactile or olfactory defects.

Tissue and Cellular Expression of *Gigyf2*

The gene trap construct contains a β -galactosidase sequence under control of the endogenous *Gigyf2* promoter. By staining sections of *Gigyf2* +/- and -/- embryos with X-gal, it thus is possible to determine the tissue and cellular sites of *Gigyf2* promoter activity as a surrogate for *Gigyf2* gene expression at different stages of development. As shown in Figure 3A, *Gigyf2* is broadly expressed in embryonic mouse tissues, including

strong expression in the central nervous system. X-gal staining is more intense in *-/-* compared to *+/-* mice, consistent with the presence of two vs. one copy of the reporter allele, respectively. Wild type mice had absent X-gal staining (not shown). *Gigyf2* gene expression was observed throughout the CNS in adult *Gigyf2* *-/-* mice (Fig. 3B, panel a). Animals of this genotype were used, since they were confirmed to have no histological changes from wild type or *Gigyf2* *+/-* mice by Luxol Fast Blue H&E staining, and they have a strong *Gigyf2* promoter-driven β -galactosidase signal. *Gigyf2* expression was noted to be especially marked in cerebellar Purkinje cells, the pons, the choroid plexus, and the olfactory bulb region (Fig. 3B, panels b-e). High intensity staining was evident in both neuronal and glial cells in multiple brain regions (Fig. 3C).

Motor Dysfunction in Heterozygous *Gigyf2* *+/-* Mice

Given the association of the *Gigyf2* gene with human Parkinson's disease (18), which most frequently has onset in adulthood and increases in prevalence with advancing age, we examined neurological function in postnatal *Gigyf2* *+/-* mice up to 18 months of age. Abnormalities of neurological function were not evident until animals reached approximately 12 months of age, at which point mild truncal ataxia became apparent as an exaggerated splaying of the hindlimbs when animals were dropped 15 cm onto a flat surface. This abnormal motor response was present in approximately 50% of 12-month-old *Gigyf2* *+/-* mice and absent from wild type (*Gigyf2* *+/+*) littermates, as assessed by an investigator unaware of the genotype of individual animals. Balance performance on a rotating horizontal rod was used to further quantify the apparent motor ataxia in the *Gigyf2* *+/-* mice. As shown for 15-month-old mice in Figure 4, animals with inactivation

of one allele of the *Gigyf2* gene exhibited an approximately 50% decrease in staying time on a rod rotating at 5 rpm compared to wild type controls ($p < 0.05$).

Neurodegeneration in *Gigyf2* +/- Mice

Histologic sections prepared from the cerebrum, midbrain, and cerebellum showed no overt neuronal or glial cellular abnormalities on staining Luxol Fast Blue H&E staining (not shown). In particular, there was no apparent cell loss in the substantia nigra. There also were no abnormalities apparent in sections from these brain regions immunostained with antibodies to glial fibrillary acid protein (GFAP), ubiquitin, or tyrosine hydroxylase. By contrast, there was evidence of motor neuron degeneration in Luxol Fast Blue H&E stained sections from multiple spinal cord levels in *Gigyf2* +/- mice. Specific pathological findings included eosinophilic motor neuron cell bodies, swollen axons, and scattered inclusion body-like structures (Fig. 5A). Histomorphometric quantitation of neuronal and glial cell number in lumbar region spinal cord sections confirmed a significant decrease in the neuronal/glial cell ratio (Fig. 5B).

Immunostaining with α -synuclein antibody yielded additional evidence of neuronal abnormalities and neurodegeneration. This included α -synuclein positive, coarse neurites in the cerebellar granule cell layer of the *Gigyf2* +/- mice, but not in age-matched (*Gigyf2* +/+) controls (Fig 6A). Increased numbers of α -synuclein-positive neurites were diffusely present throughout the brain and spinal cord of *Gigyf2* +/- animals in comparison with controls (Fig 6B). Quantitative histomorphometry by an observer unaware of the genotypes of individual animals confirmed a statistically significant increase in α -synuclein-positive neuritic plaques in *Gigyf2* +/- cerebrum and spinal cord

(Fig. 6C). The mean number of plaques also was higher in midbrain and cerebellum of *Gigyf2* +/- vs. control animals, but this did not reach statistical significance. These findings reveal evidence of neurodegeneration in multiple regions of the central nervous system in *Gigyf2* +/- mice. This includes motor neuron degeneration associated with a small number of inclusion body-like structures in the spinal cord, and an absence of apparent changes in the substantia nigra.

IGF Signaling in MEFs from *Gigyf2* Null Mice

GIGYF2 was initially identified in a yeast two-hybrid screen as a binding partner for the Grb10 adapter protein (20). Since Grb10 is recruited to activated IGF-I and insulin receptors and negatively regulates IGF and insulin signaling (21,22), we considered the potential role of decreased GIGYF2 levels in modifying the actions of these hormones. As evident in Figure 2 A-C, the *Gigyf2* null mice exhibited normal embryonic and fetal growth. In addition, when examined postnatally, both the *Gigyf2* +/- heterozygotes and the small number of surviving *Gigyf2* -/- mice were indistinguishable from wild type littermates in growth, adult body weight, and blood glucose levels (data not shown). To more specifically examine the effects of *Gigyf2* gene disruption on receptor signaling, IGF-I stimulation of IGF-I receptor tyrosine phosphorylation and the activation of the downstream signaling intermediates Akt and ERK1/2 were assessed in multiple isolates of primary mouse embryonic fibroblasts (MEFs) from *Gigyf2* +/+ and -/- mice. IGF-I receptor abundance was shown to be similar in the wild type and *Gigyf2* null MEFs by immunoblotting with receptor antibody, and IGF-I was shown to stimulate receptor tyrosine phosphorylation assessed by phospho-specific antibody in a time-

dependent manner, with a maximum effect at 5 min (Fig. 7A). Quantitation at the 5-min time point in multiple MEF isolates confirmed a modest but statistically significant (approximately 25%) lower level of IGF-I-stimulated receptor tyrosine phosphorylation in the *Gigyf2* null cells compared to wild type (Fig. 7B). Determination of Akt and ERK1/2 activation by immunoblotting of the same cell extracts with phospho-specific antibodies for each of these intracellular signaling proteins demonstrated similar time-dependent activation by IGF-I with a maximum effect at 5 min (Fig. 7A). In contrast with the inhibition of IGF-I-stimulated receptor tyrosine phosphorylation in *Gigyf2* null MEFs, disruption of the *Gigyf2* gene resulted in a modest but significant increase in IGF-I stimulation of ERK1/2 (25%, $p < 0.02$) (Fig. 7B). There was a similar magnitude increase in IGF-I-stimulated Akt activation in the *Gigyf2* null MEFs, but there was larger variation in the level of phospho-Akt, and the increase was not statistically significant (Fig. 7B). These experiments provide evidence of a role for the GIGYF2 protein in regulation of IGF-I receptor signaling. The decrease in IGF-I-stimulated receptor tyrosine phosphorylation and oppositely-directed augmentation of IGF-I effect on ERK1/2 and possibly Akt activation in *Gigyf2* null MEFs is similar to the previously described effects of knockdown of the GIGYF2 binding partner, Grb10, in cultured fibroblasts (22).

DISCUSSION

We recently have identified multiple mutations in the *GIGYF2* gene in patients with familial Parkinson's disease (18), and we have proposed that *GIGYF2* may represent the specific gene responsible for the previously described linkage of the chromosome 2q37 region to Parkinson's disease (PARK11 locus) (19). Mutations in the coding region of *GIGYF2* were found in approximately 5 percent of individuals in two distinct populations of familial Parkinson's disease patients. Only one other gene, *LRRK2*, has been shown to have a similarly high mutation frequency in Parkinson's disease (7). This report describes a mouse model of *Gigyf2* gene disruption that provides substantial further evidence for a role of the GIGYF2 protein in neurodegeneration.

The absence of detectable GIGYF2 mRNA and protein levels was confirmed in mice in which both alleles of the *Gigyf2* gene were disrupted by a gene trap insert. These *Gigyf2* null mice exhibited early perinatal lethality, with 85 percent mortality during the first two days after birth, indicating a critical role for the GIGYF2 protein in postnatal survival. The homologous GIGYF1 protein, which is encoded by a distinct gene, was shown to be present but not up-regulated in the mice with *Gigyf2* gene disruption, and the lethal phenotype confirmed a lack of compensation for GIGYF2 function by GIGYF1. *Gigyf2* null mice exhibited normal intrauterine growth, and there were no evident anatomic or histological abnormalities in full-term fetuses. However, newborn mice lacking GIGYF2 failed to feed normally, as evidenced by reduced gastric milk spots together with postnatal weight loss, and the most likely cause of perinatal death was consequent dehydration. The *Gigyf2* null animals had normal self-righting behavior, an

absence of gross abnormalities in motility, and no apparent defects in oropharyngeal or nasal structures. We observed very strong *Gigyl2* gene expression in the olfactory bulb region of fetal and newborn mice, and previous studies from other groups have demonstrated a phenotype in mice with olfactory dysfunction similar to the *Gigyl2* null mice, including absent gastric milk spot, perinatal weight loss, and high early mortality (27,28). We therefore speculate that the failure of *Gigyl2* null mice to feed may be a consequence of defective olfactory sensory input. As in the *Gigyl2* deficient mice, approximately 15 percent of mice with initial feeding deficiency survive the perinatal period and thereafter have growth and survival indistinguishable from control animals (28). It is thought that mice lacking normal olfaction are unable to locate the maternal nipple during the initial postnatal period, when they are lacking in sight. The 15 percent survivors may represent individual animals that initially locate the maternal food source by chance and later can achieve this with visual cues. Given the presence of *GIGYF2* mutations in human Parkinson's disease, it is of interest that defects in olfaction have been described as frequent and early abnormalities in Parkinson's disease (29). Direct measures of olfactory function in *Gigyl2* null mice will require electrophysiological monitoring methods that are beyond the scope of the current study (30).

Mice with gene trap disruption of a single *Gigyl2* allele have an approximately 50 percent decrease in abundance of the normal 7 kb *GIGYF2* mRNA transcript. They do not express lower molecular weight transcripts, and thus do not appear to generate alternative transcripts from splicing around the gene trap or utilization of downstream transcription start sites. A similar magnitude decrease in *GIGYF2* protein abundance (approximately 50 percent) in heterozygous *Gigyl2* +/- embryos and cultured MEFs from

these animals was demonstrated by immunoblotting. The *Gigyf2* +/- mice survive embryonic life at the expected Mendelian ratio and are indistinguishable from *Gigyf2* +/+ littermate controls in growth and development. Neurological deficits were not evident in growing or young adult mice. However, by 12 months of age, approximately 50 percent of the *Gigyf2* +/- mice developed motor ataxia, which was quantified as a 50 percent decrease in balance time on a horizontal rotating rod compared to wild type controls.

Histological examination of 12-month-old *Gigyf2* +/- mice demonstrated central nervous system abnormalities that included coarse neurites in the cerebellar granule cell layer, α -synuclein positive neuritic plaques in the brain and spinal cord, scattered inclusion body-like structures, and spinal cord motor neurons with eosinophilic-stained cell bodies and swollen axons. Quantitative histomorphometry confirmed a significant increase in α -synuclein positive neurites in the cerebrum and spinal cord, plus a decreased ratio of neurons to glial cells in the lumbar spinal cord. These findings are consistent with neurodegeneration in the *Gigyf2* +/- mice that either has its onset or progresses with aging in adult animals. Consistent with the observed effects of *Gigyf2* gene disruption on cells in the cerebrum, midbrain, cerebellum, and spinal cord, GIGYF2 mRNA is expressed at multiple sites throughout the central nervous system. Although the *Gigyf2* +/- mice manifested motor ataxia, they not have other features typical for human Parkinson's disease, such as an evident tremor or neuronal loss in the substantia nigra. This may reflect species differences, since other mouse models with disruption of genes linked to Parkinson's disease also do not fully recapitulate the functional and pathological features of the human disorder. For example, disruption of the *Parkin* or *DJ-1* genes in mice results in neurodegeneration and motor function deficits, but these

animals also do not have cellular deficits in the nigrostriatal dopaminergic pathway (31-33). *SNCA* null mice have synaptic vesicle depletion from nerve terminals, but only subtle defects in dopamine release and no overt substantia nigra cell loss (34,35). By contrast, over-expressing *SNCA* transgenic animals have a more marked phenotype, especially when the transgene is specifically driven by a dopaminergic neuron-targeted tyrosine hydroxylase promoter (36). *Nurr1* null mice have decreased motility and an early lethal phenotype associated with failure to feed, and *Nurr1* +/- mice do develop decreased dopamine levels in the striatum and decreased dopaminergic striatal neurons associated with diminished motor activity and performance (37). However, the human ortholog of *Nurr1*, *NR4A2*, has thus far been linked to Parkinson's disease in only a single family, and the human phenotypic consequences of *NR4A2* mutations therefore requires further investigation (16). The substantial variations in mouse phenotypes following disruption of different genes, in addition to the differences from Parkinson's disease, suggests that the distinctions from human Parkinson's disease may result from both species differences and the specific functional properties of individual genes.

The occurrence of neurodegeneration in mice with partial loss of expression of the GIGYF2 protein provides important support for a causal link between the mutations identified in the human *GIGYF2* gene and Parkinson's disease. The finding of neurodegeneration with decreased protein abundance in the mice also suggests that the described mutations in human Parkinson's disease likely result in loss of expression or function of the GIGYF2 protein. The observation of inclusion body-like structures suggestive of Lewy bodies in the brain and spinal cord is a distinct feature of the *Gigyl2* +/- mice, which has not been described in other mouse models with disruption of

Parkinson's disease associated genes. It would be of interest in future studies to generate mice with greater than 50 percent GIGYF2 functional loss, and determine whether this results in additional clinical or histological features of human Parkinson's disease, including more extensive inclusion body formation. In this case, further characterization of the composition of these structures would be feasible and might give insight into GIGYF2 protein function.

We previously demonstrated in a yeast two-hybrid assay that the 1299 amino acid protein encoded by the *GIGYF2* gene is a binding partner for the Grb10 adapter protein (20). Grb10 has been widely studied as a protein that is recruited to activated IGF-I and insulin receptors, as well as other receptor tyrosine kinases (38). Studies on *Grb10* null mice and cultured cells with siRNA knockdown of Grb10 strongly support a role for the endogenous Grb10 protein in negatively regulating IGF-I and insulin receptor signaling (21,22). GIGYF2 and the homologous GIGYF1 protein, which is encoded by a distinct gene on human chromosome 7, may be recruited to activated IGF-I and insulin receptors through binding of the Grb10 C-terminus to the receptors and its N-terminus to the GIGYF proteins. We have provided evidence for IGF-I stimulation of GIGYF1 recruitment to Grb10 and activated IGF-I receptors (20). However, studies on this or other aspects of GIGYF1 and GIGYF2 function have been limited by a propensity of these proteins to form toxic aggregates when over-expressed in cultured cells.

As an alternative approach to investigating the potential interaction between the GIGYF2 protein and the IGF-I signaling pathway, we compared IGF-I signaling in cultured MEFs from *Gigyf2* $-/-$ and control $+/+$ mice. The *Gigyf2* $-/-$ cells exhibited a modest but significant decrease in IGF-I-stimulated receptor phosphorylation, with no

change in receptor abundance in comparison with control cells. By contrast, there was a significant increase in IGF-I-stimulated ERK1/2 phosphorylation and a suggestive, non-significant increase in Akt phosphorylation in the *Gigyf2* null cells. In previous studies, Grb10 knockdown in cultured fibroblasts also resulted in decreased IGF-I receptor tyrosine phosphorylation and increased downstream signaling from the IGF-I receptor, and similar effects of Grb10 on insulin receptor phosphorylation and downstream signaling (22). It has been hypothesized that recruitment of endogenous Grb10 to activated IGF-I and insulin receptors has a dual function in blocking receptor dephosphorylation by phosphoprotein phosphatases and inhibiting downstream signals that are generated by the tyrosine kinase activity of the receptors (22). This raises the interesting possibility that Grb10 might function physiologically by initially inhibiting IGF-I and insulin signaling, and then releasing latent active (tyrosine phosphorylated) receptors through its dissociation from the receptors at specifically targeted sites of hormone signaling. Since GIGYF2 was cloned as a protein interactive with Grb10 in a yeast two-hybrid assay and lacks domains known to bind IGF-I or insulin receptors, it will be important in future studies to determine whether the observed effects of GIGYF2 on IGF-I receptor phosphorylation and signaling are mediated via a GIGYF2-receptor protein complex with Grb10 functioning as a linker, or by GIGYF2 modulating the capacity of Grb10 to bind the receptors.

The identification of GIGYF2 as a gene linked to neurodegeneration as well as IGF-I and insulin signaling is of interest because of emerging direct and indirect evidence for a role of both of these hormones in Parkinson's disease and other human neurodegenerative disorders, such as Alzheimer's disease (23,24,26,39,40). IGF-I

promotes brain growth in vivo (41), with effects on neurons that include augmented proliferation, stimulation of differentiation, and inhibition of apoptosis (25). Among its multiple actions in the central nervous system, IGF-I receptors are abundantly expressed in the midbrain, and IGF-I has specifically been shown to decrease dopamine-induced cell death in rat cerebellar cell cultures and a human neuroblastoma cell line (42). Insulin receptors are particularly abundant in the substantia nigra and basal ganglia, in addition to the cerebral cortex (43). A selective decrease in insulin receptor mRNA and protein in the substantia nigra in Parkinson's disease has been described in one study (44), and intracerebroventricular insulin infusion in an animal model resulted in increased dopamine transporter mRNA in the substantia nigra (45). A recent study has reported an approximately two-fold increased risk of Parkinson's disease in patients with type 2 diabetes mellitus (26). Further studies in the molecular function of the GIGYF2 protein and the mechanism through which mutations or decreased expression of GIGYF2 result in neurodegeneration may provide insight into not only Parkinson's disease, but also the co-occurrence of neurodegenerative diseases with metabolic disorders such as diabetes mellitus.

ACKNOWLEDGEMENTS

We acknowledge Bay Genomics for providing the gene-trap ES cell clone. A special thank you goes to Ginny Hovanesian, Paul Monfills, Raziell Pereira and Penny Clouiter-Lyon for the technical support. This work was supported by the COBRE Genomics Center (J. Klysik), the Hallett Center for Diabetes and Endocrinology (R.J. Smith), and NIH Grant DK43038 (R.J. Smith).

REFERENCES

1. Wood-Kaszmar A, Gandhi S, Wood NW (2006) Understanding the molecular causes of Parkinson's disease. *Trends Mol Med* 12: 521-528.
2. Hughes AJ, Daniel SE, Kilford L, Lees AJ (1992) Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. *J Neurol Neurosurg Psychiatry* 55: 181-184.
3. Hughes AJ, Daniel SE, Lees AJ (2001) Improved accuracy of clinical diagnosis of Lewy body Parkinson's disease. *Neurology* 57:1497-1499.
4. Braak H, Del Tredici K, Rüb U, de Vos RAI, Jansen Steur, ENH, Braak E (2003) Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol Aging* 24: 197-211.
5. Becker G, Muller A, Braune S, Buttner T, Benecke R, Greulich W, Klein W, Mark G, Rieke J, Thumler R (2002) Early diagnosis of Parkinson's disease. *J Neurol* 249 (Suppl 1): 40-48.
6. Gosal D, Ross OA, Toft M (2006) Parkinson's disease: the genetics of a heterogeneous disorder. *Eur J Neurol* 13: 616-627.
7. Khan NL, Jain S, Lynch JM, Pavese N, Abou-Sleiman P, Holton JL, Healy DG, Gilks WP, Sweeney MG, Ganguly M, Gibbons V, Gandhi S, Vaughan J, Eunson LH, Katzenschlager R, Gayton J, Lennox G, Revesz T, Nicholl D, Bhatia KP, Quinn N, Brooks D, Lees AJ, Davis MB, Piccini P, Singleton AB, Wood NW (2005) Mutations in the gene *LRRK2* encoding dardarin (PARK8) cause familial Parkinson's disease:

- clinical, pathological, olfactory and functional imaging and genetic data. *Brain* 128: 2786-2796.
8. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 276: 2045-2047.
 9. Kitada D, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, Shimizu M (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 392: 605-608.
 10. Valente EM, Abou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, Ali Z, Del Turco D, Bentivoglio AR, Healy DG, Albanese A, Nussbaum R, Gonzalez-Maldonado R, Deller T, Salvi S, Cortelli P, Gilks WP, Latchman DS, Harvey RJ, Dallapiccola B, Auburger G, Wood NW (2004) Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* 304: 1158-1160.
 11. Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, Dekker MC, Squitieri F, Ibanez P, Joosse M, van Dongen JW, Vanacore N, van Swieten JC, Brice A, Meco G, van Duijn CM, Oostra BA, Heutink P (2003) Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science* 299: 256-259.
 12. Ramirez A, Heimbach A, Grundemann J, Stiller B, Hampshire D, Cid LP, Goebel I, Mubaidin AF, Wriekat AL, Roeper J, Al-Din A, Hillmer AM, Karasak M, Liss B, Woods CG, Behrens MI, Kubisch C (2006) Hereditary parkinsonism with dementia is

- caused by mutations in ATP13A2, encoding a lysosomal type 5 P-type ATPase. *Nat Genet* 38: 1184-1191.
13. Leroy E, Boyer R, Auburger G, Leube B, Ulm G, Mezey E, Harta G, Brownstein MJ, Jonnalagata S, Chernova T, Dehejia A, Lavedan C, Gasser T, Steinbach PJ, Wilkinson KD, Polymeropoulos MH (1998) The ubiquitin pathway in Parkinson's disease. *Nature* 395: 451-452.
 14. Strauss KM, Martins LM, Plun-Favreau H, Marx FP, Kautzmann S, Berg D, Gasser T, Wszolek Z, Muller T, Bornemann A, Wolburg H, Downward J, Riess O, Schulz JB, Kruger R (2005) Loss of function mutations in the gene encoding Omi/HtrA2 in Parkinson's disease. *Hum Mol Genet* 14: 2099-2111.
 15. Marx FP, Holzmann C, Strauss KM, Li L, Eberhardt O, Gerhardt E, Cookson MR, Hernandez D, Farrer MJ, Kachergus J, Engelender S, Ross CA, Berger K, Schols L, Schulz JB, Riess O, Kruger R. (2003) Identification and functional characterization of a novel R621C mutation in the synphilin-1 gene in Parkinson's disease. *Hum Mol Genet* 12: 1223-1231.
 16. Le WD, Xu P, Jankovic J, Jiang H, Appel S, Smith RG, Vassilatis DK (2003) Mutations in NR4A2 associated with familial Parkinson disease. *Nat Genet* 33: 85-89.
 17. Davidzon G, Greene P, Mancuso M, Klos KJ, Ahlskog JE, Hirano M, DiMauro S (2006) Early-onset familial parkinsonism due to POLG mutations. *Ann Neurol* 59: 859-862.

18. Lautier C, Goldwurm S, Dürr A, Giovannone B, Tsiras WG, Pezzoli G, Brice A, Smith RJ (2008) Mutations in the *GIGYF2 (TNRC15)* Gene at the PARK11 locus in familial Parkinson's disease *Am J Hum Genet* Mar 19; [Epub ahead of print].
19. Pankratz N, Nichols WC, Uniacke SK, Halter C, Rudolph A, Shults C, Conneally PM, Foroud T (2002) Genome screen to identify susceptibility genes for Parkinson disease in a sample without parkin mutations. *Am J Hum Genet* 71: 124-135.
20. Giovannone B, Lee E, Laviola L, Giorgino F, Cleveland KA, Smith RJ (2003) Two novel proteins that are linked to insulin-like growth factor (IGF-I) receptors by the Grb10 adapter and modulate IGF-I signaling. *J Biol Chem* 278: 31564-31573.
21. Langlais P, Dong LQ, Ramos FJ, Hu D, Li Y, Quon MJ, Liu F (2004) Negative regulation of insulin-stimulated mitogen-activated protein kinase signaling by Grb10. *Mol Endocrinol* 18: 350-358.
22. Dufresne AM, Smith RJ (2005) The adapter protein GRB10 is an endogenous negative regulator of insulin-like growth factor signaling. *Endocrinology* 146: 4399-4409.
23. Trejo JL, Carro E, Garcia-Galloway E, Torres-Alemann I (2004) Role of insulin-like growth factor I signaling in neurodegenerative diseases. *J Mol Med* 82: 156-162.
24. Craft S, Wastson GS (2004) Insulin and neurodegenerative disease: shared and specific mechanisms. *Lancet Neurol* 3: 169-178.
25. Russo VC, Gluckman PD, Feldman EL, Werther GA (2005) The insulin-like growth factor system and its pleiotropic functions in brain. *Endo Rev* 26: 916-943.
26. Hu G, Jousilahti P, Bidel S, Antikainen R, Tuomilehto J (2007) Type 2 diabetes and the risk of Parkinson's disease. *Diab Care* 30: 842-847.

