Characterising the function of a novel Embryonic Stem cell-associated signal transducer, Gab1β

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DECLARATION

I declare that the work presented in this thesis is my own, except where otherwise stated. The contribution of others has been clearly indicated. No part of this thesis has been, or will be submitted for any other degree, diploma or qualification.

Daniela Gattegno Ho

December 2008
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Activation of Ras/mitogen-activated protein kinase (ERK MAPK) signalling controls the differentiation of mouse embryonic stem (ES) cells. An established modulator of the ERK MAPK pathway is the IRS-1 (Insulin Receptor Substrate 1) family adaptor protein Gab1 (Grb2-associated binder 1). Gab1 is ubiquitously expressed and is activated by a wide range of cell surface receptors, mediating growth factor, cell-cell and cell-substratum interactions. The N-terminal region of Gab1 contains a pleckstrin homology (PH) domain required for membrane binding and a nuclear localisation sequence (NLS) that facilitates nuclear translocation. Undifferentiated mouse ES cells preferentially express high levels of a novel form of Gab1 (Gab1β) lacking the N-terminal region. Based on its novel structure and abundance, Gab1β may act in a dominant negative manner by binding and mislocalising downstream effectors. Alternatively, it may have a deregulated function unrestrained by the PH or NLS domains. Data presented here shows that Gab1β is tyrosine phosphorylated in response to the self-renewal factor Leukemia Inhibitory Factor (LIF) and/or Foetal Bovine Serum (FBS) stimulation. This then leads to the formation of complexes with Shp2 and the p85 subunit of PI3K. Experiments comparing the responses of wild-type and Gab1β knock-out ES cells indicate that Gab1β enhances ERK and potentially AKT phosphorylation in response to LIF. In contrast, Gab1β has a negative effect on ERK and AKT phosphorylation in response to IGF-1 (Insulin...
Growth Factor 1). These results suggest that the contribution of Gab1β to signalling activity is receptor specific and may imply that the response of ES cells to ERK activation is context specific. By reintroducing fluorescently tagged Gab1 proteins into Gab1β knockout ES cells, I investigated the localisation of Gab1β in ES cells. Gab1β localised at the cell membrane as well as in a perinuclear body. I next investigated the potential role of Gab1β in the differentiation of ES cells into neural precursors. A monolayer differentiation protocol was used to differentiate Gab1β wild-type and knock-out cells into neural precursors. Furthermore, the effect of insulin on the emergence of neural precursors from Gab1β-targeted cells was also explored.
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CHAPTER 1: INTRODUCTION

1.1 Embryonic Stem cells

Multicellular organisms require the existence and precise control of stem cells, which maintain tissue homeostasis by replacing terminally, differentiated, aged or injured cells (Boiani and Scholer, 2005). Stem cells are cells that have the ability to divide for indefinite periods in the undifferentiated state but also retain the potential to give rise to specialised cells (Boiani and Scholer, 2005). In addition to somatic stem cell systems in the foetus and the adult, stem cells can also be obtained from inner cell mass (ICM) of peri- and early post-implantation-stage embryos. Such cells are called embryonic stem cells (ES cells) (Evans and Kaufman, 1981; Martin, 1981). ES cells can be readily propagated in culture, unlike most somatic stem cells. As the in vitro counterpart of epiblast cells (Smith, 2001), ES cells have the capacity to differentiate into cells of all three germ layers (endoderm, mesoderm, ectoderm), a property referred to as pluripotency. This can be demonstrated by their injection into a mouse blastocyst, and their contribution to all the tissues of the developing mouse including colonisation of the germline (Bradley et al., 1992). Because pluripotency is a fundamental biological function in multicellular organisms, it is probably evolutionarily secured by numerous genetic backup systems and conserved among different types of stem cell (Boiani and Scholer, 2005). Another property of ES cells is their continuous self-renewal, which requires the unique transcriptional profile of the pluripotent state to be maintained (Chen and
Daley, 2008). Despite the considerable interest in the use and manipulation of ES cells, some of the factors that regulate their unique properties are still to be elucidated.

1.2 Regulation of Embryonic Stem cell self-renewal and pluripotency

In early experimental approaches to address the molecular mechanism of pluripotency, it was hypothesised that pluripotent cells express a unique set of factors that underlie their “stemness” (Chen and Daley, 2008). This is supported by a study, which set out to characterise the transcriptional profile of embryonic and adult stem cells (Ramalho-Santos et al., 2002). In the latter study, the authors demonstrated that self-renewing cells do share common genetic profiles, but that ES cells express a significantly higher number of genes. Understanding ES cell self-renewal and the factors that underlie these mechanisms is important for the future therapeutic use of ES cells. In addition to general pathways of signal transduction that operate in most cell types, several exogenous factors are known to modulate stem cell pluripotency and self-renewal (Boiani and Scholer, 2005). Therefore, ES cell self-renewal is directed by both intrinsic and extrinsic factors. The best-characterised factors regulating ES cell self-renewal are outlined below.
1.2.1 Endogenous self-renewal signals

Several key factors regulating ES cell self-renewal have been identified. They include the homeodomain transcription factors Oct4 and Nanog, as well as the HMG-box transcription factor Sox2. These three transcription factors have been shown to co-occupy the promoters of a large population of regulatory genes potentially involved in specialised circuits in human ES cells (Boyer et al., 2005).

1.2.1.1 Oct4

The murine Oct4 gene encodes a Class V POU transcription factor containing a POU-specific domain and a homeodomain (Okamoto et al., 1990; Rosner et al., 1990). The Oct4 protein is a member of the POU family of transcription factors that bind the octamer sequence ATGCAAAT and is expressed in early embryo cells and germ cells (Okamoto et al., 1990; Pesce and Scholer, 2001; Rosner et al., 1990). Oct4 is highly expressed in pluripotent ES cells, but is absent from all differentiated somatic cell types in vivo and in vitro (Niwa et al., 2002; Niwa et al., 2000; Rosner et al., 1990). Oct4 was recently shown to be involved in the maintenance of ES cell survival and antiapoptosis mechanisms (Guo et al., 2008). In this study, using a tetracycline-inducible ES cell line (ZHBtc4) and its parental control line (CGR8), Oct4 was demonstrated to be protect ES cells from apoptosis, induced by etoposide, UV or heat shock. Upon reduction of Oct4, the percentage of caspase-positive
cells, a measure of apoptotic cells, increased, in contrast to parental cells. Therefore, not only is Oct4 involved in the maintenance of ES cell self-renewal and pluripotency, but in the survival of ES cells too. This suggests a more global role for Oct4 in ES cell biology. The feature common to all cells expressing Oct4 in the early embryo is that they retain the capacity for differentiation along multiple lineages (Rosner et al., 1990). When Oct4 is deleted from ES cells, they transdifferentiate into trophectodermal cells, indicating that Oct4 is required for ES cells to maintain their pluripotent identity (Chambers and Smith, 2004; Niwa et al., 2000). Similarly, Oct4-/- embryos die at peri-implantation stages because of the conversion of ICM into trophectoderm (Nichols et al., 1998). Interestingly, a PouV gene from Xenopus laevis, Xlpou91, has the capacity to maintain murine ES cells in the absence of Oct4 (Morrison and Brickman, 2006). This suggests that Oct4 may act as a conserved transcription factor in the maintenance of pluripotency in both mammals and amphibians. It is the amount of Oct4, however, that controls the fate of ES cells (Niwa et al., 2000). A less than 2-fold increase from the normal expression levels causes differentiation into ectoderm and mesoderm, whereas a reduction to less than 50% leads to de-differentiation into trophectoderm (Niwa et al., 2000; Okumura-Nakanishi et al., 2005). This reduction in Oct4 is coupled with the upregulation of Cdx2 (Caudal-related homeobox 2) and Eomes (Eomesodermin) (Niwa et al., 2000; Niwa et al., 2005). Ectopic expression of Oct4 in immortalised murine cells results in their transformation (Gidekel et al., 2003). This suggests that the reactivation of a pluripotency factor such as Oct4 may contribute to tumourigenesis in somatic tissues (Hochedlinger et al., 2005). Furthermore, Oct4 expression is
downregulated in ES cells upon withdrawal of LIF (Chambers and Smith, 2004; Nichols et al., 1998), which suggests that Oct4 may be a target of the LIF/Stat3 signalling cascade. However, when Oct4 expression in ES cells is maintained via a tetracycline-dependent transgene and LIF is withdrawn, the cells differentiate (Niwa et al., 2000). This suggests that other factors may be acting along with Oct4 in order to maintain pluripotency. Many ES cell-expressed genes (e.g. the zinc-finger protein Zfp42/Rex-1) have Oct4 binding domains within their regulatory elements and so Oct4 can act directly to activate these target genes (Friel et al., 2005). It may also act by forming complexes with transcriptional co-factors. For example the Sry-related factor Sox2 is thought to cooperate with Oct4 in order to maintain self-renewal (Avilion et al., 2003).

1.2.1.2 Sox2

Sox2 is a member of the SRY-related HMG box gene family that encode transcription factors with a single HMG DNA-binding domain (Avilion et al., 2003). Like Oct4, it is expressed in the ICM, epiblast and germ cells, but, unlike Oct4, it is also expressed in the multipotential cells of the extraembryonic ectoderm of the mouse embryo (Avilion et al., 2003). Sox2-null mice have primary defects in the pluripotent epiblast and the null blastocysts are incapable of giving rise to pluripotent ES cells (Avilion et al., 2003). Small increases in the level of Sox2 have been shown to trigger the differentiation of ES cells (Kopp et al., 2008). Although Sox2 homozygous
mutants have been shown to undergo early embryonic lethality (Avilion et al., 2003), it is thought that the requirement of ES cells for Sox2 is based on its indirect association with Oct4 on a pluripotency-associated gene enhancer containing Oct4 and Sox2 binding motifs. Sox2 and Oct4 have been shown to physically interact and to cooperatively regulate their own expression by binding to adjacent HMG and POU motifs within enhancers required for the transcription of each gene (Okumura-Nakanishi et al., 2005; Tomioka et al., 2002). Furthermore, the expression of most pluripotency-associated genes, such as Fgf4 (Ambrosetti et al., 2000; Yuan et al., 1995), Utf1 (Nishimoto et al., 1999), Fbx15 (Tokuzawa et al., 2003), Lefty1 (Nakatake et al., 2006) and Nanog (Kuroda et al., 2005; Rodda et al., 2005), is regulated by enhancers, which are highly active in undifferentiated ES cells, but not in differentiated cells. New evidence has shown that, using an inducible Sox2-null ES cell line, Sox2 is indispensable for maintaining ES cell pluripotency, as Sox2 null cells differentiate into trophectoderm-like cells (Masui et al., 2007). Interestingly, however, by monitoring the levels of Oct4/Sox2 enhancer-dependent genes (such as Fgf4, Nanog, Lefty1 etc), it was shown that Sox2 is not required for the activation of the Oct4/Sox2 enhancers. These enhancers remained active even after the depletion of Sox2 protein. The authors therefore suggested that Sox2 regulates the expression of Oct4 through the regulation of multiple transcription factors. Furthermore, in a study trying to identify the regulatory interactions between the fundamental regulators (Oct4, Sox2 and Nanog), and additional transcription factors (such as Stat3, Klf4 and others), it was found that the three “master regulators” worked in conjunction with other transcription factors to control ES cell biology (Zhou et al., 2007). These
additional factors may act in a repressive or positive manner on the control of target genes. These data suggest that ES cells are governed by a complex regulatory network of many factors, whose repressive and enhancing actions result in the maintenance of self-renewal and pluripotency.

1.2.1.3 Nanog

Nanog was initially described as a novel divergent homeobox transcription factor which promotes ES self-renewal, pluripotency and epiblast formation (Chambers et al., 2003; Mitsui et al., 2003). Nanog-null embryos fail to form epiblasts, and are mostly composed of disorganised extraembryonic tissue (Chambers et al., 2003; Mitsui et al., 2003). Nanog is specifically expressed in ES cells but not in differentiated cells. Furthermore, Nanog has been shown to act independently of the LIF cascade (the cytokine required to maintain ES cell self-renewal in culture), as overexpression of Nanog leads to LIF-independent self-renewal of ES cells (Chambers et al., 2003). Nanog has been shown to require dimerisation in order to exert its positive effects on the maintenance of self-renewal and pluripotency (Mullin et al., 2008; Wang et al., 2008). A Nanog monomer was shown to be unable to replace wild-type Nanog to sustain LIF-independent self-renewal of ES cells. How Nanog is able to sustain ES cells self-renewal independently of LIF is still unclear. Nanog was recently demonstrated to bind to NFκB, which then represses its pro-differentiative effects (Torres and Watt, 2008). The authors also demonstrated that Nanog does this in cooperation with Stat3, the
transcription factor activated by LIF, to maintain pluripotency. Once Nanog is downregulated, human and mouse ES cells undergo differentiation by showing morphological changes characteristic of differentiated cells, as well as a decrease in proliferation rate (Ivanova et al., 2006; Liu et al., 2008; Zaehres et al., 2005). In contrast, Nanog overexpressing cells show a marked increase in reprogramming activity after fusion with neural stem cells (Silva et al., 2006). A series of differentially expressed genes has been identified upon the downregulation of Nanog in ES cells (Liu et al., 2008). The general trend was that genes known to be involved in ES cell self-renewal and pluripotency, such as Utf1 and Klf4, were downregulated upon Nanog silencing. Similarly, genes known to be involved in ES cell differentiation, such as Lefty1, were upregulated during Nanog silencing. Interestingly, Nanog function requires the continued presence of Oct4. As described above, when ES cells that express Oct4 from the tetracycline-regulated locus have their Oct4 transgene repressed, ES cells differentiate along the trophectoderm lineage, an effect rescued by the transfection of Oct4 cDNA into the cells (Niwa et al., 2002). This however cannot be prevented by the transfection of Nanog cDNA (Chambers et al., 2003). Nanog and Oct4 have also been shown to interact with each other as well as with proteins from multiple repression complexes, such as the NuRD, Sin3A and Pml complexes (Liang et al., 2008). Furthermore, Zfp143, a selenocysteine tRNA gene transcription-activating factor, was recently shown to interact with Oct4 on a cis-regulatory element of Nanog, thereby activating Nanog transcription (Chen et al., 2008). This was also true for another zinc finger transcription factor, Zfp281, which was shown to physically interact with Oct4, Nanog and Sox2. Therefore, despite
the fact that Oct4 and Nanog may act independently, their signals must occur simultaneously in order to maintain pluripotency in the epiblast. This is sure to be dependent on various repressive and activating transcription factor networks, which act in concert to maintain ES cell self-renewal and pluripotency. More recently, however, Nanog was shown to be undetectable in around 20% of cells that express Oct4 (Chambers et al., 2007). Nanog null cells were shown to produce teratomas containing tissues representative of all three germ layers. Furthermore, Nanog null cells are fully capable of forming undifferentiated AP positive colonies. This observation lead to the discovery that the permanent loss of Nanog in ES cells does not mark commitment, but it merely predisposes them to towards the differentiation into germ cells (Chambers et al., 2007).

1.2.2 Induced Pluripotent Stem cells

The property of ES cells to self-renew indefinitely while maintaining pluripotency has lead to the expectation of their potential use in the treatment of various diseases as well as drug screening and genetic therapy. The use of human embryos, however, has shown to be ethically questionable and therefore the use of human ES cells has been hindered. A seminal study successfully demonstrated that the pluripotent state could be induced from a differentiated cell. Takahashi et al. demonstrated that ES-like cells can be generated from both mouse (Takahashi et al., 2007; Takahashi and Yamanaka, 2006) and human (Takahashi et al., 2007) fibroblasts by the
reprogramming of their Oct4, Sox2, Klf4 and cMyc genes. Such cells are now known as induced pluripotent stem (iPS) cells. The use of iPS cells not only bypasses the ethically challenging use of embryos, but the risk of immune rejection as well during the treatment of disease. iPS cells are comparable to ES cells, as they exhibit the growth and morphology of ES cells and express ES cells marker genes such as Nanog and ERα (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Furthermore, iPS cells stain positive for alkaline phosphatase and the ES cell-specific surface marker SSEA1, differentiate into all three germ layers in vitro and form teratomas when injected into immunodeficient mice (Takahashi and Yamanaka, 2006; Zhao and Daley, 2008).

iPS cells are generated by the retroviral transduction of four defined factors: Oct4, Sox2, Klf4 and c-Myc. Klf4 (gut-enriched Kruppel-like factor) belongs to the Kruppel-like factor (Klf) family of evolutionarily conserved zinc finger transcription factors that regulate numerous biological processes such as proliferation, differentiation, development and apoptosis (Jiang et al., 2008; McConnell et al., 2007). Although Klf4 is not required for the maintenance of the undifferentiated state in ES cells, simultaneous depletion of Klf2, Klf4 and Klf5 in ES cells leads to differentiation (Jiang et al., 2008). Klf4 has also been shown to repress p53 directly (Rowland et al., 2005), and p53 has been demonstrated to suppress Nanog during ES cell differentiation (Lin et al., 2005). This suggests that Klf4, although not directly required for the maintenance of pluripotency, is still an important factor in a wide network of signalling proteins controlling ES cell biology.
c-Myc is part of the Myc family of oncogenes encoding a basic helix-loop-helix leucine zipper transcription factor (de Alboran et al., 2001), and contributes to many human tumours. Other members of the family include N-Myc, L-Myc, S-Myc and B-Myc (Adhikary and Eilers, 2005). Activated c-Myc has been shown to be important in the progression of Burkitt’s lymphoma (Spencer and Groudine, 1991). Its expression has also been demonstrated to be elevated or deregulated in a variety of other human cancers, such as breast, prostate and gastrointestinal cancers. (Nesbit et al., 1999; Pelengaris and Khan, 2003; Schlagbauer-Wadl et al., 1999). c-Myc is expressed in the whole embryo and in adult tissues where cells have a high proliferative capacity. As well promoting cell growth and proliferation (de Alboran et al., 2001), c-Myc inhibits terminal differentiation of most cell types and sensitizes cells to apoptosis (Pelengaris and Khan, 2003). Interestingly, c-Myc has been shown to suppress p21\textsuperscript{CIP1/WAF1} expression in colonic epithelial cells, as the re-expression of p21\textsuperscript{CIP1/WAF1} restores both cell cycle arrest as well as cellular differentiation in cells that express c-Myc (Adhikary and Eilers, 2005; van de Wetering et al., 2002). This suggests that c-Myc may have a role in the maintenance of ES cell proliferation as well as an anti-differentiation factor. c-Myc null embryos die by embryonic day 10.5 with defects in growth, cardiac and neural development. c-Myc null ES cells are compromised in their differentiative and growth potential as well as their ability to give rise to tumours (Baudino et al., 2002). Furthermore, Myc has been shown to be a target of Stat3 in ES cells (Cartwright et al., 2005). This suggests that Myc activity is involved to the maintenance of self-renewal and
pluripotency. However, limited Myc expression in response to MAPK/Erk inhibition cannot be rescued by the addition of LIF (Ying et al., 2008). This suggests that increased levels of Myc are not required for ES cell propagation, although some requirement for basal Myc activity cannot be excluded.