27. Wang SS, Lewcock JW, Feinstein P, Mombaerts P, Reed RR (2004) Genetic disruptions of *O/E2* and *O/E3* genes reveal involvement in olfactory receptor neuron projection. *Development* 131: 1377-1388.
28. Parrish M, Ott T, Lance-Jones C, Schuetz G, Schwaeger-Nickolenko A, Monaghan AP (2004) Loss of the *Sall3* gene leads to palate deficiency, abnormalities in cranial nerves, and perinatal lethality. *Mol Cell Biol* 24: 7102-7112.
29. Hawkes C (2003) Olfaction in neurodegenerative disorder. *Mov Disorders* 18: 364-372.
30. Scolnick JA, Cui K, Duggan CD, Xuan S, Yuan XB, Efstratiadis A, Ngai J (2008) Role of IGF signaling in olfactory sensory map formation and axon guidance. *Neuron* 57: 847-857.
31. Goldberg MS, Fleming SM, Palacino JL, Cepeda C, Lam HA, Bhatnagar A, Meloni EG, Wu N, Ackerson LC, Klapstein GJ, Gajendiran M, Roth BL, Chesselet MF, Maidment NT, Levine, MS, Shen J (2003) Parkin-deficient mice exhibit nigrostriatal deficits but not loss of dopaminergic neurons. *J Biol Chem* 278: 43628-43635.
32. Goldberg MS, Pisani A, Haburcak M, Vortherms TA, Kitada T, Costa C, Tong Y, Martella G, Tschertter A, Martins A, Bernardi G, Roth BL, Pothos EN, Calabresi P, Shen J (2005) Nigrostriatal dopaminergic deficits and hypokinesia caused by inactivation of the familial Parkinsonism-linked gene DJ-1. *Neuron* 45: 489-496.
33. Kim RH, Smith PD, Aleyasin H, Hayley S, Mount MP, Pownall S, Wakeham A, You-Ten AJ, Kalia SK, Horne P, Westaway D, Lozano AM, Anisman H, Park DS, Mak TW (2005) Hypersensitivity of DJ-1-deficient mice to 1-methyl-4-phenyl-

- 1,2,3,6-tetrahydropyridine (MPTP) and oxidative stress. *Proc Natl Acad Sci (USA)* 102: 5215-5220.
- 34.** Abeliovich A, Schimtz Y, Farinas I, Choi-Lundberg D, Ho WH, Castillo PE, Shinsky N, Verdugo JMC, Armanini M, Ryan A, Hynes M, Phillips H, Sulzer D, Rosenthal A (2000) Mice lacking α -synuclein display functional deficits in the nigrostriatal dopamine system. *Neuron* 25: 239-252.
- 35.** Cabin DE, Shimazu K, Murphy D, Cole NB, Gottschalk W, McIlwain KL, Orrison B, Chen A, Ellis CE, Paylor R, Lu B, Nussbaum RL (2002) Synaptic vesicle depletion correlates with attenuated synaptic response to prolonged repetitive stimulation in mice lacking α -synuclein. *J Neurosci* 22: 8797-8807.
- 36.** Thiruchelvam MJ, Powers JM, Cory-Slechta DA, Richfield EK (2004) Risk factors for dopaminergic neuron loss in human α -synuclein transgenic mice. *Eur J Neurosci* 19: 845-854.
- 37.** Jiang C, Wan X, He Y, Pan T, Jankovic J, Le W (2005) Age-dependent dopaminergic dysfunction in Nurr1 knockout mice. *Exp Neurol* 191: 154-162.
- 38.** Holt LJ, Siddle K (2005) Grb10 and Grb14: enigmatic regulators of insulin action--and more? *Biochem J* 388: 393-406
- 39.** Scheele C, Nielsen AR, Walden TB, Sewell DA, Fischer CP, Brogan RJ, Petrovic N, Larsson O, Tesch PA, Wennmalm K, Hutchinson DS, Cannon B, Wahlestedt C, Pedersen BK, Timmons JA (2007) Altered regulation of the PINK1 locus: a link between type 2 diabetes and neurodegeneration. *FASEB J* 21: 3653-3665.

40. de la Monte SM, Wands JR (2005) Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: relevance to Alzheimer's disease. *J Alzheim Dis* 7: 45-61.
41. Carson MJ, Behringer RR, Brinster RL, McMorris FA (1993) Insulin-like growth factor I increases brain growth and central nervous system myelination in transgenic mice. *Neuron* 10: 729-740.
42. Offen D, Shtaf B, Hadad D, Weizman A, Melamed E, Gil-Ad I (2001) Protective effect of insulin-like-growth-factor-1 against dopamine-induced neurotoxicity in human and rodent neuronal cultures: possible implications for Parkinson's disease. *Neurosci Lett* 316: 129-132.
43. Unger JW, Livingston JN, Moss AM (1991) Insulin receptors in the central nervous system: localization, signaling, mechanisms, and functional aspects. *Prog Neurobiol* 36: 343-362.
44. Moroo I, Yamada T, Makino H, Tooyama I, McGeer PL, Hirayama K (1994) Loss of insulin receptor immunoreactivity from the substantia nigra pars compacta neurons in Parkinson's disease. *Acta Neuropathol (Berl)* 87: 343-348.
45. Figlewicz DP, Szot P, Chavez M, Woods SC, Veith RC (1994) Intraventricular insulin increases dopamine transporter mRNA in rat VTA/substantia nigra. *Brain Res* 644: 331-334.

FIGURE LEGENDS

Figure 1. Gene-Trap disruption of *Gigyf2* gene locus. (A) Schematic representation of the partial genomic structure of the mouse *Gigyf2* gene before and after the gene-trap integration. (B) PCR analysis of DNA from E17.5 embryos obtained in crosses of *Gigyf2* heterozygous parents. Primers P2-F and P2-R were used to amplify a 639bp fragment (wt allele), while primers P1-F and GT2-R were used to amplify a 290bp (mutated allele). (C) Northern blot of total RNA from primary MEFs isolated from *Gigyf2* *+/+*, *+/-*, *-/-* mice using a 450 bp probe of the first 4 exons of the mouse *Gigyf2* gene upstream from the gene trap and (D) using a C-terminus probe. (E) Northern blot of total RNA from primary MEFs isolated from *Gigyf2* *+/+*, *+/-*, *-/-* mice using a 450 bp probe of mouse *Gigyf1* gene. (F) Western blot of total proteins from primary MEFs isolated from *Gigyf2* *+/+*, *+/-*, *-/-* mice and (G) E14.5 embryos using GIGYF2 antibody and β -Actin antibody to control for loading. (H) Western blot of total proteins from *Gigyf2* *+/+*, *+/-*, *-/-* MEFs using GIGYF1 antibody

Figure 2. Phenotype of *Gigyf2* knockout mice. (A-G) Size of *Gigyf2* *+/+* (WT), *+/-* (Het), and *-/-* (KO) mice was assessed at embryonic day 17.5 (E17.5) and postnatal day 1 (P0-P1) by measuring crown-rump length and weight. Milk spot size in P0-P1 mice was assessed as length across the spot's longest diagonal. For E17.5 mice WT n=5, Het n=5, and KO n=7; for P0-P1 mice WT n=5, Het n=18 and KO n=4. Plotted data are means $\pm$ SD, subjected to a two-tailed *t* test for comparison of Het and KO mice to WT (* $p < 0.005$; # $p < 0.005$; ** $p < 0.002$).

Figure 3. *Gigyf2* expression in knockout mice. (A) Whole mount embryos of different stages expressing β -galactosidase from the *Gigyf2* promoter as an indirect measure of GIGYF2 tissue distribution during development. (B) β -gal staining of sections of *Gigyf2*^{-/-} mouse brain: sagittal section (1x) of full brain (a), cerebellar region (10x) (b), pons region (4x) (c), (10x) (d), and coronal section of hippocampal region (4x) (e). (C) Cell type expression of β -gal in *Gigyf2*^{-/-} mouse brain: neurons (top panel) and glia (bottom panel).

Figure 4. Motor ataxia in 15-month-old *Gigyf2* heterozygous mice. Rotating rod performance of 15-month-old male *Gigyf2*^{+/-} (Het) mice compared to age matched *Gigyf2*^{+/+} (WT) controls. Four independent trials were conducted in which time on a 5-rpm rotating rod was recorded for all mice (WT n=4, Het n=10). Data plotted are means $\pm$ SD, subjected to a one-tailed *t* test for comparison between WT and Het mice (*p*<0.05).

Figure 5. Motor neuron degeneration in *Gigyf2*^{+/-} mice. (A) Luxol Fast Blue and Hematoxylin & Eosin staining of spinal cord from 15-month-old *Gigyf2*^{+/-} mice. Arrows show hypoxic motor neurons (b), swollen axons (c-d), and inclusion body like structures (e and inset window) compared to a healthy motor neuron in *Gigyf2*^{+/+} control animal (a). (B) Number of neurons and glial cells per unit of surface area were counted from 2-3 independent sections from sacral and lumbar regions of spinal cords from *Gigyf2*^{+/+} (n=4) and *Gigyf2*^{+/-} (n=4) mice. Data plotted are means $\pm$ SD, subjected to a Mann-Whitney rank sum test for comparison between WT and Het animals (* *p*<0.05).

Figure 6. Alpha-synuclein positive neuritic plaques. (A) α -synuclein staining of cerebellar granule cell layer in 12-month-old *Gigyl2* +/- mouse. Arrows indicate examples of coarse neurites, which are absent in the age matched *Gigyl2* +/+ mouse. (B) Immunohistochemical staining of cerebral cortex (top panels) and midbrain (bottom panels) with an α -synuclein antibody shows neuritic plaques in the brain of a 15-month-old heterozygous male mouse. (C) Number of neuritic plaques per section across several regions of brain (CRB= cerebrum, MidBR= midbrain, CBL= cerebellum and SC= spinal cord) in 15-month-old mice. Data are means $\pm$ SD from WT n=4 and Het n=10, subjected to a two-tailed *t* test for comparison between WT and Het groups (# $P < 0.05$, * $p < 0.02$, and ** $p < 0.05$).

Figure 7. IGF-I stimulated cell signaling in *Gigyl2* -/- mouse embryonic fibroblasts. Passage 4 mouse embryonic fibroblasts (MEFs) derived from *Gigyl2* +/+ (WT) and -/- (KO) mice were treated with IGF-I for the length of time indicated, and total cell lysates were used for immunoblotting. (A) IGF-I receptor (IGF-IR), Akt and ERK1/2 activation was assessed in two littermate-matched pairs of WT and KO MEFs using phospho-specific antibodies (representative blot from a single pair shown). (B) Six littermate-matched pairs of WT and KO MEFs from three independent litters were treated with IGF-I for 0 or 5 minutes and analyzed as described in (A). Phosphorylation was quantified by densitometry and normalized to protein content. Fold increase in phosphorylation at 5 minutes compared to basal was determined for each cell line. Relative units were obtained by normalizing each fold increase to a mean WT value of

100. Data plotted are means $\pm$ SE. The data were subjected to two-tailed t test analysis for comparison between WT and KO MEFs (* $p < 0.02$, ** $p < 0.002$).

Genotype	E17.5-E18.5	P0-P1	P21
WT (+/+)	23	12	87
HET (+/-)	47 (203%)	22 (183%)	172 (198%)
KO (-/-)	20 (87%)	9 (75%)	14 (16%)
Total	90	43	273

Table 1. Perinatal lethality of *Gigyf2* ^{-/-} mice.

Numbers of individual genotyped progeny at each stage are shown (E, embryonic day; P, postnatal day). Indicated in parenthesis is percent of each genotype compared to *Gigyf2* ^{+/+} at each stage. Assuming equal viability, the expected Mendelian ratio would be 100% *Gigyf2* ^{+/+}, 200% *Gigyf2* ^{+/-}, 100% *Gigyf2* ^{-/-}.

Figure 1

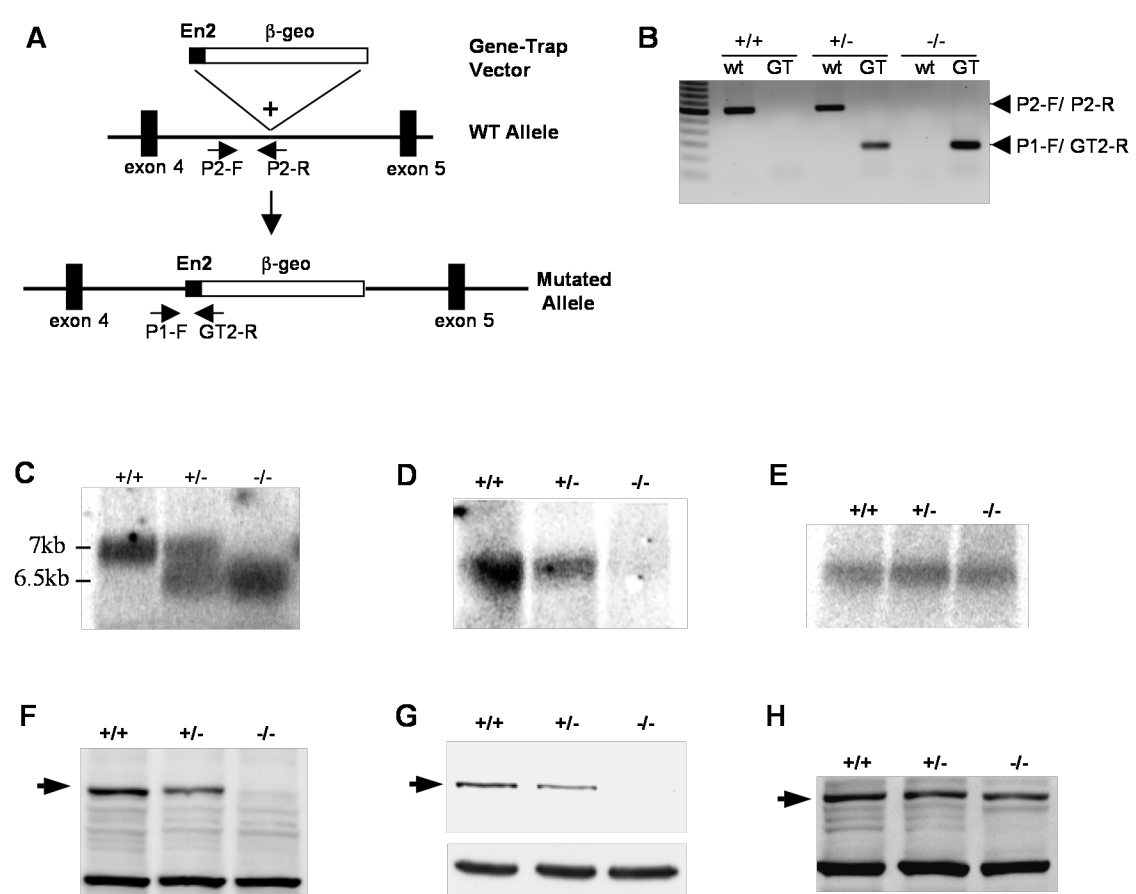


Figure 2

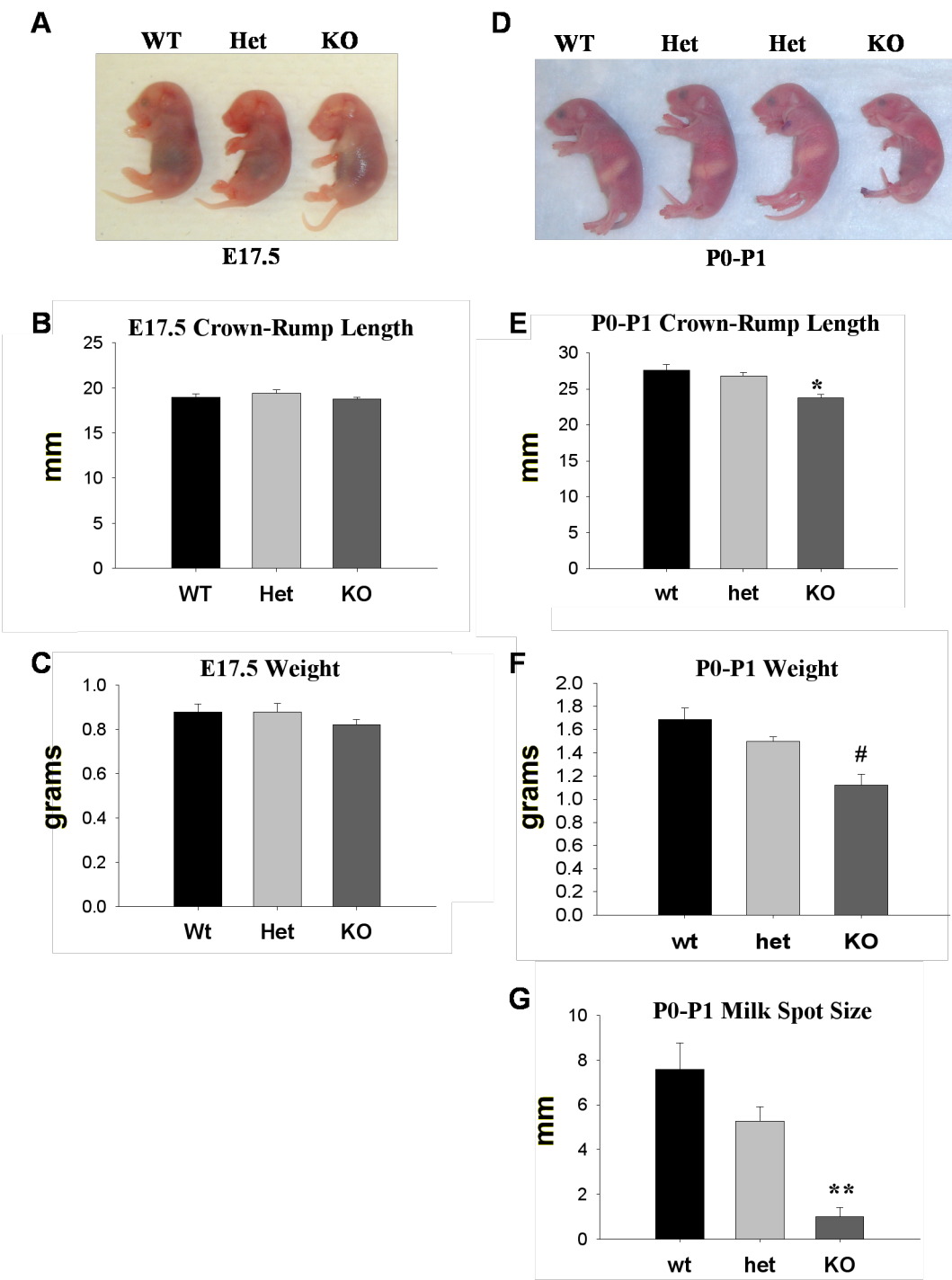


Figure 3

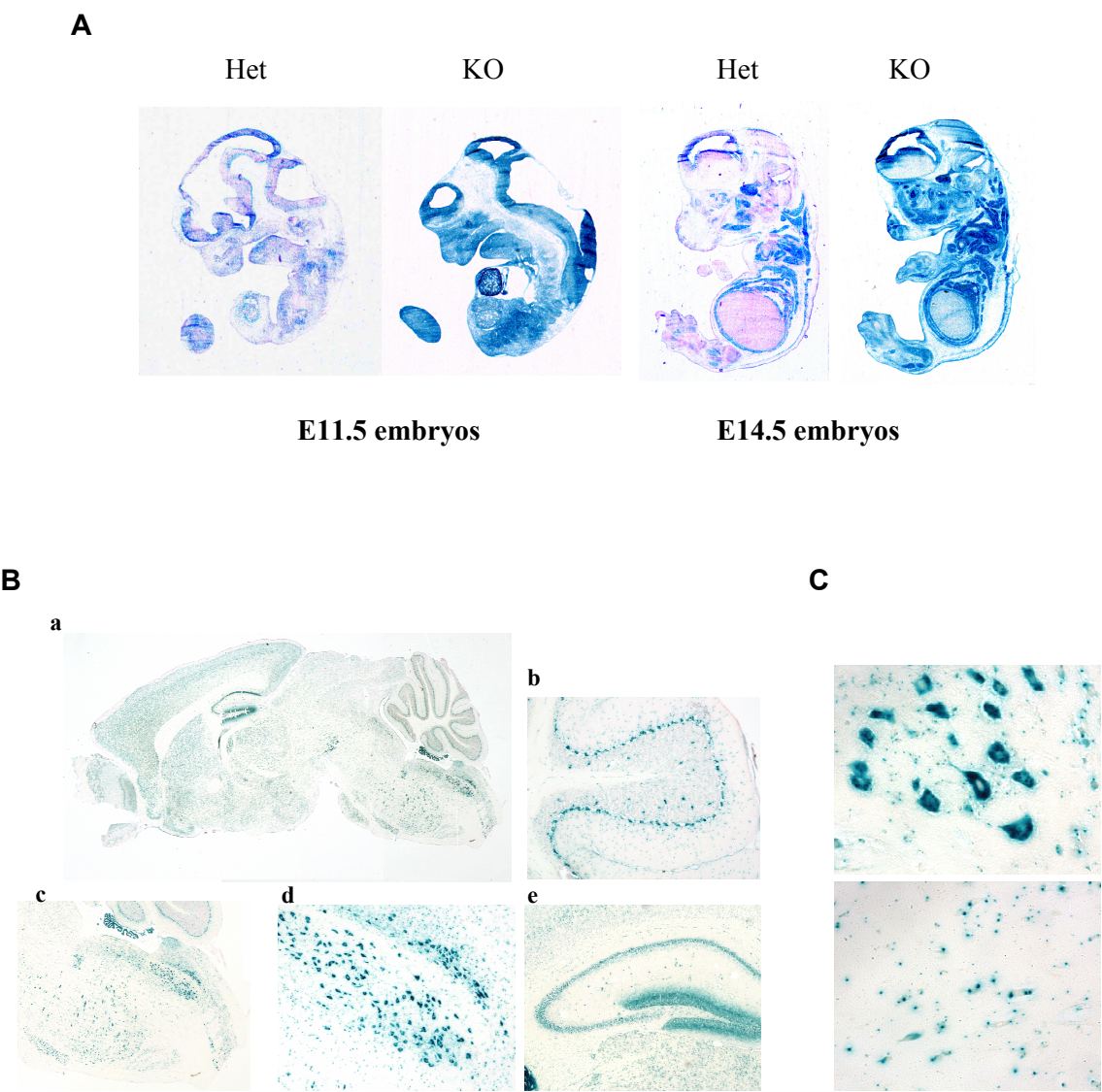


Figure 4

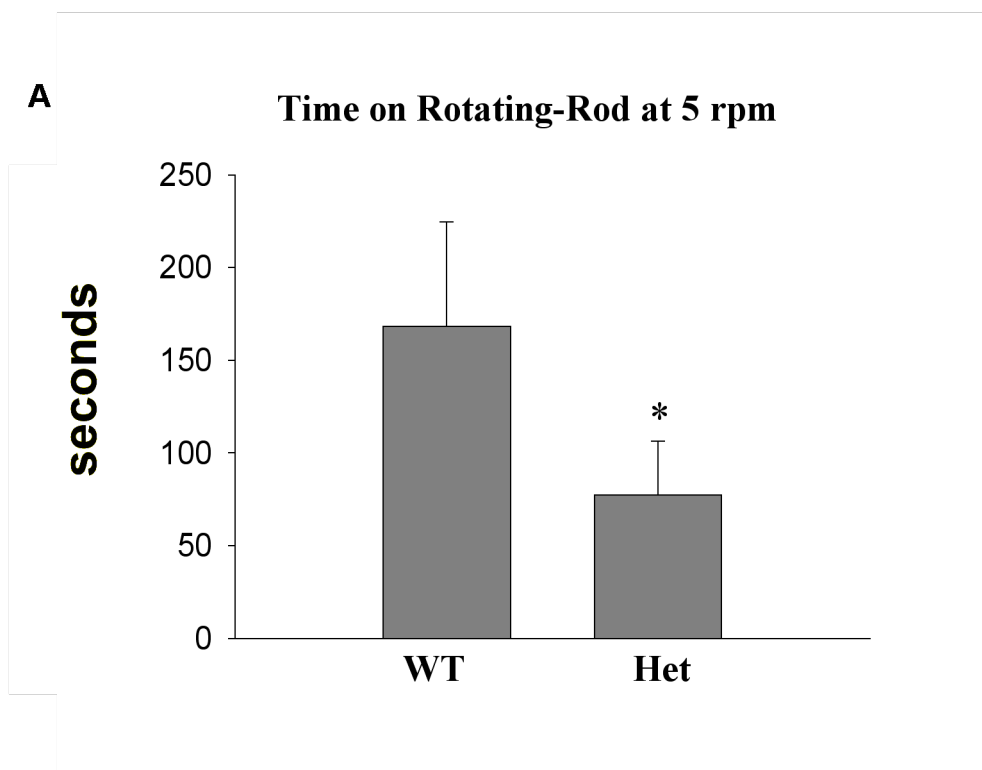


Figure 5

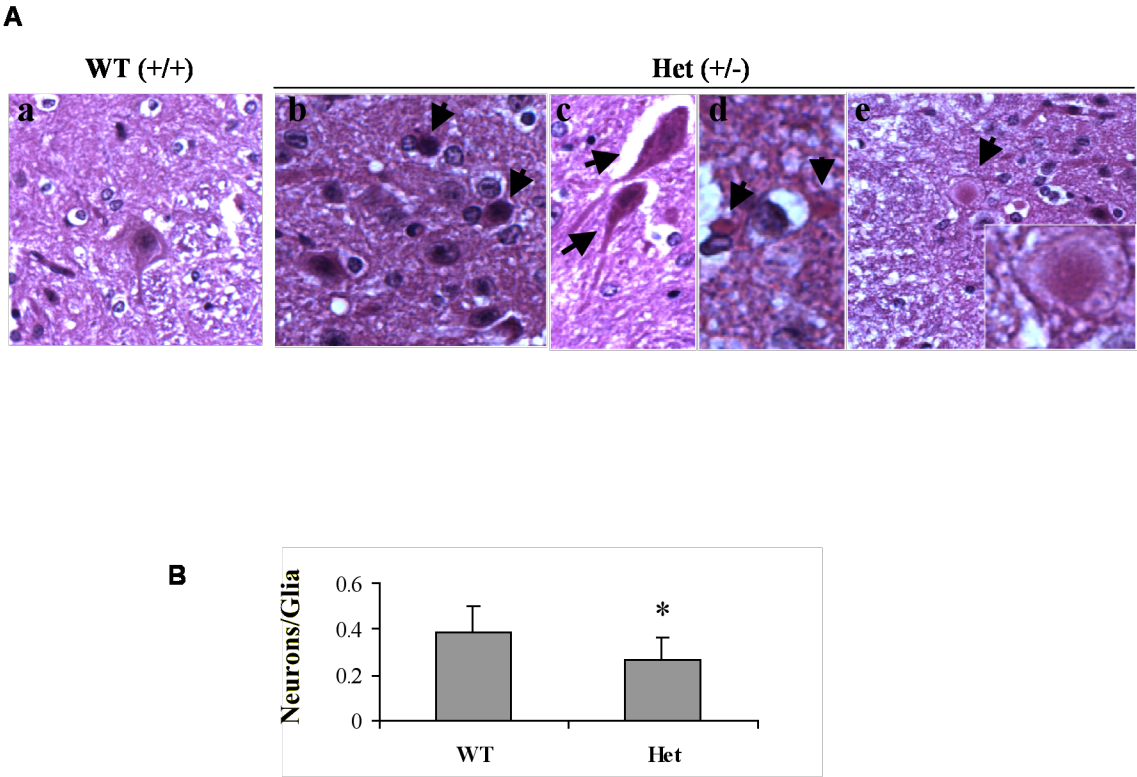


Figure 6

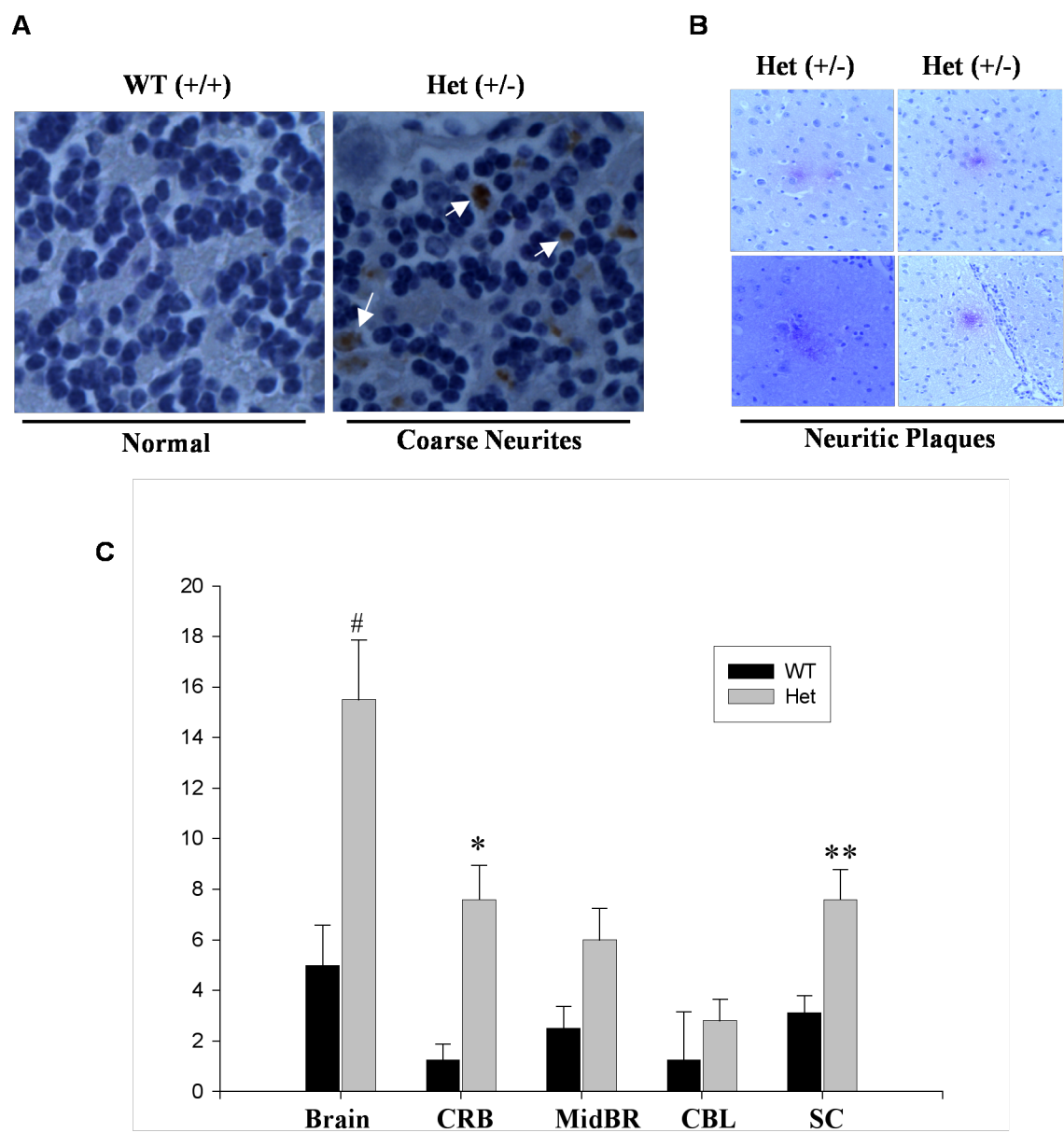
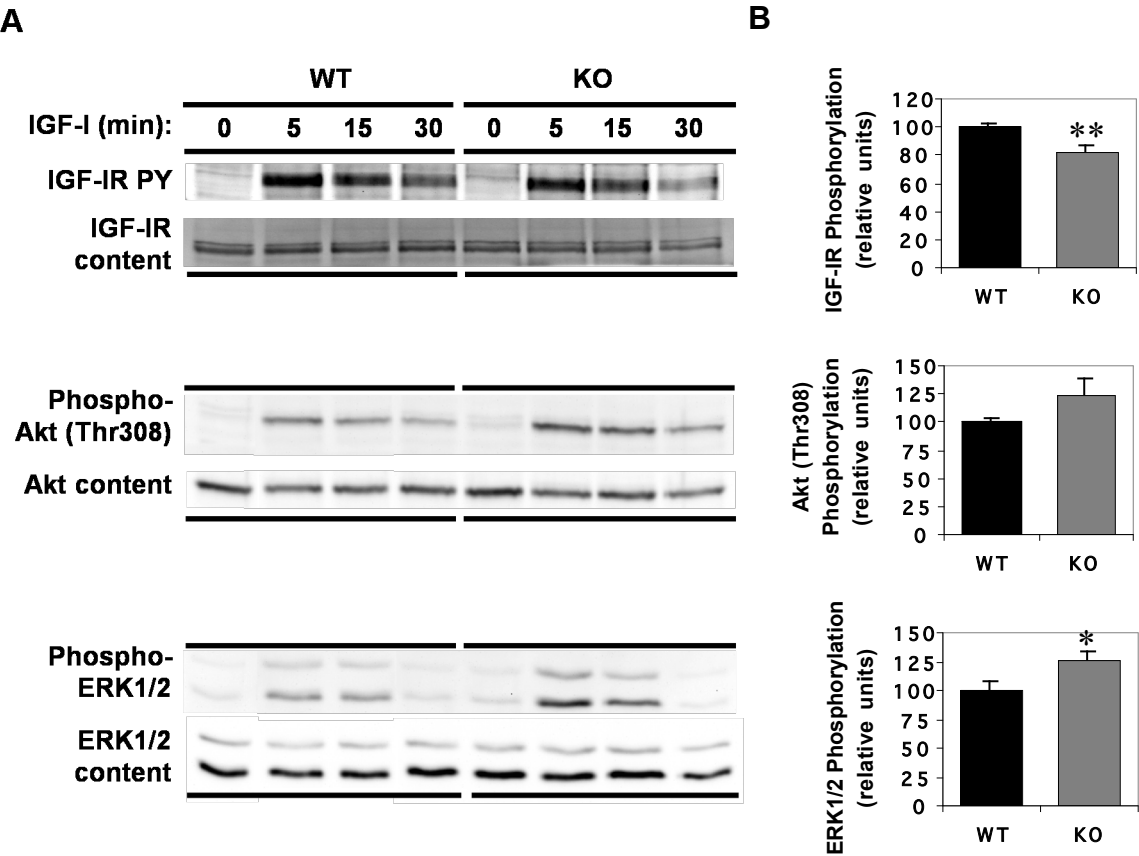


Figure 7



CHAPTER 4

The Parkinson's Disease Protein GIGYF2

Regulates Cyclin D1 and the CUL7

Ubiquitin Ligase Complex

Title: The Parkinson's disease protein GIGYF2 regulates cyclin D1 and the CUL7 ubiquitin ligase complex.

William G. Tsiaras, Barbara Giovannone and Robert J. Smith

From the Division of Endocrinology, Hallett Center, Alpert Medical School of Brown University, Rhode Island Hospital, Providence, RI, 02903, USA

Address for correspondence and reprints: Dr. Robert J. Smith, Division of Endocrinology, Alpert Medical School of Brown University, Rhode Island Hospital, One Hoppin Street, Suite 200, Providence, RI, 02903, USA. E-mail: rsmith4@lifespan.org

Phone: 401-444-3420

Fax: 401-444-4921

This chapter is a manuscript detailing biochemical analysis of *Gigyf2*^{-/-} mouse embryonic fibroblasts and GIGYF2-interacting proteins in an effort to further define the molecular function of GIGYF2. I wrote the manuscript and performed all of the experiments with the exception of the confocal microscopy in figure 6B. Barbara Giovannone generated the *Gigyf2*^{-/-} mice and cell lines, the tetracycline inducible Myc-GIGYF2 HEK-293 cells, and performed the experiments shown in figure 6B.

INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disorder affecting an estimated 1-2% of the population above the age of 60 (21, 22). The characteristic pathology of PD is loss of dopaminergic neurons in the substantia nigra and accumulation of intracellular proteinaceous inclusions called Lewy bodies (27). The molecular mechanisms responsible for this neuropathology remain largely unknown; however, advances have been made through the study of proteins genetically linked to PD. One of the most recently identified of these is the Grb10-Interacting GYF Protein-2 (GIGYF2). In humans, the *GIGYF2* gene is located on chromosome 2q37.1 in a region that shows significant linkage to autosomal dominant familial PD (PARK11 locus) (26, 31-33). Candidate gene analysis at the PARK11 locus identified mutations in *GIGYF2* in approximately 5% of patients with familial PD, and partial disruption of the *Gigyf2* gene in mice resulted in the development of age dependent motor ataxia and neurodegeneration (11, 23). These data strongly support a role for GIGYF2 in the health and survival of the central nervous system, yet little is known about the function of this protein.

GIGYF2 was originally identified in a yeast two-hybrid screen using the growth factor receptor-bound protein Grb10 as bait (10). Grb10 binds to the intracellular portion of the insulin-like growth factor (IGF-I) receptor and functions as an important suppressor of IGF-I action (9). IGF-I plays a critical role in the modulation of a number of biological processes including cell growth, proliferation, survival, migration and differentiation (40). In the central nervous system, IGF-I acts as a neurotrophic factor,

positively regulating the proliferation and differentiation of adult neural progenitor cells (16, 43). More importantly, low levels of IGF-I and/or IGF-I receptor activity have been linked to the progression of various neurodegenerative diseases and may play a specific role in the development of PD (29, 43).

Mouse embryonic fibroblasts (MEFs) derived from *Gigyl2*^{-/-} animals show altered IGF-I receptor signaling, indicating that GIGYF2 may contribute to Grb10 mediated IGF-I receptor regulation (11). Upon ligand binding, activated IGF-I receptor *trans*-autophosphorylates specific intracellular tyrosine residues, which serve as docking sites for the recruitment and subsequent phosphorylation of proximal receptor substrates. These events initiate a signaling cascade that ultimately results in the activation of the mitogen-activated protein kinases ERK1 and ERK2 and the serine/threonine kinase Akt. In response to IGF-I stimulation *Gigyl2*^{-/-} MEFs show a decrease in IGF-I receptor phosphorylation, an increase in ERK1/2 activation, and a trend towards increased Akt activation (11). ERK1/2 and Akt are critical mediators of IGF-1 action; therefore, it is likely that specific IGF-I functions are altered in GIGYF2 deficient cells.

One of the best-characterized functions of IGF-I is its ability to modulate the expression of cyclin D1 (34). In proliferating cells, cyclin D1 plays an important role in the regulation of cell cycle progression. In contrast to this, recent evidence suggests that in post-mitotic neurons, elevation of cyclin D1 and reactivation of the cell cycle results in induction of apoptosis (25). Deregulation of cyclin D1 levels may also contribute to the mechanism of neurodegeneration seen in PD. In the 6-hydroxydopamine animal model of PD, there was significant upregulation of cyclin D1 mRNA in the substantia nigra. This increase in cyclin D1 correlated with dopaminergic neuron degeneration and cell

death (13). Also, evidence of increased cyclin D1 activity has been detected in dopaminergic neurons in patients with PD (14).

The molecular function of GIGYF2 is not known, nor is it clear how mutations in the *GIGYF2* gene contribute to the development or progression of PD. The objective of this study was to investigate functional properties of the GIGYF2 protein. Because of the link between GIGYF2 and the IGF-I system, and the importance of cell cycle regulatory mechanisms in PD, we examined the expression of cyclin D1 in *Gigyf2*^{-/-} MEFs. Here we show that in the absence of GIGYF2, the level of cyclin D1 protein is reduced as a result of increased cyclin D1 protein degradation. Cyclin D1 degradation is regulated by two distinct cullin-based E3 ubiquitin ligases, SKP1-CUL1-FBX4 and SKP1-CUL7-FBXW8 (24, 30). We demonstrate that GIGYF2 forms a complex with CUL7 and that overexpression of GIGYF2 disrupts the SKP1-CUL7-FBXW8 E3 ubiquitin ligase resulting in elevated levels of cyclin D1 protein. Furthermore, we show that GIGYF2 preferentially associates with a unique CUL7 complex consisting of the tumor suppressor protein p53 and the Parkin-like cytoplasmic p53 binding protein PARC.

MATERIALS AND METHODS

Plasmids

Full-length human HA-CUL7, His-SKP1 and FLAG-FBXW8, each in pcDNA 3.0 mammalian expression vector, were provided by Daniele Guardavaccaro, New York University School of Medicine, New York, NY. N-terminal Myc (EQKLISEEDL) and C-terminal FLAG (DYKDDDDK) epitope tags were inserted into full-length human GIGYF2 cDNA sequence by PCR. Epitope tagged GIGYF2 cDNA constructs were transferred from pBluescript SK(+) (Stratagene, La Jolla, CA) into the NotI/XbaI sites of the pcDNA 3.1/Zeo(+) vector (Invitrogen, Carlsbad, CA) or into the NotI/XhoI sites of the pcDNA5/FRT/TO vector (Stratagene). To generate Myc tagged C-terminal GIGYF2 construct, full-length human GIGYF2 in pBluescript was cut with BclI/XhoI to generate a 1.9 kb C-terminal fragment of GIGYF2, which was then shuttled into the BamHI/XhoI sites of the pCMV-Tag 3a Myc tag mammalian expression vector (Stratagene). The 1.9 kb C-terminal fragment of GIGYF2 was also cloned into the BamHI site of the pSos vector (Stratagene) using BamHI linkers (New England BioLabs, Ipswich, MA) to generate an in-frame human SOS-C-terminal GIGYF2 construct for use in the yeast two-hybrid screen. The N-terminal Myc tagged GYF domain fragment of GIGYF2 was generated by PCR from the original mouse clone identified in the Grb10 yeast two-hybrid screen (10). The PCR product was cloned into the XhoI/XbaI sites of the pcDNA 3.1/Zeo(+) vector for mammalian expression. All PCR generated constructs were verified by sequencing.

Cell culture

The generation of *Gigyf2* gene disrupted mice using the gene-trap method and protocol for genotyping have been previously described (11). Animal procedures were performed according to institutional regulations of the Rhode Island Hospital animal facility (IACUC protocol #0221-06). Mouse embryonic fibroblasts (MEFs) were cultured from embryos isolated at embryonic day 12.5. All MEF experiments were performed with primary cells prior to passage 5. MEF and HEK-293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) containing 4 mM L-glutamine, 4.5 g/L glucose, 3.7 g/L sodium bicarbonate, and supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO). U-2 OS cells were maintained in McCoy's 5a medium (modified) (Invitrogen) with 1.5 mM L-glutamine, 2.2 g/L sodium bicarbonate, and supplemented with 10% FBS. Myc-GIGYF2 or GIGYF2-FLAG tetracycline (Tet) inducible HEK-293 cells were generated using the Flp-In T-REx system (Invitrogen) in accordance with the manufacturer's instructions. Briefly, full-length Myc-GIGYF2 or GIGYF2-FLAG pcDNA5/FRT/TO constructs were cotransfected with pOG44 Flp recombinase expression plasmid into Flp-In T-REx-293 cells. Stable transfectants were selected in DMEM, 10% FBS with 15 µg/ml blasticidin (Invitrogen) and 100 µg/ml hygromycin B (Sigma). Tet-regulated expression of epitope tagged GIGYF2 and GIGYF2 inclusion body formation were assessed by immunoblot and immunofluorescence following a time course of Tet induction and titration of Tet concentration (0.01-1.0 µg/ml). Cell lines were cultured in a 37°C incubator with 5% CO₂. All transfections were performed using FuGENE 6 transfection reagent (Roche, Indianapolis, IN) according to the manufacturer's instructions. Total amount of plasmid

DNA per transfection was kept constant through addition of varying amounts of pcDNA 3.1 empty vector. For proteasome inhibition, cells were treated with 25 μ M MG132 (Peptide Institute, Osaka, Japan) or DMSO for 6 hr prior to processing.

Immunoprecipitation and immunoblotting

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) before lysis. MEFs were lysed in 1% NP-40 lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM NaF, 10% glycerol, 1% NP-40). All other cell lines were lysed in NET-N buffer (20 mM Tris, pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40). Day 14.5 and 17.5 mouse embryos from timed matings were obtained by caesarean section, washed in PBS and snap frozen in liquid nitrogen. Frozen embryos were broken up with a hammer, transferred to 1% NP-40 lysis buffer and homogenized using a Polytron homogenizer. All lysis buffers were supplemented with 2 mM phenylmethylsulfonyl fluoride and 1X Protease Inhibitor Cocktail (Sigma) immediately before use. Cell and embryo extracts were clarified by centrifugation, and protein concentrations were determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Lysates were used for immunoprecipitation (1-3 mg total protein) or 50-80 μ g protein per sample (whole cell lysate) were boiled in sodium dodecyl sulfate (SDS) sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Immunoprecipitations were performed for 4-16 hr at 4°C with 2-5 μ g antibody and Protein A Agarose (Pierce, Rockford, IL) or Protein G Agarose (Upstate, Lake Placid, NY). Immunoprecipitates were washed 4 times with NET-N lysis buffer and boiled in SDS sample buffer. Whole cell lysates and immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose

membranes (PerkinElmer, Boston, MA). Membranes were blocked in 5% milk-Tris-buffered saline-Tween 20 (TBST), immunoblotted with primary antibodies in 5% bovine serum albumin (BSA) TBST and appropriate secondary antibodies conjugated to horseradish peroxidase (Jackson Immuno Research, West Grove, PA) at 1:5000 dilution in 5% BSA TBST. Immunoblots were developed using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer). Digital image acquisition and densitometry were performed using an Alpha Innotech FluorChem Imaging System (San Leandro, CA). Immunoprecipitations and immunoblotting were performed using the following antibodies: rabbit anti-GIGYF2 polyclonal antibody generated against a human C-terminal GIGYF2 peptide (amino acids 1284-1299) and purified on a peptide-affinity column, anti- β -actin (C4; Santa Cruz Biotechnology, Santa Cruz, CA), anti-CUL1 (2H4C9; Invitrogen), anti-CUL7 (A300-223A; Bethyl Laboratories, Montgomery, TX), anti-Cyclin D1 (AM29; Invitrogen), anti-Cyclin D3 (610279; BD Biosciences, San Jose, CA), anti-FLAG (M2; Sigma), anti-HA (16B12; Covance, Berkeley, CA), anti-HA (A190-108A; Bethyl Laboratories), anti-c-Myc (9E10; Roche), anti-p53 (DO-1 and FL-393; Santa Cruz Biotechnology), anti-PARC (A300-098A; Bethyl Laboratories), anti-p19 SKP1 (610530; BD Biosciences), anti- β -tubulin (TUB 2.1; Sigma) and anti- γ -tubulin (GTU-88; Sigma).

Quantitative real-time PCR

MEFs were plated at 1×10^4 cells/cm², cultured for 48 hr and harvested for RNA (60-70% confluent at time of harvest). Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's

instructions. cDNA synthesis was performed using 1 µg DNase treated total RNA, random hexamers and TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Quantitative real-time PCR reactions for specific cDNA were performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and the Applied Biosystems 7500 Fast Real-Time PCR System. Reactions were analyzed using 7500 Fast System Software v1.3.1 (Applied Biosystems). The following primers were used for real-time PCR reactions: cyclin D1 forward primer 5'-GCGTACCCTGACACC AATCTC-3'; cyclin D1 reverse primer 5'-CTCCTCTTCGCACTTCTGCTC-3'; 28S forward primer 5'-TTCACCAAGCGTTGGATTGTT-3'; 28S reverse primer 5'-TGTCTG AACCTGCGGTTTCCT-3'.

Protein half-life determination

MEFs were plated at 1×10^4 cells/cm² and cultured for 48 hr prior to the addition of cycloheximide (CHX) (Sigma). Cells were treated with CHX (100 µg/ml) and harvested at the indicated time points in 1% NP-40 lysis buffer. Whole cell lysates were analyzed by immunoblot using anti-Cyclin D1 antibody and anti-β-actin or anti-β-tubulin antibody to control for loading. Cyclin D1 levels at each time point were normalized to loading control and divided by amount at time 0 to obtain percent cyclin D1 remaining. Percent cyclin D1 remaining over time were plotted on a semi-log graph. Data were fit to exponential decay curves using non-linear regression to obtain decay constants (K) for each MEF line. Cyclin D1 half-lives were calculated using the formula: $T_{1/2} = \ln(2)/K$. All data analysis was performed using Prism 5.0 software (GraphPad Software, San Diego, CA).

Yeast two-hybrid screening

The CytoTrap two-hybrid system (Stratagene) was used to identify potential GIGYF2 interacting proteins. Briefly, pSOS-C-terminal GIGYF2 bait construct and day 9.5 FVB mouse embryo cDNA library, cloned unidirectionally into the CytoTrap pMyr XR vector, were cotransformed into the yeast *S. cerevisiae* temperature-sensitive mutant strain cdc25H. Cotransformants were selected and screened for interacting clones by galactose-dependent growth at a 37°C. pMyr library cDNA plasmids were isolated from positive interacting yeast clones, transferred to *E. coli*, amplified, extracted and sequenced. The identities of putative GIGYF2-interacting cDNAs were determined using the Basic Local Alignment Search Tool (BLAST) to search available mouse nucleotide sequences (2).

Immunofluorescence microscopy

HEK293 or Myc-GIGYF2 Tet inducible HEK-293 cells were plated on Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY) or 6 well plates. Cells were transfected and Tet induced as indicated. After incubation for 24-48 h, cells were fixed in 2% paraformaldehyde, permeabilized with 0.2% Triton-X100-PBS and blocked with 5% BSA-PBS followed by 2% normal goat serum-PBS (Sigma). Cells were incubated with the indicated primary antibodies and FITC-conjugated goat anti-rabbit IgG (1:500) or Rhodamine-conjugated goat anti-mouse IgG (1:250) secondary antibodies (Vector Laboratories, Burlingame, CA). Slides were coated with Vectashield mounting medium (Vector Laboratories) and analyzed on a Nikon Eclipse C1si confocal microscope system

(Nikon, Melville, NY). Plates were analyzed on an Olympus IX70 fluorescence microscope (Olympus, Center Valley, PA).

Statistical analysis

Paired and unpaired, two-tailed t tests were performed in Sigma Stat 3.0 software (Systat Software, San Jose, CA). Graphs represent arithmetic means $\pm$ standard error of the mean (SEM), normalized to controls as indicated for each figure. Comparison of cyclin D1 decay constants (K) between *Gigyl2*^{+/+} and *Gigyl2*^{-/-} MEFs was performed using the Extra-Sum-of-Squares F Test in Prism 5.0 software.

RESULTS

Decreased cyclin D1 protein in the absence of GIGYF2. IGF-I is an important regulator of cyclin D1 protein levels (34). We have previously shown that MEFs derived from *Gigyf2*^{-/-} mice have altered IGF-I signaling. To determine if this altered IGF-I signaling affects regulation of cyclin D1, we analyzed the level of cyclin D1 protein in *Gigyf2*^{-/-} MEFs compared to littermate matched *Gigyf2*^{+/+} controls. Immunoblots of whole cell extracts prepared from littermate matched *Gigyf2*^{+/+} and *Gigyf2*^{-/-} MEFs using an antibody specific for cyclin D1 revealed an approximately 35% decrease in the level of cyclin D1 protein in cells lacking GIGYF2 ($P < 0.001$). This appears to be specific for cyclin D1, as we did not detect a similar decrease in the level of another D-type cyclin, cyclin D3 (Fig. 1, A and B). The same result was observed in whole embryo extracts prepared from mid- (E 14.5) and late- (E 17.5) gestation embryos. *Gigyf2*^{+/+}, *Gigyf2*^{+/-} and *Gigyf2*^{-/-} embryos showed decreasing levels of cyclin D1 with a corresponding decrease in GIGYF2 protein levels across the three genotypes (Fig. 1C).

Increased cyclin D1 protein degradation in *Gigyf2*^{-/-} cells. IGF-I can regulate cyclin D1 levels through changes in both the amount of cyclin D1 mRNA and the rate of cyclin D1 protein turnover (34). To determine if the decrease in cyclin D1 protein observed in *Gigyf2*^{-/-} MEFs is due to a change in mRNA quantity, we determined the relative cyclin D1 transcript levels in *Gigyf2*^{-/-} MEFs compared to wild type controls using quantitative real-time PCR. Quantitative mRNA data from early-passage MEFs was analyzed using the comparative C_T method to obtain relative cyclin D1 mRNA

expression ranges (Fig. 2A). The results indicate that the level of cyclin D1 mRNA in *Gigyf2*^{-/-} MEFs is approximately the same as in wild type controls.

Given that the amount of cyclin D1 mRNA is not changed in *Gigyf2*^{-/-} MEFs, we hypothesized that the decrease in cyclin D1 protein in these cells is due to a change in cyclin D1 protein turnover. To address this, we determined the amount of cyclin D1 protein at various time points after addition of the translation inhibitor cycloheximide to randomly growing early-passage MEFs. The level of cyclin D1 protein at each time point was determined by immunoblot (Fig. 2B) followed by quantitative densitometry. Percent cyclin D1 remaining was calculated for all time points and data were analyzed using non-linear regression to obtain the decay constant (K) for cyclin D1 in *Gigyf2*^{+/+} and *Gigyf2*^{-/-} MEFs. Using this method, we determined that the rate of cyclin D1 turnover is significantly faster in *Gigyf2*^{-/-} MEFs compared to wild type control cells (K different, $P < 0.0005$). The half-life of cyclin D1 in *Gigyf2*^{-/-} MEFs is 41.6 minutes (95% confidence range: 35.8 – 49.5 min.) compared to a half-life of 65.8 minutes (95% confidence range: 56.2 – 79.5 min.) in *Gigyf2*^{+/+} MEFs (Fig. 2C). We conclude from these results that the decrease in cyclin D1 observed in the absence of GIGYF2 is due to an increase in cyclin D1 protein degradation.

GIGYF2 interaction with the ubiquitin-proteasome system. In mouse fibroblasts, control of cyclin D1 turnover is regulated by the Akt pathway. In these cells, activation of Akt in response to growth factor stimulation results in decreased turnover of cyclin D1 protein (8). In *Gigyf2*^{-/-} MEFs, cyclin D1 turnover is increased despite the fact that we see no change or a slight increase in IGF-I stimulated Akt activation. In light of this, we hypothesize that GIGYF2 is acting through an alternative mechanism to regulate

cyclin D1 protein degradation. One possibility is that GIGYF2 exerts its effects by interacting directly with proteins involved in the targeted degradation of cyclin D1. To test this hypothesis, we used yeast two-hybrid cloning to identify potential GIGYF2 interacting proteins.