1.2.3 The LIF signal cascade

When they were originally isolated, ES cells required co-culture with mitotically inactive fibroblasts in order to maintain self-renewal. Since then, however, it has been demonstrated that murine ES cells will self-renew indefinitely if grown in medium containing the differentiation inhibitor called Leukaemia Inhibitory Factor (LIF) (Smith et al., 1988; Williams et al., 1988). LIF is a cytokine belonging to the IL-6 family and was initially characterised by its ability to induce differentiation of M1 leukaemia cells (Gearing et al., 1987; Tomida et al., 1984). It exerts its effect by binding to the LIF receptor (LIFR) – gp130 receptor heterodimer on the cell membrane and activating the transcription factor signal transducer and activator of transcription-3 (Stat3). The addition of LIF to ES cell cultures activates the LIFR-gp130 heterodimer. Stat3 then binds to phosphotyrosine residues on the dimeric receptor (Davis et al., 1993) and undergoes phosphorylation and dimerisation itself. Phosphorylated Stat3 dimers then translocate to the nucleus, where they regulate target genes (Boiani and Scholer, 2005; Niwa et al., 1998). Stat3 is the critical signalling target of LIF in allowing ES cell self-
renewal. Inactivation of Stat3 function in LIF-maintained ES cells by overexpression of a Stat3 dominant negative mutant triggers their morphological differentiation in the presence of LIF (Boeuf et al., 1997; Niwa et al., 1998). Furthermore, expression of a conditionally active form of Stat3 in ES cells in the absence of LIF allows ES cells to retain their undifferentiated state (Matsuda et al., 1999), thereby directly identifying Stat3 as the key element in LIF-mediated maintenance of ES cell self-renewal. LIF however, does not act alone as in the absence of serum it fails to block neural differentiation and to maintain pluripotency (Ying et al., 2003a). This serum factor is likely to be Bone Morphogenetic Protein 4 (BMP4).

1.2.4 Bone Morphogenetic Proteins

Bone Morphogenetic Proteins (BMPs) are members of the TGF-β (Transforming Growth Factor β) superfamily of secreted polypeptides (Shi and Massague, 2003). BMPs have been shown to participate in the activation of many key cellular responses during growth and differentiation (Feng and Derynck, 2005; Varga and Wrana, 2005). BMP proteins were originally isolated as critical components of bone protein extracts directing cartilage and bone formation (Wozney et al., 1988). More recently, Ying et al (Ying et al., 2003) demonstrated that ES cells required LIF in conjunction with BMP4 in the absence of serum, in order to block neural differentiation and to maintain pluripotency. The authors demonstrated the success of serum-free self-renewal in LIF plus BMP by isolating stable transfectants, by single cell
cloning as well as by deriving de novo ES cells in these conditions. BMP proteins signal through cell surface complexes of “type I” and “type II” receptors, which are structurally similar transmembrane serine/threonine kinases. The binding of BMP proteins to the receptor complex results in the phosphorylation of Smad proteins, which are ligand-induced transcription regulators of BMP responses (Feng and Derynck, 2005; Murakami et al., 2003; Shi and Massague, 2003). BMP mediates its positive effects on ES cell self-renewal through activation of members of the Id (inhibitor of differentiation) gene family via the Smad pathway and not through potentiating gp130/Stat3 signalling (Hollnagel et al., 1999; Ying et al., 2003). Id proteins, inhibitors for basic helix-loop-helix transcription factors, have been shown to be involved in various cell fate decisions, including neural differentiation (Ruzinova and Benezra, 2003). Their constitutive expression bypasses BMP or FBS requirement for the maintenance of ES cell self-renewal (Ying et al., 2003a). Under these conditions, and in the absence of LIF, Id transfected ES cells do not differentiate into neural-like cells. This suggests that Id expression specifically blocks neural lineage commitment.

1.2.5 Balancing LIF/Stat3 and BMP/Id signalling

In summary, LIF and BMP act together in order to maintain ES cell self-renewal, despite there being no evidence of common targets between their downstream transducers, Stat3 and Id, as yet. Nevertheless, the fact that overexpression of BMP, even in the presence of LIF, leads to differentiation
into non-neural fates, indicates that a balance between Stat3 and Smad signalling determines the choice between self-renewal and differentiation (reviewed in Temple, 2003). Although this synergism may be independently regulated, it may involve the coassembly of Stat3 and Smad with the transcriptional coactivators CREB-binding protein (CPB)/p300 in order to regulate binding to appropriate signalling sites. It has been demonstrated that this complex, bridged by p300 and induced upon LIF and BMP stimulation of neural progenitors, is involved in the cooperative signalling of LIF and BMP2 (Nakashima et al., 1999; Rao, 2004; Varga and Wrana, 2005). This complex may be active in ES cells, thereby inducing ES cell-specific target genes (Ying et al., 2003).

Evidence for independent regulation of ES cell self-renewal by LIF and BMP is provided by two studies concentrating on potential targets of these two effectors. Firstly, using chromatin immunoprecipitation (ChIP), Cartwright et al. (Cartwright et al., 2005) have demonstrated that Myc, a helix-loop-helix/leucine zipper transcription factor, is a direct target gene of Stat3 in ES cells. The authors also demonstrated that sustained levels of Myc, using a constitutively expressed form of Myc, promoted self-renewal in the absence of LIF by blocking differentiation. Secondly, in a study identifying factors that are produced by mouse embryonic fibroblast cells required for the maintenance of pluripotency of ES cells, Qi et al. (Qi et al., 2004) demonstrated that BMP4 inhibits the mitogen activated protein kinase (MAPK) Erk (extracellular signal-related kinase) which is involved in ES cell differentiation (Burdon et al., 1999). Therefore, the fact that the LIF/Stat3
cascade has been shown to activate Phosphoinositide 3-kinase (PI3K) (Paling et al., 2004), a pro-pluripotency effector, and BMP has been demonstrated to inhibit the pro-differentiative effectors, Erk1 and Erk2, is indicative of a system where LIF and BMP, rather than having the same targets, act cooperatively towards the maintenance of self-renewal.

1.2.6 The Ras/mitogen-activated protein kinase

Signal transduction via the gp130/LIF pathway induces the activation of the mitogen activated protein kinases (MAPK) Erk1 and Erk2 (extracellular signal-related kinase), in addition to Stat3. This activation results from the association of gp130 with the protein tyrosine phosphatase Shp2 (Fukada et al., 1996). The function of Shp2 in ES cell self-renewal is still unclear. Studies have shown that ES cells expressing a granulocyte colony stimulating factor (G-CSF)-gp130 chimeric receptor bearing a mutation at the Shp2 binding tyrosine residue (Y757), required lower levels of gp130 stimulation for the maintenance of pluripotency and demonstrated increased gp130-stimulated levels of activated Stat3 compared to ES cells bearing a wild type chimeric receptor (Burdon et al., 1999). This suggests that Shp2 participation is required in the LIF cascade in order to balance ES cell self-renewal and differentiation (Chan et al., 2003). Additionally, the MEK inhibitor PD098059-treatment of ES cells leads to an increase in self-renewal (Burdon et al., 1999), which is indicative of Erk having a pro-differentiative effect. This study, in combination with the observation that lack of normal Shp2 function increases
an ES cell’s tendency to remain uncommitted and undifferentiated (Qu et al., 1997; Qu and Feng, 1998), indicates that the balance between Stat3 activation, which promotes ES cell self-renewal, and Erk inhibition by BMP (Qi et al., 2004), determine stem cell fate (Chan et al., 2003). More recently, however, FGF4 (Fibroblast Growth Factor 4) has been shown to act as an autoinductive stimulus that pushes ES cells towards lineage specification by activating Erk (Kunath et al., 2007). The authors suggest that the FGF4/Erk signal does not specify lineage but renders ES cells responsive to differentiative signals. This was confirmed by the observation that FGF4 null ES cells or ES cells deficient in downstream MAPK/Erk activation, exhibit a decreased ability to commit to any specific lineage, or indeed differentiate.

1.2.7 PI3-Kinase/Akt Signal cascade

Phosphoinositide 3-kinase (PI3K) generates phosphorylated phosphoinositides (phosphatidylinositol (3,4)-bipshosphate and phosphatidylinositol (3,4,5)-tripshosphate) at the cell membrane, which serve as crucial second messengers for a wide range of biological functions including mitogenesis, survival, differentiation, and cytoskeletal organisation (Gross et al., 2005; Hallmann et al., 2003). PI3Ks are regulated and activated by growth factors usually via receptor tyrosine kinases. Members of the I \(_{A}\) family of PI3Ks, comprising a regulatory subunit (typically 85 or 55 kDa) and a 110 kDa catalytic subunit are known to be activated via the gp130/LIF/Stat3 pathway (Lehr et al., 2004; Vanhaesebroeck et al., 2001;
Vanhaesebroeck and Waterfield, 1999). Phosphorylated phosphoinositides transmit the signals through downstream effectors including Akt, a PH (pleckstrin homology) domain-containing serine/threonine kinase implicated in the regulation of cell cycle progression, cell death, adhesion, migration, metabolism and tumourigenesis (Brazil et al., 2004; Takahashi et al., 2005; Watanabe et al., 2006). A previous study has demonstrated that LIF induces the PI3K-dependent phosphorylation of Akt, and that expression of a dominant negative form of the p85 subunit of PI3K leads to a reduction of self-renewal (Paling et al., 2004). This is indicative of PI3K having a direct positive effect on the maintenance of ES cell self-renewal and that PI3K is a target of the LIF/Stat3 pathway. Inhibition of PI3K using chemical inhibitors, has also been shown to increase Erk phosphorylation in response to LIF (Paling et al., 2004). Furthermore, a more recent study using a myristoylated, constitutively active form of Akt expressed in ES cells, demonstrated that constitutive expression of Akt allows ES cells to self-renew with undifferentiated characteristics, even in the absence of LIF (Watanabe et al., 2006). The authors also demonstrated that Akt signalling is able to maintain the undifferentiated state independently of the Stat3 and Erk signalling pathways. Nevertheless, Williams et al (Williams et al., 2000) have shown that PDK1 (3-phosphoinositide-dependent protein kinase 1; upstream activator of Akt) knockout mouse ES cells are viable, which indicates that the activation of PI3K signalling via PDK is not essential for ES cell self-renewal. Deletion of Akt1 in PTEN -/- ES cells has been shown to decrease the PTEN-induced tumorigenesis (Stiles et al., 2002). PTEN is a tumour suppressor gene, whose deleted or mutated expression is associated with various human
cancers (Li et al., 1997). The deletion of PTEN leads to increased PIP3 and AKT expression (Franke et al., 1997; Sun et al., 1999). The further deletion of Akt1 in PTEN -/- ES cells partially rescues the increased uncontrolled cell growth. These data suggest that Akt1 may take part in PTEN-controlled tumorigenesis.

1.2.8 Balancing internal and external signalling for efficient self-renewal

ES cells are thought to represent the in vitro counterparts of in vivo epiblast cells. They are pluripotent and able to self renew indefinitely, but whether they depend on the same signals as pluripotent epiblast cells in order to maintain these characteristics, is still of great interest. The cues that are required to maintain this pluripotent state are still unclear, despite the fact that ES cells can routinely be propagated by adding LIF and BMP. The addition of these cytokines however is an attempt to mimic the environment in which these cells would find themselves in vivo. During normal development however, LIF is not required for the maintenance of the ICM in mouse epiblasts. Embryos lacking LIF, LIFR (LIF receptor) or gp130 develop normally, at least until mid-gestation. LIF null mice are viable into adulthood, although homozygous females are sterile due to a failure of the uterus to support implantation (Nichols et al., 2001; Stewart et al., 1992). A recent study has demonstrated the minimal signalling requirements for self-renewal. Ying et al. (Ying et al., 2008) have shown that the addition of LIF and BMP to ES cell culture medium can be dispensed with, when the Erk and
GSK3β signalling pathways are inhibited. Replacing LIF and BMP with inhibitors against Erk (PD0325901) and GSK3β (CHIR99021), allowed ES cells to be expanded, as well as to maintain the appropriate levels of ES cell-associated signal transducers such as Oct4, Nanog and Rex1. This maintenance of ES cell propagation was not dependent upon Stat3. Furthermore, whilst their pluripotency was maintained, multilineage commitment was also demonstrated. The authors suggested that ES cells represent a basal state of cellular existence and that they intrinsically maintain their pluripotent status until the correct inducing signal presents itself, such as FGF4. This suggests that ES cells are only capable of maintaining their self-renewal and pluripotency, as long as they are not placed in an environment that induces them to differentiate. This is representative of what happens in vivo, where pluripotent cells of the ICM of the epiblast maintain their ES cell status only until the right differentiative signals arise and instruct them to commit to a certain lineage.

1.3 Gab adaptor proteins and their role in signalling

The PI3 kinase (PI3K) and the Ras/mitogen-activated protein kinase (Erk MAPK) pathways are key regulators of cell proliferation and survival and are known to influence the fate of mouse embryonic stem (ES) cells. An established modulator of both these pathways is the IRS-1 (Insulin Receptor Substrate 1) family adaptor protein Gab1 (Grb2-associated binder 1). Scaffolding adaptor proteins contribute to the specification and/or
amplification of signal transduction pathways (Pawson and Scott, 1997). Although adaptors themselves do not contain any enzymatic or other direct effector function they contain modular domains that allow them to mediate multiple protein-protein and protein-lipid interactions (Schlessinger, 2000).

Adaptor proteins are typically composed of interaction domains and binding motifs, and link cell-surface receptors to intracellular targets, which in turn regulate specific downstream signalling pathways (Koch et al., 1991). Adaptor proteins couple receptors to their proximal targets, and they also direct the flow of information in the intracellular networks that control cellular responses (Pawson, 2007).

The Gab (Grb2-associated binding) proteins belong to a family of multifunctional docking proteins that play a central role in the integration of Receptor Tyrosine Kinases (RTKs). They are closely related to insulin receptor substrates, which function as binding partners of intracellular signalling proteins in response to receptor stimulation in order to bring them into close proximity to their targets (Jordan et al., 2003). Gab proteins are tyrosine phosphorylated in response to various growth factors, such as EGF, HGF and insulin, and serve as docking proteins that recruit a number of downstream signalling proteins (Itoh et al., 2000; Lehr et al., 2004; Rodrigues et al., 2000). Gab1 is ubiquitously expressed and is activated by a wide range of cell surface receptors, mediating growth factor, cell-cell and cell-substratum interactions (Itoh et al., 2000).
All Gab proteins are characterised by similar overall structural organisation and share 40-50% sequence similarity. The mammalian genes (Gab1, Gab2, Gab3) are homologous to the Drosophila melanogaster DOS (Daughter of Sevenless) gene, as well as to the C.elegans Soc1 (Suppressor-of-Clear) gene (reviewed in Gu and Neel, 2003). The molecular weights of the mammalian homologues Gab1, Gab2 and Gab3 are 100-120kDa, 97-100kDa and 90kDa respectively. Gab1 and Gab2 are expressed ubiquitously, but are most highly expressed in the brain, kidney, lung, heart, testis and ovary. Gab3 null mice develop normally, despite the protein’s widespread expression pattern. It is, however, predominantly expressed in lymphoid (Nishida and Hirano, 2003) and haematopoietic (Seiffert et al., 2003; Wolf et al., 2002) tissues. This is indicative of a role in macrophage cell development and differentiation.

Gab2 was originally cloned from haematopoietic cells and was shown to be an important effector of cytokine receptor activation in association with Shp2 (Gu et al., 1998). Gab2 overexpression has been shown to potentiate Neu-evoked breast carcinogenesis in mice, an effect mediated by the hyperactivation of the Shp2-Erk pathway (Bentires-Alj et al., 2006). Gab2, along with Gab1 has also been demonstrated to mediate EGF-stimulated Erk activation and cell migration in breast carcinoma cells (Meng et al., 2005). The increased proliferation observed during mammary tumorigenesis in response to EGF stimulation has been linked to overexpression of Gab2 in human mammary epithelial cells (Brummer et al., 2006). This altered response to EGF is mediated by Gab2 potentiating Akt and Erk activation. Increased Gab2 expression has also been observed in malignant cancer cells
This suggests that Gab2 may be a tumour-related adaptor protein because of its potentiating effect on Erk signalling in response to growth factor activation. Gab2 has also been shown to be an essential, cell-autonomous signalling molecule in FcγR- (a high affinity IgG receptor) mediated signal transduction during phagocytosis in macrophages (Gu et al., 2003). A central function of Gab2 in the phagocytic machinery is to activate PI3K by binding its p85 subunit and recruiting it to its substrate lipids, thereby generating more PIP3 lipids at the phagocytic cup. This potentiation of PI3K signalling via Gab2 has also been shown in mast cells. Biochemical assays have shown that PI3K signalling downstream from the FcεR1 (a high affinity IgE receptor) receptor, is defective in Gab2 null mast cells (Gu et al., 2001). Consequently, Gab2 null mice, though viable, are defected in their allergic response. In the same study, Gab2 expression increased dramatically during embryonic neural differentiation. Furthermore, Gab2 has been shown to mediate macrophage differentiation, mediated by enhanced MAPK signalling. In addition to these central roles in immune responses as well as tumourigenesis, Gab2 suppression has been shown to reduce bFGF (basic Fibroblast Growth Factor) – mediated Akt activity during RA- (retinoic acid) induced neuronal differentiation (Mao and Lee, 2005).
1.3.1 Gab1: Structure, Recruitment and Function

Gab1 was originally isolated from a cDNA expression library derived from a glial tumour, and was found to be most similar to IRS-1 (Insulin Receptor Substrate 1), a major substrate of the insulin receptor (Holgado-Madruga et al., 1996). The Gab1 gene is localised to chromosome 4q31.1 in human and 8C3 in mouse (Sarmay et al., 2006). As a scaffold protein upstream of RTK (Receptor Tyrosine Kinase) signal transduction, it has multiple tyrosine phosphorylation sites to dock downstream signalling proteins. Furthermore, it is a membrane-linked docking protein (MLDP) as it is localised in the lipid component of plasma membranes and contains a pleckstrin homology (PH) domain (Gotoh, 2008). Gab1 couples receptors to the cell membrane, but also amplifies the downstream signals emanating from the receptors themselves. What allows Gab1 to relay such complex signals from receptors is its structure. Its ability to bind to a variety of receptors and proteins allows Gab1 to dynamically regulate signalling, and to act as more than just a relay for signalling.

1.3.1.1 Gab1 Structure

The notable regions of the Gab1 protein that are important for its function are: the N-terminal PH (Pleckstrin Homology) domain, the N-terminal NLS (Nuclear Localisation Signal), a central series of proline-rich sequences, the MBD (Met Binding Domain) and multiple phosphotyrosine binding sites
(Figure 1.1). The PH domain recognises membrane components, most notably phosphoinositides, thus targeting Gab1 to the membrane, at points of cell-cell contact (Maroun et al., 1999a).

**Figure 1.1:** Structure of the Gab1 protein. The Gab1 protein contains five basic domains that are important for its function as an adaptor protein. The PH (Pleckstrin Homology) domain acts as a lipid membrane-binding domain which brings Gab1 close to the membrane. The NLS (Nuclear Localisation Sequence) allows Gab1 to move in and out of the nucleus. Proline-rich sequences provide binding sites for SH3 domain-containing proteins. The MBD (Met Binding Domain) is a fragment responsible for the binding of phosphorylated Met receptor and is located within the proline-rich domain. The phosphotyrosine binding sites, once phosphorylated upon stimulation, act as docking sites to mediate interactions with SH2 domain-containing proteins such as Shp2 and the p85 subunit of PI3K. There are 16 phosphotyrosine binding sites in total.

The PH domain has been shown to be critical for EGF-induced activation of Erk and PI3K in epithelial cells (Itoh et al., 2000). It has also been shown to be required for its tyrosine phosphorylation and association with Shp2 after insulin stimulation (Rocchi et al., 1998). Furthermore, a PH domain deletion mutant of Gab1 was defective in EGFR signalling (Rodrigues et al., 2000) whilst it also fails to support tubulogenesis in MDCK cells (Maroun et al., 1999a). The NLS is a short amino acid sequence, which is found within the
PH domain. It is recognised by cytosolic nuclear receptors, and then acts to retain or to direct Gab1 into the cell nucleus. An NLS has been classically defined as a short primary amino acid sequence motif, consisting mainly of charged lysines or arginines, that is both necessary and sufficient for transport of the protein to the nucleus (Terry et al., 2007).