Significant overexpression of full-length GIGYF2 results in protein aggregation and cytotoxic inclusion body formation in the cytoplasm of cells. The mechanism of GIGYF2 aggregation and the protein domains responsible for this behavior are not known (10). The largest fragment of GIGYF2, which can be expressed at high levels and does not form toxic inclusion bodies, is a 625 amino acid fragment in the C-terminus of the protein (data not shown). This construct is approximately the full C-terminal half of GIGYF2 from amino acid 674 to the C-terminal end at amino acid 1299 (amino acid numbers are based on human GIGYF2 sequence). Analysis of primary amino acid sequence revealed that this portion of GIGYF2 contains a predicted bipartite nuclear localization sequence, coiled-coil domain and several glutamine and glutamic acid repeats of varying length. This GIGYF2 fragment was cloned into the pSOS vector of the CytoTrap yeast two-hybrid system and used to screen a day 9.5-mouse embryo cDNA expression library (5). Twelve independent GIGYF2-interactive clones were identified from the embryonic cDNA library. The 12 clones were sequenced and 9 were found to contain open reading frames and the remaining consisted of only 3' UTR sequence. One of these clones contained the complete coding sequence of mouse S-phase kinase-associated protein 1A (SKP1). The identification of SKP1 as a potential GIGYF2-interacting protein was of particular interest because of SKP1's role in targeted protein degradation as a component of specific E3 ubiquitin ligase complexes (6).

E3 enzyme complexes mediate transfer of the small soluble protein ubiquitin to target substrates. Polyubiquitylation of proteins results in their association with and degradation by the 26S proteasome whereas mono- or diubiquitylation appears to have a regulatory, non-proteolytic effect. In mammals, SKP1 containing complexes consist of a cullin scaffold, either CUL1 or CUL7, an F-box protein and the small RING-finger protein RBX1. The F-box component of the complex uses distinct protein-protein interaction domains to provide substrate specificity while the RBX1 subunit facilitates ubiquitylation through the recruitment of E2 ubiquitin-conjugating enzymes (6). Recently, the F-box proteins FBX4 and FBXW8 in complex with SKP1 and either CUL1 or CUL7 respectively, were identified as mediators of cyclin D1 ubiquitylation and degradation (24, 30). Interaction with one or both of these complexes represents a potential mechanism for GIGYF2 effects on cyclin D1 protein turnover.

To efficiently test for interaction between GIGYF2 and CUL1 or CUL7 based complexes, we performed co-immunoprecipitation experiments using transient overexpression of epitope-tagged constructs. Because significant overexpression of GIGYF2 results in cytotoxic inclusion body formation, we developed a tetracycline (Tet) inducible system in HEK-293 cells to allow controlled expression of an N-terminal Myc- or C-terminal FLAG-epitope-tagged full-length GIGYF2 protein. Transient induction (12 - 48 hours) of epitope-tagged full-length GIGYF2 with low concentrations of Tet yields detectable levels of soluble GIGYF2 with no evidence of inclusion body formation (see Fig. 6). To test for protein-protein interaction, His-tagged SKP1 and HA-tagged CUL1 or CUL7 constructs were transiently transfected into the full-length Myc-GIGYF2 Tet inducible HEK-293 cells. Following 12 hour Tet induction, interaction was assessed

by immunoprecipitation with anti-Myc antibody and immunoblotting with either anti-SKP1 or anti-HA antibodies. Immunoprecipitation of Myc-GIGYF2 revealed co-precipitation of HA-CUL7 (Fig. 3). Similar results were obtained with the C-terminal FLAG-GIGYF2 Tet inducible system (data not shown). Under these same conditions, we were unable to detect co-precipitation of CUL1 and GIGYF2 (see Fig. 5; also data not shown). Surprisingly, neither endogenous nor overexpressed SKP1 co-precipitated with the GIGYF2-CUL7 complex (Fig. 3).

Characterization of the GIGYF2-CUL7 complex. As noted earlier, CUL7 binds to FBXW8, SKP1 and RBX1 to form an E3 ubiquitin ligase that can target cyclin D1 for ubiquitin dependent degradation (7, 30). CUL7 has also been shown to form a second, unique E3 ubiquitin ligase complex through association with RBX1, the tumor suppressor protein p53 and the parkin-like cytoplasmic p53 binding protein (PARC). The formation of these two CUL7-based complexes appears to be mutually exclusive (38, 39).

The absence of SKP1 in GIGYF2-CUL7 co-immunoprecipitations suggests that GIGYF2 may be differentially associating with CUL7-based complexes. To better characterize this, we performed co-immunoprecipitation experiments with endogenous GIGYF2, both endogenous and overexpressed CUL7, and endogenous p53. In non-Tet inducible HEK-293 cells, immunoprecipitation of GIGYF2 using a GIGYF2 specific antibody co-precipitated endogenous CUL7 and p53. Immunoprecipitation of either overexpressed CUL7 or endogenous p53 confirmed that GIGYF2, CUL7 and p53 can form a complex in HEK-293 cells and that association is not dependent on transient overexpression of the components (Fig. 4A). We further confirmed GIGYF2-CUL7-p53 complex formation through co-immunoprecipitation experiments in a different human

cell line, the U-2 OS osteosarcoma cell. U-2 OS cells have been used extensively for analysis of CUL7-based complexes (3, 4, 17, 18, 38). In this cell type, association between GIGYF2 and p53 appeared to be dependent on CUL7 as co-precipitation of p53 with GIGYF2 was detected above background only after overexpression of CUL7 (Fig. 4B).

We next determined if PARC is also a component of the GIGYF2-CUL7 complex. In the Myc-GIGYF2 Tet inducible HEK-293 cells, immunoprecipitation of GIGYF2 with an anti-Myc antibody co-precipitated overexpressed CUL7 as well as endogenous p53 and PARC. As noted earlier, this GIGYF2 complex is specific for CUL7, as we did not detect CUL1 in any of the co-precipitates (Fig. 5A). Again, we observed that SKP1 was consistently absent from this GIGYF2 complex. Furthermore, immunoprecipitation of the SKP1-CUL7-FBXW8 complex using an antibody against overexpressed FBXW8 failed to co-precipitate Myc-GIGYF2 (Fig. 5B). These data indicate that GIGYF2 preferentially associates with the CUL7-PARC-p53 complex and not with SKP1-CUL7-FBXW8.

Many of the cullin family members localize to both nuclear and cytoplasmic compartments of the cell. CUL7 and PARC, however, are found exclusively in the cytoplasm and association between PARC and p53 has been shown to stabilize cytoplasmic p53 localization (3, 28). Based on the localization of both CUL7 and PARC, we suspect that GIGYF2 forms a complex with these proteins in the cytoplasm. To confirm this, we assessed co-localization of CUL7 and GIGYF2 by indirect immunofluorescence in the Myc-GIGYF2 Tet inducible HEK-293 cells. As expected, after 24 hours of low concentration Tet induction, Myc-GIGYF2 and transiently

transfected HA-CUL7 appeared to co-localize in the cytoplasm. Diffuse cytoplasmic co-localization was seen in all cells with detectable expression of both constructs. In a number of cells, GIGYF2 and CUL7 appeared to concentrate into single discreet perinuclear foci (Fig. 6A). Analysis of endogenous GIGYF2 localization in HEK-293 cells using a GIGYF2 specific antibody revealed that these perinuclear foci co-localized with the centrosome marker γ -tubulin (Fig. 6B). These results indicate that GIGYF2-CUL7 complex formation occurs diffusely in the cytoplasm with potential for additional association of the two proteins at or near the centrosome.

To further characterize GIGYF2-CUL7 complex formation we sought to identify the region(s) of GIGYF2 that are sufficient for CUL7 interaction. There are two regions within GIGYF2 that have established protein-protein interaction capacity. The first is the C-terminal half of the protein, which has been shown in this study to have the ability to form protein-protein interactions in yeast. The second is the GYF domain in the N-terminus of the protein. The GYF domain is a small adaptor domain that has the capacity to recognize and associate with proline-rich sequences either inter- or intramolecularly (19). The GYF domain of GIGYF2 is necessary and sufficient for the interaction between GIGYF2 and Grb10 (9, 10). To test the contribution of these GIGYF2 domains to CUL7 interaction we generated Myc-tagged C-terminal (Myc-674-1299) and GYF (Myc-494-622) GIGYF2 mammalian expression constructs. Both the GYF domain and the C-terminus of GIGYF2 can be transiently transfected into cells to yield overexpression without inclusion body formation (Fig. 7; also data not shown). CUL7 and GIGYF2 fragments were transiently transfected into HEK-293 cells and interaction was assessed by immunoprecipitation with an anti-Myc antibody and immunoblotting

with an anti-CUL7 antibody. Immunoprecipitation of the Myc-C-terminal GIGYF2 construct revealed co-precipitation of CUL7. We did not detect interaction between CUL7 and the GYF fragment of GIGYF2. However, we consistently failed to obtain overexpression levels of this construct that were comparable to those seen with the C-terminal construct (Fig. 7). Therefore, we conclude that the C-terminal half of GIGYF2 is sufficient for CUL7 interaction, but we cannot rule out a contribution of the GYF domain or other regions of GIGYF2 to complex formation.

Regulation of CUL7 complex formation and cyclin D1 protein levels by GIGYF2. Two possible outcomes of GIGYF2-CUL7 interaction are regulation of CUL7 function or targeting of GIGYF2 for ubiquitylation and proteasome degradation. Formation of a GIGYF2-CUL7-PARC-p53 complex that excludes SKP1 indicates that GIGYF2 is unlikely to be a substrate of the SKP1-CUL7-FBXW8 E3 ubiquitin ligase. All CUL7-based complexes that have been identified exhibit ubiquitin ligase activity *in vitro* (39). Therefore, GIGYF2 could still be a substrate of the CUL7-PARC-p53 complex. Arguing against this, we found that overexpression of CUL7 alone or in combination with p53 did not decrease the level of GIGYF2 protein, inhibition of proteasome activity with MG132 did not increase the level of GIGYF2 protein and inhibition of protein synthesis using cycloheximide revealed that the GIGYF2 protein is not rapidly turned over (Fig. 4, A and B; Fig. 8, A-D; also data not shown). Instead we hypothesize that GIGYF2 regulates CUL7 function by interfering with the formation of the SKP1-CUL7-FBXW8 E3 ubiquitin ligase complex. This hypothesis is consistent with the observation that in cells deficient in GIGYF2 there is increased turnover of the SKP1-CUL7-FBXW8 E3 substrate cyclin D1. The hypothesis also predicts that

increasing the level of GIGYF2 protein in the cell should [1] disrupt CUL7 interaction with SKP1 and FBXW8 and [2] interfere with the ability of this complex to target cyclin D1 for degradation resulting in an increase in the level of cyclin D1.

To test these predictions we first assessed the ability of CUL7 to co-immunoprecipitate SKP1 and FBXW8 in the presence of overexpressed CUL7-interacting C-terminal GIGYF2 fragment. Indeed, after C-terminal GIGYF2 overexpression, the amount of FBXW8 that co-precipitated with CUL7 was reduced by 23% ($P < 0.005$) and the amount of SKP1 that co-precipitated with CUL7 was reduced by 37% ($P < 0.001$) (Fig. 9, A and B). It has been reported, that the stability of FBXW8 protein is dependent on the co-expression of CUL7 (4, 44). We confirmed this observation in the present experiment. In the absence of co-overexpression of CUL7 the level of transiently transfected FBXW8 was always reduced when compared to cells transfected with both CUL7 and FBXW8 (Fig. 9A).

Our second prediction is that disruption of SKP1-CUL7-FBXW8 complex formation by GIGYF2 should result in an increase in the level of cyclin D1 protein. To test this, the level of GIGYF2 was increased in Myc-GIGYF2 Tet inducible HEK-293 cells with increasing amounts of Tet and the level of cyclin D1 was assessed by immunoblot. Induction of Myc-GIGYF2 with 0.05 or 0.1 $\mu\text{g/ml}$ Tet for 24 hours resulted in a 2 to 2.5-fold increase in GIGYF2 expression over endogenous (Fig. 10A, see GIGYF2 Blot). This level of overexpression does not result in cytotoxic inclusion body formation (See Fig. 6; also data not shown). As predicted, increasing the level of GIGYF2 resulted in a dose dependent increase in cyclin D1 protein (Fig. 10A). This effect was most consistent at the higher level of Tet induction. Quantification of cyclin

D1 protein amount by densitometry following 0.1 µg/ml Tet induction of GIGYF2 showed an approximately 2-fold increase in the level of cyclin D1 as compared to 0 µg/ml Tet treated cells ($P < 0.05$) (Fig. 10B).

DISCUSSION

The precise etiology of PD is unknown, but it is clear that genetic factors play a role in the development of the disease. To date, thirteen genetic loci (PARK1-13) have been linked to Mendelian forms of PD (42). For a number of these loci, the specific gene product linked to PD susceptibility has been identified. Analysis of these proteins has yielded important information about mechanisms of neurodegeneration seen in both familial as well as in the more common sporadic form of PD. Recently, we provided evidence linking the GIGYF2 protein to neurodegenerative disease. Mutation of the *GIGYF2* gene at the PARK11 locus is a potentially frequent cause of familial PD, and partial loss of this gene in mice results in an age-dependent neurodegenerative phenotype (11, 23). In this study, through characterization of *Gigyf2*^{-/-} cells and biochemical analysis of GIGYF2 interacting proteins, we have provided the first evidence for a potential molecular function of the GIGYF2 protein.

GIGYF2 and the homologous protein, GIGYF1, were originally cloned from mouse cDNA expression libraries by yeast two-hybrid screening using the Grb10 adaptor protein as bait. Grb10 is a physiologically important regulator of IGF-I receptor action and both GIGYF1 and GIGYF2 may participate in Grb10 regulation of the IGF-I system. We previously reported that MEFs derived from *Gigyf2*^{-/-} animals showed a decrease in IGF-I stimulated receptor phosphorylation, an increase in ERK1/2 activation and a trend towards increased Akt activation. Upon further examination, we found that the level of the cell cycle regulatory protein cyclin D1 was reduced in *Gigyf2*^{-/-} MEFs compared to controls. While significant, the magnitude of this effect was relatively mild, with the

average decrease in cyclin D1 protein being roughly 35% below wild type. This may be due to the fact that GIGYF1 is expressed in these cells and could potentially be compensating for the loss of GIGYF2 function. The two proteins have an overall sequence similarity of approximately 40% but show extensive homology within specific domains. It is also possible that the effect on cyclin D1 levels is influenced by residual expression of GIGYF2. *Gigyf2*^{-/-} mice were generated using the gene trap method (11, 41). Integration of the gene trap construct into a target allele has previously been shown to effectively eliminate expression of wild type mRNA from >96% of trapped genes (45). However, some investigators have documented that expression of a small amount of full length mRNA (1-10% of wild type) can occur from gene-trapped alleles (44, 45). Immunoblots of *Gigyf2*^{-/-} MEF and embryo extracts using a GIGYF2 specific antibody consistently showed faint but detectable protein expression (see Fig. 1C; also data not shown) and northern blot analysis of *Gigyf2*^{-/-} MEFs revealed a small amount of wild type GIGYF2 mRNA expression (11). Thus, it is possible that the amount of GIGYF2 protein that is still expressed in *Gigyf2*^{-/-} animals is sufficient to temper the effect on cyclin D1 levels.

Mitogens like IGF-I stimulate cell cycle progression in part by increasing the amount of cyclin D1 protein. Despite the fact that *Gigyf2*^{-/-} MEFs have lower levels of cyclin D1, we did not detect any proliferative abnormalities in these cells (data not shown). This is not entirely surprising considering that the level of another D-type cyclin, cyclin D3 was unaffected in *Gigyf2*^{-/-} MEFs. There is considerable redundancy between the D-type cyclins and thus preservation of cyclin D3 levels may adequately compensate for the observed decrease in cyclin D1 (36). It has also been shown that

MEFs that lack all three D-type cyclins have only mild proliferative defects (20).

Therefore, the relatively modest decrease in cyclin D1 levels that we observed would not be expected to have a significant impact on the cell cycle.

Cyclin D1 levels are regulated through changes in gene transcription, mRNA translation and stability, and protein degradation (34). Here we showed that in the absence of GIGYF2 there was a statistically significant decrease in cyclin D1 protein half-life with no change in cyclin D1 mRNA, indicating that the reduced cyclin D1 level in these cells was due to an increase in protein degradation. Turnover of cyclin D1 protein is highly regulated and involves both ubiquitin-proteasome dependent and independent mechanisms (1). Using the C-terminus of GIGYF2 as bait in a yeast two-hybrid screen we identified SKP1 as a potential GIGYF2-interacting protein. SKP1 is an important component of specific E3 ubiquitin ligases. Two different SKP1-containing complexes, SKP1-CUL1-FBX4 and SKP1-CUL7-FBXW8, are capable of targeting cyclin D1 for polyubiquitylation and 26S proteasome dependent degradation. Through co-immunoprecipitation analysis we found that GIGYF2 formed a complex with CUL7 but that this GIGYF2-CUL7 complex did not contain SKP1. Thus far, we have been unable to confirm GIGYF2-SKP1 interaction in mammalian cells. It is possible that GIGYF2 interacts with SKP1 in a cell type or tissue specific manner or that GIGYF2-SKP1 interaction occurs under specific, unidentified physiologic conditions. Alternatively, GIGYF2-SKP1 interaction in the yeast two-hybrid screen could have occurred through complex formation with the endogenous yeast cullin Cdc53. While ultimately leading to the identification of the GIGYF2-CUL7 interaction, the significance of our yeast two-hybrid result is at this point not clear.

Previous reports indicate that CUL7 is capable of forming two distinct and mutually exclusive complexes *in vivo*. CUL7 forms a classic cullin-based E3 ubiquitin ligase by interacting with SKP1, FBXW8 and RBX1 and a more unique cullin-based complex through interaction with PARC, p53 and RBX1. The SKP1-CUL7-FBXW8 complex has been shown to target cyclin D1 for ubiquitylation and degradation through direct interaction between FBXW8 and phosphorylated cyclin D1. Here we showed that overexpression of a CUL7-interacting fragment of GIGYF2 disrupted or inhibited formation of the SKP1-CUL7-FBXW8 complex, suggesting a potential mechanism for GIGYF2 regulation of cyclin D1 levels. It is possible that GIGYF2 physically displaces SKP1-FBXW8 from CUL7 through competition for the same binding site. Alternatively, GIGYF2 may promote and/or stabilize formation of the CUL7-PARC-p53 complex, which has previously been shown to exclude SKP1 and FBXW8. The region of CUL7 responsible for FBXW8 and SKP1 association has been localized and it remains to be determined whether the CUL7-GIGYF2 interaction motif overlaps with this.

Our data indicate that GIGYF2 preferentially associates with the CUL7-PARC-p53 complex and that interaction likely occurs in the cytoplasm either diffusely or near the centrosome. The CUL7-PARC-p53 complex demonstrates ubiquitin ligase activity *in vitro*, although substrates of this complex have not yet been identified. We did not find any evidence to suggest that GIGYF2 is targeted for degradation by this CUL7-based complex. Instead, we found that GIGYF2 was quite stable with no change in the level of the protein up to twelve hours following addition of the translation inhibitor cycloheximide. The stability of GIGYF2 may in fact be a contributing factor in its propensity to form aggregates and large intracellular inclusion bodies when

overexpressed. We cannot rule out that under specific conditions, the CUL7-PARC-p53 complex does indeed target GIGYF2 for polyubiquitylation and degradation. However, it is also possible that this complex facilitates mono- or diubiquitylation of GIGYF2 resulting in regulatory, non-proteolytic effects. Interpretation of our data is complicated by the fact that a clear function for the CUL7-PARC-p53 complex has not yet been determined. Both CUL7 and PARC can bind directly to p53 and a number of studies have indicated that the two proteins are capable of inhibiting p53 (3, 15, 18). However, cells derived from mice in which the genes for either CUL7 or PARC have been disrupted do not have any apparent defects in p53 function (17, 38). In future experiments, it will be important to determine what role GIGYF2 plays in the CUL7-PARC-p53 complex and specifically what effect GIGYF2 has on p53 activity.

Currently, there is no genetic evidence linking CUL7 to PD or other neurodegenerative diseases. However, mutations in the *CUL7* gene have been identified in the human 3-M syndrome. This is an autosomal recessive disorder characterized by pre- and postnatal growth retardation with otherwise normal endocrine function (12). Consistent with this, disruption of *Cul7* or *Fbxw8* genes in mice results in growth failure and cell proliferation defects. Furthermore, loss of either CUL7 or FBXW8 in mice is associated with decreased IGF-I receptor tyrosine phosphorylation, and CUL7 deficient MEFs show increased IGF-I stimulated Akt and ERK1/2 activation (4, 35, 44). Thus, both GIGYF2 and CUL7 appear to be connected to the IGF-I system, and defects in IGF-I signaling seen in CUL7 deficient cells mirror those seen in cells lacking GIGYF2. While no growth defects were observed in *Gigyf2*^{-/-} embryos, both GIGYF2 and CUL7

are expressed in the brain and may function together to regulate IGF-I actions in the central nervous system.

Seven *GIGYF2* missense mutations resulting in single amino acid substitutions were found in patients with familial PD (23). Six of these single amino acid substitutions are located in the N-terminus of GIGYF2 (Asn56Ser, Thr112Ala, Ile278Val, Ser335Thr, Asn457Thr and Asp606Glu). Because the C-terminal half of GIGYF2, from amino acid 674 to 1299, was found to be sufficient for CUL7 interaction, it is not expected that these 6 mutations would directly interfere with GIGYF2-CUL7 association. However, they may indirectly affect GIGYF2-CUL7 interaction through changes in GIGYF2 localization or protein folding and stability. The remaining single amino acid substitution (Val1242Ile) is located close to the C-terminal end of GIGYF2 and therefore could impact direct interaction between GIGYF2 and CUL7. It is also possible that the mutations in *GIGYF2* and resulting amino acid substitutions affect the capacity of GIGYF2 to disrupt SKP1-FBXW8 binding to CUL7 or GIGYF2's ability to interact with the CUL7-PARC-p53 complex. Further studies will be required to determine if and how these *GIGYF2* mutations influence CUL7 function and cyclin D1 degradation.

Deregulation of cyclin D1 contributes to the induction of neuronal apoptosis in neurodegenerative disease. In addition to this, despite considerable redundancy between the D-type cyclins, mice in which only cyclin D1 expression has been disrupted display neurologic abnormalities (37). Thus, maintenance of appropriate cyclin D1 levels appears to be an important component of normal function and survival of the central nervous system. Therefore, it is conceivable, that altered GIGYF2 function and subsequent loss of regulated cyclin D1 proteolysis plays a role in the development or

progression of PD. *GIGYF2* mutations identified thus far appear to be autosomal dominant and *Gigyf2*^{+/-} mice develop age dependent neurodegeneration (11, 23). These data indicate that *GIGYF2* mutations in familial PD result in loss of GIGYF2 function and would, based on our model, lead to a decrease in cyclin D1 levels. In post-mitotic neurons, decreased cyclin D1 may protect cells from induction of apoptosis. On the other hand, it would also impact the proliferation and differentiation capacity of neural progenitor cells. The role of neurogenesis in PD has not been clearly established. However, defects in progenitor cell proliferation in the primary sites of adult neurogenesis have been observed in both animal models of PD and in the human disease (46). Further studies are required to determine precisely how the reduction of cyclin D1 levels in *Gigyf2* gene disrupted mice contributes to the neurodegenerative phenotype observed in these animals.

In conclusion, we have shown that GIGYF2 modulates cyclin D1 levels through changes in the rate of cyclin D1 degradation. GIGYF2 has the capacity to disrupt SKP1-CUL7-FBXW8 complex formation, providing potential mechanisms for regulation of both cyclin D1 levels and IGF-I receptor signaling. Furthermore, interaction between GIGYF2 and CUL7-PARC-p53 suggests that GIGYF2 might participate in the function of this unique cullin complex. Disruption of these functions represents a potential mechanism by which mutations in the *GIGYF2* gene result in PD. Further work is needed to clarify the role that GIGYF2 plays in the development or progression of PD. It is anticipated that this work will lead to valuable insights into the mechanism of neurodegeneration in PD and to new therapies for the treatment of this important disease.

REFERENCES

1. **Alao, J. P.** 2007. The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. *Mol Cancer* **6**:24.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. *J Mol Biol* **215**:403-410.
3. **Andrews, P., Y. J. He, and Y. Xiong.** 2006. Cytoplasmic localized ubiquitin ligase cullin 7 binds to p53 and promotes cell growth by antagonizing p53 function. *Oncogene* **25**:4534-4548.
4. **Arai, T., J. S. Kasper, J. R. Skaar, S. H. Ali, C. Takahashi, and J. A. DeCaprio.** 2003. Targeted disruption of p185/Cul7 gene results in abnormal vascular morphogenesis. *Proc Natl Acad Sci U S A* **100**:9855-9860.
5. **Aronheim, A., E. Zandi, H. Hennemann, S. J. Elledge, and M. Karin.** 1997. Isolation of an AP-1 repressor by a novel method for detecting protein-protein interactions. *Mol Cell Biol* **17**:3094-3102.
6. **Cardozo, T., and M. Pagano.** 2004. The SCF ubiquitin ligase: insights into a molecular machine. *Nat Rev Mol Cell Biol* **5**:739-751.
7. **Dias, D. C., G. Dolios, R. Wang, and Z. Q. Pan.** 2002. CUL7: A DOC domain-containing cullin selectively binds Skp1.Fbx29 to form an SCF-like complex. *Proc Natl Acad Sci U S A* **99**:16601-16606.
8. **Diehl, J. A., M. Cheng, M. F. Roussel, and C. J. Sherr.** 1998. Glycogen synthase kinase-3 β regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev* **12**:3499-3511.

9. **Dufresne, A. M., and R. J. Smith.** 2005. The adapter protein GRB10 is an endogenous negative regulator of insulin-like growth factor signaling. *Endocrinology* **146**:4399-4409.
10. **Giovannone, B., E. Lee, L. Laviola, F. Giorgino, K. A. Cleveland, and R. J. Smith.** 2003. Two novel proteins that are linked to insulin-like growth factor (IGF-I) receptors by the Grb10 adapter and modulate IGF-I signaling. *J Biol Chem* **278**:31564-31573.
11. **Giovannone, B., W. G. Tsiaras, S. de la Monte, J. Klysik, C. Lautier, S. Goldwurm, and R. J. Smith.** Partial Loss of the *GIGYF2* (PARK11) Parkinson's Disease Gene in Mice Results in Age-Dependent Neurodegeneration. Manuscript in preparation
12. **Huber, C., D. Dias-Santagata, A. Glaser, J. O'Sullivan, R. Brauner, K. Wu, X. Xu, K. Pearce, R. Wang, M. L. Uzielli, N. Dagoneau, W. Chemaitilly, A. Superti-Furga, H. Dos Santos, A. Megarbane, G. Morin, G. Gillessen-Kaesbach, R. Hennekam, I. Van der Burgt, G. C. Black, P. E. Clayton, A. Read, M. Le Merrer, P. J. Scambler, A. Munnich, Z. Q. Pan, R. Winter, and V. Cormier-Daire.** 2005. Identification of mutations in CUL7 in 3-M syndrome. *Nat Genet* **37**:1119-1124.
13. **Iwata, S., M. Nomoto, H. Morioka, and A. Miyata.** 2004. Gene expression profiling in the midbrain of striatal 6-hydroxydopamine-injected mice. *Synapse* **51**:279-286.

14. **Jordan-Sciutto, K. L., R. Dorsey, E. M. Chalovich, R. R. Hammond, and C. L. Achim.** 2003. Expression patterns of retinoblastoma protein in Parkinson disease. *J Neuropathol Exp Neurol* **62**:68-74.
15. **Jung, P., B. Verdoodt, A. Bailey, J. R. r. Yates, A. Menssen, and H. Hermeking.** 2007. Induction of cullin 7 by DNA damage attenuates p53 function. *Proc Natl Acad Sci U S A* **104**:11388-11393.
16. **Kalluri, H. S., R. Vemuganti, and R. J. Dempsey.** 2007. Mechanism of insulin-like growth factor I-mediated proliferation of adult neural progenitor cells: role of Akt. *Eur J Neurosci* **25**:1041-1048.
17. **Kasper, J. S., T. Arai, and J. A. DeCaprio.** 2006. A novel p53-binding domain in CUL7. *Biochem Biophys Res Commun* **348**:132-138.
18. **Kim, S. S., M. Shago, L. Kaustov, P. C. Boutros, J. W. Clendening, Y. Sheng, G. A. Trentin, D. Barsyte-Lovejoy, D. Y. Mao, R. Kay, I. Jurisica, C. H. Arrowsmith, and L. Z. Penn.** 2007. CUL7 is a novel antiapoptotic oncogene. *Cancer Res* **67**:9616-9622.
19. **Kofler, M. M., and C. Freund.** 2006. The GYF domain. *FEBS J* **273**:245-256.
20. **Kozar, K., M. A. Ciemerych, V. I. Rebel, H. Shigematsu, A. Zagozdzon, E. Sicinska, Y. Geng, Q. Yu, S. Bhattacharya, R. T. Bronson, K. Akashi, and P. Sicinski.** 2004. Mouse development and cell proliferation in the absence of D-cyclins. *Cell* **118**:477-491.
21. **Lang, A. E., and A. M. Lozano.** 1998. Parkinson's disease. Second of two parts. *N Engl J Med* **339**:1130-1143.

22. **Lang, A. E., and A. M. Lozano.** 1998. Parkinson's disease. First of two parts. *N Engl J Med* **339**:1044-1053.
23. **Lautier, C., S. Goldwurm, A. Durr, B. Giovannone, W. G. Tsiaras, G. Pezzoli, A. Brice, and R. J. Smith.** 2008. Mutations in the GIGYF2 (TNRC15) Gene at the PARK11 Locus in Familial Parkinson Disease. *Am J Hum Genet*
doi:10.1016/j.ajhg.2008.01.015
24. **Lin, D. I., O. Barbash, K. G. Kumar, J. D. Weber, J. W. Harper, A. J. Klein-Szanto, A. Rustgi, S. Y. Fuchs, and J. A. Diehl.** 2006. Phosphorylation-dependent ubiquitination of cyclin D1 by the SCF(FBX4-alphaB crystallin) complex. *Mol Cell* **24**:355-366.
25. **Liu, D. X., and L. A. Greene.** 2001. Neuronal apoptosis at the G1/S cell cycle checkpoint. *Cell Tissue Res* **305**:217-228.
26. **Maraganore, D. M., M. de Andrade, T. G. Lesnick, K. J. Strain, M. J. Farrer, W. A. Rocca, P. V. Pant, K. A. Frazer, D. R. Cox, and D. G. Ballinger.** 2005. High-resolution whole-genome association study of Parkinson disease. *Am J Hum Genet* **77**:685-693.
27. **Moore, D. J., A. B. West, V. L. Dawson, and T. M. Dawson.** 2005. Molecular pathophysiology of Parkinson's disease. *Annu Rev Neurosci* **28**:57-87.
28. **Nikolaev, A. Y., M. Li, N. Puskas, J. Qin, and W. Gu.** 2003. Parc: a cytoplasmic anchor for p53. *Cell* **112**:29-40.
29. **Offen, D., B. Shtatif, D. Hadad, A. Weizman, E. Melamed, and I. Gil-Ad.** 2001. Protective effect of insulin-like-growth-factor-1 against dopamine-induced

- neurotoxicity in human and rodent neuronal cultures: possible implications for Parkinson's disease. *Neurosci Lett* **316**:129-132.
30. **Okabe, H., S. H. Lee, J. Phuchareon, D. G. Albertson, F. McCormick, and O. Tetsu.** 2006. A critical role for FBXW8 and MAPK in cyclin D1 degradation and cancer cell proliferation. *PLoS ONE* **1**:e128.
 31. **Pankratz, N., W. C. Nichols, S. K. Uniacke, C. Halter, J. Murrell, A. Rudolph, C. W. Shults, P. M. Conneally, and T. Foroud.** 2003. Genome-wide linkage analysis and evidence of gene-by-gene interactions in a sample of 362 multiplex Parkinson disease families. *Hum Mol Genet* **12**:2599-2608.
 32. **Pankratz, N., W. C. Nichols, S. K. Uniacke, C. Halter, A. Rudolph, C. Shults, P. M. Conneally, and T. Foroud.** 2002. Genome screen to identify susceptibility genes for Parkinson disease in a sample without parkin mutations. *Am J Hum Genet* **71**:124-135.
 33. **Pankratz, N., W. C. Nichols, S. K. Uniacke, C. Halter, A. Rudolph, C. Shults, P. M. Conneally, and T. Foroud.** 2003. Significant linkage of Parkinson disease to chromosome 2q36-37. *Am J Hum Genet* **72**:1053-1057.
 34. **Samani, A. A., S. Yakar, D. LeRoith, and P. Brodt.** 2007. The role of the IGF system in cancer growth and metastasis: overview and recent insights. *Endocr Rev* **28**:20-47.
 35. **Sarikas, A., X. Xu, L. Field, and Z. Q. Pan.** 2007. 535. THE CUL7 E3 UBIQUITIN LIGASE IS A NEGATIVE REGULATOR OF ONCOGENE-INDUCED SENESCENCE. European Life Scientist Organization (ELSO) 2007

Poster Abstracts

<http://www.elsevier.com/locate/ymbs>

36. **Sherr, C. J., and J. M. Roberts.** 2004. Living with or without cyclins and cyclin-dependent kinases. *Genes Dev* **18**:2699-2711.
37. **Sicinski, P., J. L. Donaher, S. B. Parker, T. Li, A. Fazeli, H. Gardner, S. Z. Haslam, R. T. Bronson, S. J. Elledge, and R. A. Weinberg.** 1995. Cyclin D1 provides a link between development and oncogenesis in the retina and breast. *Cell* **82**:621-630.
38. **Skaar, J. R., T. Arai, and J. A. DeCaprio.** 2005. Dimerization of CUL7 and PARC is not required for all CUL7 functions and mouse development. *Mol Cell Biol* **25**:5579-5589.
39. **Skaar, J. R., L. Florens, T. Tsutsumi, T. Arai, A. Tron, S. K. Swanson, M. P. Washburn, and J. A. DeCaprio.** 2007. PARC and CUL7 form atypical cullin RING ligase complexes. *Cancer Res* **67**:2006-2014.
40. **Stewart, C. E., and P. Rotwein.** 1996. Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors. *Physiol Rev* **76**:1005-1026.
41. **Stryke, D., M. Kawamoto, C. C. Huang, S. J. Johns, L. A. King, C. A. Harper, E. C. Meng, R. E. Lee, A. Yee, L. L'Italien, P. T. Chuang, S. G. Young, W. C. Skarnes, P. C. Babbitt, and T. E. Ferrin.** 2003. BayGenomics: a resource of insertional mutations in mouse embryonic stem cells. *Nucleic Acids Res* **31**:278-281.

42. **Thomas, B., and M. F. Beal.** 2007. Parkinson's disease. *Hum Mol Genet* **16 Spec No. 2**:R183-94.
43. **Trejo, J. L., E. Carro, E. Garcia-Galloway, and I. Torres-Aleman.** 2004. Role of insulin-like growth factor I signaling in neurodegenerative diseases. *J Mol Med* **82**:156-162.
44. **Tsutsumi, T., H. Kuwabara, T. Arai, Y. Xiao, and J. A. Decaprio.** 2008. Disruption of the Fbxw8 gene results in pre- and postnatal growth retardation in mice. *Mol Cell Biol* **28**:743-751.
45. **Zambrowicz, B. P., A. Abuin, R. Ramirez-Solis, L. J. Richter, J. Piggott, H. BeltrandelRio, E. C. Buxton, J. Edwards, R. A. Finch, C. J. Friddle, A. Gupta, G. Hansen, Y. Hu, W. Huang, C. Jaing, B. W. J. Key, P. Kipp, B. Kohlhauff, Z. Q. Ma, D. Markesich, R. Payne, D. G. Potter, N. Qian, J. Shaw, J. Schrick, Z. Z. Shi, M. J. Sparks, I. Van Sligtenhorst, P. Vogel, W. Walke, N. Xu, Q. Zhu, C. Person, and A. T. Sands.** 2003. Wnk1 kinase deficiency lowers blood pressure in mice: a gene-trap screen to identify potential targets for therapeutic intervention. *Proc Natl Acad Sci U S A* **100**:14109-14114.
46. **Zhao, C., W. Deng, and F. H. Gage.** 2008. Mechanisms and functional implications of adult neurogenesis. *Cell* **132**:645-660.

FIGURE LEGENDS

Figure 1. Loss of GIGYF2 results in decreased cyclin D1 protein levels. (A) Primary MEFs derived from *Gigyf2*^{+/+} and *Gigyf2*^{-/-} mice were harvested at passage 2. GIGYF2 and cyclin D levels were assessed by immunoblot of whole cell lysate (WCL). Cyclin D1 and cyclin D3 protein levels are shown in two littermate matched MEF pairs from two independent litters. (B) Littermate matched *Gigyf2*^{+/+} (WT) and *Gigyf2*^{-/-} (KO) MEF pairs from 4 separate litters were processed as in (A). Cyclin D1 immunoblots were quantified by densitometry and normalized to a loading control (e.g. β -actin level) to determine amount of cyclin D1. Relative units were obtained by normalizing cyclin D1 amounts to a mean WT value of 100. Data plotted are means $\pm$ SEM. The data were subjected to two-tailed paired *t* test analysis for comparison between WT and KO MEFs (N=9 for each genotype, * *P* < 0.001). (C) Immunoblot of whole embryo extracts prepared from littermate matched *Gigyf2*^{+/+}, *Gigyf2*^{+/-} and *Gigyf2*^{-/-} embryos at embryonic days 14.5 (E 14.5) and 17.5 showing levels of GIGYF2 and cyclin D1. Immunoblot of β -actin is shown as a loading control.

Figure 2. Increased cyclin D1 protein degradation in *Gigyf2*^{-/-} MEFs. (A) Total RNA was isolated from primary *Gigyf2*^{+/+} (WT) and *Gigyf2*^{-/-} (KO) MEFs and relative levels of cyclin D1 mRNA were determined by quantitative real-time PCR. Plot illustrates the range of cyclin D1 expression relative to WT as determined by the comparative C_T method (N=3 for each genotype). (B) *Gigyf2*^{+/+} and *Gigyf2*^{-/-} MEFs were treated with 100 μ g/ml cycloheximide (CHX) for the amount of time indicated and cyclin D1 levels

were assessed by immunoblot of whole cell lysate. (C) MEFs were processed as in (B).

Data plotted are means $\pm$ SEM of percent cyclin D1 protein remaining over time

(*Gigyf2*^{+/+} N=6, *Gigyf2*^{-/-} N=4). The half-lives of cyclin D1 in *Gigyf2*^{+/+} and *Gigyf2*^{-/-}

MEFs were calculated using the formula: $T_{1/2} = \ln(2)/K$, where K = the decay constant

(see text and material and methods for details).

Figure 3. Co-immunoprecipitation of cullin protein CUL7 with GIGYF2. HEK-293 cells stably expressing full-length Myc-GIGYF2 under the control of a tetracycline (Tet) inducible promoter were transiently transfected with His-SKP1 and HA-CUL7 constructs. Myc-GIGYF2 expression was induced $\pm$ 0.1 μ g/ml Tet for 12 hours. Cell lysates (2mg) were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-HA, anti-Myc, or anti-SKP1 antibodies. Anti-SKP1 antibody recognizes both endogenous SKP1 and overexpressed His-SKP1. Expression of each construct was assessed by immunoblot of 50 μ g of whole cell lysate (WCL). Asterisk indicates position of IgG light chain in the immunoblot.

Figure 4. GIGYF2 forms an endogenous complex with CUL7 and p53. (A) HEK-293 cells were transiently transfected with HA-CUL7 or empty vector. Forty-eight hours after transfection, cells were harvested and lysates (2.5 mg) were subjected to immunoprecipitation with protein-A agarose alone (—) or in combination with anti-GIGYF2, anti-HA or anti-p53 antibodies. Immunoprecipitations and 50 μ g of whole cell lysate (WCL) were immunoblotted with anti-GIGYF2, anti-CUL7 and anti-p53

antibodies. (B) U-2 OS cells were prepared and analyzed as in (A). Both short and long exposures of the p53 immunoblot are shown.

Figure 5. GIGYF2 forms a complex with CUL7-PARC-p53 that excludes SKP1 and FBXW8. (A) HEK-293 cells stably expressing full-length Myc-GIGYF2 under the control of a tetracycline (Tet) inducible promoter were transiently transfected with His-SKP1 and HA-CUL7 or empty vector. Myc-GIGYF2 expression was induced ± 0.05 $\mu\text{g/ml}$ Tet for 24 hours. Cell lysates (2mg) were immunoprecipitated with anti-Myc antibody. Immunoprecipitations and 50 μg of whole cell lysate (WCL) were immunoblotted with the indicated antibodies. Asterisk indicates position of IgG light chain in the immunoblot. (B) Myc-GIGYF2 Tet inducible HEK-293 cells were transiently transfected with HA-CUL7, His-SKP1 and FLAG-FBXW8 or empty vector. Myc-GIGYF2 expression was induced ± 0.1 $\mu\text{g/ml}$ Tet for 12 hours. Cell lysates (2mg) were immunoprecipitated with anti-FLAG antibody and analyzed as in (A).

Figure 6. Cytoplasmic localization of the GIGYF2-CUL7 complex. (A) Myc-GIGYF2 tetracycline (Tet) inducible HEK-293 cells were transiently transfected with HA-CUL7. Myc-GIGYF2 expression was induced ± 0.1 $\mu\text{g/ml}$ Tet for 12 hours. The subcellular localization of Myc-GIGYF2 and HA-CUL7 was determined by indirect immunofluorescence with anti-Myc and anti-HA antibodies. (B) Co-localization of endogenous GIGYF2 and the centrosome marker gamma-tubulin in HEK-293 cells. Subcellular localization was assessed by indirect immunofluorescence with anti-GIGYF2 and anti-gamma-tubulin antibodies.

Figure 7. A C-terminal fragment of GIGYF2 is sufficient for CUL7 interaction. HEK-293 cells were transiently transfected with HA-CUL7 and Myc tagged C-terminal GIGYF2 fragment (Myc-674-1299), Myc tagged GYF domain GIGYF2 fragment (Myc-494-622) or empty vector. Forty-eight hours after transfection, cells were harvested and lysates (2 mg) were subjected to immunoprecipitation with anti-Myc antibody and immunoblotted with anti-Myc and anti-CUL7 antibodies. Expression of each construct was assessed by immunoblot of 50 μ g of whole cell lysate (WCL).

Figure 8. GIGYF2 protein is not rapidly degraded by the ubiquitin-proteasome system. (A) HEK-293 cells were transiently transfected with HA-CUL7 or empty vector. Six hours prior to harvest, cells were treated with 25 μ M MG132 or DMSO to inhibit 26S proteasome activity. The level of endogenous GIGYF2 was assessed by immunoblot of 50 μ g of whole cell lysate with anti-GIGYF2 antibody. (B) U2-OS cells were treated with 25 μ M MG132 or DMSO for 6 hours. Cells were harvested and whole cell lysates were immunoblotted with anti-GIGYF2 and anti-CUL7 antibodies. Increase in p53 protein level shown as positive control for 26S proteasome inhibition by MG132 treatment. (C) *Gigyf2*^{+/+} MEFs were treated with 100 μ g/ml cycloheximide (CHX) for the amount of time indicated and GIGYF2 levels were assessed by immunoblot of 50 μ g of whole cell lysate. Cyclin D1 immunoblot included as positive control for CHX treatment and immunoblot of β -actin is shown as a loading control. (D) HEK-293 cells were treated with 30 μ g/ml CHX for the amount of time indicated. The level of GIGYF2 at each time point was assessed by immunoblot of whole cell lysate with anti-GIGYF2 antibody (arrow = GIGYF2, asterisk = non-specific band).

Figure 9. GIGYF2 C-terminal fragment overexpression disrupts the SKP1-CUL7-

FBXW8 complex. (A) HEK-293 cells were transiently transfected with the indicated constructs. Total DNA per transfection was kept constant through addition of varying amounts of empty vector. Twenty-four hours after transfection, cells were harvested and lysates (1 mg) were subjected to immunoprecipitation with anti-HA antibody. Immunoprecipitations and 50 µg of whole cell lysate (WCL) were immunoblotted with the indicated antibodies. Asterisk indicates position of a non-specific band in the anti-FLAG immunoblot. (B) The experiment described in (A) was repeated three times and the levels of FLAG-FBXW8 and SKP1 that co-precipitated with HA-CUL7 minus/plus Myc-CT-GIGYF2 overexpression were quantified by densitometry. Relative units were obtained by normalizing FBXW8 and SKP1 amounts to a mean minus Myc-CT-GIGYF2 value of 100. Data plotted are means $\pm$ SEM. The data were subjected to two-tailed *t* test analysis for comparison between minus and plus Myc-CT-GIGYF2 (* $P < 0.005$, ** $P < 0.001$).

Figure 10. Overexpression of GIGYF2 increases the level of cyclin D1 protein. (A) Myc-GIGYF2 tetracycline inducible HEK-293 cells were treated with the indicated amounts of tetracycline or vehicle alone for 24 hours. The level of Myc-GIGYF2, total GIGYF2 and cyclin D1 were assessed by immunoblot of whole cell lysate (WCL). Immunoblot of β -tubulin is shown as a loading control. (B) Myc-GIGYF2 tetracycline inducible HEK-293 cells were treated with 0.1 µg/ml tetracycline (plus GIGYF2) or vehicle alone (minus GIGYF2) for 24 hours. Cyclin D1 immunoblots were quantified by densitometry and normalized to a loading control (β -actin or β -tubulin) to determine amount of cyclin D1.

Relative units were obtained by normalizing cyclin D1 amounts to a mean minus GIGYF2 value of 100. Data plotted are means $\pm$ SEM. The data were subjected to two-tailed *t* test analysis for comparison between minus and plus GIGYF2 (N=6, * $P < 0.05$).