The three proline-rich domains consist of PxxP motifs (proline-rich sequences), which are signature binding sites for SH3 (Src homology 3) domain-containing proteins (Feng et al., 1994), such as the adaptor protein Grb2 (Holgado-Madruga et al., 1996). Grb2 (growth factor receptor-bound protein 2) provides scaffold for the indirect binding of Gab1 to phosphorylated receptors. Grb2 has been demonstrated to be important in the differentiation of endodermal cells and formation of the epiblast (Cheng et al., 1998). The MBD is responsible for the direct binding of Gab1 to the tyrosyl phosphorylated c-Met receptor (Gu and Neel, 2003; Sachs et al., 2000). Gab1 can also, however, bind to the c-Met receptor indirectly thorough Grb2 (Schaeper et al., 2000). The c-Met receptor is a tyrosine kinase receptor, and its activation by HGF (Hepatocte Growth Factor) stimulates a variety of cellular responses including cell motility (Stoker et al., 1987), growth (Maina et al., 1997) and branching tubulogenesis (Rosario and Birchmeier, 2003). Interestingly, the development of the embryo requires the direct and indirect binding of Gab1 to the c-Met receptor for different processes. For example, by analysing knockin mice with mutations in either the Grb2 or Met binding sites of Gab1, either mode of binding has been shown to be sufficient for Met-dependent muscle precursor cell migration, whereas both modes are
required for placenta development, liver growth and palatal shelf closure (Schaeper et al., 2007). Upon the stimulation by growth factors or cytokines, Gab1 undergoes phosphorylation. These tyrosine phosphorylation sites provide docking sites for other downstream effectors of the signalling cascade to the RTK. The 16 phosphotyrosine sites located on this protein (Rocchi et al., 1998) allow for a diverse range of downstream effects. These phosphotyrosine sites, with the aid of the previously described domains within Gab1, provides docking sites for the recruitment and activation of downstream effectors.

1.3.1.2 Recruitment of Gab1 and downstream effectors to the RTK

Gab1 is tyrosine phosphorylated in response to various growth factors, cytokines and chemicals activating RTKs, such as: EGF (epidermal growth factor) (Holgado-Madruga et al., 1996; Kameda et al., 2001b; Montagner et al., 2005), PDGF (Platelet Derived Growth Factor) (Kallin et al., 2004), HGF (Hepatocyte Growth Factor) (Hoffmann et al., 2005), Interleukin-6 (Podar et al., 2004), LIF (Takahashi-Tezuka et al., 1998) and hydrogen peroxide (Holgado-Madruga and Wong, 2003). The interactions that Gab1 elicits with RTKs occur both indirectly via the Grb2 adaptor protein and directly through the proline-rich MBD in Gab1. The direct interaction entails Gab1 acting as a substrate for the RTK c-Met, activated by the mesenchymally-derived HGF (Hepatocyte Growth Factor), where it is involved in the c-Met-specific cell dissociation and branching morphogenesis (Lock et al., 2002; Maroun et al.,
2003; Montesano et al., 1991; Sachs et al., 2000; Schaeper et al., 2000). This interaction is very specific and the c-Met receptor is the only receptor to which Gab1 has been shown to bind to directly. The adaptor protein Grb2 however, may act as a bridge between Gab1 and activated RTKs.

### 1.3.1.2.1 The Gab1-Grb2 interaction

Signalling by protein-tyrosine kinases involves a regulated series of protein-protein interactions mediated by modules such as SH2 and SH3 domains (Pawson and Scott, 1997). As mentioned before, SH2 domains recognise phosphotyrosines (Anderson et al., 1990; Songyang et al., 1993), whilst SH3 domains recognise proline-rich motifs (Feng et al., 1994). An example of an SH2-domain containing protein is the adaptor protein Grb2 (Cheng et al., 1998). Grb2 has no catalytic domain, but recognises the proline-rich motifs of Gab1, therefore allowing Gab1 to be recruited to a RTK (Buday, 1999; de Mol et al., 2004; Kallin et al., 2004). Once a RTK is activated by a ligand, Grb2 binds to specific phosphotyrosines of the RTK (such as the EGF receptor) and then associates with Gab1 constitutively via its SH3 domains. The interaction between Grb2 and Gab1 is essential for the ability of Gab1 to convey the message to downstream signalling effectors. For example, a mutation in the Grb2 binding site of Gab1 inhibits with the association between Gab1 and the EGF receptor (Lock et al., 2000). Furthermore, Gab1 fails to be tyrosine phosphorylated in response to EGF in fibroblasts isolated from mice expressing a null mutant of Grb2, which demonstrates the importance of
Grb2 for the efficient tyrosine phosphorylation of Gab1 (Saxton et al., 2001). The direct or indirect interaction between Gab1 and a RTK elicits the recruitment and activation of various downstream effectors, the most notable ones being Shp2, PI3K, PLCγ and CrkII (Sun et al., 2005). Grb2 null ES cells proliferate normally but are unable to differentiate into the endodermal lineage (Cheng et al., 1998).

1.3.1.2.2 The Gab1-Shp2 interaction

Shp2 (SH2 domain-containing protein tyrosine phosphatase 2) is a ubiquitously-expressed protein tyrosine phosphatase containing SH2 (Src homolog 2) domains, and takes part in the regulation of various biological functions in response to growth factors, cytokines and hormones (Dance et al., 2008). For example, signal transduction via the gp130/LIF pathway induces the activation of the mitogen activated protein kinases (MAPK) Erk1 and Erk2 (extracellular signal-related kinase), which results from the association of gp130 with Shp2 (Bradley et al., 1992). Shp2 null mice die between days 8.5 and 10.5 in gestation with multiple defects in the patterning of axial mesodermal tissues and posterior development during gastrulation (Qu and Feng, 1998; Raabe et al., 1996; Sun et al., 1993). Furthermore, an activation mutation of Shp2 causes Noonan syndrome, an autosomal dominant disorder characterised by dysmorphic facial features, proportionate short stature and heart disease (Tartaglia et al., 2001). This gain-of-function mutation is also associated with sporadic juvenile
myelomonocytic leukaemia, myelodysplasic syndrome, acute lymphoblastic leukaemia and acute myelogenous leukaemia (Bentires-Alj et al., 2004; Loh et al., 2005; Tartaglia et al., 2004; Tartaglia et al., 2003; Wang et al., 2006). The general consensus is that the oncogenic potential of Shp2 is largely due to the resulting enhanced MAPK activation (Edouard et al., 2007). Shp2 has also been shown to be important for the β4-mediated induction of anchorage-independent growth of epithelial and carcinoma cells (Bertotti et al., 2006). It is also involved in haematopoiesis (Qu et al., 1997), focal adhesion (Herrera Abreu et al., 2006), as well as ES cell differentiation to mesoderm and haemangioblasts (Chan et al., 2003). Shp2-mutant ES cells, being more sensitive to LIF, are more efficient in producing secondary embryoid bodies (EBs) compared to wild type ES cells (Qu and Feng, 1998). The pro-differentiative effect of Shp2, is supported by the demonstration that expression of a dominant-negative form of Shp2 leads to a decrease in the differentiation of ES cells (Burdon et al., 1999). Furthermore, inhibition of Erk activation by chemical inhibitors, enhances ES cell self-renewal (Burdon et al., 1999). Shp2 has been shown to be important in many biological processes, a trait that probably derives itself from its ability to promote MAPK stimulation in response to diverse agonists (Dance et al., 2008).

Shp2 has two SH2 domains in the amino-terminal region (N-SH2, C-SH2), a protein tyrosine phosphatase (PTP) domain and a C-terminal hydrophilic tail containing phosphotyrosines sites (‘C’ tail) (Dance et al., 2008). The latter contains two tyrosyl phosphorylation sites, which are differentially phosphorylated by receptor and non-receptor protein tyrosine kinases (Neel
et al., 2003). Shp2 shows low basal activity, principally due to an auto-inhibition mechanism controlled by its own N-SH2 and PTP domains (Cunnick et al., 2002; Dance et al., 2008). The subsequent activation of Erk activation by LIF (Nakaoka et al., 2003; Takahashi-Tezuka et al., 1998) or EGF (Cai et al., 2002; Cunnick et al., 2001; Meng et al., 2005) for example, requires the association between Shp2 and Gab1.

There are two Shp2 binding sites located in the C-terminal of Gab1 (Tyr-627, Tyr-659) (Cunnick et al., 2001; Rocchi et al., 1998). It has been suggested that the association between Shp2 and Gab1 acts to locate Shp2 to the plasma membrane in order to activate an upstream signalling step (such as activation of Src or Ras GTPase), as a Gab1-Shp2 fusion protein was shown to lead to growth factor-independent Erk activation (Cunnick et al., 2001; Cunnick et al., 2002). In the same studies, Gab1 has been shown to activate Shp2 by targeting it to the membrane in a PH domain-dependent manner in response to EGF, thereby activating Erk. The association between Gab1 and Shp2 has been demonstrated to be critical in promoting Ras/MAPK signalling to enhance epidermal cell proliferation and oppose differentiation (Cai et al., 2002; Montagner et al., 2005; Yamasaki et al., 2003). In another report, by mutating the phosphotyrosine 627 binding site in Gab1, Holgado-Madruga & Wong demonstrated that this complex is essential for cell growth and transformation, as mutated cells exhibited an increase in stress fiber cytoskeletal organisation, and a decrease in cell proliferation and anchorage-independent growth (Holgado-Madruga and Wong, 2004). The association between Shp2 and Gab1 has also been shown to be important for the
maturation of focal adhesions mediated by the cytokine IL-1β (Interleukin 1β) (Herrera Abreu et al., 2006). Additionally, the Gab1-Shp2 complex has been demonstrated to regulate the phosphorylation and activity of eNOS (endothelial NO synthase), an enzyme involved in the response of endothelial cells to fluid shear stress (Dixit et al., 2005). In the embryo, the association between Gab1 and Shp2 in response to EGF is required for Met receptor-directed placental development and muscle progenitor cell migration to the limbs (Schaeper et al., 2007). This was demonstrated by generating knockin mice that carried a point mutation in the Shp2 binding site of Gab1.

1.3.1.2.3 The Gab1-PI3K interaction

Another well-defined Gab1 partner is the p85 subunit of PI3K (Holgado-Madruga et al., 1997; Laffargue et al., 1999). This complex has been established through experiments mutating either the p85 subunit or the Gab1-p85 phosphotyrosine site on Gab1 itself (Holgado-Madruga et al., 1997). These experiments demonstrate that Gab1 is important in the recruitment and activation of PI3K, as well as the downstream effector Akt, and the subsequent effects of this activation. In the embryo, recruitment of PI3K to the c-Met receptor by Gab1 has been shown to be important for EGF receptor-mediated embryonic eyelid closure and keratinocyte migration (Schaeper et al., 2007). Gab1 has been shown to associate with PI3K in response to various signals such as: fluid shear stress in endothelial cells (Jin
et al., 2005), NGF (nerve growth factor) (Holgado-Madruga et al., 1997), lysophosphatidic acid (Laffargue et al., 1999), FGF (fibroblast growth factor) (Lamothe et al., 2004), EGF (epidermal growth factor) (Mattoon et al., 2004; Rodrigues et al., 2000), VEGF (vascular endothelial growth factor) (Dance et al., 2006), HGF (hepatocyte growth factor) (Maroun et al., 1999a) and members of the GDNF (glial cell line-derived neurotrophic factor) family of ligands (De, V et al., 2005; Maeda et al., 2004). Using a GST fusion protein of the Gab1 PH domain, Rodrigues et al. demonstrated that the Gab1 PH domain bound most strongly to PtdIns(3,4,5)P3 (phosphatidylinositol 3,4,5-triphosphates), membrane lipids, in response to PDGF (platelet-derived growth factor) (Rodrigues et al., 2000). This paper demonstrated firstly, that the PH domain of Gab1 is directed to the membrane via PI3K activity. This is also true in the case of the activation of the c-Met receptor by HGF, whereby the inhibition of PI3K by a chemical inhibitor abrogates the PH domain-dependent Gab1 subcellular localisation (Maroun et al., 1999a). Secondly and most importantly, Rodrigues et al. demonstrated that Gab1 may participate in a positive feedback loop with respect to PI3K activation, to enhance signalling through the RTK. Blocking the PH domain-mediated translocation of Gab1 to the plasma membrane using Wortmanin, also inhibited the MAPK pathway, which in this instance is not thought to be downstream of PI3K. Therefore, PI3K acts in two ways: it mediates EGFR signalling downstream of Gab1 but it is also required for the downstream signalling emanating from Gab1. In order to relate PI3K and Erk activation via Gab1, Lehr et al (Lehr et al., 2004) observed that insulin induction of CHO (chinese hamster ovarian) cells overexpressing Gab1, resulted in a substantial elevation of Gab1-
associated PI(3)K binding activity. However, in the presence of activated Erk1/2, no significant change in PI(3)K binding/activity was detectable in response to insulin treatment. This is indicative of Gab1 having a modulating role within these major signalling pathways.

1.3.1.3 Gab1: Biological Function

The importance of this molecule is emphasised by the observation that Gab1 null mice die in utero and display developmental defects in trophoblast differentiation and disruption of liver size, migration of myogenic precursor cells (due to altered c-Met receptor signalling) (Sachs et al., 2000), as well as disruptions of the heart (Nakaoka et al., 2007), placenta and skin (Itoh et al., 2000). Many defects, are due to disrupted c-Met receptor downstream signalling. The lack of direct or indirect binding of Gab1 to the receptor has grave effects. Interestingly, the development of the embryo requires both the direct and indirect binding of Gab1 to the c-Met receptor for different processes. For example, either mode of binding is sufficient for Met-dependent muscle precursor cell migration, whereas both modes are required for placenta development, liver growth and palatal shelf closure (Schaeper et al., 2007). Gab1 has also been shown to have a role in several cellular processes, such as: cell survival (Holgado-Madruga et al., 1997; Holgado-Madruga and Wong, 2003), proliferation and survival of myeloma cells (Podar et al., 2004), cytoskeletal reorganisation and chemotaxis (Kallin et al., 2004), glucose tolerance and hepatic insulin action (Bard-Chapeau et al., 2004).
flow shear stress (Jin et al., 2005), development of migratory muscle progenitor cells (Vasyutina et al., 2005), epithelial morphogenesis (Maroun et al., 1999a; Maroun et al., 1999b), growth and regeneration of pancreatic cells (Hoffmann et al., 2005), mesangial cell morphogenesis (Kolatsi-Joannou et al., 1995), cell cycle transition, cell proliferation and transformation (Kostenko et al., 2006; Mood et al., 2006), cardiomyocyte elongation (Nakaoka et al., 2003). Gab1 was also demonstrated to localise at adherens junctions and undergo tyrosine phosphorylation in response to cell-cell adhesion in an E-cadherin-dependent manner in cultured epithelial cells (Shinohara et al., 2001). Furthermore, Gab1 is expressed in the marginal zone of B cells, where, in association with Shp2, it negatively regulates their differentiation in response to TI-2 (thymus independent 2 responses) (Sarmay et al., 2006).

### 1.3.2 Gab1 Variants

The Pleckstrin Homology (PH) domain is a region that shares homology with an internal repeat of pleckstrin, a major substrate of protein kinase C in platelets (Lemmon et al., 1996; Rebecchi and Scarlata, 1998). PH domains are believed to mediate intermolecular interactions, primarily protein-phospholipid interactions (Kameda et al., 2001a). Rocchi et al. (Rocchi et al., 1998) demonstrated that the PH domain plays a critical role in the tyrosine phosphorylation of Gab1, as well as its subsequent association with Shp2 after insulin stimulation. Recently, Kameda et al. (Kameda et al., 2001a) demonstrated the expression of a novel 87 kDa Gab1 lacking its PH domain
during the neoplastic progression of carcinogen-transformed SHE (Syrian Hamster Embryo) cells. The authors showed that this short form of Gab1 undergoes an EGF-dependent association with Shp2, as the long form would normally, thereby demonstrating that the lack of a PH domain does not significantly affect the protein’s ability to convey signalling. In addition to this, it was demonstrated that the expression of the short form of Gab1 was associated with an increase in growth-factor-dependent colony formation in soft agar.

One of the most significant observations Kameda et al. (Kameda et al., 2001a) made, was the fact that the long form of Gab1 was localised sub-cellularly at cell membranes, whilst the short one was expressed diffusely in the cell cytoplasm. This observation was attributed to the N terminal of Gab1 containing the PH domain. These differences in localisation between the two forms of Gab1 are not surprising, as it has been shown that the PH domain is essential for the targeting of Gab1 to the proximity of the cellular membrane at sites of cell-cell contact, this localisation being dependent on PI(3)K activity (Maroun et al., 1999a; Maroun et al., 2000). Furthermore, the long form of Gab1 has been shown to be involved in the nuclear translocation of ERK (Osawa et al., 2004). The authors demonstrated that the NLS contained within the PH domain of Gab1 is involved in the nuclear translocation of Erk, which then activates the downstream effector Egr1 (early growth response factor 1) in response to re-endothelialisation. As Erk does not possess a Nuclear Localisation Signal (NLS), but Gab1 does, the authors proposed that Erk interacts with Gab1 via the latter’s MBD domain.
However, the PH domain of Gab2 has been shown to be redundant for its localisation and tyrosine phosphorylation in response to IL3 (interleukin 3) (Edmead et al., 2005).
1.4 Gab1β: an ES cell-associated signal transducer

Unexpectedly, mouse ES cells preferentially express a novel form of Gab1 called Gab1β (Burdon T, unpublished). Gab1α and β expression levels were compared in protein lysates obtained from ES cells, embryonal carcinoma (EC) cells, mouse embryonic fibroblasts (MEF) and pooled tissues from a whole mid-gestation embryo (Figure 1.2).

![Figure 1.2: Western blot showing that ES cells express a novel 95KDa variant of the adaptor protein Gab1 called Gab1β. Protein lysates obtained from ES (ES cells), EC (Embryonic Carcinoma cells), MEF (Mouse Embryonic Fibroblasts) and 11dpc (11dpc whole mouse embryo) were analysed on a Western blot probed with a Gab1-specific antibody, which recognises both Gab1α and Gab1β (Burdon T., unpublished).](image)

The common form of Gab1, Gab1α, was expressed in all four protein lysate samples, whilst the ES and EC cell lysates contained predominantly a shorter form, Gab1β. This suggests that the expression of the smaller, novel Gab1β is downregulated upon the differentiation of ES cells. The Western blot in Figure 1.2 was probed with an antibody raised against an epitope common to
both isoforms. The novel isoform of Gab1 protein, called Gab1β, is readily detected in the mouse testes, at levels comparable to those of ES cells. In addition, it is also detected, albeit at lower levels, in the heart, muscle, stomach epithelium, lung and most strikingly in the pancreas, areas of the kidney and the dentate gyrus of the brain. Gab1β null mice are healthy and fertile and show no signs of abnormal development (Burdon T., unpublished). Structurally, Gab1β lacks the N-terminal PH domain and NLS but retains the majority of protein-protein interaction motifs (Figure 1.3), whilst the Gab1β protein contains a novel 50 nucleotide sequence at the 5′ end (Burdon T, unpublished). An alignment of mouse Gab1α and Gab1β protein sequences can be seen in Figure 1.4.

![Figure 1.3: Structure of the two Gab1 isoforms, Gab1α and Gab1β. The two proteins differ only in that Gab1β lacks part of the N-terminal of the protein, including the PH domain, which encompasses the NLS.](image-url)
Figure 1.4: Alignment of mouse Gab1α and Gab1β protein sequences. Gab1β lacks the PH domain (red), as well the NLS (grey) contained within it. The proline rich regions (PxxP) to which Grb2 binds are coloured in blue. The Met Binding Sequence is coloured in green. Phosphotyrosine binding sites are indicated in pink.
Interestingly, these two different isoforms arise from alternative transcripts rather than splice variants, due to separate start sites (Figure 1.5). Gab1β protein lacks the N terminal PHD and NLS due to the lack of an ATG start until after the PH domain in the mRNA sequence, and therefore the latter is not translated into protein (Burdon T., unpublished). What regulates the generation of these alternative transcripts is unclear. It is possible that the transcription factors that regulate ES cells may also be involved in the upregulation of Gab1β protein transcription rather than that of Gab1α. Alternatively, different epigenetic effects such as methylation may also be involved in silencing one or the other in specific cell types. For example, Gab1β seems to be silenced in most differentiated cell types.

![Figure 1.5: Generation of alternative Gab1 transcripts. Gab1β transcription initiates at a different promoter site. Gab1β lacks the N terminal domains of Gab1α because of the elimination of the upstream initiation ATG codon.](image-url)
1.5 Project aim and objectives

Gab1β is a highly abundant and novel ES cell-associated protein. Gaining insight into its potential role, will allow the broadening of our understanding of the complex network of genes that regulate ES cell biology. In order to gain more knowledge of this novel signal transducer, the effect of the lack of a PH domain on the ability of Gab1β to act as an adaptor protein was investigated. Since the PH domain plays a critical role in membrane binding the function of the protein may be expected to differ. It is possible that Gab1β might act in a dominant negative manner, by binding and mislocalising downstream effectors that Gab1α proteins complex with. This would firstly alter what signalling Gab1β participates in and where. Alternatively, Gab1β might have a deregulated function and localisation, unrestrained by the PHD. Therefore, how Gab1β participates in Gab1-dependent signalling was investigated.

The role of an NLS is to allow a protein to enter/exit from the nucleus of a cell, and clearly determines the intracellular localisation of a protein. This along with the PH domain have been shown to influence the localisation of Gab1α and therefore its function. The PH domain, affects what signalling pathways the protein participates in as well as the anchoring of the protein to RTKs and the membrane, and the other, the NLS, is involved in the protein’s intracellular localisation as well as its interaction with downstream effectors, such as Erk (Osawa et al., 2004). Therefore, the lack of these two crucial
localisation determination domains may affect the areas of the cell that Gab1β finds itself in. This in turn may have an effect on the signalling capacity of Gab1β. The localisation of the novel Gab1β isoform was therefore investigated in ES cells.

The abundance of a protein in ES cells does not necessarily indicate a role in the maintenance of the cells’ “stemness”. Therefore, although highly expressed, Gab1β may not be involved in the regulation of self-renewal or pluripotency, the two defining characteristics of ES cells. It may indeed be involved in the transition of ES cells into differentiation. The neural path of differentiation is the default route that ES cells will follow once the exogenous factors that maintain them are removed. Gab1β may be part of this default differentiation pathway. Furthermore, Gab1β expression has been detected in the dentate gyrus of the brain (Burdon T., unpublished), an area of the brain where neural stem cells are thought to reside in (Gage et al., 1995). The effect of Gab1β on the differentiation of ES cells into the neural lineage was therefore investigated.
CHAPTER 2: MATERIALS & METHODS

2.1 Molecular Biology Methods

2.1.1 DNA manipulation and modification

2.1.1.1 Restriction Enzyme Digest
A standard 50µl restriction digest normally contained 5µl of the appropriate 10x buffer, 12µl DNA (100-200 ng), 29µl distilled H₂O and 4µl enzyme. The optimum incubation time for most enzymes was 2 hours at 37°C, and the appropriate incubation temperature was used for enzymes with different optimum temperature conditions. For digestions with larger or smaller amounts of DNA, this standard digest was scaled up or down as required. Enzymes were predominantly purchased from New England Biolabs, Roche and Promega.

2.1.1.2 Alkaline Phosphatase Digest
This was performed using shrimp alkaline phosphatase (Promega). 10µl of 10x alkaline phosphatase buffer was added to the DNA solution. The solution was then vortexed and then spun for a couple of seconds. The solution was split into two eppendorf tubes and 1/10 volume of alkaline phosphatase enzyme was added to one, whilst the other was used as an undigested control. The solutions were then left at 37°C for 1 hour and then stored at -20°C, ready for purification.
2.1.1.2 Ligation

Ligations of cohesive ends were performed using T4 DNA Ligase (New England Biolabs) which is supplied with a 10x T4 DNA Ligase buffer. A typical 20µl reaction contained: 2µl 10xligase buffer, 1µl ligase, ~50ng linearised vector DNA, 5-100ng insert DNA, and then up to 20µl with nuclease-free water. A typical insert:vector ration was 3:1 and the following equation was used to calculate the correct amount of insert to be used, as this depended on the size of both the insert and the vector:

\[
\frac{\text{vector (ng) } \times \text{ size of insert (kb)}}{\text{size of vector (kb)}} \times 3 \equiv \frac{\text{? ng of insert}}{1}
\]

The reaction was then placed at 4°C overnight. The next day, the ligation reactions were used to transform competent cells as described in section 2.1.3.1.

2.1.2 Electrophoresis

Agarose gel electrophoresis was used to visualise DNA fragments produced by restriction enzyme digests and PCR (Sambrook et al., 2001). Gels were made by dissolving (by microwave heating) the appropriate amount of agarose (normal or low melt agarose) in 1xTBE (10xTBE buffer/1 litre: 108g Tris base, 55g Orthoboric acid, 40ml 0.5M EDTA (pH8.0)) or 1xTAE (10xTAE
buffer/1 litre: 48.4g Tris base, 10.9g Glacial Acetic acid, 2.92g EDTA) and
pouring the molten agarose into a plastic mould, after adding the
appropriate amount of Ethidium Bromide (5µl / 100ml agarose –Sigma stock
solution: 10mg/ml). A 0.8% gel was used to visualise DNA fragments
between 1-10 kb. For smaller fragments the percentage of agarose used
varied between 1-2%. The sizes of the gels used were: 6x5cm and 6x10cm and
were typically run at 50 or 100V. DNA was loaded onto the gels using a Gel
Loading Solution (Sigma) (2µl for 10µl DNA). The size of DNA fragments
was visualised using a range of molecular weight markers, such as Molecular
Weight Marker X (Roche), Generuler 50bp DNA ladder (Fermentas). Gels
were visualised using a short wavelength UV transiluminator and
photographed using the Gel Doc analysis system (Kodak).

2.1.3 Plasmid DNA propagation

2.1.3.1 Transformation of competent cells

2.1.3.1.1 Subcloning Efficiency DH5α Competent Cells (Invitrogen)

The competent cells were thawed on wet ice and the required number of
eppendorf tubes were placed on ice. The cells were gently mixed and 50µl of
them were aliquoted into the chilled tubes. Any unused cells were refrozen
in an ethanol bath for 5 minutes before returning to the -80°C freezer. 5µl of
the DNA (plasmid, linear or circular) was added undiluted to the cells and
incubated on ice for 30 minutes. 2.5µl of the pUC19 control DNA was also added to an aliquot of cells. The cells were then heat-shocked for 20 seconds in a 37°C water bath without shaking, and then placed on ice for 2 minutes. 950µl of room temperature LB medium (Luria Bertani medium: 1% Bacto-triptone (Difco), 0.5% Bacot-yeast (Difco) extract, 125nM NaCl) was added to the cells and the tubes were then shaken at 225rpm (37°C) for 1 hour. The cells were then spun down for 10 seconds at 1,000rpm in a microcentrifuge, the supernatant was discarded, leaving a small amount behind. The pellet was then resuspended in the remaining LB supernatant and spread on LB agar (LB medium containing 1.5% agar (Difco)) plates with 100µg/ml ampicillin and incubated overnight at 37°C. The next day, one colony per propylene tube was set up with 3ml of LB (100 µg/ml ampicillin) and left to grow, swirling overnight (37°C, 225 rpm), after streaking on a LB plate with 100µg/ml ampicillin and incubated overnight at 37°C. If the bacterial mix in the polypropylene tube was cloudy the next day, this would indicate growth of the vector, and therefore, the bacterial mixture was purified (Section 2.1.4.1 or 2.1.4.2) and tested by enzymatic digests and then, if positive, used to make a larger bacterial preparation. The large culture was set up by adding 2ml of the small culture to 500ml and left swirling overnight, or by using some of the re-streaked colony. Again, this culture was cleaned (Section 2.1.4.3) and then the OD$_{260}$ was measured. Glycerol stocks of the reaction mixture were also made (850µl bacterial culture + 150µl 100% glycerol) and stored at -80°C.
The competent cells were thawed on wet ice and the required number of polypropylene tubes was placed on ice. The cells were gently mixed and 50µl of them were aliquoted into the chilled tubes. 1µl of the provided β-mercaptoethanol was added to the cells, the tubes were gently swirled and incubated on ice for 10 minutes, swirling every 2 minutes. 5µl (~50ng) of the DNA (plasmid, linear or circular) was added undiluted to the cells. 1µl of the pUC18 control DNA, previously diluted 1 in 10 in distilled water, was also added to an aliquot of cells. The cells were gently swirled and then left on ice for 30 minutes. The cells were then heat-shocked for 30 seconds in a 42°C water bath without shaking, and then placed on ice for 2 minutes. 900µl of room temperature LB medium was added to the cells and the tubes were then shaken at 225rpm (37°C) for 1 hour. The cells were then spun down for 10 seconds at 1,000rpm in a microcentrifuge, the supernatant was discarded, leaving a small amount behind. The pellet was resuspended in the remaining LB supernatant and spread on LB plates with 100µg/ml ampicillin and incubated overnight at 37°C. The next day, one colony per propylene tube was set up with 3ml of LB (100 µg/ml ampicillin) and left to grow, swirling overnight (37°C, 225 rpm), after streaking on a LB plate with 100µg/ml ampicillin and incubated overnight at 37°C. If the bacterial mix in the polypropylene tube was cloudy the next day, this would indicate growth of the vector, and therefore, the bacterial mixture was purified (Section 2.1.4.1 or 2.1.4.2) and tested by enzymatic digests and then, if positive, used to make a larger bacterial preparation. The large culture was set up by adding 2ml of
the small culture to 500ml and left swirling overnight, or by using some of the re-streaked colony. Again, this culture was cleaned (Section 2.1.4.3) and then the OD$_{260}$ was measured. Glycerol stocks of the reaction mixture were also made (850µl bacterial culture + 150µl 100% glycerol) and stored at -80°C.

2.1.4 DNA Purification

2.1.4.1 QIAprep miniprep method (Qiagen)

1.5 ml of a small bacterial cell culture was centrifuged in a tabletop centrifuge for 2 min at 13,000 rpm. The supernatant was removed and the bacterial cell pellet was resuspended in 250µl Buffer P1 and transferred to a 1.5 ml microcentrifuge tube. 250µl buffer P2 was added and the tube was gently inverted 4-6 times to mix. 350µl of buffer N3 was added and the tube was inverted immediately 4-6 time to mix. The solution was centrifuged for 10 minutes at 13,200 rpm in a tabletop microcentrifuge, and a compact white pellet was formed. The supernatants were applied to a QIAprep Spin Column by pipetting. The mix was centrifuged for 30-60 sec, and the flow-through was discarded. The QIAprep Spin Column was washed by adding 0.5ml Buffer PB and centrifuging for 30-60 seconds and the flow-through was discarded. The column was then washed by adding 0.75ml Buffer PE and centrifuging for 30-60 seconds. The flow-through was discarded and the column was centrifuged for an additional 1 minute to remove residual wash buffer. The QIAprep Spin Column was placed in a clean 1.5 ml
microcentrifuge tube and to elute the DNA, 50µl of nuclease free water was added to the centre of the column and left to stand for 1 minute. The QIAprep Spin Column was then centrifuged for a final minute to elute the DNA, which was collected in the microcentrifuge tube. The DNA was then stored at -20°C.

2.1.4.2 Wizard Plus SV minipreps DNA purification system (Promega)

The bacterial cells grown overnight from a small culture were harvested by centrifugation for 5 minutes at 13,200rpm in a tabletop centrifuge. The supernatant was poured off and the inverted tube was blotted on a paper towel to remove the excess media. 250µl of Cell Resuspension Solution was added to the pellet which was completely resuspended by vortexing or pipetting. 250µl of Cell Lysis Solution was added and the lysate was mixed by inverting the tube four times. The cell suspension was incubated until it was clear for 5 minutes. 10µl of Alkaline Protease Solution was added and mixed by inverting the tube four times. The lysate was then incubated for 5 minutes at room temperature. 350µl of Neutralisation Solution was added and immediately mixed by inverting the tube four times. The bacterial lysate was then centrifuged at 13,200rpm in a microcentrifuge for 10 minutes at room temperature. The cleared lysate was then decanted into the prepared spin column provided with the kit, leaving the white precipitate behind. The
spin column with the supernatant was then centrifuged at maximum speed for 1 minute. The spin column was then removed from the tube and the flowthrough was then discarded from the collection tube. The spin column was then reinserted into the collection tube. 750μl of Column Wash Solution, previously diluted with 95% ethanol (as instructed in the product manual) was added to the column. The column was then centrifuged at maximum speed for 1 minute at room temperature. The spin column was removed and the flowthrough was discarded. The wash procedure was then repeated using 250μl of Column Wash Solution. The spin column was then transferred to a new sterile 1.5ml eppendorf tube and the plasmid was eluted using 50μl of nuclease free water to the spin column. The tube was then centrifuged at maximum speed for 1 minute at room temperature. The purified plasmid DNA was then stored at -20°C.

2.1.4.3 QIAprep maxiprep method (Qiagen)

The bacterial cells from a large culture were harvested by centrifugation at 6000 x g for 15 min at 4°C. All traces of supernatant were removed by inverting the open centrifuge tube until the entire medium had been drained (the protocol could be stopped here and continued later by freezing the pellets at -20°C). The bacterial pellet was then resuspended completely in 10ml of Buffer P1 by vortexing and/or pipetting up and down until no cell
clumps remained. 10ml buffer P2 were added and mixed gently by inverting 4-6 times and then incubated at room temperature for 5 minutes. The lysate appeared viscous at this stage of the protocol. 10ml of chilled buffer P3 were added to the lysate, mixed immediately by inverting 4-6 times and then incubated on ice for 20 minutes. The lysate was then mixed and centrifuged at 20,000 x g for 30 minutes at 4°C (in non-glass tubes). The supernatant containing the plasmid DNA was removed promptly. Once again, the supernatant was centrifuged at 20,000xg for 15 minutes at 4°C and the supernatant containing the DNA plasmid was removed promptly. Adding 10ml of buffer QBT equilibrated a QIAGEN-tip 500, and the column was allowed to empty by gravity flow. The supernatant from the last centrifugation was then added to the QIAGEN-tip and allowed to enter the resin by gravity flow. The column was then washed with 2x30 ml of buffer QC. The DNA was then eluted by adding 15ml of buffer QF. The DNA was then precipitated by adding 10.5ml (0.7 volumes) room temperature isopropanol, mixed immediately and centrifuged at 15,000xg for 30 minutes at 4°C. The supernatant was carefully decanted and the DNA pellet was washed with 5ml of room temperature 70% ethanol. The mixture was then centrifuged at 15,000 x g for 10 minutes at 4°C, and then the supernatant was carefully decanted without disturbing the pellet. The pellet was air-dried for 10 minutes, and redissolved in 500µl nuclease-free water, incubated at 37°C for 30 minutes to allow effective resuspension and then stored at -20°C.
2.1.4.4 QIAquick Gel Extraction Kit (Qiagen)

This method was used to extract DNA from an agarose gel (Section 2.1.2). The gel slice containing the DNA band was excised with a clean, sharp scalpel. The gel slice was weighed and 3 volumes of buffer QG were added to 1 volume of gel (i.e., 100–200µl for each 100mg of gel). The sample was incubated at 50°C for 30 min until the gel slice had completely dissolved. The tube was vortexed every 2-3 minutes. 1xgel volume of isopropanol was added and mixed. A QIAquick spin column was placed in a 2ml collection tube and the sample was applied to the column and centrifuged at 13,200rpm on a tabletop centrifuge for 1 minute. For sample volumes more that 800µl, the remaining sample was loaded and spun again. The flow-through was discarded and the QIAquick Spin Column was placed back into the same collection tube. 0.5ml of buffer QG was added to the column and centrifuged for 1 minute (optional) to remove all traces of agarose. To wash, 0.75ml Buffer PE was added to the column and centrifuged for 1 minute. The flow-through was discarded, and the column was spun for an additional minute. Residual ethanol from Buffer PE was not completely removed unless the flow-through was discarded before this additional centrifugation. The QIAquick Spin Column was placed into a clean 1.5ml microcentrifuge tube. To elute the DNA, 50µl of nuclease-free water was added to the center of the QIAquick Spin Column and centrifuged for 1 min. Alternatively, for increased DNA concentration, 30µl of water was added to the center of the column, let to stand for 1 min, and then centrifuged. The DNA was then stored at 4°C for immediate use and at -20°C for long-term storage.
2.1.4.5 Ethanol precipitation of DNA fragments
Two volumes of 96% ethanol were added to the DNA solution along with
1/10 volume of 3M Sodium Acetate (pH5.2) and mixed by inverting. The
DNA was normally visible as a white precipitate. If this was not the case, the
solution was placed at -20°C from 1 hour-overnight. The solution was then
spun at maximum speed for 10 minutes at 4°C (for low concentrations) or at
room temperature (when the precipitate was clearly visible). The pellet was
then washed twice in 80% ethanol, by adding the latter and centrifuging for 5
minutes. The pellet was then centrifuged for 2 minutes and the remaining
ethanol was removed. The DNA pellet was then left to dry under saran rap
for 5-10 minutes. The DNA was resuspended in the appropriate amount of
nuclease-free water, typically 500µl for DNA extracted from a large bacterial
culture.

2.1.4.6 Chlorophorm extraction of DNA fragments
An equal volume of chlorophorm was added to the DNA solution and then
vortexed to mix. The solution was then spun at maximum speed in a tabletop
microcentrifuge for 2 minutes. The supernatant containing the DNA was
removed and placed in a new eppendorf tube. Then an ethanol precipitation
was performed as described above in section 2.1.4.5.
2.1.4.7 Phenol-chlorophorm extraction of DNA fragments

An equal volume of phenol was added to the DNA solution which was then vortexed to mix. The solution was then spun at maximum speed in a tabletop microcentrifuge for 2 minutes. The supernatant containing the DNA was removed and placed in a new eppendorf tube. An equal volume of chlorophorm was added, the solution was vortexed and then centrifuged for 2 minutes. The supernatant was transferred to a new eppendorf tube and an ethanol precipitation was performed as described in section 2.1.4.5.

2.1.5 DNA quantification

The concentration of nucleic acids was determined using ultraviolet (UV) absorbance spectrophotometry (GeneQuant spectrophotometer). The absorbance of light of 260nm wavelength was measured for each sample. This is directly proportional to the amount of DNA in a solution. 1 O.D. at 260nm for double-stranded (ds) DNA is equal to 50µg/ml of dsDNA. Two independent measurements of the samples were always taken and distilled water was used as a blank.

2.1.6 Polymerase Chain Reaction

Each 50µl reaction contained: 25-100ng of template DNA, 5µl 10xPCR buffer (Stratagene), 0.5µl 25mM dNTP, 0.5µl 20µM forward primer, 0.5µl 20µM reverse primer, 1µl pFu Turbo DNA polymersase (Stratagene), and then made up to 50µl with PCR grade water. Thermal cycle conditions to amplify EGFP cDNA from the pEGF-C1 vector (Clontech) were as follows: 95°C for 4
minutes (denaturation), 95°C for 45 seconds (separation), gradient of annealing temperature (10-15°C below melting temperature of primers) for 30 seconds (for example, a gradient from 55°C to 64°C: 55.2°C, 56.6°C, 59.3°C, 62.4°C), 72°C for 1 minute (extraction), 24 thermal cycles were used to amplify EGFP DNA from pEGFP-C1 vector (Clontech). 5µl of PCR product was used for agarose gel electrophoresis to visualise the amplified DNA. In the case of the polyglycine linker, the 2 primers were annealed by combining: 10µg of each oligo, 7µl 5M NaCl, up to 200µl PCR TE. This mix was placed at 98°C for 5 minutes, the heating block was switched off and allowed to cool down. The poluglycine linker insert was then placed at 20°C for use in a ligation.