Figure 1

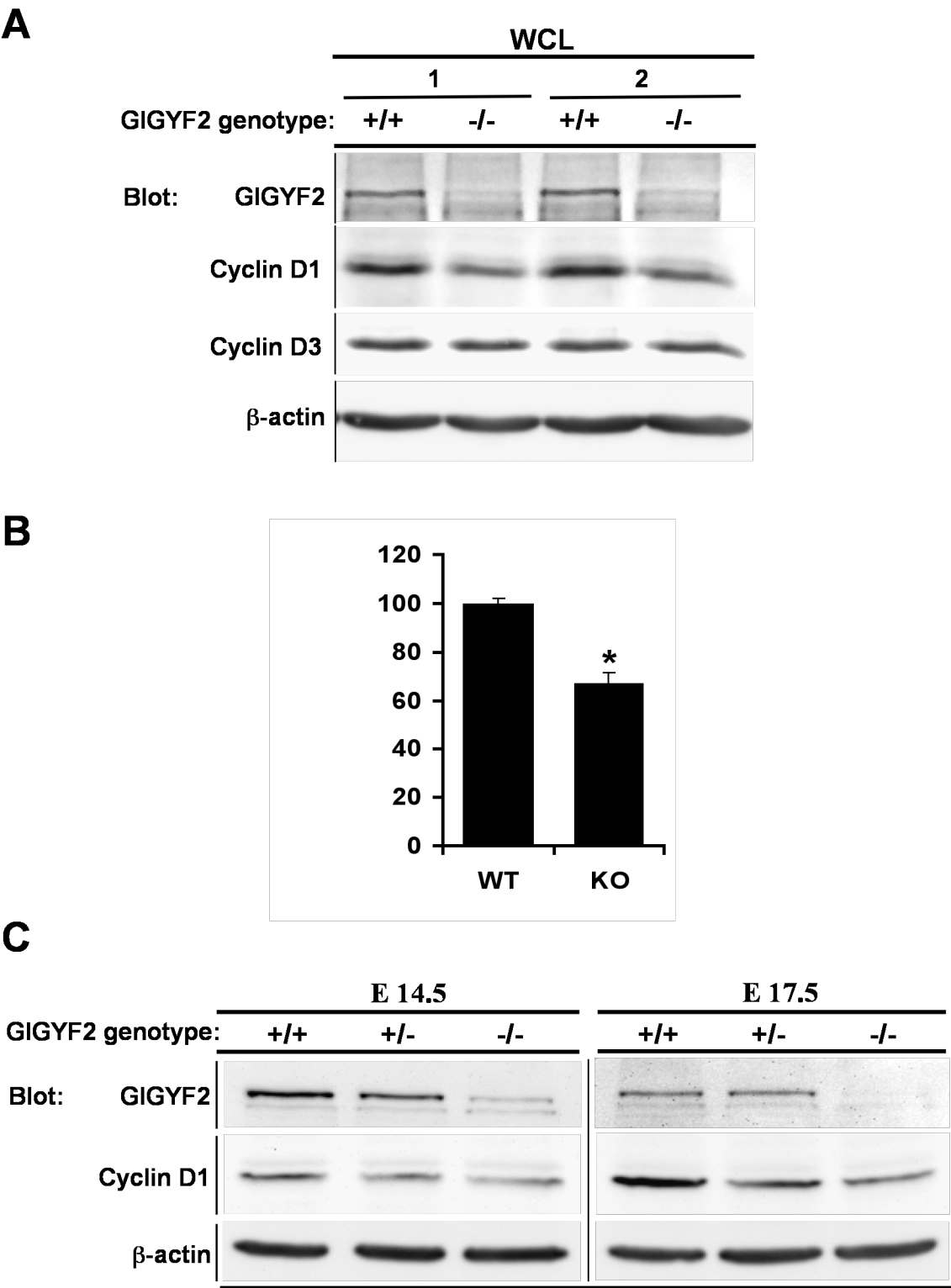


Figure 2

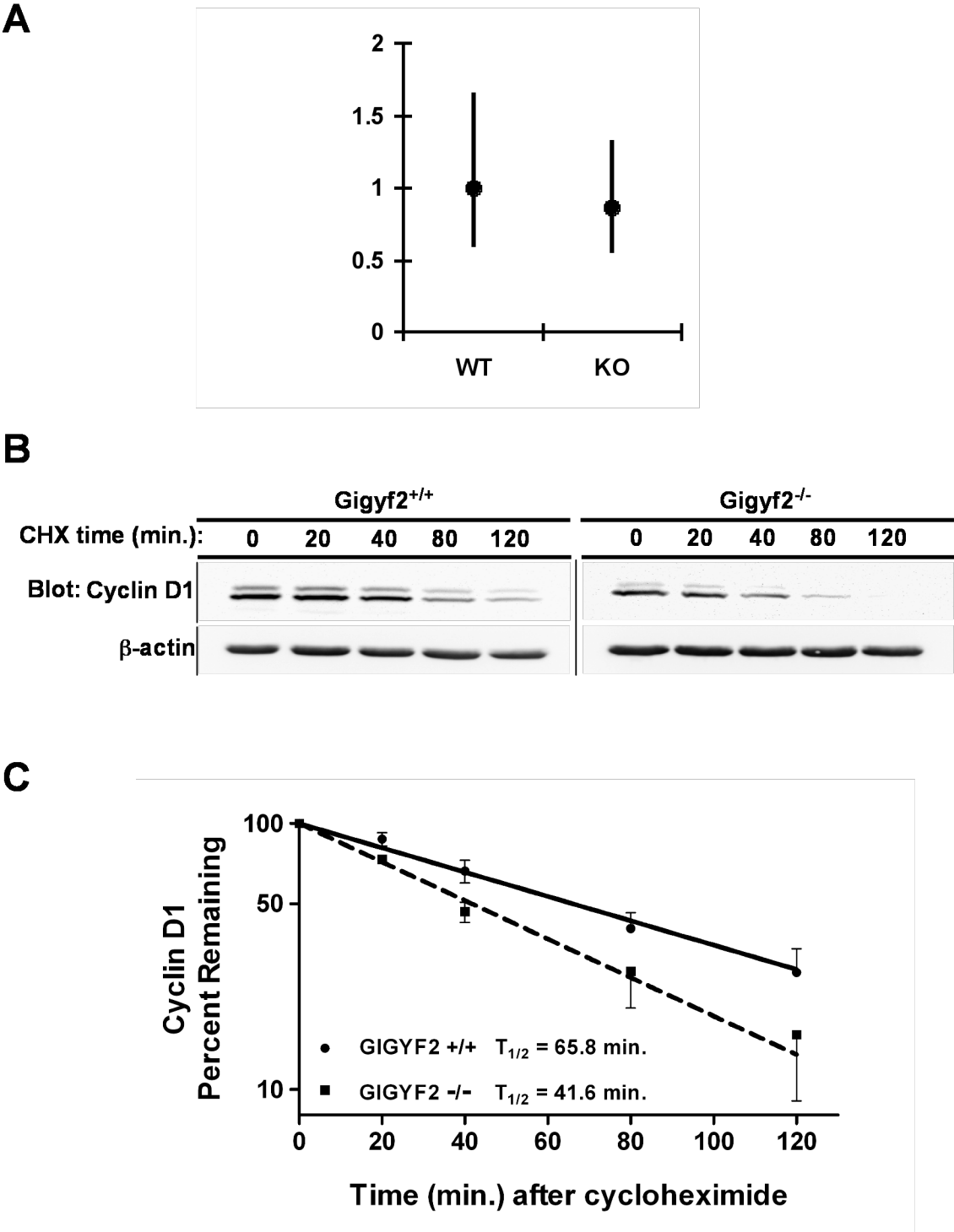


Figure 3

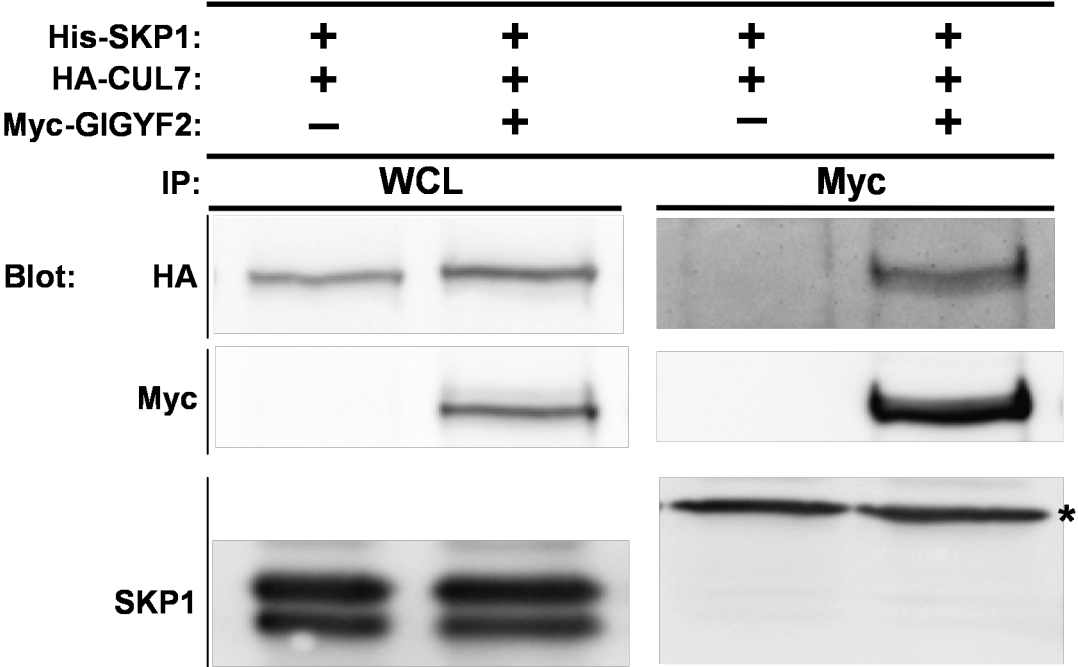
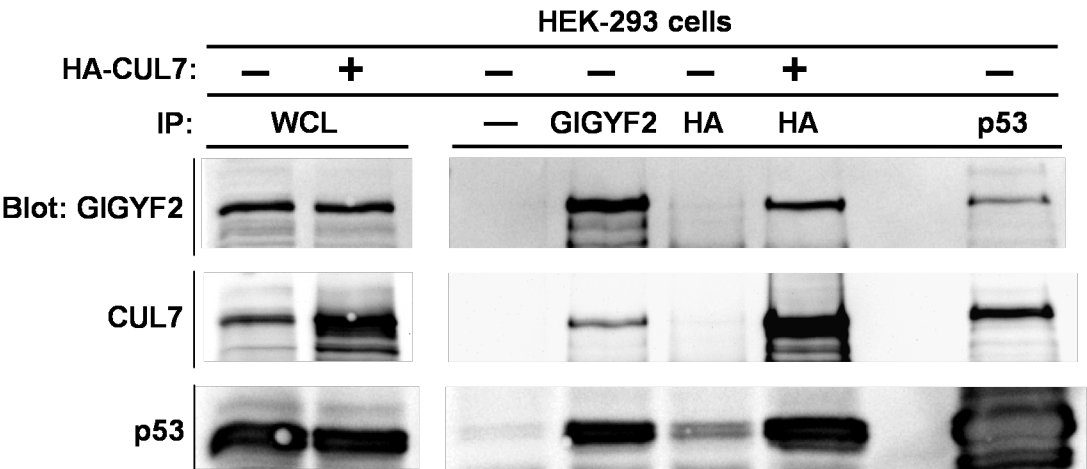


Figure 4

A



B

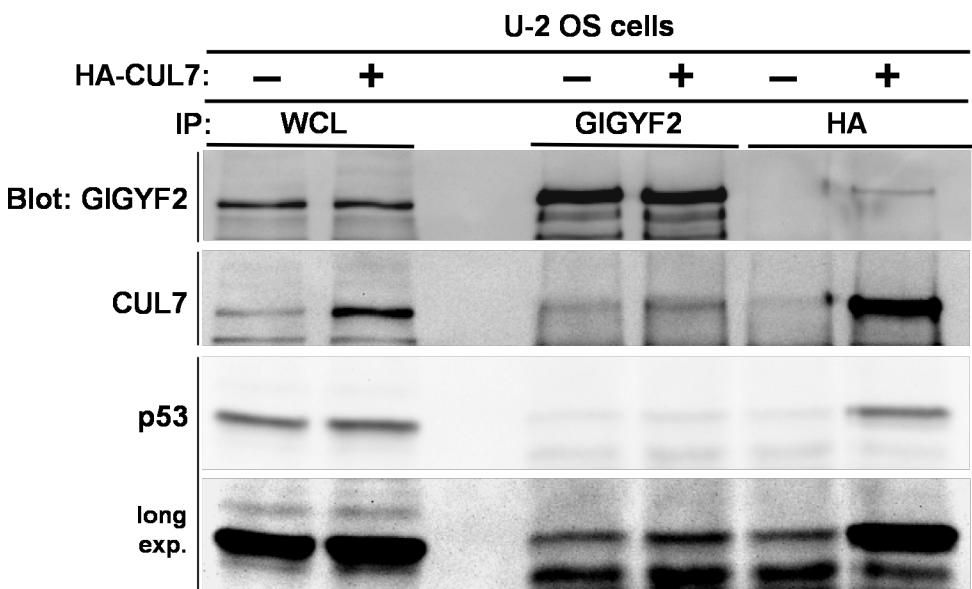
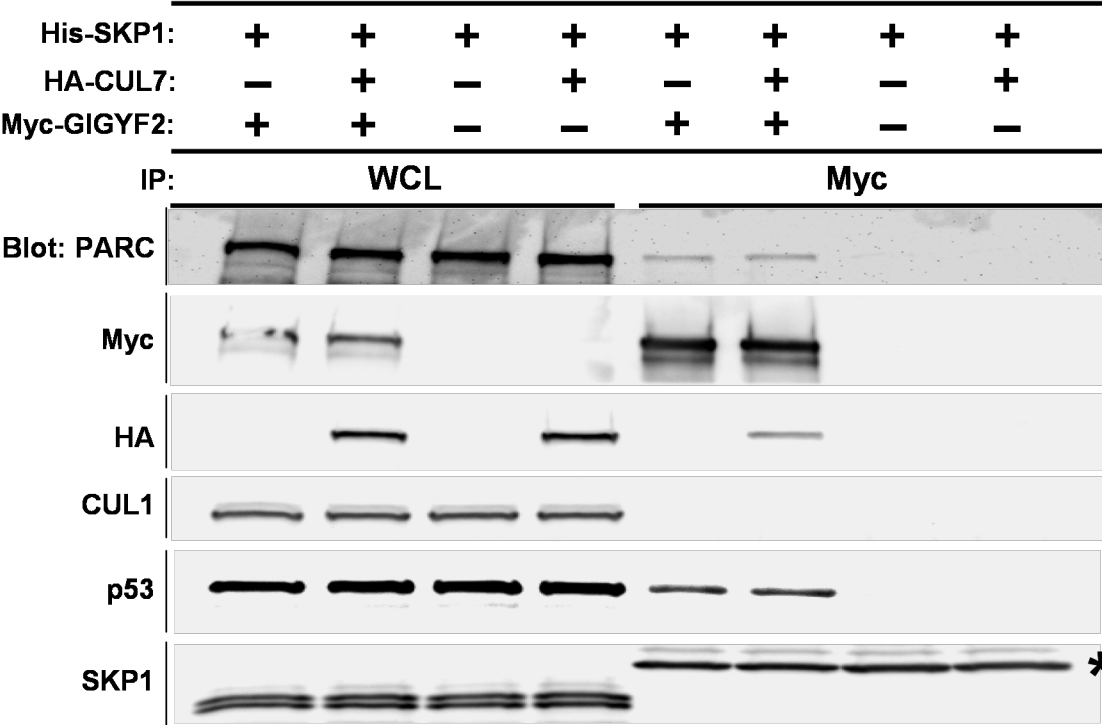


Figure 5

A



B

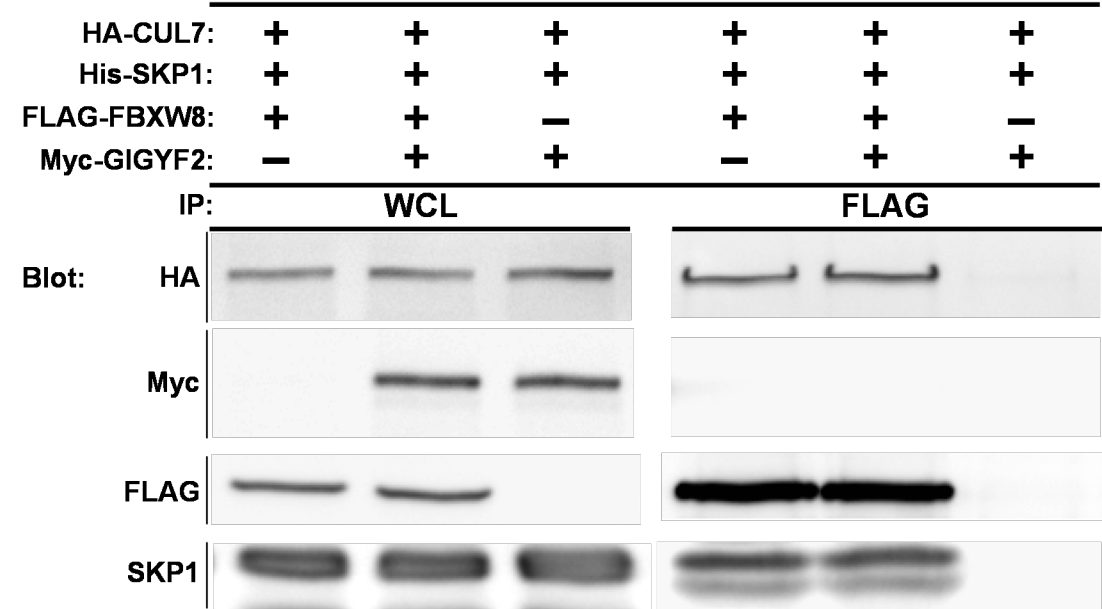


Figure 6

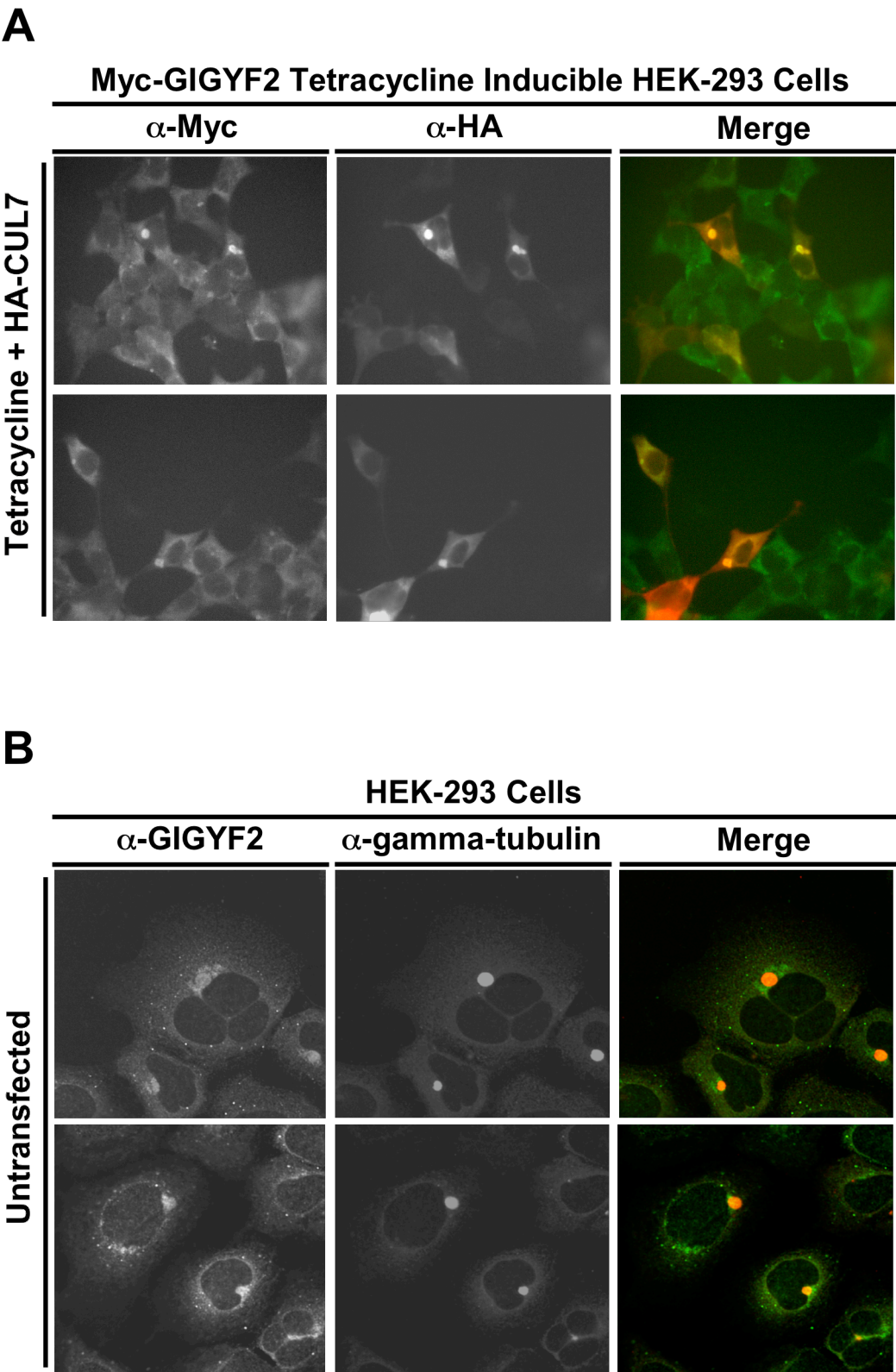


Figure 7

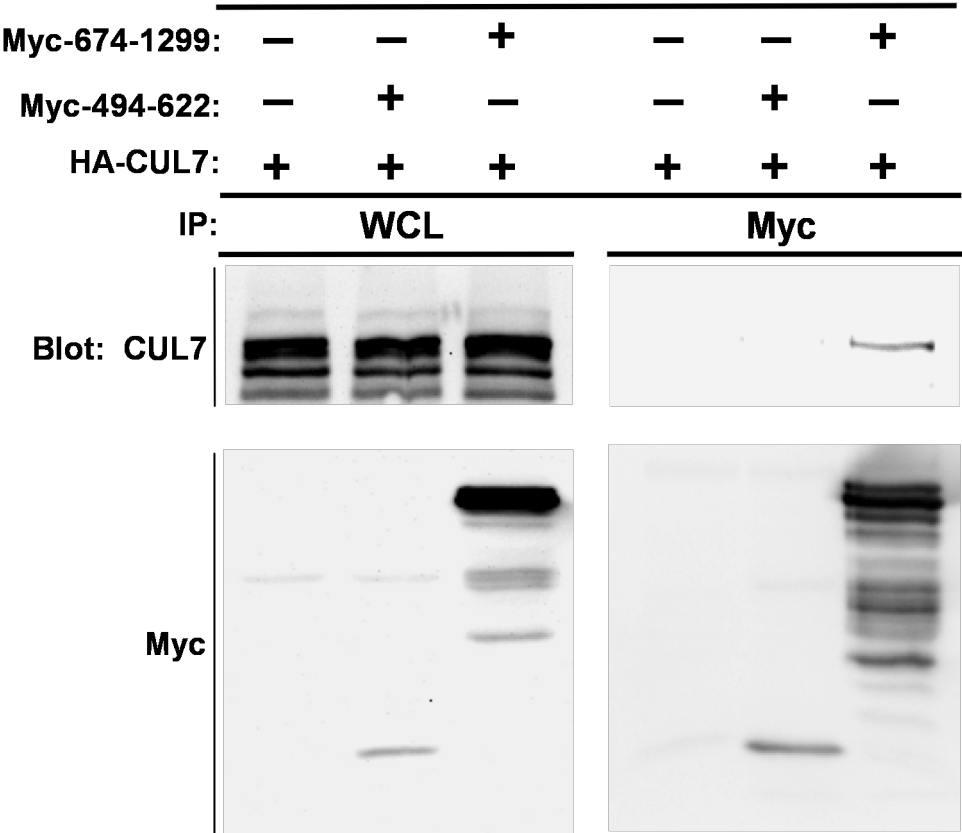


Figure 8

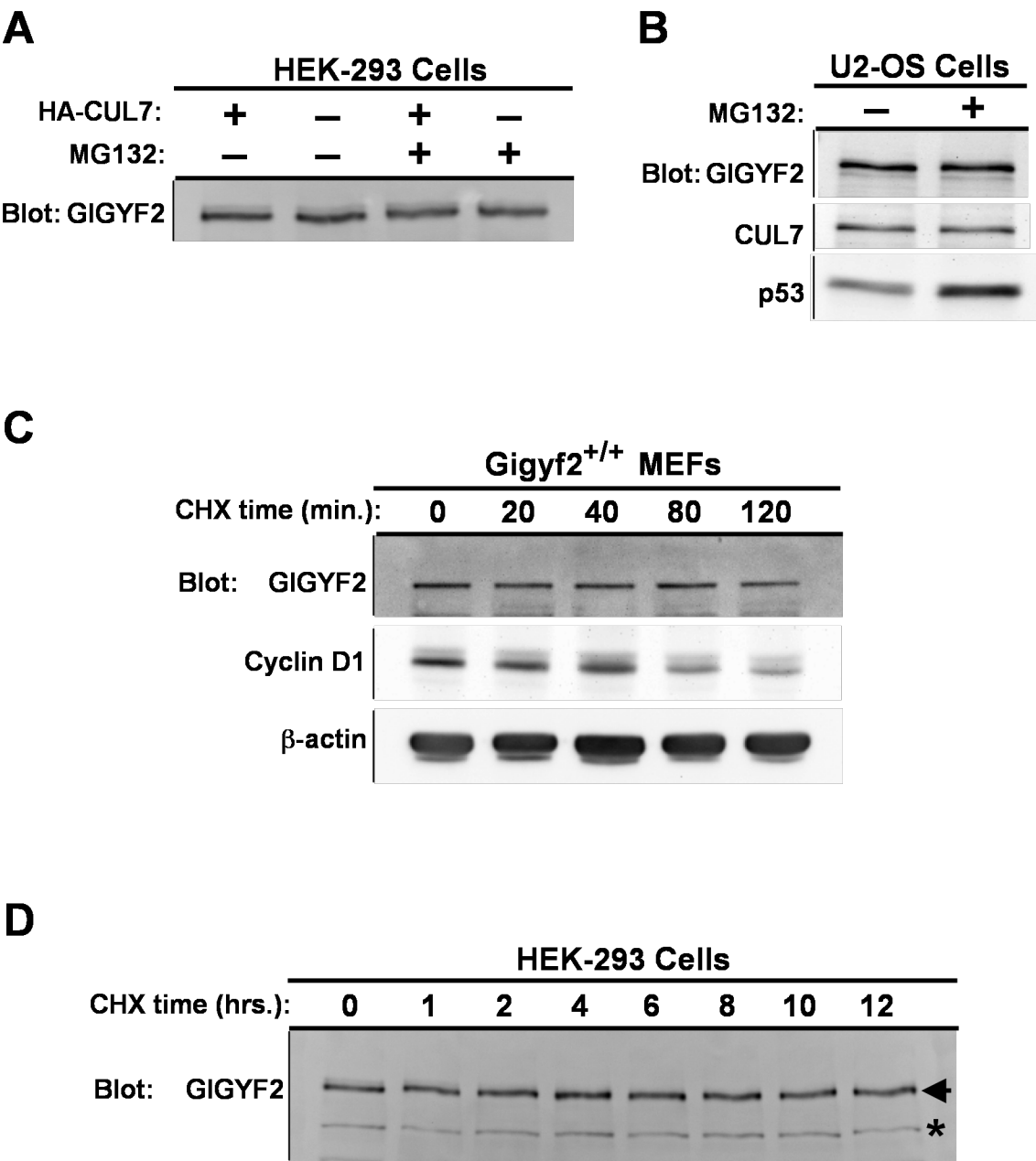
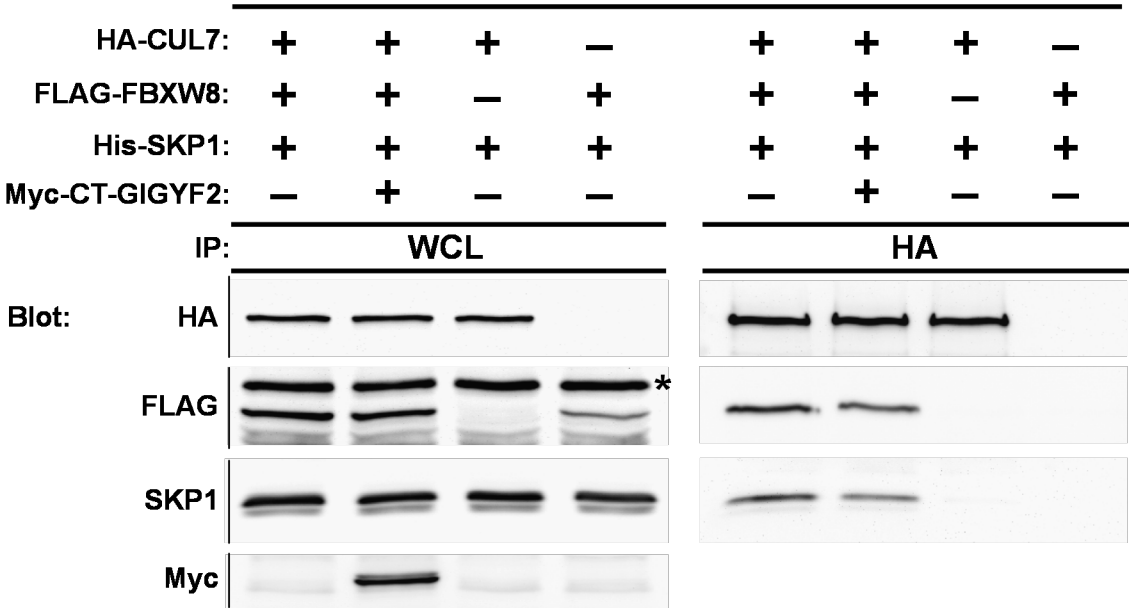


Figure 9

A



B

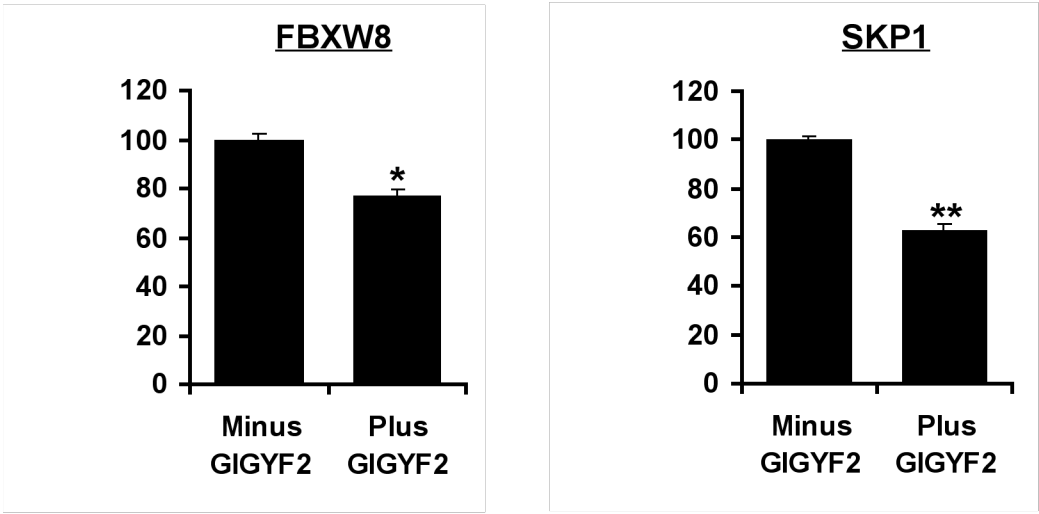
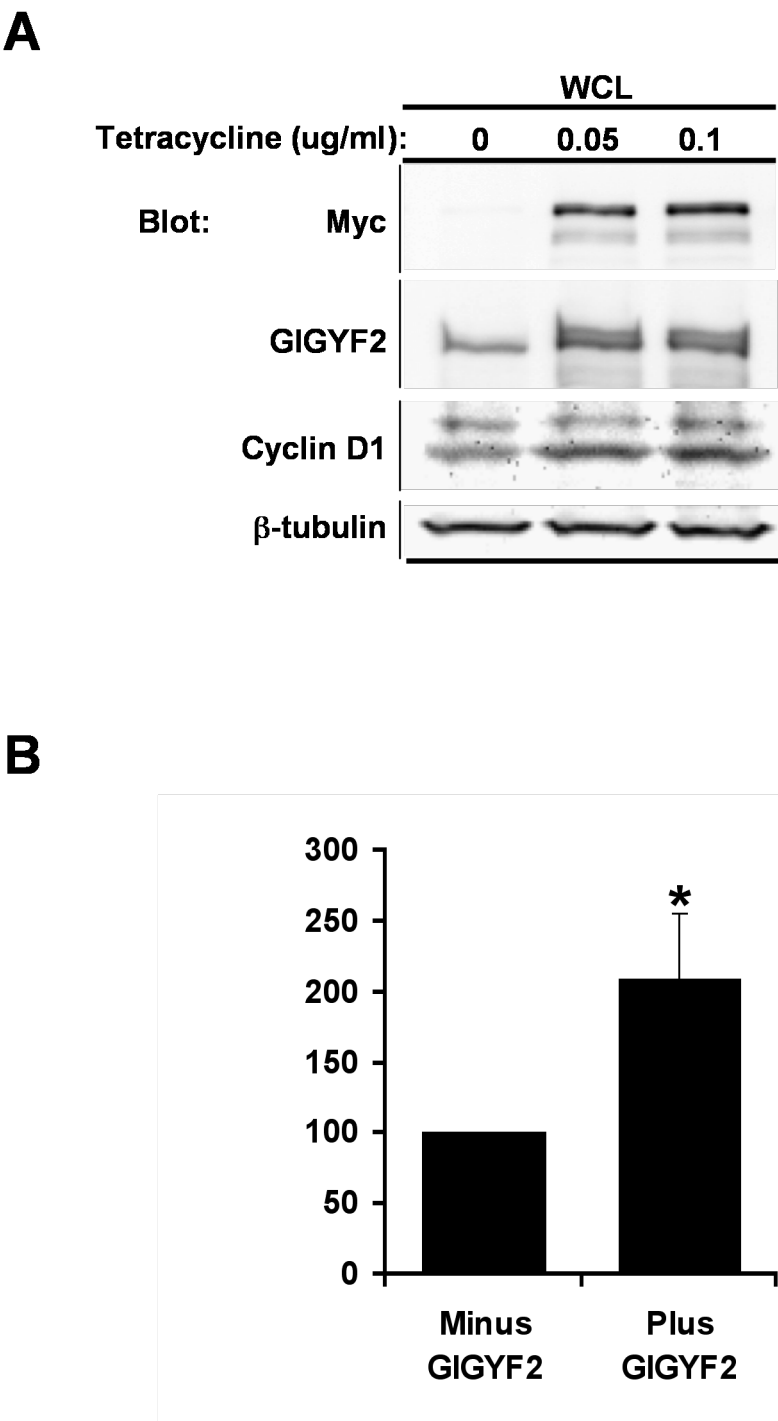


Figure 10



CHAPTER 5

Discussion

OVERVIEW

The insulin receptor (IR) and the insulin-like growth factor receptor (IGF-IR) are critical mediators of mammalian growth, development and survival. Given their immense biological significance, a complex system of inter- and intracellular molecules has evolved to facilitate precise regulation of these hormone receptors. The adaptor protein Grb10 is an important component of this regulatory system. Work over the last decade has established Grb10 as a potent suppressor of IR and IGF-IR signaling (33, 46, 67). Disruption of Grb10 function results in significantly altered IR and IGF-IR physiology, and has been linked to human diseases of metabolism and growth (14, 19, 66, 88, 90). Grb10 action is mediated, in part, through interaction with and recruitment of additional effector molecules to activated receptors. Recently, two homologous Grb10-interacting proteins, GIGYF1 and GIGYF2, were identified based on their capacity to bind to the N-terminal proline-rich region of Grb10. GIGYF1 protein forms a transient complex with Grb10 and activated IGF-IR, and plays a role in the modulation of receptor signaling (29). GIGYF2 exhibits similar affinity and specificity for Grb10 interaction, yet the biological significance and molecular function of this protein have not been established. The work presented in this thesis represents the first effort to address these questions.