2.1.6.1 Primer sequences

i) EGFP (750bp) from pEGFP-C1 vector
for: 5’-GGGCCGGCCGTCGCCACCATGGTGAGCAAGGGCGAGGAGC-3’
rev: 5’-CCCGCGGCCGCCTTGTACAGCTCGTCCATG-3’

ii) PH domain (480bp) from Gab1α-puro vector
for: 5’-GGGGCGGCCGCCATGAGCGGCGGCGAA-3’
rev: 5’-AAGCTTCCATCTGACTGGAGGCTG-3’
iii) EGFP (750bp) from EGFP-C1 vector

for:

5’-
GGGGGGCCTCGAGGCGGCGGCGGCGGCGTGAGCAAGGGCGAG
-3’

rev: 5’-CCCCCCCCGTCGACCCTTGTACAGCTCGTC-3’

iv) polyglycine linker (annealed oligos)

for: 5’-GGCCACTAGTGCGCGCGCGCGCGCGCGCGCGCG-3’

rev: 5’-GGCCGCGCAGCGCCGCCGCCGCCACTAGT-3’

2.1.6.2 ZERO Blunt TOPO PCR cloning Kit (Invitrogen)

This cloning kit was used for the direct insertion of blunt-end PCR products into a supplied plasmid vector containing a multiple cloning site. The blunt PCR product was produced by PCR, as described above in section 2.1.6. The TOPO cloning reaction was set up as follows: 1-4µl PCR product (previously phenol/chlorophorm extracted), 1µl salt solution (provided with the kit), 1µl pCR II-Blunt-TOPO vector, distilled water up to 6µl. The reaction was gently mixed and incubated at room temperature for 5 minutes. The reaction was then placed on ice and was immediately used to transform One Shot
competent cells provided with the kit. 2µl of the reaction was added to one vial of cells which were then gently mixed and incubated on ice for 30 minutes. The cells were then heat-shocked for 30 seconds at 42°C without shaking and then immediately transferred to ice. 250µl of room temperature LB medium was added to the cells, the tube was tightly capped and the shaken horizontally for 1 hour at 37°C at 225rpm. 10-50µl of the transformation was spread onto pre-warmed LB plates containing 50µg/ml kanamycin antibiotic. The plates were then incubated overnight at 37°C. an efficient reaction produced several hundred colonies which were then picked and screened.

2.1.7 Cloning Strategy

The purpose of this experiment was to create EGFP fusion proteins of Gab1α and Gab1β, and to then transfet these into Gab1β -/- mouse ES cells. The first fusion that was created was EGFP-Gab1β, where EGFP was fused to the N-terminal of Gab1β. EGFP was PCR-amplified from the vector pEGFP-C1 (BD Biosciences Clontech) (Figure 2.1), with added NotI sites at either end of the PCR-amplified EGFP. The blunt PCR-amplified EGFP fragment was then inserted into the TOPO PCR vector as previously described (Section 2.1.6.2). The EGFP fragment was then cut out of the TOPO vector using NotI and then inserted in frame into a NotI site found between the CAG promoter and the Gab1β cDNA in the vector called pCAGIP-Gab1β (previously generated in the laboratory by T. Burdon and L. Sutherland) (Figure 2.2). The EGab1α
vector was constructed by PCR-amplifying the PH domain from a vector called pCAGIP-Gab1α (T. Burdon & L. Sutherland) and inserted into the NotI site between the EGFP and Gab1β cDNA in the vector EGFP-Gab1β. The second variant of the fusion vectors was constructed by inserting a polyglycine linker between the EGFP and Gab1 proteins in the EGab1β and EGab1α vectors. The polyglycine linker was made by annealing 2 polyglycine linkers. The oligonucleotides were annealed by mixing them at a 1:1 molar ratio in a microcentrifuge tube (200pmol each).

![Figure 2.1: Map of pEGFP-C1 vector (BD Biosciences Clontech).](image)

The mixture was then made up to 7µl with dH2O and heated to 70°C for 5 minutes. Then 1µl of 10xDNA polymerase buffer (0.5M Tris, 0.1M MgCl₂, 10mM DTT, 0.5mg/ml BSA) was added. The mixture was allowed to cool to room temperature and incubated for 1 hour. The annealed oligonucleotides
were then digested with NotI and ligated to the linearised EGab1β or EGab1α vectors. The third variant of the fusion vectors was constructed by fusing EGFP to the C-terminal of Gab1β separated by a polyglycine linker. A similar approach as before was used, only EGFP was PCR-amplified from the pEGFP-C1 vector using primers that would add an XhoI site at either end of the fragment. The PCR-amplified fragment was then digested with XhoI and inserted into an XhoI site found at the C-terminal end of Gab1β in the pCAGIP-Gab1β vector (Figure 2.4).
Figure 2.2: Cloning strategy for EGFP-Gab1β. EGFP was PCR amplified and then inserted into a NotI site between the CAG promoter and the Gab1β cDNA in the pCAGIP-Gab1β vector.
**Figure 2.3:** Cloning strategy for EGFP-GLY<sup>6</sup>-Gab1β. The two oligonucleotides were annealed and inserted into the NotI site between the EGFP and Gab1β cDNA of the EGFP-Gab1β vector.
Figure 2.4: Cloning strategy for Gab1β-GLYα-EGFP. EGFP was PCR amplified and inserted into an XhoI site at the C-terminal end of Gab1β of the pCAGIP-Gab1β vector.
2.2 Embryonic Stem Cell culture

Unless stated, all Embryonic Stem cell culture was performed following a standard protocol (Pollard and Walker, 2004). ES cells were maintained in BHK-21 (Glasgow MEM) medium (Sigma). To 500 ml ES cell medium 50 ml Foetal Bovine Serum (FBS, FSB001141, Cambrex) 5.6 ml L-glutamine (200mM), 5.6 ml non-essential amino acids, 5.6 ml sodium pyruvate (100mM), 1.1 ml β-mercaptoethanol (50mM) and 300 µl ESGRO (LIF - 10⁶ units) was added using a sterile filter. The medium was then stored at 4°C.

2.2.1 Treatment of plastics

All flasks and petri dishes were treated with 0.1% gelatin for 5-30 minutes before the addition of ES cells, in order to facilitate their adhesion. 5 ml of gelatin were added to a standard 25 cm² flask (T25). 1 ml was added to each well of a 24-well plate, whilst 14 ml were added to a 75 cm² (T75) and 10 ml to a 10 cm petri dish. The gelatin was removed by aspiration before the addition of the ES cells.

2.2.2 Resuscitation of frozen ES cell stocks

Vials (cryotubes) containing ES cells were removed from the -150°C storage. The sealed vial was then placed into a waterbath at 37°C for 2 minutes, or until all cells were thawed. The cells were then transferred to a tissue culture hood after the vial had been washed with 70% ethanol, and were then added into a sterile 15ml tube containing 5ml ES medium. The cells were pelleted
by centrifugation for 3 minutes at 1000 rpm, and the medium was then
removed by aspiration. The cells were then resuspended in 6ml fresh
medium and then transferred to a previously gelatinised T25 flask.

2.2.3 Propagation of ES cells

ES cells were propagated in T25 flasks until the cells were 90-95% confluent. The cells were then split into either two T25 flasks or moved to one T75 flask. The medium was changed at least 2 hours before splitting the cells. The medium was then removed by aspiration and the cells were washed with 5 ml 1xPBS (0.17M NaCl, 3.4mM KCl, 4mM Na<sub>2</sub>HPO<sub>4</sub>, 2.4mM KH<sub>2</sub>PO<sub>4</sub>). 0.5ml of TVP (1 ml 2.5% Trypsin, 0.0037g Na<sub>2</sub>EDTA, 1ml chicken serum – made up to 10 ml with PBS, filtered) was added to the flask and the cells were incubated for 3 minutes at 37°C to lift them from the flask. The cells were then removed from the surface of the flask by vigorous tapping and 5 ml fresh medium were added to the flask to wash any remaining cells off the surface. The cells were then transferred to a sterile 15 ml tube and the cells were pelleted for 3 minutes at 1000 rpm. The medium was aspirated off, the cells were resuspended in 5 ml of medium to create a single-cell suspension. To cells that were transferred to 2 T25 flasks, 1/6 of the cell mixture was added to each flask already containing 5ml of new medium. For cells being moved to a T57 flask, 1/3 of the mixture was added to the flask, which already contained 9 ml of fresh medium. The cells were then grown overnight at 37°C.
2.2.4 Preparation of frozen ES cell stocks

Storage of ES cell lines was done when the cells reached confluence and did not require further propagation. Cells were stored at -150°C in a filtered freezing mixture, which retained the integrity of the cells. Cell lines intended to be frozen were treated in the same way as propagating cell lines were. The cells were removed as previously described in Section 2.2.3. The pelleted cells were then resuspended in 1 ml ES medium before 1ml of freezing mix (3ml ES cell medium, 1 ml DMSO, 1ml foetal calf serum, filtered and stored at -20°C until use) was added, and the cells were gently mixed. The cell mixture was then aliquoted into pre-chilled cryotubes and stored overnight at -80°C, before transferring them to 150°C for long term storage.

2.2.5 Introduction of DNA into cells

2.2.5.1 Preparation of DNA

The DNA was always prepared as described in Section 2.1.4. The DNA (around 30µg) from a maxiperep would then be used in an enzymatic digest (Section 2.1.1.1) in order to linearise the vector in question. The digest was then run on an agarose gel, the required band was cut out and cleaned as indicated in Section 2.1.4.4 and the OD$_{260}$ was measured.
2.2.5.2 Transfection of DNA using Lipofectamine 2000 (Invitrogen)

0.5x10^6 cells were plated into 9.6cm² wells in 3ml of medium to ensure even plating. The next day, the cells was fed with 2ml of medium. Two hours later, 5µl lipofectamine/well was mixed with 250µl optimem and left to stand for minutes. Then, 2µg of DNA/welll was added to 250µl of optimem (Invitrogen). The DNA mixture was added to the lipofectamine solution, mixed and left to stand for 30 minutes. The total 500µl was then added to the 9.6cm² well containing the plated ES cells, and left overnight.

2.2.5.3 Selection of ES clones

The next day, the medium was changed after 12 hours incubation with the lipofectamine/vector mix. The transfected cells were then split ¼ and ¾ into 10cm dishes. The next day, the medium on the plates was changed to a medium containing a selection antibiotic, typically puromycin at a concentration of 1.5 µg/ml. The cells were grown for 9 days under selection. The cells had a media change at 48 hour intervals, until colonies of ~5mm were observed. The colonies were then transferred to 2-3 previously-gelatinised 24 well plates in the following way. 100µl of trypsin was added to wells of a 96 well plate. The medium of the 10cm dishes was changed to 5ml. then, using the a pipette tip, colonies were picked, added to the trypsin and left at 37°C for 2-3 minutes until dissociated. The dissociated cells were then transferred to a well of a 24 well plate containing 1ml of selection medium. The cells were then placed in the incubator to grow until confluent.
2.2.5.4 Expansion of clones

Each clone was then expanded to a 6well plate. The cells were PBS-washed, 200µl of trypsin was added to the 24-well, and the cells were left in the incubator for 5 minutes. During this incubation time, 3 ml of fresh selection medium was added to each well of previously-gelatinised 6 well plates. The cells were vigorously resuspended in the trypsin by pipetting up and down in the 24 well plate, and were then added to a well of the 6 well plate, resuspending again. The cells were then left to grow until confluent. They were then propagated into T25 culture flasks as described in Section 2.2.3. Subsequently, once cells were routinely cultured, protein was extracted and analysed on Western blots.

2.2.6 Neural differentiation of ES cells

An adherent monoculture system was used to differentiation ES cells into neuron-like precursors (Ying and Smith, 2003; Ying et al., 2003b). A mixed formulation of basal media and supplements provides optimum cell viability and efficient neural differentiation. This formulation called N2B27 contained DMEM/F12 medium (Gibco) supplemented with modified N2 (Gibco), which contains insulin, apo-transferin, progesterone, putrescine and sodium selenite. This mixture was then supplemented with BSA combined with Neurobasal (Gibco) medium with added B27 (Gibco).
0.7 \times 10^5 \text{ or } 1 \times 10^5 \text{ cells were plated in 90mm wells at varying densities in N2B27 plus 100 units/ml LIF and allowed to plate overnight. Cells were then transferred to N2B27 medium alone or containing 2ng/ml FGF4 with or without 1\mu g/m l heparin. The medium supplemented with FGF4 and heparin was then changed every two days. Once the cells looked like neural precursor-like cells, they were fixed and stained for Tuji and or Oct4, as well as DAPI. In order to investigate the effect of insulin on the emergence of neural precursors, Helen Murray provided me with a cocktail of N2B27 supplement without added insulin. The concentrations of insulin used were: 0 \mu g/ml (zero), 0.25 \mu g/ml (low) and 25 \mu g/ml (high).

2.3 Analytical methods

2.3.1 Western blotting

Cells were lysed using a 1\% SDS solution (4xlysis buffer: 40ml glycerol, 12g SDS, 25mls 1M TrisHCl pH 6.8, 0.02\% Bromophenol Blue, 20ml \beta-mercaptoethanol; store at -20\degree C). Cells were washed once in PBS and 1ml of 1x lysis buffer/9.6cm² well of a 6-well plate was added on ice. The gloopy protein lysate was then scraped off the well and into an eppendorf tube and stored on ice. The lysate was then sonicated, and stored at -20\degree C. When ready to run on a Western blot, the protein lysate was thawed at 37\degree C and then heated to 98\degree C for 5 minutes. The samples were then spun and vortexed twice to ensure efficient mixing.
One dimensional gel electrophoresis under denaturing conditions (in the presence of Sodium Dodecyl Sulphate - SDS -) separates proteins based on their molecular size as they move through a polyacrylamide gel matrix toward the anode (Ansubel et al., 1994). The polyacrylamide gel is cast as a separating gel topped by a stacking gel, in glass plates which are secured in an electrophoresis apparatus. The glass plate sandwich of the electrophoresis apparatus (Biorad) was assembled according to the manufacturer’s instructions using two clean glass plates and 0.75mm spacers and locked to the casting stand. The separating gel solution (standard 10% seperating gel: 6ml dH20, 5ml 40% Acrylamide, 3.2ml 2% Bis solution, 5ml 1.5M Tris pH 8.8, 200µl 10% SDS, 200µl 10% APS, 12µl TEMED) was applied (immediately after making to avoid polymerisation) to the sandwich along an edge of one of the spacers until the height of the solution was around 5 cm (or around 1 cm below the comb). The desired percentage of the acrylamide gel in the separating gel depended on the molecular size of the protein. Generally, 8% gels (7.6ml dH2O, 4ml 40% acrylamide –Sigma-, 2.6ml 2% Bis solution – Biorad-, 5ml 1.5M Tris pH 8.8, 200µl 10% SDS, 200µl 10% APS, 12µl Temed – Sigma-) were used for SDS-denatured proteins of 60-200 KDa, 10% for 16-70 KDa and 15% gels for SDS-denatured proteins of 12-45 KDa. The gel was then covered with a layer of isopropanol to provide a barrier to oxygen, which inhibits polymerisation, and also allows a flat interface to form during gel formation. The gel was allowed to set for 30-60 minutes. Then, the layer of isopropanol was poured off and the stacking gel (same for all: 6.6ml d H2O, 0.625ml 40% acrylamide, 0.4ml 2% Bis solution, 0.65ml 1M Tris pH6.8, 50µl 10% SDS, 50µl 10% APS, 5µl TEMED) was poured gently
into the assembly and the comb was inserted. The mixture was allowed to set for at least 10 minutes before removing the comb and placing the assembly in the electrophoresis tank, which was filled with running buffer (5x Running Buffer: 30gr Tris, 188gr Glycine, 10gr SDS, up to 2 litres with distilled H₂O). The sample wells were washed with running buffer before loading the samples, gel and a prestained protein standard was included (SeeBlue Plus 2, Invitrogen) The power supply was then connected and run at a constant current (usually ~15 mA for a mini gel). After the bromophenol blue tracking dye had reached the bottom of the separating gel, the power supply was disconnected and the gel was disassembled. Proteins were transferred to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia biotech) for Western blotting using a wet blotting device (Criterion blotter, Biorad). The nitrocellulose membrane was cut to size, soaked in dH₂O for 10 minutes and then in 1x transfer buffer (10x Transfer buffer: 116gr Tris, 58.6gr Glycine, 75.2ml 10% SDS, up to 2 litres with distilled H₂O) for another 10 minutes. The gel and nitrocellulose membrane were sandwiched between 3M filter paper and placed into the folding plastic cassettes. The cell was filled with 1xTransfer buffer (100ml 10xTransfer buffer, 200ml 100%ethanol, 700ml distilled H₂O), an ice pack and magnetic stirrer were added, and the whole blotter was placed on ice on a magnetic block. The transfer was run for 75 minutes at 90Volts. Once the transfer was complete, the membrane was blocked overnight in 5% fat-free milk powder (Marvel, Nestle) made up in 1xTBST (25xTBS: 125gr Tris, 400gr NaCl, pH 7.6, up to 2 litres with distilled H₂O; TBST: 80ml TBS, 2x1ml Tween 20, up to 2 litres with distilled H₂O). The membrane was probed with the required antibody in a 5% BSA (Sigma)
made in TBST for 1 hour. After washing 3x20 minutes in TBST, the membrane was incubated in 1:5000 diluted anti-rabbit IgG HRP conjugate (Amersham Pharmacia biotech) in a 5% mixture of blocking buffer (1ml 5% milk block diluted in 10ml TBST) for 1 hour. The membranes were then washed 3x20 minutes in TBST. Detection of HRP labeled antibodies was done using ECL western blotting detection reagents (Amersham Pharmacia biotech) following manufacturers instructions and exposed to BioMax Light-1 film (Eastman Kodak). To strip, membranes were washed 2x5 minutes in TBST and then incubated with Restore Western blot Stripping buffer (Pierce) for 20-30 minutes at room temperature.

2.3.2 Immunoprecipitation

Lysis buffer
150mM NaCl
10mM Tris HCl pH7.4
0.5% NP40

6x10^6 cells were plated in a 10cm^2 dish and allowed to grow overnight. On the day of the experiment, to 50ml lysis buffer the following were added: 10µl/ml phosphatase inhibitor cocktail 2 (Sigma) and 1µl/ml protease inhibitor cocktail (Sigma; dilute stock powder in 1000µl). The medium was removed from the plate of cells, which were then washed once with PBS. In
order to induce the signalling pathways, the cells were starved of growth factors, using a serum-free medium for 3 hours. An induction of 10 minutes with FCS and/or LIF was followed by a wash with PBS. 1ml of lysis buffer was added, and the plates of lysing cells were immediately placed on ice. The cells were scraped off, transferred into eppendorfs and then vortexed for 30 seconds. Lysates were then spun for 10 minutes and the supernatant containing the protein was transferred to another eppendorf tube. The amount of protein was then measured by a BCA protein assay (Pierce), and all samples were made up to the same concentration in a total volume of around 1ml (1µg/ml minimum concentration was achieved). Equally, a small amount (usually around same concentration, but around 100µl total volume) from each sample was put aside, loading buffer was added, the sample was boiled and then stored in the freezer (control lysates sample). To the immunoprecipitation sample, 5-10µl (depending on the antibody, different amounts of antibody were used; manufacturers instructions were followed with all antibodies) of antibody was added, and was then allowed to rotate for 1 hour at 4°C. Then, 30µl of a 50% slurry of Protein Sepharose A (Amersham) was added, and the lysates were left to rotate at 4°C overnight. The next day, the sepharose beads were collected by spinning the lysates for 5-10 seconds at 5000rpm. The beads were then washed 4 times with lysis buffer leftover from the day before (spin, remove supernatant, add lysis buffer, mix gently, spin again). It was important not to lose the pellet of sepharose beads, which had the immunoprecipitates bound to them. Eventually, after the last wash, the supernatant was removed and the agarose beads were resuspended in 2x sample buffer and lysis buffer and
made up to a volume of around 70-100µl. The lysates were then stored at -20°C. When the samples were analysed on Western blots, the immunoprecipitates were mixed, and the amount required to run on a gel was removed and heated at 98°C for 5 minutes. The samples were then analysed on Western blots.

2.3.3 Immunohistochemistry

2.3.3.1 Protocol A (Gab1 staining)

1x10⁵ cells were plated in a 1.9cm² well of a 4-well plate and left to grow overnight. The next day, the cells were washed in PBS and then fixed in 4% paraformaldehyde (made in PBS) for 10 minutes. The fixed cells were washed twice in PBS and either stored at 4°C or blocked straight away. The plate was washed 4x5 minutes in PBST (1xPBS, 0.3% Triton X-100 –Sigma-), and then blocked (blocking solution: 10% goat serum –Sigma- in PBST, 1% BSA -Sigma-) for 2 hours at room temperature. Once blocked, the primary antibody diluted in blocking solution was added (e.g. Gab1: 1/500) straight after removing the block without washing, and left overnight at 4°C. The next day, the stained plates were washed 4x5 minutes with PBST. The secondary antibody diluted in blocking solution (e.g. Alexa 568 - IgG: 1/1000) was then added and left for 2 hours at room temperature and in the dark. After 2 hours, the plates were washed 4x5 minutes with PBST in the dark to ensure minimum loss of fluorescence. The plates were then stained
with 1/500 DAPI (1 \( \mu \text{g/ml} \); Amersham) in PBS for 5 minutes and then washed with PBS for another 5 minutes.

2.3.3.2 Protocol B (Oct4 staining)

5x10^5 cells were plated in a 9.6cm^2 well of a 6-well plate and left to grow overnight. The next day, the cells were washed twice in PBS and then fixed in 4% paraformaldehyde (made in PBS) for 20 minutes. The fixed cells were washed twice in PBS. The cells were then permeabilised for 5 minutes in 100% ethanol. The plates were then washed 1x5 minutes with PBS and blocked (blocking solution: 10% goat serum in PBS-T; PBS-T: 10% Tween 20 – Sigma- in PBS) for 30 minutes at room temperature. Once blocked, the primary antibody diluted in 1% goat serum in PBS-T was added (e.g. Oct4: 1/200) straight after removing the block without washing, and left overnight at 4°C. The next day, the stained plates were washed 3x5 minutes with PBS-T. The secondary antibody diluted in 1% goat serum in PBS-T was then added and left for 1 hours at room temperature and in the dark with shaking. The plates were then washed 3x10 minutes with PBS-T in the dark to ensure minimum loss of fluorescence. The plates were then stained with 1/500 DAPI diluted in PBS for 5 minutes and then washed with PBS for another 5 minutes.
2.3.4 List of Antibodies

p44/42 MAPK (Cell Signalling; #9101)
ERK2 (BD Transduction; 610104)
pAKT (Serine 473) (Cell Signalling; #9271)
AKT (Cell signalling, #9272)
pGSK3β (Serine 9) (Cell Signalling; #9336)
GSK3β (Cell Signalling; #9315)
SHP2 (Santa Cruz; sc-280)
p85 subunit (PI3K) (Upstate; 06-195)
pSTAT3 (Serine 705) (Cell Signalling; #9131)
STAT3 (Santa Cruz; sc-482)
pTyr (Upstate; 05-321)
OCT3/4 (Santa Cruz; sc-5279)
TUJI (Covance; MMS-435P)
pGab1 (Tyrosine 627) (Cell Signalling; #3231)
pGab1 (Tyrosine 307) (Cell Signalling; #3234)
Gab1 (rabbit polyclonal) (Dr Toshio Hirano, Osaka University, Japan)
ECL-HRP linked Secondary antibodies (Amersham)
Alexa-Fluor Secondary antibodies (Invitrogen)
CHAPTER 3: CHARACTERISING THE BIOCHEMICAL EFFECTS OF GAB1β ON ES CELLS

3.1 Introduction

Gab1α is a scaffolding adapter molecule that contributes to the specification and/or amplification of signal transduction pathways (Nishida and Hirano, 2003; Pawson and Scott, 1997). Gab1α is tyrosine phosphorylated in response to various growth factors, cytokines and chemicals activating RTKs. Growth factors include: EGF (epidermal growth factor) (Holgado-Madruga et al., 1996; Kameda et al., 2001b; Montagner et al., 2005), PDGF (Platelet Derived Growth Factor) (Kallin et al., 2004), HGF (Hepatocyte Growth Factor) (Hoffmann et al., 2005). Gab1α is also phosphorylated in response to the cytokines Interleukin-6 (Podar et al., 2004) and LIF (Takahashi-Tezuka et al., 1998). It is also phosphorylated in response to oxidative stress caused by hydrogen peroxide (Holgado-Madruga and Wong, 2003). The tyrosine phosphorylation of Gab1α and its recruitment to receptors results in the activation of two major signalling pathways, the Shp2/ERk and PI3K/Akt cascades. For example, Gab1α in complex with Shp2, has been shown to promote Erk signalling to enhance epidermal cell proliferation and oppose terminal differentiation of stratified epithelial cells in response to EGF (Cai et al., 2002). Using Gab1α-deficient immortalised fibroblasts, Gab1α has also been shown to act upstream of Ras for EGF receptor/ErB-mediated Erk activation (Yamasaki et al., 2003). Furthermore, the activation of Erk in response to EGF, PDGF and HGF is reduced in Gab1α-deficient primary
fibroblasts (Itoh et al., 2000). Interestingly, expression of a Gab1 mutant lacking the PH domain in fibroblasts does not interfere with EGF-induced Erk activation (Cunnick et al., 2000). This suggests that the Gab1 PH domain is not required for EGF-induced Erk activation. Therefore, Gab1β, which lacks the PH domain, should also be able to mediate EGF-induced Erk activation in ES cells.

As Gab1α has been shown to be phosphorylated in response to various growth factors and cytokines, one may expect that Gab1β may also have this ability as it still retains motifs that are required to do so, such as the tyrosine phosphorylation sites for Shp2 and PI3K. FBS, which is routinely used to culture mouse ES cells, is known to contain a myriad of growth factors, EGF possibly being one of them. Although the levels of EGF in bovine serum have not been reported, human serum has been reported to contain EGF in the range of 1±0.5 ng/ml (Sporn, 1991). The cytokine LIF, routinely used to maintain ES cell self-renewal, is also involved in Gab1α-mediated signal transduction (Takahashi-Tezuka et al., 1998). Gab1α has been shown to link the cytokine receptor gp130 to the activation of the Mapk Erk in response to IL6 (Interleukin 6), a close relative to LIF (Takahashi-Tezuka et al., 1998). In this study, the authors demonstrated that Gab1α is tyrosine phosphorylated and forms a complex with both Shp2 and PI3K in response to IL6, via the gp130 receptor. As previously described, gp130 forms a heterodimer receptor complex with the LIF receptor (LIFR) in response to LIF, and this leads to activation of Stat3, a signal required for the maintenance of ES cell self-
renewal (Niwa et al., 1998). Furthermore, Gab1α has been shown to interact with Shp2 and PI3K in cultured cardiomyocytes in response to LIF and the Gab1α-Shp2 interaction leads to the activation of the Mapk Erk5 (Nakaoka et al., 2003). Gab1α-deficient embryonic fibroblasts have been demonstrated to have markedly reduced levels of activated Erk1 and Erk2 in response to IL6 (Takahashi-Tezuka et al., 1998). These data suggest that Gab1α is an important regulator of Erk signalling in response to IL6, and therefore potentially in response to LIF. Whether the PH domain is crucial for these signalling cascades is still not clear. The PH domain of Gab1α has been shown to be both essential and non-essential for the mediation of signalling. For example, the PH domain is not required for EGF-induced Erk2 activation in fibroblasts (Cunnick et al., 2000). This is also true for a naturally occurring PH domain-deficient form of Gab1α, that arises during the neoplastic progression of SHE (Syrian hamster embryo) cells, which is tyrosine phosphorylated in response to EGF activation, and also forms a complex with the EGFR (Kameda et al., 2001). The PH domain however, has also been shown to be required for LPA (lysophosphatidic acid)-induced Erk2 activation (Cunnick et al., 2000). Therefore, whether the PH domain is required for efficient signalling via Gab1 proteins may depend on the receptors Gab1 is conveying signals from. This means that whether Gab1β will have similar effects on the signalling pathways that Gab1α takes part in, may depend on the receptors from which the signals are emanating from.
Apart from inducing Erk activation, Gab1α has also been shown to activate Akt, the PI3K effector, in response to various cytokines and growth factors. For example, Gab1α−/− fibroblasts, lack the ability the mediate the same high levels of EGF-induced Akt activation, compared to +/+ fibroblasts (Mattoon et al., 2004). Furthermore, Gab1α has been shown to potentiate the PI3K/Akt signalling cascade in response to VEGF (Dance et al., 2006) and NGF (Holgado-Madruga et al., 1997). Gab1α has also been shown to take part in a transactivation pathway with the EGF receptor (Laffargue et al., 1999). The authors of this study demonstrated that the actovation of PI3K by LPA in non-myeloid-derived cell lines is conditioned by the ability of LPA to transactivate the EGFR/Gab1α pathway. Apart from all the above, Gab1α has also been shown to mediate PI3K activation in response to Fibroblast Growth Factor (FGF) in mouse embryonic fibroblasts (Lamothe et al., 2004) and Glial cell line-Derived Neurotrophic Factor (GDNF) in human primitive neuroectodermal tumour cells (Maeda et al., 2004). Although there is no data linking Gab1α and PI3K in response to LIF, it would be interesting to see whether Gab1β, the predominant Gab1 protein in ES cells, is actually involved in the regulation of the PI3K pathway and whether this is in response to LIF or any other growth factor or cytokine. LIF has been shown to induce the activation of the PI3K pathway in ES cells, which leads to the phosphorylation of Akt, GSK3α, Gsk3β and S6 protein (Paling et al., 2004). Interestingly, using pharmacological inhibitors, blocking PI3K-dependent signalling reduces ES cell self-renewal by increasing the activation of the MAPK Erk (Paling et al., 2004). Furthermore, Akt has been identified in an RNA interference-based screen for positive regulators of ES cell self-renewal
(Pritsker et al., 2006). These data are supported by the demonstration that a myristoylated, active form of Akt can maintain the undifferentiated phenotype in mouse ES cells, without the addition of LIF (Watanabe et al., 2006). The precise mechanism of how this occurs, however, is still elusive. Apart from Akt, Glycogen Synthase Kinase 3 β (GSK3β) has also been shown to play a key role in the maintenance of mouse ES cell self-renewal by regulating Nanog expression (Storm et al., 2007). All the above data suggest that the PI3K pathway may participate in the maintenance of self-renewal in response to LIF. Though a clear link has not been demonstrated, as an adaptor protein Gab1β may act as a bridge between LIF and PI3K in ES cells.

Gab1α was initially discovered as an IRS-1-like adaptor protein (Holgado-Madruga et al., 1996) and is phosphorylated at PI3K and Shp2 binding sites in response to signals from the insulin receptor (Rocchi et al., 1998). Insulin signalling is important for normal growth and development as well as for normal homeostasis of glucose, fat and protein metabolism (Le Roith and Zick, 2001). Insulin and IGF1 are closely related cytokines, whose biological effects are mediated by binding to their respective cell surface type 1 tyrosine receptors (Nguyen et al., 2007). In the presence of insulin for example, the insulin receptor (IR) phosphorylates insulin receptor substrate (IRS) proteins that are linked to the activation of two main signalling pathways: the PI3K pathway, which is responsible for most of the metabolic actions of insulin, and the MAPK pathway, which regulates expression of genes and cooperates with the PI3K pathway to control cell growth and differentiation (Avruch, 1998; Taniguchi et al., 2006). PI3K is important for many insulin responses,
including glucose uptake, general and growth-specific protein synthesis, and glycogen synthesis (White, 1998). In ES cells, the insulin pathway activates PI3K (Welham et al., 2007), a signal important for the maintenance of ES cell proliferation and pluripotency, through the activation of Akt-dependent inactivation of GSK3β (Paling et al., 2004; Welham et al., 2007). Furthermore, the inhibition of GSK3β has been demonstrated to be important in the maintenance of the undifferentiated state of mouse ES cells (Sato et al., 2004). More recently, IRS1 has been claimed to regulate murine ES cell self-renewal (Rubin et al., 2007). In this study, the authors demonstrated that the differentiation of ES cells by LIF withdrawal was associated with a reduction in IRS1 expression and a decrease in Akt and GSK3 phosphorylation. The suggestion that IRS1 may directly interfere with differentiation means that IRS1 may also have an effect on the MAPK signalling pathway, as the latter is a pro-differentiative pathway (Burdon et al., 1999b). This is supported by the fact that IGF1 and insulin have been shown to activate Erk in mouse ES cells (Nguyen et al., 2007). During development however, the physiological roles of IGF1 and insulin are not clear (Kaye, 1997) and the biological roles of insulin and IGF1 in ES cells are of interest. In a study aimed at unravelling the biochemical factors that control ES cell self-renewal in culture, the role of insulin in ES cells was questioned (Ying et al., 2008). In this study, the authors developed a medium that contained 3 basic components: inhibitors against Erk, FGF and GSK3. This medium was able to delineate the minimum requirements for the maintenance of ES cell self-renewal and which are capable of eliminating differentiation-inducing signals coming from Erk, FGF and GSK3. When insulin was omitted, ES cells were more
sensitive to the FGF and Erk inhibitors, and although they remained mostly undifferentiated over four weeks of culture, after the first passage the propagation rate declined steadily. This suggests that the presence of insulin sensitised the cells to differentiation-inducing signals. Furthermore, it suggests that insulin promotes long-term growth capability but does not dictate the fate choice between self-renewal and lineage commitment (Ying et al., 2008).

IRS proteins contain several common structures with Gab1 proteins. For example, they contain: an NH$_2$-terminal PH domain and/or a PTB domain that mediates protein-lipid or protein-protein interactions; multiple COOH-terminal tyrosine residues that create SH2-protein binding sites; proline rich regions to engage SH3 domains; and serine/threonine-rich regions which regulate overall function through other protein-protein interactions (White, 1998). The insulin receptor substrate, IRS-1, was the first docking protein identified and serves as the prototype for this class of molecules (Sun et al., 1991). These common features are what lead to the discovery of Gab1a as an IRS-1-like protein (Holgado-Madruga et al., 1996). All the data on the effects of insulin, IGF1 and IRS1 on the PI3K and Erk pathways in ES cells, suggest that Gab1β may take part in the regulation of these pathways, as it is so closely related to IRS1. Furthermore, considering that insulin has been shown to have a background role in the propagation of ES cells, rather than a direct one in the fate-decision process of ES cells, Gab1β may take part in this signalling pathway. Insulin proved non-essential to an extent, but not completely dispensable for ES cells when the minimum requirements for the
The maintenance of ES cell self-renewal were determined (Ying et al., 2008). This is similar to Gab1β in that it is expressed and takes part in major signalling pathways, but it is not required for the maintenance of self-renewal and pluripotency of ES cells.

The requirement for the PH domain for the function of Gab1α is still not clear. For example, the PH domain of Gab1α has been shown to be essential for the ability of Gab1α to support branching morphogenesis downstream from the Met receptor tyrosine kinase in epithelial cells, in concert with PI3K activity (Maroun et al., 1999a). The Gab1α PH domain is even more important for recruitment in BCR signalling as a PH domain deletion mutant fails to become tyrosyl phosphorylated upon BCR engagement (Ingham et al., 2001). In other pathways, however, the PH domain appears to be dispensable for recruitment. For example, expression of a Gab1α mutant lacking the PH domain in fibroblasts does not interfere with EGF-induced Erk activation (Cunnick et al., 2000). The majority, however, of the data point to a requirement of the PH domain for the function of Gab1α. Studies in other species also suggest this. For example, PH domain mutants of the Drosophila melanogaster Gab1α homologue, DOS, fail to function in Sevenless signalling and to rescue the lethal phenotype of DOS loss-of-function mutant flies (Bausenwein et al., 2000). Similarly, a PH domain mutant of C. elegans SOC1, the Gab1α nematode homologue, is non-functional (Liu and Rohrschneider, 2002; Schutzman et al., 2001).
In terms of insulin signalling, the IRS1 PH domain has been shown to be required for insulin-stimulated tyrosine phosphorylation of IRS1 and IRS1-associated PI3K activity (Myers et al., 1995; Yenush et al., 1996). Furthermore, the Gab1α PH domain has been suggested to be important for Gab1α tyrosine phosphorylation and subsequent Shp2 association after insulin stimulation (Rocchi et al., 1998). Based on these data, Gab1β would not be expected to be tyrosine phosphorylated or form a complex with Shp2 in response to insulin or IGF. In the same publication however, the authors suggest that the PH domain of Gab1α is required only when low levels of receptor are present. This may mean that the role of the PH domain depends on the environment of receptors and activators that the protein is present. Gab1β may have a completely separate and distinct biochemical role in ES cell insulin signalling compared to Gab1α, which is independent of the PH domain. Therefore, as well as providing information on the biological role of Gab1β, investigating its biochemical activity in response to insulin/IGF will prove useful in understanding the importance of the PH domain for this protein.