As an initial approach to the analysis of GIGYF2, we examined human genetic data for potential linkage of the *GIGYF2* gene to human disease. This work revealed significant association between the *GIGYF2* gene region (PARK11 locus) and familial Parkinson's disease (PD) (49, 59-61). In chapter 2, we describe the results of a candidate-gene analysis investigating the involvement of the *GIGYF2* gene in PD. This

study identified mutations in the *GIGYF2* gene in close to 5% of the PD population analyzed. These results suggest that mutations in *GIGYF2* are a frequent cause of familial PD and reveal an important role for the GIGYF2 protein in the central nervous system. To further explore the link between GIGYF2 and neurodegeneration and the involvement of GIGYF2 in growth and development, we used a gene disruption strategy in mice. Mice that are homozygous for a gene trap insertion in *Gigyf2* exhibit significant early postnatal lethality. *Gigyf2*^{+/-} mice develop normally, but show evidence of age-dependent motor ataxia and neurodegeneration. Mouse embryonic fibroblasts derived from *Gigyf2*^{-/-} animals show altered IGF-IR signaling, indicating that GIGYF2 may contribute to Grb10-mediated receptor regulation. Together, these findings support a causal role for GIGYF2 in PD, and establish the first functional link between a PD gene and the IGF hormonal system.

To better define potential molecular actions of GIGYF2 we further characterized GIGYF2 deficient cells. This work revealed that in the absence of GIGYF2, the level of the cell cycle regulatory protein cyclin D1 is reduced as a result of increased cyclin D1 protein degradation. Through biochemical analysis, we determined that GIGYF2 interacts with the CUL7-PARC-p53 complex and has the capacity to disrupt the SKP1-CUL7-FBXW8 E3 ubiquitin ligase. These results provide a mechanism for GIGYF2-regulation of cyclin D1 degradation and suggest participation of the GIGYF2 protein in potential CUL7-PARC-p53 functions. In the remainder of this chapter some of the implications of this work will be discussed and a model will be proposed for how GIGYF2, Grb10, and CUL7 may jointly participate in regulation of IR/IGF-IR activity.

GIGYF2: a link between insulin/IGFs and Parkinson's disease

Perhaps the most significant aspect of this work is the identification of a functional link between an IGF-IR regulatory protein and Parkinson's disease. Chapters 2 and 3 of this thesis provide evidence for the involvement of GIGYF2 in neurodegeneration and show that loss of GIGYF2 alters IGF-IR signaling. The IGF hormonal system plays an important role in central nervous system (CNS) growth, development and function (69). Both IGF-I and IGF-II are expressed in the brain during embryonic development and throughout adulthood. High expression of IGF-I is seen in neuronal-rich regions of the CNS including the spinal cord, midbrain, cerebral cortex, hippocampus, and olfactory bulb (24, 26, 68). IGF-II expression has been detected in multiple regions of the CNS, although it appears to be enriched in structures of neuroepithelial origin such as the choroid plexus (10). The expression pattern of the IGF-IR resembles that of its ligands, with high expression seen in cerebellum, midbrain, olfactory bulb and choroid plexus (8, 10, 68, 89). The co-expression of IGFs and the IGF-IR in the CNS suggests important autocrine and paracrine actions of this hormonal system in the brain. IGFs are also transported across the blood-brain-barrier, and circulating IGFs have been shown to exert neurogenic and neuroprotective activity within the CNS (85).

Whether activated by local or systemic IGFs, the IGF-IR exerts potent and pleiotropic actions within the CNS. Acting primarily through the MAPK and PI3K signaling pathways, the IGF-IR promotes mitogenesis and differentiation of embryonic neurons, glial cells, and adult neural stem cells, and regulates axon growth, myelination, dendritic arborization and synaptogenesis. In the adult brain, the IGF-IR provides trophic

support to existing neurons, inhibits neuronal apoptosis, and stimulates both continuous and injury-induced neurogenesis (9, 17, 69). These actions are critically important for the health of the CNS, and altered IGF-IR activity has been linked to neurodegenerative disorders such as Alzheimer's disease, Ataxia telangiectasia, Huntington's disease, and Parkinson's disease (21, 35, 57, 62).

Our analysis of *Gigyf2* promoter activity using the gene-trap β -galactosidase cassette revealed widespread expression of GIGYF2 throughout the mouse brain. It is interesting to note that the highest levels of *Gigyf2* promoter activity overlap with regions of the CNS enriched for IGF and IGF-IR expression. These regions include the olfactory bulb, hippocampus, cerebellum, and choroid plexus. Therefore, it's reasonable to assume that the altered IGF-IR signaling observed in *Gigyf2*^{-/-} mouse embryonic fibroblasts also occurs in the CNS. In the absence of GIGYF2, IGF-I stimulated activation of PI3K and MAPK pathways is increased. Excessive activation of downstream signaling molecules like Akt and ERK1/2 in response to IGF stimulation may have deleterious effects on differentiation and patterning of specific neuronal structures. Indeed, recent evidence suggests that olfactory bulb development may be particularly susceptible to altered IGF-IR signaling. Scolnick et al. (2008) found that IGF acts as a chemoattractant for axon growth cones within the olfactory bulb, and that IGF signaling is required for appropriate patterning of olfactory neuron projections. Proper sensory function of the olfactory bulb depends upon this ordered assembly of olfactory neuron innervations (73). As discussed in Chapter 3, the perinatal lethality of *Gigyf2*^{-/-} mice is consistent with a defect in olfaction leading to a failure to feed. Altered IGF-IR signaling in the olfactory bulb represents a potential molecular mechanism for this phenotype. Olfactory bulb size and

structure in *Gigylf2*^{-/-} animals appears to be grossly intact. However, IGF-IR signal augmentation in the absence of GIGYF2 is relatively mild, and therefore would not be predicted to cause gross morphological changes in the olfactory bulb. Instead, it's likely that subtle changes in patterning occur such that overall sensory function is impacted. More work is needed to determine if this is in fact why *Gigylf2*^{-/-} mice exhibit failure to feed and perinatal lethality. In future experiments, it will be important to assess the olfactory capacity of *Gigylf2*^{-/-} animals and to determine if IGF-IR signaling is altered in *Gigylf2*^{-/-} olfactory neurons.

IGF-IR signaling in *Gigylf2*^{+/-} mouse embryonic fibroblasts has not been assessed. Therefore, it's not clear to what extent altered IGF-IR signaling contributes to the age-dependent neurodegenerative phenotype observed in these animals. Our characterization of *Gigylf2*^{+/+}, *Gigylf2*^{+/-} and *Gigylf2*^{-/-} embryos revealed that the heterozygous animals showed a moderate reduction in cyclin D1 levels. This reduction was not as pronounced as that seen in the *Gigylf2*^{-/-} embryos, suggesting that the heterozygous animals exhibit an intermediate phenotype. It's possible then, that *Gigylf2*^{+/-} mice have an intermediate phenotype with respect to altered IGF-IR signaling. There may be sufficient normal receptor activity in these animals to prevent perinatal lethality but not enough for sustained health of the CNS. Since IGF-IR activation is generally considered neuroprotective, it seems counterintuitive that increased signaling with reduction of GIGYF2 would lead to neurodegeneration. While IGFs do promote neuron survival, a balance of IGF-IR signaling may be required for normal proliferation and differentiation of neural progenitor cells. There is a precedent for this in other developing tissues. For example, IGF-I stimulates proliferation of preadipocytes through activation of the MAPK

pathway. Differentiation of these cells into adipocytes, however, requires down-regulation of this pathway even in the presence of continued IGF-IR activation (11). In the CNS, the effect of altered IGF-IR signaling on neurogenesis is less clear. Much of this stems from the fact that IGF-I stimulation has the potential to promote progenitor cell proliferation or differentiation depending on cell type and culture conditions (4). However, there is some evidence suggesting that regulation of IGF-IR signaling is essential for appropriate CNS development. In mice, neuron-specific disruption of the gene encoding the lipid phosphatase PTEN results in increased Akt activation. This causes premature differentiation of glia cells leading to layering defects in the brain (91).

It is now generally accepted that neurogenesis in the mammalian CNS is not restricted to embryonic development. Neural progenitor cells have been isolated from multiple regions of the adult brain and their proliferation and differentiation occurs throughout life. The precise role of neurogenesis in neurodegenerative diseases has not been determined (92). However, there is evidence of altered neurogenesis in specific neurodegenerative diseases such Alzheimer's disease and Parkinson's disease (28, 92). The clinical manifestations of PD result primarily from loss of dopamine producing neurons in the substantia nigral region of the midbrain. In animal models of PD, there is increased proliferation of neural progenitor cells in several areas of the brain, including the substantia nigra (74, 93). It appears, however, that very few of these proliferating progenitor cells differentiate correctly into dopaminergic neurons (39, 45, 48, 81). Since regulated IGF-IR activity is required for appropriate neural progenitor cell proliferation, altered IGF-IR signaling may contribute to the defects in neurogenesis seen in animal

models of PD. This may be an important mechanism by which loss or mutation of GIGYF2 results in neurodegeneration in the mouse and in human PD.

While the work presented in this thesis primarily focused on the IGF-IR, partial loss of GIGYF2 may also affect insulin receptor signaling. GIGYF2 does not appear to contain structural domains that would allow it to interact directly with receptors. Therefore, modulation of receptor activity by GIGYF2 presumably occurs through Grb10-receptor complex formation. Grb10 interacts with and regulates both the IGF-IR and the IR. In cell culture systems, Grb10 exhibits a higher affinity for the IR, and may preferentially interact with the IR over the IGF-IR (44). Like the IGF system, insulin and the IR are expressed in the brain and play important roles in CNS function (13, 89). Insulin has been shown to regulate metabolism and survival of neurons as well as specific cognitive functions like learning and memory (15, 69). Insulin action may also contribute to neurogenesis as IR activation promotes both proliferation and differentiation of neural stem cells in culture (4). Perhaps most relevant to this discussion is the observed association between type 2 diabetes mellitus and Parkinson's disease. There appears to be a higher prevalence of diabetes among patients with PD, and diabetes is associated with an increased risk of developing PD (34, 63, 64, 70, 72). Even in the absence of overt diabetes, studies have shown reduced insulin-stimulated glucose uptake in PD patients (15). Given the importance of insulin function in the CNS, it's possible that impaired IR activity actually contributes to the development of PD. As with IGFs and the IGF-IR, the regions of highest IR expression are also enriched for GIGYF2. We therefore speculate that altered IR signaling may occur as a result of loss or mutation of GIGYF2, and that this may play a role in the development or progression of PD. In

future experiments, it will be important to determine if IR signaling is affected in *Gigyl2*^{-/-} and *Gigyl2*^{+/-} animals, and to assess whether PD patients with *GIGYF2* mutations exhibit more pronounced insulin system dysfunction.

Potential effect of *GIGYF2* mutations

Our analysis of patients with familial PD identified seven different *GIGYF2* mutations resulting in single amino acid substitutions. All were found in the heterozygous state, consistent with *GIGYF2* mutations being autosomal dominant with respect to familial PD. In mice, partial loss of the *Gigyl2* gene results in the neurodegenerative phenotype. Together, these data suggest that *GIGYF2* mutations result in loss of GIGYF2 protein function. Six of the mutations we have identified are found within the N-terminal half of the protein (Asn56Ser, Thr112Ala, Ile278Val, Ser335Thr, Asn457Thr and Asp606Glu), and the seventh (Val1242Ile) is located at the extreme C-terminal end of GIGYF2. None of the mutations occur within predicted functional motifs such as the GYF domain or bipartite nuclear localization signal; however, they are located in evolutionarily conserved regions of the protein. We know from overexpression studies that high levels of GIGYF2 result in self-aggregation, inclusion body formation, and a decrease in the amount of detergent soluble GIGYF2 protein (data not shown; also (29)). The exact mechanism by which this occurs is not known, but it suggests that GIGYF2 solubility is easily disrupted. A significant possibility, then, is that GIGYF2 mutations alter the folding and stability of the protein resulting in self-aggregation. This would lead to a decrease in soluble, and presumably functional, GIGYF2 protein. While our mouse data suggest that loss of GIGYF2 function is

sufficient to cause neurodegeneration, aggregation of mutant GIGYF2 may have additional consequences for neuron survival. Accumulation of misfolded proteins occurs in a number of neurodegenerative diseases including PD, and is suspected to play a role in disease progression (43). Therefore, it is possible that GIGYF2 mutations may cause neurodegeneration through both loss of normal GIGYF2 function and cytotoxicity of misfolded GIGYF2 aggregates.

Analysis of the GIGYF1 protein, which displays a similar tendency towards aggregation, revealed that the N-terminal half of the protein primarily mediates this process. Various N-terminal GIGYF1 constructs form large cytoplasmic inclusion bodies, while C-terminal constructs appear to remain soluble (B. Giovannone, personal communication). The extent to which the N-terminal half of GIGYF2 mediates inclusion body formation has not been determined, but the C-terminal half of the protein when overexpressed shows no evidence of self-aggregation. The fact that the majority of GIGYF2 mutations are found within the N-terminal half of the protein supports the hypothesis that these amino acid changes affect GIGYF2 stability. This hypothesis can be readily tested using the tetracycline-inducible GIGYF2 expression system. Assessment of soluble versus insoluble amounts of wild type or mutant GIGYF2 after controlled tetracycline induction should reveal the effect of GIGYF2 mutations on protein stability. It will also be important, in future experiments, to determine if GIGYF2 mutants have an effect on IGF-IR and IR signaling, and to what extent their capacity for Grb10 interaction is altered. Ultimately, a more detailed analysis of the effect of PD-linked mutations on GIGYF2 function is needed to understand the causal role that GIGYF2 plays in the development of PD.

The GIGYF2-CUL7 complex and cyclin D1 degradation

In our efforts to uncover the molecular function of GIGYF2, we identified the cullin family member CUL7 as a GIGYF2-interacting protein. CUL7 has been shown to form two mutually exclusive complexes in cells. In association with SKP1, FBXW8 and RBX1, CUL7 functions as the structural backbone for a classic cullin-based E3 ubiquitin ligase. CUL7 also interacts with the large Parkin-like protein PARC, p53, and RBX1 to form an atypical E3 ubiquitin ligase complex (2, 20, 79, 80). Our data shows that GIGYF2 preferentially interacts with the CUL7-PARC-p53 complex and has the capacity to disrupt CUL7 interaction with SKP1 and FBXW8. Therefore, GIGYF2 may modulate CUL7 function by regulating the formation of specific CUL7-based complexes. This hypothesis predicts that decreased GIGYF2 levels would result in increased SKP1-CUL7-FBXW8 complex assembly and subsequent ubiquitin ligase activity. The only known substrate of the SKP1-CUL7-FBXW8 complex is cyclin D1 (58). Consistent with our model, in *Gigyf2*^{-/-} mouse embryonic fibroblasts there is increased degradation of cyclin D1 protein. The ultimate effect of this is a reduction in the level of cyclin D1, which we observe in both *Gigyf2*^{-/-} and *Gigyf2*^{+/-} animals.

Mammals express three D-type cyclins (D1, D2, and D3). Each is capable of promoting cell cycle progression by interacting with and activating the cyclin-dependent kinases CDK4 and CDK6. Phosphorylation of the retinoblastoma protein RB by cyclin D-CDK4/6 complexes stimulates entry into S-phase of the cell cycle. The three D-type cyclins, at a molecular level, appear to be functionally redundant. However, they do show tissue- and cell type-specific actions due to distinct patterns of expression (76).

Interestingly, cyclin D1 function appears to be particularly important in the CNS. *Cyclin D1*^{-/-} mice display abnormal neurologic function consistent with a defect in neuronal development or activity (78). These mice also exhibit significant proliferative defects in the retina and show evidence of postnatal retinal photoreceptor degeneration (47, 78). Cyclin D1 is highly expressed in the CNS and the level of cyclin D1 protein and its activity increases during neuronal maturation (82, 83). Therefore, it is likely that the CNS would be particularly susceptible to the effects of reduced cyclin D1 levels seen in *Gigyl2* gene disrupted mice.

Given the importance of cyclin D1 function in neural precursor proliferation and differentiation, it is possible that neurogenic capacity is affected in *Gigyl2*^{+/-} mice. As discussed above in relation to IGF-IR signaling dysfunction, defects in neurogenesis may play an important role in the development of neurodegeneration in these animals. Alternatively, reduced cyclin D1 levels may negatively impact neuron survival in a manner similar to that seen with retinal cell degeneration in *cyclin D1*^{-/-} mice. Future experiments should assess the level of cyclin D1 protein in the CNS of both *Gigyl2*^{+/-} and *Gigyl2*^{-/-} mice to determine the extent to which cyclin D1 is involved in the phenotypes of these animals. We have shown that increasing GIGYF2 levels leads to an increase in the amount of cyclin D1 protein, presumably through disruption of the SKP1-CUL7-FBXW8 complex. Therefore, we could also test functional effects of the PD-linked GIGYF2 mutations by assessing the ability of mutant GIGYF2 protein to modulate cyclin D1 levels. This would provide important information about the mechanism of neurodegeneration seen in patients with GIGYF2 mutations and potentially great insight into the pathophysiology of the more common sporadic form of PD.

Interaction between GIGYF2 and the CUL7-PARC-p53 complex may do more than simply modulate CUL7-targeted cyclin D1 degradation. It is possible that GIGYF2 participates directly in the function of this complex. Both PARC and CUL7 bind directly to p53, and each is capable of inhibiting p53 function (1, 38, 40). The exact mechanism by which this occurs is not known but it appears that PARC and CUL7 may sequester p53 in the cytoplasm preventing p53-regulated transcription within the nucleus (56). p53 is an important tumor suppressor protein. It plays a critical role in the regulation of cell cycle progression and apoptosis in response to cell stresses (32, 37, 75). In the CNS, p53 is widely expressed in both proliferating and postmitotic cells (87). Accumulation and activation of p53 occurs rapidly in neurons in response to a variety of stressors, including DNA damage, oxidative stress, and excitotoxicity. This leads to the initiation of apoptosis and neuronal cell death. It has been suggested that abnormalities in p53 function play a role in the progression of specific neurodegenerative diseases like PD (16). In animal models of PD, degeneration of dopaminergic cells is associated with increased levels of p53, and enhanced activation of p53 is seen in the substantia nigra of post-mortem PD brains (6, 22, 54, 55). CUL7, PARC and GIGYF2 are all expressed in the CNS, and in fact, PARC regulation of p53 was originally described in neuronal cells. This presents the intriguing possibility that the three proteins function together in the regulation of p53 activity. Loss or mutation of GIGYF2 and subsequent deregulation of p53 function would likely have significant effects on neuron survival and may be a component of GIGYF2-associated PD. Analysis of GIGYF2's role in the CUL7-PARC-p53 complex, and specifically the effect of GIGYF2 on p53 function, represents an important direction for future research.

A model for shared function of GIGYF2, Grb10 and CUL7

A primary goal of this work was to identify potential molecular functions for the GIGYF2 protein. Because GIGYF2 interacts with Grb10, an important modulator of IR and IGF-IR signaling, it was anticipated that GIGYF2 would play a role in Grb10-mediated receptor regulation. Disruption of the *Gigyf2* gene in mice revealed that loss of GIGYF2 results in altered IGF-IR signaling, confirming that GIGYF2 does indeed participate in the regulation of this receptor. We also found that GIGYF2 interacts with CUL7 and modulates CUL7 complex formation. These two activities may represent distinct functions of GIGYF2, perhaps occurring simultaneously or separately in a cell- or tissue-specific manner. Alternatively, interaction with CUL7 may facilitate GIGYF2 regulation of IGF-IR signaling. Interestingly, loss or reduction of GIGYF2, Grb10 or CUL7 protein results in identical changes in IGF-IR signaling (2, 23, 71, 86). In each case, IGF-I stimulated tyrosine phosphorylation of the IGF-IR is reduced and activation of downstream signaling molecules Akt and ERK1/2 is increased. While each of these proteins could function in parallel to regulate IGF-IR activity, it is more likely that the three proteins act coordinately in a common pathway to modulate receptor signaling.

Grb10 affects IR and IGF-IR function, in part, by regulating the endocytic trafficking of activated receptors from the plasma membrane. Increased levels of Grb10 protein promote receptor internalization and degradation through multi- or polyubiquitylation of the IGF-IR and the IR (52, 65). For the IGF-IR, this process has been shown to require the activity of the Nedd4 E3 ubiquitin ligase and direct interaction between Nedd4 and Grb10. It is thought that Grb10 functions as an adaptor protein to

recruit Nedd4 to activated IGF-IR thereby facilitating receptor ubiquitylation (51, 53).

Ubiquitin-modification is involved in multiple aspects of growth factor receptor trafficking. A number of components of the endocytic machinery contain ubiquitin-interacting motifs (UIM), and sorting and processing of endocytic vesicles involves direct interaction between UIM containing proteins and ubiquitylated cargo. Ubiquitylation of UIM containing proteins also occurs and appears to play an important role in the regulation of these proteins and subsequent trafficking of endocytic vesicles. In addition to Nedd4, a number of different E3 ubiquitin ligases have been implicated in the endocytic process. They act at multiple steps and on a variety of substrates to finely tune the internalization and trafficking of growth factor receptors (reviewed in (50)).

Given the importance of ubiquitylation in endocytic trafficking, and the established role for Grb10 in this process, it is possible that GIGYF2 modulates receptor trafficking through recruitment of the CUL7 E3 ubiquitin ligase to Grb10-receptor complexes. We have shown that the C-terminal half of GIGYF2 is sufficient for CUL7 interaction. This region is distinct from the Grb10-interacting GYF domain of GIGYF2. Therefore, it is possible that GIGYF2 binds simultaneously to Grb10 and CUL7, acting as an adaptor protein to facilitate complex formation with the activated receptor. In the absence of GIGYF, Grb10 or CUL7, appropriate attenuation of receptor signaling may not occur due to inefficient internalization and down-regulation of ligand-activated receptors, resulting in the observed increase in Akt and ERK1/2 activation. There is some debate about the subcellular location of IR and IGF-IR dephosphorylation events but recent reports suggest that receptor dephosphorylation occurs rapidly, within 30 seconds, and does not require receptor internalization (77). Thus, loss of GIGYF2,

Grb10 or CUL7 may result in an inability to traffic receptors away from sites of active dephosphorylation, explaining the decrease in receptor phosphorylation that is observed.

There is currently no direct evidence in support of this model; however, there is significant circumstantial evidence to suggest that it merits further experimentation.

First, a recent unpublished report identified GIGYF2 as a Rin1 interacting protein (84).