The aim of this chapter is to establish whether Gab1β, despite the absence of the PH domain, is an active participant in the Shp2 and PI3K pathways in ES cells, or whether it acts as a dominant negative regulator by binding and mislocalising these effectors. I aim to investigate this by determining whether Gab1β can form complexes with Shp2 and PI3K. Then, in order to determine whether it acts as a positive or a negative effector, I aim to establish the
biochemical potential of Gab1β by performing inductions with LIF, FBS and insulin on Gab1β-targeted ES cells. The biochemical output of these inductions will be measured by detecting the levels of Erk, Akt and/or GSK3β phosphorylation in response to the inductions. In addition to investigating the biochemical output of Gab1β, I also aim to establish its potential role in the growth of ES cells in response to insulin.
3.2 Results

3.2.1 Gab1β activation

Upon growth factor or cytokine stimulation, Gab1α undergoes rapid tyrosine phosphorylation, creating a number of docking sites to mediate interactions with SH2 domain-containing proteins, such as the tyrosine phosphatase Shp2 and the p85 subunit of PI3K (Liu and Rohrschneider, 2002). In this section I aim to determine whether Gab1β also undergoes rapid phosphorylation resulting in complex formation with Shp2 and PI3K in murine ES cells. Therefore, mouse ES cells, which highly express Gab1β, were starved and then induced with growth factors in order to activate the signalling pathways of interest, namely the Shp2 and PI3K pathways. Briefly, $6 \times 10^6$ cells were plated overnight in a 10cm culture dish. The next day, the cells were starved of all growth factors and cytokine for 3 hours and then induced with either 10% Foetal Bovine Serum (FBS) or 1000units/ml Leukemia Inhibitory Factor (LIF) for 10 minutes. The cells were lysed and then, a Gab1-specific antibody, which recognises both Gab1α and Gab1β, was used to isolate all Gab1 protein and protein complexes, which were isolated on protein sepharose-A beads overnight. Immunoprecipitates were run on Western blots, which were probed with antibodies against Gab1 phosphorylated at tyrosines 627 and 307, as well as an antibody raised against pan phosphotyrosine. Tyrosine 307 corresponds to the binding site for PLCγ whilst tyrosine 627 corresponds to the binding site for Shp2. The experiment was performed twice with the same wildtype cell line.
Figure 3.1: Tyrosine phosphorylation state of Gab1β protein complexes in murine ES cells in response to FBS and LIF. In both experiments (a&b, c&d), cells were induced with 10% FBS or 1000 units/ml LIF for 10 minutes. a) Gab1β, depicted by arrows, is phosphorylated at tyrosines 307 and 627 in response to FBS or LIF; Gab1β is also phosphorylated at multiple tyrosines in response to FBS; whether it is phosphorylated at multiple tyrosines in response to LIF is still unclear, as the sample is underloaded. b) Erk is phosphorylated in response to either FBS or LIF, whilst Stat3 is phosphorylated in response to only LIF. c) Gab1β is clearly phosphorylated at tyrosines 307 and 627 in response to LIF; Gab1β is also phosphorylated at multiple in response to either FBS or LIF. d) Erk is phosphorylated in response to FBS or LIF, which confirms the activation of the cells.
The tyrosine phosphorylation sites 307 and 627 on Gab1β are both phosphorylated in response to LIF (Figure 3.1a & 3.1c). Gab1β also seems to be phosphorylated in response to FBS (Figure 3.1a). Gab1β is also tyrosine phosphorylated at tyrosines in response to FBS or LIF (Figure 3.1b). Erk is phosphorylated in response to FBS or LIF in both experiments (Figure 3.1b & 3.1d). This confirms the activation of the cells in response to both FBS and LIF. As expected, Stat3 is also phosphorylated only in response to LIF (Figure 3.1b).

3.2.2 Gab1β interactions

The Gab1 immunoprecipitation experiment described in the previous section showed that Gab1β is tyrosine phosphorylated in response to either LIF or FBS. To determine whether Gab1β associates with Shp2 and PI3K, any potential Gab1β-Shp2 and Gab1β-PI3K protein complexes were isolated using Shp2 or PI3K-specific antibodies respectively. The experiment was performed as described previously in Section 3.2.1. The complexes were analysed on Western blots probed with a Gab1-specific antibody. The data shows that Gab1β forms complexes with Shp2 in response to either LIF or FBS (Figure 3.2). The Western blots showed that Gab1β binds more Shp2 in response to LIF than in response to FBS. This suggests that LIF is a more effective inducer of Gab1β-Shp2 complex formation. In combination with the results in Figure 3.1, which showed that Gab1β is phosphorylated at the binding site for Shp2 in response to LIF, these data suggest that Gab1β is
recruited to the LIFR/gp130 receptor dimer in response to LIF, is then phosphorylated at site 627, the binding site for Shp2, which then allows the formation of a Gab1β-Shp2 complex.

**Figure 3.2:** Isolation of Gab1β-Shp2 complexes in mouse ES cells using an Shp2-specific antibody. Shp2 forms a complex with Gab1β in response to both LIF and FBS as seen in two separate experiments (a&b). Gab1β forms more complexes with Shp2 in response to LIF, than in response to FBS.

Gab1β also forms a complex with the p85 subunit of PI3K in response either LIF or FBS (Figure 3.3). In both experiments, there is a higher basal level of Gab1β-PI3K complexes present in uninduced immunoprecipitates when compared to the basal level of Gab1β-Shp2 complexes. This shows that in response to either FBS or LIF, Gab1β is able to form a complex with PI3K.
Figure 3.3: Isolation of Gab1β-p85 subunit of PI3K in mouse ES cells using a PI3K-specific antibody. The p85 subunit of PI3K forms a complex with Gab1β in response to both LIF and FBS as seen in two separate experiments (a&b).

3.2.3 Effect of Gab1β on downstream signalling

The data in Figure 3.1 show that Gab1β is phosphorylated in response to its recruitment to the LIFR/gp130 dimer receptor as well as to other unknown receptors that get activated in response to FBS. Gab1β also associates with Shp2 (Figure 3.2) and PI3K (Figure 3.3) in response to either LIF or FBS. To determine whether Gab1β acts in a positive or negative manner in these complexes, the effect of this association on downstream signalling pathways from Shp2 and PI3K was investigated.

In the following experiments, previously generated Gab1β-targeted ES cell lines made by Dr. T. Burdon and L. Sutherland were used. Before investigating the role of Gab1β in downstream Shp2 and PI3K signalling, the
Gab1β expression profiles of Gab1β-deficient cell lines was confirmed. These cells were generated by homologous recombination using the IOUD2 cell line (Burdon T., unpublished). The IOUD2 cell line carries a β-galactosidase neomycin fusion gene (βgeo) inserted into the untranslated region of the Oct4 gene (Mountford et al., 1994). Two rounds of electroporation and selection for homologous recombination, using a resistance marker, were used to generate Gab1β knockout ES cells. To confirm the Gab1β expression profile of various Gab1β-targeted cells before using them in the following experiments cell protein lysates of Gab1β wildtype (+/+), heterozygous (+/-) or knockout (-/-) cells: C1 (+/+), 46 (+/-), 26 (-/-), HT (+/+), 153 (+/-) and 55 (-/-) were analysed by immunoblotting. Western analysis of protein lysates from these six targeted clones confirmed the Gab1β genotypes (Figure 3.4). As Gab1β gene expression decreased, so did the amount of Gab1β protein, with the knockouts giving rise to no Gab1β protein.

Figure 3.4: Screening of Gab1β-targeted cell lines for Gab1β protein expression. a) The expression of Gab1β is confirmed in all three cell lines b) Three other cell lines were screened and their genotype was also confirmed. The expression of Gab1α protein seems unaffected by the Gab1β targeting event. The blots were also probed with a Stat3-specific antibody as a loading control.
The effectors investigated downstream of PI3K were Akt and GSK3β, both of which have been shown to be important for the maintenance of ES cell self-renewal (Paling et al., 2004; Sato et al., 2004; Watanabe et al., 2006). PI3K has been reported to be important for the maintenance of the undifferentiated state of ES cells by forced expression of a dominant-negative p85 mutant and treatments with LY294002, a specific inhibitor of PI3K (Paling et al., 2004). Akt has been shown to be sufficient for the maintenance of pluripotency in mouse ES cells, by expressing a constitutively active myristoylated form of Akt in ES cells. This form allowed for the maintenance of the undifferentiated state of the cells in the absence of LIF (Watanabe et al., 2006). Activation of the WNT signalling pathway has been suggested to be sufficient for the maintenance of mouse and human ES cells, by the inhibition of GSK3β (Sato et al., 2004). GSK3β has also been suggested to have a role in PI3K-dependent regulation of Nanog expression (Storm et al., 2007; Welham et al., 2007). Activation of an inducible form of Nanog prevents the loss of self-renewal observed in the presence of PI3K inhibitors (Welham et al., 2007). The effector investigated downstream of Shp2 was the MAPK Erk. Signal transduction via the gp130/LIF pathway induces the activation of the mitogen activated protein kinases (MAPK) Erk1 and Erk2, in addition to Stat3 (Burdon et al., 2002; Qu and Feng, 1998). This activation results from the association of gp130 with the protein tyrosine phosphatase Shp2 (Fukada et al., 1996). In contrast to increased Stat3 phosphorylation, LIF-stimulated Erk phosphorylation levels are decreased in Shp2-deficient ES cells, compared to wildtype or Shp2-mutant cells reconstituted with wildtype Shp2 expression (Feng, 2007; Qu and Feng, 1998; Qu et al., 2001).
activation of Erk, in response to LIF-stimulated Shp2 recruitment, has been shown to promote the differentiation of ES cells (Burdon et al., 1999b).

In all the following induction experiments, 1x10⁶ Gab1β-targeted ES cells (Figure 3.4) were plated in 9.4 cm² wells and allowed to grow overnight. The next day, cells were starved of all cytokine and growth factors in a serum-free medium for 3 hours and then induced with 1000 units/ml LIF or 10% FBS for 5, 10 or 15 minutes. The cells were then lysed for protein, which was analysed on Western blots probed with phospho-specific antibodies for Erk, Akt and GSK3β. The antibody against phosphorylated Erk recognises the phosphorylation of threonines 202 and 204 on Erk1 and the phosphorylation of threonines 185 and 187 on Erk2. The antibody against phosphorylated Akt recognises the phosphorylation of serine 473, whilst that of GSK3β recognises the phosphorylation of serine 9.

3.2.3.1 Effect of Gab1β on PI3K and Shp2 signalling in response to Foetal Bovine Serum

The basal levels of Erk phosphorylation between the three different cell lines did not differ greatly, with the Gab1β +/- cell line having a slightly lower basal level of Erk phosphorylation compared to +/+ and -/- cells (Figure 3.5). Upon induction with FBS, there was no significant difference between the levels of Erk phosphorylation in Gab1β wildtype, +/- and -/- cells at any of the three timepoints. Any slight differences in the levels of Erk
phosphorylation between cell lines can be attributed to slight differences in protein loading. There is an acute increase in Erk phosphorylation upon 5 minutes of FBS induction, which then gradually decreases as the induction time increases. This suggests that the effect of FBS on the activation of Erk is acute and not sustained.

![Image](image.png)

**Figure 3.5:** The effect of FBS stimulation on Erk phosphorylation of Gab1β-targeted ES cells. Cells were induced with 10% FBS for 5, 10 and 15 minutes. The presence/absence of Gab1β does not have an effect on Erk phosphorylation in response to FBS.

When Gab1β-targeted ES cells (C1 +/+, 46 +/- and 26 -/-) were induced with FBS, Gab1β -/- cells, exhibited less Akt phosphorylation compared to wildtype cells after 10 minutes of induction, despite the similar low levels of Akt phosphorylation in the uninduced conditions (Figure 3.6a). The same result was obtained when the experiment was performed with different Gab1β-targeted ES cells (HT +/+, 153 +/- and 55 -/-) (Figure 3.6b). Interestingly, the level of Akt phosphorylation of +/- cells after induction was similar to the level of -/- cells in both experiments. This suggests that there may be an optimum amount of Gab1β that is required for the efficient
downstream potentiation of Akt, and that only Gab1β wildtype cells are capable of transmitting this effect.

**Figure 3.6:** The effect of FBS on the phosphorylation of Akt of Gab1β-targeted ES cells. Cells were induced with 10% FBS for 10 minutes. a) (C1 +/+, 46 +/-, 26 -/-): Gab1β potentiates Akt phosphorylation, but not GSK3β phosphorylation in response to FBS. b) (HT +/-, 153+/-, 55 -/-): Gab1β may potentiate Akt phosphorylation.

These data demonstrate that, in response to FBS, Gab1β potentiates the phosphorylation of Akt. Although the magnitude of AKT activation may vary somewhat between cell lines, this could mean that Gab1β is a positive regulator of PI3K signalling despite the lack of a PH domain. Most importantly however, these data indicate that Gab1β does not act in a dominant negative manner by binding and mislocalising PI3K. Interestingly, Gsk3β phosphorylation does not differ between Gab1β wildtype and knockout cell lines, upon FBS stimulation. Although phosphorylated in
response to PI3K activation, Akt and Gsk3β may not necessarily translate into the same biochemical effect. Gsk3β phosphorylation may be independent of Akt phosphorylation. Furthermore, the kinetics of Gsk3β activation may be different to that of Akt.

3.2.3.2 Effect of Gab1β on PI3K and Shp2 signalling in response to Leukaemia Inhibitory Factor

Inductions were again performed as described in section 3.2.3. After starvation however, Gab1β-targeted cells (C1 +/-, 46 +/-, 26 --/) were induced with 1000 units/ml LIF for 5, 10 and 15 minutes. Protein lysates were analysed on Western blots probed with antibodies against phosphorylated Erk, Akt or GSK3β. Similar low levels of phosphorylated Erk were observed in uninduced conditions in all three cell lines (Figure 3.7). At all three induction timepoints, however, Gab1β --/ cells exhibited lower levels of Erk phosphorylation when compared to wildtype and +/- cells. Importantly, unlike in Figure 3.6, where the --/ and +/- cells had a similar pattern of Akt phosphorylation in response to serum, in response to LIF, there is a gradual decrease in Erk phosphorylation as the amount of Gab1β decreases from wildtype cells to --/ cells. This suggests that there is a correlation between the amount of Gab1β and the levels of Erk phosphorylation.
Gab1β +/+ cells exhibited slightly higher levels of Stat3 phosphorylation at tyrosine 705 upon induction with LIF at all 3 timepoints. This however could be attributed to the higher levels of total Stat3 protein. Densitometric analysis of the Western blots in Figure 3.7 probed with phosphorylated Erk and unphosphorylated Erk2 supports this data. There is a clear decrease in the quantity of phosphorylated Erk when normalised to total levels of Erk2 (Figure 3.8). This confirms that Gab1β does indeed potentiate Erk phosphorylation in response to LIF.
Figure 3.8: Densitometric analysis of Western blots in Figure 3.7. Values were obtained by dividing the total optical density of the phosphorylated Erk signal over the optical density of the unphosphorylated Erk2 signal. The densitometric data confirm the Western blot data in Figure 13. Therefore, Gab1β does indeed potentiate Erk phosphorylation in response to LIF, based on this data alone.

These data demonstrate that Gab1β is a positive regulator of Erk phosphorylation in response to LIF, and not a dominant negative adaptor protein. This means that the PH domain is not required for the biochemical potential of Gab1β to potentiate Erk phosphorylation. This also suggests that the PH domain is potentially redundant for the protein’s ability to take part in signalling pathways, and that Gab1β can indeed convey downstream signalling without it.
The same experiment was repeated with different Gab1β wildtype, +/- and -/- cell lines (HT +/-, 153 +/-, 55 -/-) in order to confirm the data in Figure 3.7. Again, low levels of unphosphorylated Erk were observed in uninduced conditions. At all three timepoints, Gab1β -/- cells exhibited lower levels of Erk phosphorylation compared to wildtype and +/- cells (Figure 3.9).

![Figure 3.9: The effect of LIF stimulation on the levels of Erk phosphorylation in Gab1β-targeted ES cells (HT +/-, 153 +/-, 55 -/-). Cells were induced with 1000 units/ml LIF for 5, 10 and 15 minutes. Gab1β may enhance Erk phosphorylation in response to LIF.](image)

Densitometric analysis of the Western blots in Figure 3.9 probed with phosphorylated Erk and unphosphorylated Erk2 supports this data. There is a decrease in the amount of phosphorylated Erk when normalised to the total levels of Erk2 (Figure 3.10). This confirms that Gab1β potentiates Erk phosphorylation in response to LIF in ES cells.
**Figure 3.10**: Densitometric analysis of Western blots in Figure 3.9. Values were obtained by dividing the total optical density of the phosphorylated Erk signal over that of unphosphorylated Erk2. The analysis confirms that Gab1β potentiates Erk phosphorylation in response to LIF.

The data so far indicate that Gab1β is an active signalling protein in the LIF-Shp2-Erk signalling cascade. To avoid the possibility that what has been observed is due to clonal variations of the cell lines used, the induction experiment was repeated using a Gab1β knockout cell line transfected with a Gab1β expression vector. This cell line, called Gab1β restored, was generated in the lab by Dr. Thomas Burdon, by transfecting the −/− cells with a vector that had the Gab1β cDNA under the control of a strong CAG promoter, along with a resistance gene under the control of an IRES element.
Figure 3.11: The effect of LIF on the levels of Erk phosphorylation in Gab1β and Gab1α restored cells. Cells were induced with 1000 units/ml LIF for 10 minutes. a) Both Gab1α and Gab1β restored cells exhibit similar levels of Erk phosphorylation as +/+ cells. Gab1β -/- cells exhibit lower levels of Erk phosphorylation compared to all 3 other cell lines. b) Gab1β +/+, Gab1β and Gab1α restored cell lines exhibit higher levels of Erk phosphorylation compared to Gab1β -/- cells.

The expression of Gab1β in these cells was demonstrated by Western blot (Figure 3.11a). The re-expression of Gab1β in these cells should in theory also restore their ability to potentiate Erk phosphorylation in response to LIF. The induction experiment was repeated using these cells, as well as Gab1β -/- that had Gab1α protein introduced into them (Gab1α restored). When compared to Gab1β +/+ (C1), Gab1β restored cells did indeed reinstate the
levels of Erk phosphorylation when induced with 1000 units/ml LIF for 10 minutes (Figure 3.11). Interestingly, overexpressing Gab1α in Gab1β -/- cells also restored the levels of Erk phosphorylation. This suggests that even though Gab1α is not a “native” protein in ES cells, it can still act biochemically in response to LIF. Therefore, although the levels of Gab1α are negligible in ES cells, it still has the ability to participate in the same signalling pathways as Gab1β does in ES cells, when overexpressed. Their common structure allows this, despite the presence of a PH domain in Gab1α. Gab1α has in fact been shown to participate in the LIF signalling pathway. It acts as an adapter molecule in transmitting signals to the Erk MAPK for the cytokine receptor gp130 (Takahashi-Tezuka et al., 1998). This study demonstrated that Gab1α forms a complex with Shp2 as well as the p85 subunit of PI3K in response to IL6. The authors showed that the complex between Gab1α and Shp2 acts upstream of Erk in gp130 signalling.

Upon induction with LIF, the levels of Akt phosphorylation did not differ greatly between Gab1β wildtype and -/- cells (Figure 3.12). This was in stark contrast to the increase in Akt phosphorylation in response to FBS in Gab1β wildtype cells (Figure 3.6). Though not clear, there was a slight increase in the levels of Akt phosphorylation in the wildtype cells, but this could be attributed to increased levels of unphosphorylated Akt, in the samples. Though there seems to be a slight increase in the levels of GSK3β phosphorylation in the Gab1β wildtype cells compared to the -/- cells, this could be attributed to an increase in the levels of unphosphorylated GSK3β,
which has not been demonstrated. Therefore, one would suggest that Gab1β does not have a positive or a negative effect on Akt or GSK3β phosphorylation in response to LIF induction.

![Figure 3.12: The effect of Gab1β on the levels of Akt and GSK3β phosphorylation in response to LIF. Cells were induced with 1000 units/ml for 10 minutes. Gab1β does not significantly potentiate Akt or GSK3β phosphorylation in response to LIF. Gab1β cell lines used: C1 +/+ , 46 +/- , 26 -/-.](image)

3.2.3.3 Effect of Gab1β on Shp2 signalling in response to co-stimulation with Leukemia Inhibitory Factor and Foetal Bovine Serum

Murine ES cells are routinely cultured in a growth medium that contains LIF and FBS. It is the combination of these two supplements that maintains their self-renewal and pluripotency in culture, although BMP has been shown to replace the requirement for FBS (Qi et al., 2004; Ying et al., 2003a). Nevertheless, in order to simulate the effect of LIF and FBS on the biochemical pathways that Gab1β regulates in ES cells, the next set of inductions were performed using complete ES cells medium containing LIF and FBS as an inducer. Induction experiments were performed as previously
described. The cells were induced with complete ES cell growth medium containing LIF and FBS for 5, 10 and 15 minutes. Protein lysates were screened on Western blots probed with an antibody against phosphorylated Erk. The results suggest that Gab1β enhances Erk phosphorylation at 10 minutes of LIF and FBS induction (Figure 3.13). At 5 and 15 minutes however, there was no difference in the levels of Erk phosphorylation between Gab1β wildtype, +/- and -/- cells.