Rin1 is a guanine nucleotide exchange factor for Rab5, which is an important regulator of endocytic protein traffic (7). Rin1 has been shown to bind directly to the epidermal growth factor receptor (EGFR) and modulate its trafficking and signaling (3, 42); and more importantly, Rin1 participates in insulin receptor internalization and signal transduction (36). Second, the GYF domain structure of GIGYF2 is most homologous to the yeast Smy2 family of GYF domains (41). The Smy2 protein was originally identified as a high copy suppressor of a mutant protein involved in COPII-dependent vesicle transport (31). A yeast paralog of Smy2 called Myr1 interacts genetically with the yeast Rab family of proteins (Ypt) and plays a role in membrane vesicle trafficking. GIGYF2 is predicted to be an evolutionary relative of Myr1 based on their shared domain organization. Each contains a highly conserved GYF domain, hydrophobic and proline-rich regions, and a coiled-coil domain of similar amino acid composition. Myr1 even exhibits similar focal, cytotoxic aggregate formation when overexpressed (27). Finally, several Parkinson's disease genes have been linked to the regulation of vesicle trafficking although the implications of this have not been explored. α -synuclein (PARK1 and 4) is involved in synaptic vesicle transport and mutant α -synuclein interacts with Rab proteins and interferes with their function (18, 30). Parkin (PARK2) interacts with and ubiquitylates the endocytic machinery UIM-containing protein Eps15. This interaction is

stimulated by epidermal growth factor and interferes with EGFR internalization, degradation and signal attenuation (25). UCH-L1 (PARK5) deficient mice show upregulation of multiple genes involved in vesicle transport (12). Lastly, LRRK2 (PARK8) localizes to the membranes of endosomes and transport vesicles, suggesting a role in regulation of vesicular structures (5).

Our model suggests that GIGYF2 may sit at the confluence of two relatively novel mechanisms of Parkinson's disease pathogenesis, alteration of vesicle transport and insulin and IGF action. If correct, it could help define new directions for the treatment and even prevention of PD. There are still many unresolved question about GIGYF2 and its link to PD. While offering a great deal of insight into the actions and biological significance of this important protein, this work represents only the beginning of our efforts to understand GIGYF2. Continued research is needed to fully appreciate the molecular function of GIGYF2 and its involvement in human disease.

REFERENCES

1. **Andrews, P., Y. J. He, and Y. Xiong.** 2006. Cytoplasmic localized ubiquitin ligase cullin 7 binds to p53 and promotes cell growth by antagonizing p53 function. *Oncogene* **25**:4534-4548.
2. **Arai, T., J. S. Kasper, J. R. Skaar, S. H. Ali, C. Takahashi, and J. A. DeCaprio.** 2003. Targeted disruption of p185/Cul7 gene results in abnormal vascular morphogenesis. *Proc Natl Acad Sci U S A* **100**:9855-9860.
3. **Barbieri, M. A., C. Kong, P. I. Chen, B. F. Horazdovsky, and P. D. Stahl.** 2003. The SRC homology 2 domain of Rin1 mediates its binding to the epidermal growth factor receptor and regulates receptor endocytosis. *J Biol Chem* **278**:32027-32036.
4. **Bateman, J. M., and H. McNeill.** 2006. Insulin/IGF signalling in neurogenesis. *Cell Mol Life Sci* **63**:1701-1705.
5. **Biskup, S., D. J. Moore, F. Celsi, S. Higashi, A. B. West, S. A. Andrabi, K. Kurkinen, S. W. Yu, J. M. Savitt, H. J. Waldvogel, R. L. Faull, P. C. Emson, R. Torp, O. P. Ottersen, T. M. Dawson, and V. L. Dawson.** 2006. Localization of LRRK2 to membranous and vesicular structures in mammalian brain. *Ann Neurol* **60**:557-569.
6. **Biswas, S. C., E. Ryu, C. Park, C. Malagelada, and L. A. Greene.** 2005. Puma and p53 play required roles in death evoked in a cellular model of Parkinson disease. *Neurochem Res* **30**:839-845.
7. **Bliss, J. M., B. Venkatesh, and J. Colicelli.** 2005. The RIN Family of Ras Effectors. *Methods Enzymol* **407**:335-344.

8. **Bondy, C., H. Werner, C. T. J. Roberts, and D. LeRoith.** 1992. Cellular pattern of type-I insulin-like growth factor receptor gene expression during maturation of the rat brain: comparison with insulin-like growth factors I and II. *Neuroscience* **46**:909-923.
9. **Bondy, C. A., and C. M. Cheng.** 2004. Signaling by insulin-like growth factor 1 in brain. *Eur J Pharmacol* **490**:25-31.
10. **Bondy, C. A., H. Werner, C. T. J. Roberts, and D. LeRoith.** 1990. Cellular pattern of insulin-like growth factor-I (IGF-I) and type I IGF receptor gene expression in early organogenesis: comparison with IGF-II gene expression. *Mol Endocrinol* **4**:1386-1398.
11. **Boney, C. M., P. A. Gruppuso, R. A. Faris, and A. R. J. Frackelton.** 2000. The critical role of Shc in insulin-like growth factor-I-mediated mitogenesis and differentiation in 3T3-L1 preadipocytes. *Mol Endocrinol* **14**:805-813.
12. **Bonin, M., S. Poths, H. Osaka, Y. L. Wang, K. Wada, and O. Riess.** 2004. Microarray expression analysis of gad mice implicates involvement of Parkinson's disease associated UCH-L1 in multiple metabolic pathways. *Brain Res Mol Brain Res* **126**:88-97.
13. **Bruning, J. C., D. Gautam, D. J. Burks, J. Gillette, M. Schubert, P. C. Orban, R. Klein, W. Krone, D. Muller-Wieland, and C. R. Kahn.** 2000. Role of brain insulin receptor in control of body weight and reproduction. *Science* **289**:2122-2125.
14. **Charalambous, M., F. M. Smith, W. R. Bennett, T. E. Crew, F. Mackenzie, and A. Ward.** 2003. Disruption of the imprinted Grb10 gene leads to disproportionate

- overgrowth by an Igf2-independent mechanism. *Proc Natl Acad Sci U S A* **100**:8292-8297.
15. **Craft, S., and G. S. Watson.** 2004. Insulin and neurodegenerative disease: shared and specific mechanisms. *Lancet Neurol* **3**:169-178.
 16. **Culmsee, C., and M. P. Mattson.** 2005. p53 in neuronal apoptosis. *Biochem Biophys Res Commun* **331**:761-777.
 17. **D'Ercole, A. J., P. Ye, and J. R. O'Kusky.** 2002. Mutant mouse models of insulin-like growth factor actions in the central nervous system. *Neuropeptides* **36**:209-220.
 18. **Dalfo, E., T. Gomez-Isla, J. L. Rosa, M. Nieto Bodelon, M. Cuadrado Tejedor, M. Barrachina, S. Ambrosio, and I. Ferrer.** 2004. Abnormal alpha-synuclein interactions with Rab proteins in alpha-synuclein A30P transgenic mice. *J Neuropathol Exp Neurol* **63**:302-313.
 19. **Di Paola, R., E. Ciociola, W. Boonyasrisawat, D. Nolan, J. Duffy, G. Miscio, C. Cisternino, G. Fini, V. Tassi, A. Doria, and V. Trischitta.** 2006. Association of hGrb10 genetic variations with type 2 diabetes in Caucasian subjects. *Diabetes Care* **29**:1181-1183.
 20. **Dias, D. C., G. Dolios, R. Wang, and Z. Q. Pan.** 2002. CUL7: A DOC domain-containing cullin selectively binds Skp1.Fbx29 to form an SCF-like complex. *Proc Natl Acad Sci U S A* **99**:16601-16606.
 21. **Dore, S., S. Kar, and R. Quirion.** 1997. Rediscovering an old friend, IGF-I: potential use in the treatment of neurodegenerative diseases. *Trends Neurosci* **20**:326-331.

22. **Duan, W., X. Zhu, B. Ladenheim, Q. S. Yu, Z. Guo, J. Oyler, R. G. Cutler, J. L. Cadet, N. H. Greig, and M. P. Mattson.** 2002. p53 inhibitors preserve dopamine neurons and motor function in experimental parkinsonism. *Ann Neurol* **52**:597-606.
23. **Dufresne, A. M., and R. J. Smith.** 2005. The adapter protein GRB10 is an endogenous negative regulator of insulin-like growth factor signaling. *Endocrinology* **146**:4399-4409.
24. **Edmondson, S. R., G. A. Werther, A. Russell, D. LeRoith, C. T. J. Roberts, and F. Beck.** 1995. Localization of growth hormone receptor/binding protein messenger ribonucleic acid (mRNA) during rat fetal development: relationship to insulin-like growth factor-I mRNA. *Endocrinology* **136**:4602-4609.
25. **Fallon, L., C. M. Belanger, A. T. Corera, M. Kontogiannia, E. Regan-Klapisz, F. Moreau, J. Voortman, M. Haber, G. Rouleau, T. Thorarinsdottir, A. Brice, P. M. van Bergen En Henegouwen, and E. A. Fon.** 2006. A regulated interaction with the UIM protein Eps15 implicates parkin in EGF receptor trafficking and PI(3)K-Akt signalling. *Nat Cell Biol* **8**:834-842.
26. **Garcia-Segura, L. M., J. Perez, S. Pons, M. T. Rejas, and I. Torres-Aleman.** 1991. Localization of insulin-like growth factor I (IGF-I)-like immunoreactivity in the developing and adult rat brain. *Brain Res* **560**:167-174.
27. **Georgiev, A., A. Leipus, I. Olsson, J. M. Berrez, and A. Mutvei.** 2008. Characterization of MYR1, a dosage suppressor of YPT6 and RIC1 deficient mutants. *Curr Genet* **53**:235-247.

28. **Geraerts, M., O. Krylyshkina, Z. Debyser, and V. Baekelandt.** 2007. Concise review: therapeutic strategies for Parkinson disease based on the modulation of adult neurogenesis. *Stem Cells* **25**:263-270.
29. **Giovannone, B., E. Lee, L. Laviola, F. Giorgino, K. A. Cleveland, and R. J. Smith.** 2003. Two novel proteins that are linked to insulin-like growth factor (IGF-I) receptors by the Grb10 adapter and modulate IGF-I signaling. *J Biol Chem* **278**:31564-31573.
30. **Gitler, A. D., B. J. Bevis, J. Shorter, K. E. Strathearn, S. Hamamichi, L. J. Su, K. A. Caldwell, G. A. Caldwell, J. C. Rochet, J. M. McCaffery, C. Barlowe, and S. Lindquist.** 2008. The Parkinson's disease protein alpha-synuclein disrupts cellular Rab homeostasis. *Proc Natl Acad Sci U S A* **105**:145-150.
31. **Higashio, H., K. Sato, and A. Nakano.** 2008. Smy2p participates in COPII vesicle formation through the interaction with Sec23p/Sec24p subcomplex. *Traffic* **9**:79-93.
32. **Hofseth, L. J., S. P. Hussain, and C. C. Harris.** 2004. p53: 25 years after its discovery. *Trends Pharmacol Sci* **25**:177-181.
33. **Holt, L. J., and K. Siddle.** 2005. Grb10 and Grb14: enigmatic regulators of insulin action--and more? *Biochem J* **388**:393-406.
34. **Hu, G., P. Jousilahti, S. Bidel, R. Antikainen, and J. Tuomilehto.** 2007. Type 2 diabetes and the risk of Parkinson's disease. *Diabetes Care* **30**:842-847.
35. **Humbert, S., E. A. Bryson, F. P. Cordelieres, N. C. Connors, S. R. Datta, S. Finkbeiner, M. E. Greenberg, and F. Saudou.** 2002. The IGF-1/Akt pathway is neuroprotective in Huntington's disease and involves Huntingtin phosphorylation by Akt. *Dev Cell* **2**:831-837.

36. **Hunker, C. M., H. Giambini, A. Galvis, J. Hall, I. Kruk, M. L. Veisaga, and M. A. Barbieri.** 2006. Rin1 regulates insulin receptor signal transduction pathways. *Exp Cell Res* **312**:1106-1118.
37. **Joers, A., V. Jaks, J. Kase, and T. Maimets.** 2004. p53-dependent transcription can exhibit both on/off and graded response after genotoxic stress. *Oncogene* **23**:6175-6185.
38. **Jung, P., B. Verdoodt, A. Bailey, J. R. r. Yates, A. Menssen, and H. Hermeking.** 2007. Induction of cullin 7 by DNA damage attenuates p53 function. *Proc Natl Acad Sci U S A* **104**:11388-11393.
39. **Kay, J. N., and M. Blum.** 2000. Differential response of ventral midbrain and striatal progenitor cells to lesions of the nigrostriatal dopaminergic projection. *Dev Neurosci* **22**:56-67.
40. **Kim, S. S., M. Shago, L. Kaustov, P. C. Boutros, J. W. Clendening, Y. Sheng, G. A. Trentin, D. Barsyte-Lovejoy, D. Y. Mao, R. Kay, I. Jurisica, C. H. Arrowsmith, and L. Z. Penn.** 2007. CUL7 is a novel antiapoptotic oncogene. *Cancer Res* **67**:9616-9622.
41. **Kofler, M. M., and C. Freund.** 2006. The GYF domain. *FEBS J* **273**:245-256.
42. **Kong, C., X. Su, P. I. Chen, and P. D. Stahl.** 2007. Rin1 interacts with signal-transducing adaptor molecule (STAM) and mediates epidermal growth factor receptor trafficking and degradation. *J Biol Chem* **282**:15294-15301.
43. **Lansbury, P. T., and H. A. Lashuel.** 2006. A century-old debate on protein aggregation and neurodegeneration enters the clinic. *Nature* **443**:774-779.

44. **Laviola, L., F. Giorgino, J. C. Chow, J. A. Baquero, H. Hansen, J. Ooi, J. Zhu, H. Riedel, and R. J. Smith.** 1997. The adapter protein Grb10 associates preferentially with the insulin receptor as compared with the IGF-I receptor in mouse fibroblasts. *J Clin Invest* **99**:830-837.
45. **Lie, D. C., G. Dziewczapolski, A. R. Willhoite, B. K. Kaspar, C. W. Shults, and F. H. Gage.** 2002. The adult substantia nigra contains progenitor cells with neurogenic potential. *J Neurosci* **22**:6639-6649.
46. **Lim, M. A., H. Riedel, and F. Liu.** 2004. Grb10: more than a simple adaptor protein. *Front Biosci* **9**:387-403.
47. **Ma, C., D. Papermaster, and C. L. Cepko.** 1998. A unique pattern of photoreceptor degeneration in cyclin D1 mutant mice. *Proc Natl Acad Sci U S A* **95**:9938-9943.
48. **Mao, L., Y. S. Lau, E. Petroske, and J. Q. Wang.** 2001. Profound astrogenesis in the striatum of adult mice following nigrostriatal dopaminergic lesion by repeated MPTP administration. *Brain Res Dev Brain Res* **131**:57-65.
49. **Maraganore, D. M., M. de Andrade, T. G. Lesnick, K. J. Strain, M. J. Farrer, W. A. Rocca, P. V. Pant, K. A. Frazer, D. R. Cox, and D. G. Ballinger.** 2005. High-resolution whole-genome association study of Parkinson disease. *Am J Hum Genet* **77**:685-693.
50. **Marmor, M. D., and Y. Yarden.** 2004. Role of protein ubiquitylation in regulating endocytosis of receptor tyrosine kinases. *Oncogene* **23**:2057-2070.

51. **Monami, G., V. Emiliozzi, and A. Morrione.** 2008. Grb10/Nedd4-mediated multiubiquitination of the insulin-like growth factor receptor regulates receptor internalization. *J Cell Physiol*
52. **Morrione, A.** 2003. Grb10 adapter protein as regulator of insulin-like growth factor receptor signaling. *J Cell Physiol* **197**:307-311.
53. **Morrione, A., P. Plant, B. Valentinis, O. Staub, S. Kumar, D. Rotin, and R. Baserga.** 1999. mGrb10 interacts with Nedd4. *J Biol Chem* **274**:24094-24099.
54. **Nair, V. D.** 2006. Activation of p53 signaling initiates apoptotic death in a cellular model of Parkinson's disease. *Apoptosis* **11**:955-966.
55. **Nair, V. D., K. S. McNaught, J. Gonzalez-Maeso, S. C. Sealfon, and C. W. Olanow.** 2006. p53 mediates nontranscriptional cell death in dopaminergic cells in response to proteasome inhibition. *J Biol Chem* **281**:39550-39560.
56. **Nikolaev, A. Y., M. Li, N. Puskas, J. Qin, and W. Gu.** 2003. Parc: a cytoplasmic anchor for p53. *Cell* **112**:29-40.
57. **Offen, D., B. Shtaif, D. Hadad, A. Weizman, E. Melamed, and I. Gil-Ad.** 2001. Protective effect of insulin-like-growth-factor-1 against dopamine-induced neurotoxicity in human and rodent neuronal cultures: possible implications for Parkinson's disease. *Neurosci Lett* **316**:129-132.
58. **Okabe, H., S. H. Lee, J. Phuchareon, D. G. Albertson, F. McCormick, and O. Tetsu.** 2006. A critical role for FBXW8 and MAPK in cyclin D1 degradation and cancer cell proliferation. *PLoS ONE* **1**:e128.
59. **Pankratz, N., W. C. Nichols, S. K. Uniacke, C. Halter, J. Murrell, A. Rudolph, C. W. Shults, P. M. Conneally, and T. Foroud.** 2003. Genome-wide linkage

- analysis and evidence of gene-by-gene interactions in a sample of 362 multiplex Parkinson disease families. *Hum Mol Genet* **12**:2599-2608.
60. **Pankratz, N., W. C. Nichols, S. K. Uniacke, C. Halter, A. Rudolph, C. Shults, P. M. Conneally, and T. Foroud.** 2002. Genome screen to identify susceptibility genes for Parkinson disease in a sample without parkin mutations. *Am J Hum Genet* **71**:124-135.
 61. **Pankratz, N., W. C. Nichols, S. K. Uniacke, C. Halter, A. Rudolph, C. Shults, P. M. Conneally, and T. Foroud.** 2003. Significant linkage of Parkinson disease to chromosome 2q36-37. *Am J Hum Genet* **72**:1053-1057.
 62. **Peretz, S., R. Jensen, R. Baserga, and P. M. Glazer.** 2001. ATM-dependent expression of the insulin-like growth factor-I receptor in a pathway regulating radiation response. *Proc Natl Acad Sci U S A* **98**:1676-1681.
 63. **Powers, K. M., T. Smith-Weller, G. M. Franklin, W. T. J. Longstreth, P. D. Swanson, and H. Checkoway.** 2006. Diabetes, smoking, and other medical conditions in relation to Parkinson's disease risk. *Parkinsonism Relat Disord* **12**:185-189.
 64. **Pressley, J. C., E. D. Louis, M. X. Tang, L. Cote, P. D. Cohen, S. Glied, and R. Mayeux.** 2003. The impact of comorbid disease and injuries on resource use and expenditures in parkinsonism. *Neurology* **60**:87-93.
 65. **Ramos, F. J., P. R. Langlais, D. Hu, L. Q. Dong, and F. Liu.** 2006. Grb10 mediates insulin-stimulated degradation of the insulin receptor: a mechanism of negative regulation. *Am J Physiol Endocrinol Metab* **290**:E1262-6.

66. **Rampersaud, E., C. M. Damcott, M. Fu, H. Shen, P. McArdle, X. Shi, J. Shelton, J. Yin, Y. P. Chang, S. H. Ott, L. Zhang, Y. Zhao, B. D. Mitchell, J. O'Connell, and A. R. Shuldiner.** 2007. Identification of novel candidate genes for type 2 diabetes from a genome-wide association scan in the Old Order Amish: evidence for replication from diabetes-related quantitative traits and from independent populations. *Diabetes* **56**:3053-3062.
67. **Riedel, H.** 2004. Grb10 exceeding the boundaries of a common signaling adapter. *Front Biosci* **9**:603-618.
68. **Rotwein, P., S. K. Burgess, J. D. Milbrandt, and J. E. Krause.** 1988. Differential expression of insulin-like growth factor genes in rat central nervous system. *Proc Natl Acad Sci U S A* **85**:265-269.
69. **Russo, V. C., P. D. Gluckman, E. L. Feldman, and G. A. Werther.** 2005. The insulin-like growth factor system and its pleiotropic functions in brain. *Endocr Rev* **26**:916-943.
70. **Sandyk, R.** 1993. The relationship between diabetes mellitus and Parkinson's disease. *Int J Neurosci* **69**:125-130.
71. **Sarikas, A., X. Xu, L. Field, and Z. Q. Pan.** 2007. 535. THE CUL7 E3 UBIQUITIN LIGASE IS A NEGATIVE REGULATOR OF ONCOGENE-INDUCED SENESENCE. European Life Scientist Organization (ELSO) 2007 Poster Abstracts

<http://www.elseo.org/index.php?id=abstrlist2007&lid=616>

72. **Scigliano, G., M. Musicco, P. Soliveri, I. Piccolo, G. Ronchetti, and F. Girotti.** 2006. Reduced risk factors for vascular disorders in Parkinson disease patients: a case-control study. *Stroke* **37**:1184-1188.
73. **Scolnick, J. A., K. Cui, C. D. Duggan, S. Xuan, X. B. Yuan, A. Efstratiadis, and J. Ngai.** 2008. Role of IGF signaling in olfactory sensory map formation and axon guidance. *Neuron* **57**:847-857.
74. **Shan, X., L. Chi, M. Bishop, C. Luo, L. Lien, Z. Zhang, and R. Liu.** 2006. Enhanced de novo neurogenesis and dopaminergic neurogenesis in the substantia nigra of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease-like mice. *Stem Cells* **24**:1280-1287.
75. **Sherr, C. J., and F. McCormick.** 2002. The RB and p53 pathways in cancer. *Cancer Cell* **2**:103-112.
76. **Sherr, C. J., and J. M. Roberts.** 2004. Living with or without cyclins and cyclin-dependent kinases. *Genes Dev* **18**:2699-2711.
77. **Shi, K., K. Egawa, H. Maegawa, T. Nakamura, S. Ugi, Y. Nishio, and A. Kashiwagi.** 2004. Protein-tyrosine phosphatase 1B associates with insulin receptor and negatively regulates insulin signaling without receptor internalization. *J Biochem* **136**:89-96.
78. **Sicinski, P., J. L. Donaher, S. B. Parker, T. Li, A. Fazeli, H. Gardner, S. Z. Haslam, R. T. Bronson, S. J. Elledge, and R. A. Weinberg.** 1995. Cyclin D1 provides a link between development and oncogenesis in the retina and breast. *Cell* **82**:621-630.

79. **Skaar, J. R., T. Arai, and J. A. DeCaprio.** 2005. Dimerization of CUL7 and PARC is not required for all CUL7 functions and mouse development. *Mol Cell Biol* **25**:5579-5589.
80. **Skaar, J. R., L. Florens, T. Tsutsumi, T. Arai, A. Tron, S. K. Swanson, M. P. Washburn, and J. A. DeCaprio.** 2007. PARC and CUL7 form atypical cullin RING ligase complexes. *Cancer Res* **67**:2006-2014.
81. **Steiner, B., C. Winter, K. Hosman, E. Siebert, G. Kempermann, D. S. Petrus, and A. Kupsch.** 2006. Enriched environment induces cellular plasticity in the adult substantia nigra and improves motor behavior function in the 6-OHDA rat model of Parkinson's disease. *Exp Neurol* **199**:291-300.
82. **Tamaru, T., M. Okada, and H. Nakagawa.** 1994. Differential expression of D type cyclins during neuronal maturation. *Neurosci Lett* **168**:229-232.
83. **Tamaru, T., S. K. Trigun, M. Okada, and H. Nakagawa.** 1993. Identification of cells expressing a D type G1 cyclin in matured brain: implication for its role in neuronal function. *Neurosci Lett* **153**:169-172.
84. **Tomshine, J. C., and B. F. Horazdovsky.** 2007. Identification of New Rin1 Interaction Partners. The American Society for Cell Biology 47th Annual Meeting **2007 ASCB Late Abstracts**:71.
85. **Trejo, J. L., E. Carro, E. Garcia-Galloway, and I. Torres-Aleman.** 2004. Role of insulin-like growth factor I signaling in neurodegenerative diseases. *J Mol Med* **82**:156-162.

86. **Tsutsumi, T., H. Kuwabara, T. Arai, Y. Xiao, and J. A. Decaprio.** 2008. Disruption of the Fbxw8 gene results in pre- and postnatal growth retardation in mice. *Mol Cell Biol* **28**:743-751.
87. **van Lookeren Campagne, M., and R. Gill.** 1998. Tumor-suppressor p53 is expressed in proliferating and newly formed neurons of the embryonic and postnatal rat brain: comparison with expression of the cell cycle regulators p21Waf1/Cip1, p27Kip1, p57Kip2, p16Ink4a, cyclin G1, and the proto-oncogene Bax. *J Comp Neurol* **397**:181-198.
88. **Wang, L., B. Balas, C. Y. Christ-Roberts, R. Y. Kim, F. J. Ramos, C. K. Kikani, C. Li, C. Deng, S. Reyna, N. Musi, L. Q. Dong, R. A. DeFronzo, and F. Liu.** 2007. Peripheral disruption of the Grb10 gene enhances insulin signaling and sensitivity in vivo. *Mol Cell Biol* **27**:6497-6505.
89. **Werther, G. A., A. Hogg, B. J. Oldfield, M. J. McKinley, R. Figdor, A. M. Allen, and F. A. Mendelsohn.** 1987. Localization and characterization of insulin receptors in rat brain and pituitary gland using in vitro autoradiography and computerized densitometry. *Endocrinology* **121**:1562-1570.
90. **Yoshihashi, H., K. Maeyama, R. Kosaki, T. Ogata, M. Tsukahara, Y. Goto, J. Hata, N. Matsuo, R. J. Smith, and K. Kosaki.** 2000. Imprinting of human GRB10 and its mutations in two patients with Russell-Silver syndrome. *Am J Hum Genet* **67**:476-482.
91. **Yue, Q., M. Groszer, J. S. Gil, A. J. Berk, A. Messing, H. Wu, and X. Liu.** 2005. PTEN deletion in Bergmann glia leads to premature differentiation and affects laminar organization. *Development* **132**:3281-3291.

92. **Zhao, C., W. Deng, and F. H. Gage.** 2008. Mechanisms and functional implications of adult neurogenesis. *Cell* **132**:645-660.
93. **Zhao, M., S. Momba, K. Delfani, M. Carlen, R. M. Cassidy, C. B. Johansson, H. Brismar, O. Shupliakov, J. Frisen, and A. M. Janson.** 2003. Evidence for neurogenesis in the adult mammalian substantia nigra. *Proc Natl Acad Sci U S A* **100**:7925-7930.