Figure 3.13: The effect of Gab1β on the levels of Erk phosphorylation in response to FBS and LIF combined. Cells were induced with 1000 units/ml LIF and 10% FBS for 5, 10 and 15 minutes. a) At 5 and 15 minutes of induction, there is no significant difference in the levels of Erk phosphorylation between Gab1β-targeted ES cells (C1 +/+, 46 +/-, 26 -/ -). At 10 minutes, however, Gab1β may enhance Erk phosphorylation upon induction, as the -/- cells seem to exhibit less Erk phosphorylation compared to +/+ and +/- cells. b) In the second experiment, Gab1β -/- cells exhibited slightly less Erk phosphorylation in response to co-stimulation with LIF and FBS at all 3 timepoints.
Figure 3.14: Densitometric analysis of Western blot in Figure 3.13a. Values were obtained by dividing the total optical density of the phosphorylated Erk signal over that of unphosphorylated Erk2. The densitometric data confirm that at 5 minutes there is no significant difference in the levels of Erk phosphorylation between Gab1β-targeted ES cells. At 10 and 15 minutes, Gab1β potentiates Erk phosphorylation in response to co-stimulation with LIF and FBS.

Interestingly, in response to LIF and FBS combined, Gab1β still potentiates Erk phosphorylation. The induction timepoint at which it does so, however, is still not clear. The two experiments produced differing results. In the first one, Gab1β clearly enhanced Erk phosphorylation after 10 minutes of induction (Figure 3.13a). This was confirmed by densitometric analysis, which also revealed that Gab1β -/- cells potentiate Erk phosphorylation at the 15 minute timepoint as well, in response to co-stimulation. In the second one, however Gab1β seemed to potentiate Erk phosphorylation at all 3
timepoints (Figure 3.13b). In light of the denitometric data, however, Gab1β did not seem to have an effect on Erk phosphorylation in response to co-stimulation (Figure 3.15). This suggests that whether Gab1β has an effect on Erk activation in response to LIF and FBS co-stimulation, is still not clear.

Figure 3.15: Densitometric analysis of Western blot in Figure 3.13b. Values were obtained by dividing the total optical density of the phosphorylated Erk signal over that of unphosphorylated Erk2. The densitometric data indicate that Gab1β may not potentiate Erk phosphorylation in response to co-stimulation with LIF and FBS.

Independently of Gab1β, the co-stimulation with LIF and FBS caused a double wave of Erk phosphorylation. The highest activation was exhibited at 5 minutes, whilst the lowest was at 10 minutes; at 15 minutes, cells exhibited increased levels of Erk phosphorylation. This could be due to LIF and FBS activating Erk at different timepoints and the wave we are seeing is a culmination of both these signals. This suggests that although in FBS inductions the loss of Gab1β did not have an effect on the phosphorylation of
Erk, some factors contained within it may have an effect. It would be interesting to see which of the many factors that exist in serum actually cause this effect.

3.2.3.4 Effect of Gab1β on PI3K and Shp2 signalling in response to Insulin/Insulin Growth Factor

In order to investigate the effects of Gab1β on PI3K signalling in response to IGF in ES cells, inductions were performed as previously described in Section 3.2.3. Briefly, 1x10⁶ Gab1β-targeted ES cells plated overnight were starved of cytokines and growth factors for 3 hours, before being induced with 100ng/ml IGF for 10 minutes. Protein lysates were analysed on Western blots probed with antibodies against phosphorylated and unphosphorylated Erk and Akt. Gab1β -/- cells exhibited slightly higher levels of Erk phosphorylation compared to wildtype cells in response to IGF (Figure 3.16). The increase in Erk1 phosphorylation (upper band) was more pronounced than the increase in Erk2 phosphorylation (lower band). As the levels of Erk phosphorylation do not increase greatly upon IGF induction, and in order to confirm the stimulation of the cells, the levels of Akt phosphorylation should have been shown.
Figure 3.16: The effect of Gab1β on Erk phosphorylation in response to IGF. Cells were induced with 100ng/ml of IGF for 10 minutes. Gab1β -/- cells exhibited slightly higher levels of Erk phosphorylation upon induction.

The experiment was repeated using different Gab1β-targeted cell lines (HT +/+, 55 -/-). Results show that Gab1β seems to repress Erk activation in response to IGF in this set of cells as well, as Gab1β -/- cells exhibited lower levels of Erk phosphorylation than wildtype cells (Figure 3.17). The increase in Erk1 phosphorylation (upper band) was more pronounced than the increase in Erk2 phosphorylation (lower band).

Figure 3.17: The effect of Gab1β on Erk phosphorylation in response to IGF. The induction is not as strong as expected. However, Gab1β -/- cells exhibited slightly higher levels of Erk phosphorylation upon induction. This suggests that Gab1β may repress Erk phosphorylation in response to IGF. Cell lines used: HT +/+ and 55 -/-.
Densitometric analysis of the Western blot in Figure 3.17 confirms that Gab1β-/- cells exhibit slightly higher levels of Erk phosphorylation in response to IGF, compared to Gab1β +/+ cells (Figure 3.18). The levels of phosphorylated Akt would have indicated the level of induction in response to IGF. This would confirm the stimulation with IGF.

**Figure 3.18:** Densitometric analysis of Western blot in Figure 3.17. Values were obtained by dividing the total optical density of phosphorylated Erk over that of total Erk2. The analysis shows that the levels of phosphorylated Erk did not increase greatly upon IGF induction. Gab1β-/- cells exhibit slightly higher levels of Erk phosphorylation compared to Gab1β +/+ cells in response to IGF.

In order to confirm these data, the induction experiment was repeated using a Gab1β -/- cell line, which had Gab1β expression restored to wildtype levels. Results show that when Gab1β expression is restored in the -/- cells, they exhibit similar levels of Erk phosphorylation with Gab1β +/+ cells, which are lower than those of Gab1β -/- cells (Figure 3.19). The overexpression of Gab1α also repressed Erk activation in response to IGF.
despite the fact that it has been shown to be required for insulin-stimulated Erk activation in hepatocytes (Bard-Chapeau et al., 2005). Therefore, the expression of either Gab1α or Gab1β in ES cells results in a repression of IGF-stimulated Erk phosphorylation. Firstly, this suggests that the PH domain is not responsible for this repression. Secondly, it demonstrates the modularity of Gab1 proteins, in that either isoform can take part in the same signalling pathway and produce the same effect. This suggests that Gab1β does not act in a dominant negative way in ES cells, but that it actively participates as a repressor of IGF signalling.

\[\text{Figure 3.19:} \] The effect of Gab1β on Erk phosphorylation in response to IGF. Cells were induced with 100ng/ml IGF for 10 minutes. Gab1β restored and Gab1β +/+ cells exhibited lower levels of Erk phosphorylation compared to Gab1β -/- cells in response to IGF. The overexpression of Gab1α in Gab1β -/- cells, also restores the repression of AKT phosphorylation in response to IGF.

In order to investigate the effect of Gab1β on PI3K signalling in response to IGF in ES cells, inductions were performed as previously described in Section 3.2.3. Cells were induced with 100ng/ml IGF for 10 minutes. Gab1β -/- cells exhibited higher levels of Akt phosphorylation compared to Gab1β +/+ cells (Figure 3.20). Therefore, Gab1β represses Akt signalling in response to IGF.
This suggests that Gab1β acts as a negative regulator of Akt signalling. Whether it does so in a dominant negative fashion is still not clear.

![Figure 3.20](image1)

**Figure 3.20:** The effect of Gab1β on Akt phosphorylation in response to IGF. Upon induction. Cells were induced with 100ng/ml IGF for 10 minutes. Gab1β -/- cells exhibited less Akt phosphorylation in response to IGF, compared to Gab1β +/+ cells.

In order to examine the temporal regulation of Akt phosphorylation by Gab1β in response to IGF, the experiment was repeated over various timepoints (5, 10 and 20 minutes). At all three timepoints, Gab1β -/- cells exhibited higher levels of Akt phosphorylation compared to Gab1β +/+ cells in response to IGF (Figure 3.21).

![Figure 3.21](image2)

**Figure 3.21:** The effect of Gab1β on Akt phosphorylation in response to IGF. Cells were induced with 100ng/ml IGF for 5, 10 and 20 minutes. Upon induction at all three timepoints, Gab1β -/- cells exhibited more Akt phosphorylation compared to +/+ cells. The repression is clear despite the lower levels of total unphosphorylated Akt in Gab1β -/- lysates.
Figure 3.22: Densitometric analysis of Western blot in Figure 3.21. Gab1β -/- cells exhibit higher levels of Akt phosphorylation compared to Gab1β +/+ cells in response to IGF. Values are corrected to total unphosphorylated Akt. The analysis confirms that Gab1β represses Akt phosphorylation in response to IGF.

In order to confirm these data, the induction experiment was repeated using the Gab1β -/- cell line, which had Gab1β expression restored to wildtype levels (Gab1β restored). Results show that when Gab1β expression is restored in the -/- cells, they exhibit similar levels of Akt phosphorylation with Gab1β +/+ cells, which are lower than those of Gab1β -/- cells (Figure 3.23). This confirms the previous conclusion from Figure 3.21, that Gab1β does indeed repress Akt phosphorylation in response to IGF.
Figure 3.23: The effect of Gab1β on Akt phosphorylation in response to IGF in Gab1β restored cells. Cells were induced with 100ng/ml IGF for 10 minutes. The re-expression of Gab1β restores the repression of Akt phosphorylation upon IGF induction. Gab1β -/- cells exhibit more Akt phosphorylation in response to IGF compared to Gab1β +/+ and Gab1β restored cells. The overexpression of Gab1α in Gab1β -/- cells also restores the repression of Akt phosphorylation in response to IGF.

Interestingly, overexpressing Gab1α in Gab1β -/- cells also had an effect on the levels of Akt phosphorylation. Even though Gab1α has been shown to be a positive regulator of IGF signalling in hepatic cells (Bard-Chapeau et al., 2005), our data suggests that its overexpression in ES cells represses Akt phosphorylation. Therefore, the presence of a PH domain does not contribute to the repression of Akt in response to IGF. Most importantly, however, it suggests that Gab1β is a negative effector of Akt signalling in response to IGF, but not a dominant negative one. If it were a dominant negative effector, then one would expect the overexpressed Gab1α protein to have an opposite or an inactivating effect to that of Gab1β, and that it would potentiate Akt signalling in response to IGF. As the two isoforms have the same effect on the IGF pathway, this means that the inhibitory effect is not a dominant effect in the case of Gab1β, which lacks the PH domain. Gab1 proteins in ES cells act to repress Akt signalling in response to IGF stimulation.
3.2.4 The effect of insulin on the growth of Gab1β-targeted ES cells

In the previous sections, Gab1β was shown to repress Akt and Erk phosphorylation in response to IGF. What this means biologically for an ES cell, however, is not known. IGF1 receptor activation leads to Erk phosphorylation in mouse ES cells and this may be associated with increased growth and proliferation (Nguyen et al., 2007). This suggests that Gab1β, as it represses Erk in response to IGF, it is acting to restrict the growth of ES cells. In this next section, I investigated this hypothesis. In order to investigate the potential role of Gab1β in the growth of ES cells in response to insulin a clonal colony-forming assay was performed. In this experiment, cells were plated at very low density (300 cells/9.4 cm² area) and allowed to grow for 5 days in the presence or absence of 25 µg/ml insulin. The cells were then stained for AP. The undifferentiated state of ES cells is characterised by high level of expression of Alkaline Phosphatase (AP) (Pease et al., 1990). AP-stained colonies were counted according to the intensity of staining, which was either none (AP negative), mild (AP partial) and strong (AP positive). This clonal colony-forming assay allows the analysis of a single stem cell’s potential to self-renew and propagate, by detecting clonal growth in the presence or absence of insulin. In other words, it examines the clonogenic potential of a single pluripotent ES cell.

The medium used was a defined medium originally used to provide optimum cell viability and efficient neural differentiation (Ying and Smith,
It is a mixed formulation of basal media and supplements called N2B27, and includes DMEM/F12 medium supplemented with modified N2, which contains insulin, apo-transferin, progesterone, putrescine and sodium selenite. This mixture is then supplemented with BSA combined with Neurobasal (Gibco) medium with added B27 (Gibco). Furthermore, in order to use this medium for ES cell maintenance, LIF, BMP and insulin were added. This medium was provided by Helen Murray and was used to observe the effects of insulin on the growth of Gab1β-targeted ES cells, in a defined environment without FBS supplementation. The cells were therefore stained for AP after being grown in the presence or absence of insulin for five days.

Results show that across all cell lines used, the addition of insulin does not significantly increase the clonogenic potential of cells, as the number of AP positive cells does not change greatly when insulin is added to the cultures (Figure 3.24). Furthermore, though both Gab1β restored cell lines have a similar pattern, wildtype C1 +/- cells gave rise to much fewer colonies, mainly the positive and partial ones. Furthermore, 26 -/- cells interestingly had much fewer AP colonies of all intensities, compared to both Gab1β restored cell lines, but less compared to C1 +/- cells. This pattern did not match that of VC -/- cells (Gab1β -/- cells transfected with an empty vector), which had more AP positive cells compared to Gab1β restored cells, as well as C1 +/- cells. Gab1α restored cells gave rise to the fewest colonies of all intensities compared to the other cell lines. This suggests that Gab1α may
provide a disadvantage to the cells to respond to insulin under clonogenic conditions.

**Figure 3.24:** The effect of insulin on the growth of Gab1β-targeted ES cells, Gab1α and Gab1β restored cells. Across all cell lines used, the addition of insulin does not significantly increase the clonogenic potential of cells, as the number of AP positive cells does not change when insulin is added. Although both Gab1β restored cell lines have a similar pattern, wildtype C1 +/+ cells gave rise to much fewer colonies, mainly the positive and partial ones. 26-/- cells had much fewer AP colonies of all intensities, compared to both Gab1β restored cell lines, but less compared to C1 +/+ cells. This pattern did not match that of VC -/- cells, which had more AP positive cells compared to Gab1β restored cells, as well as C1 +/+ cells. Gab1α restored cells gave rise to the fewest colonies of all intensities compared to the other cell lines.

These results suggests that Gab1β, and its repression effect on Erk activation in response to IGF, does not have a role in the control of ES cell growth in response to insulin. This could be attributed to IGF and insulin having different effects, despite their similarities. Whilst there is receptor crosstalk,
the prevailing view is that the insulin receptor (IR) tends to regulate metabolic activities related to glucose and energy homeostasis, whilst the IGF receptor (IGF-1R) is mainly involved in regulation of cell proliferation, anti-apoptosis, differentiation and cell motility (Nguyen et al., 2007; Rother and Accili, 2000).
3.3 Discussion

In this chapter I aimed to characterise the biochemical potential of Gab1β in signalling pathways that are key to the maintenance of ES cell self-renewal, proliferation and pluripotency, namely the Shp2 and PI3K pathways. Published data suggests that the PH domain is crucial for the ability of Gab1α to function as an adaptor protein. It was therefore surprising to find that Gab1β is phosphorylated in response to LIF and FBS and that this leads to the formation of complexes with Shp2 and PI3K. Furthermore, despite the lack of this critical domain, Gab1β may potentiate Akt phosphorylation in response to FBS, but more importantly enhances Erk phosphorylation in response to LIF. Furthermore, the lack of a PH domain did not interfere with the ability of Gab1β to repress Akt and Erk phosphorylation in response to IGF.

An important question that arose early on was whether Gab1β actually had the ability to participate in the pathways in question as it lacked a domain that has been demonstrated to be crucial for its function. The PH domain has been shown to be critical for EGF-induced activation of Erk and PI3K in epithelial cells (Itoh et al., 2000). Furthermore, the Gab1α PH domain has been shown to be required for its tyrosine phosphorylation and association with Shp2 after insulin stimulation (Rocchi et al., 1998). As mentioned in the introduction of this chapter, most of the data points to the PH domain being a crucial component for Gab1α’s function as an adaptor protein. Therefore, it
was expected that the lack of the PH domain would not allow Gab1β to participate in signalling pathways. As it retains all other motifs and binding sites, it was hypothesised that it may act as a dominant negative antagonist that would be phosphorylated, bind and mislocalise proteins. For example it could act as a sink for pro-differentiative factors, which would partially explain its abundance in ES cells. It therefore came as a surprise when we found that not only is Gab1β able to be phosphorylated in response to cytokine and growth factor stimulation, but this leads to an active complex with Shp2 and PI3K, thereby potentiating Erk and Akt phosphorylation downstream. Not only does this mean that Gab1β is a participant in pathways that are important for the maintenance of ES cells self-renewal and proliferation, but that it is a positive effector within them. What is intriguing, though, is that Gab1β potentiated the activation of Erk, a pro-differentiative factor, in response to LIF. There are two questions that arise from this finding. Firstly, why would an ES cell-associated protein potentiate the activation of a pro-differentiative factor? Secondly, why would it do so in response to LIF, the very factor that is crucial for the maintenance of ES cell self-renewal?

The effect of the activation of Erk in response to LIF is still unknown in ES cells. As Erk has been shown to act as a pro-differentiative signal rather than contributing to self-renewal (Burdon et al., 1999b), one might hypothesise that the activation in response to LIF may be in charge of this effect. The addition of MEK inhibitors to growth medium for example has been shown
to promote self-renewal (Burdon et al., 1999a). Using the same inhibitors, the persistence of Oct4 positive cells increases during isolation of murine ES cells (Buehr and Smith, 2003). Furthermore, Shp2 mutant ES cells exhibit reduced LIF dependency compared to wildtype ES cells (Qu and Feng, 1998). This is supported by the fact that BMP4 has been shown to maintain self-renewal by inhibiting Erk (Qi et al., 2004). Therefore a model seems to be emerging, whereby the activation of Erk via Shp2 and in response to LIF does not contribute to self-renewal. Based on the data obtained in this chapter, where Gab1β may fit into this model has become clearer. The activation of the LIFR/gp130 dimer receptor in response to LIF recruits Shp2, where it associates with Gab1β, and this promotes the phosphorylation of Erk. Self-renewal assays using Gab1β-targeted cells show that the lack of Gab1β does not have an effect on the self-renewal of the cells (Burdon T. unpublished). This supports the finding that Gab1β potentiates Erk in response to LIF. If Gab1β was involved in the maintenance of ES cell self-renewal, the potentiation of Erk in response to LIF would be contradictory. Therefore, it is the significance of LIF-induced ERK phosphorylation which is the key issue. Why does a cytokine required for the maintenance of ES cells activate a pro-differentiative factor? Gab1β merely acts as an adaptor protein that potentiates this signalling pathway. LIF also activates Stat3, which is crucial for the self-renewal of ES cells (Niwa et al., 1998). Therefore, the efficiency of self-renewal is dependent on both the Stat3 and Erk signals activated downstream from the LIF receptor (Burdon et al., 1999a). The addition of MEK (and Gsk3) inhibitors to the growth medium of ES cells, can maintain self-renewal without the addition of LIF, BMP or serum (Ying et al., 2008).
This suggests that LIF-induced Stat3 activation may act to block Erk-induced differentiation of ES cells, and that the natural path for an ES cell, is for it to differentiate. Gab1β may be part of the natural differentiation pathway, and therefore is able to potentiate Erk activation in response to LIF.

The effect of Gab1β on Erk activation could also have nothing to do with pro-differentiative effects. Gab1β may also have a dominant negative role in the potentiation of ERK phosphorylation in response to LIF stimulation. It may act to mislocalise it in order to produce a different effect. Erk has been shown to translocate to the nucleus of cultured epithelial cells by binding to the Met Binding Domain (MBD) of Gab1α, which has a Nuclear Localisation Sequence (NLS) within its PH domain (Osawa et al., 2004). Gab1β could therefore bind activated Erk, after potentiating its phosphorylation, and then mislocalising it, due to the lack of a NLS in the N-terminal domain. This would therefore restrict Erk to the cytoplasm and inhibit its pro-differentiative effects. This suggests that Gab1β could act to bind and mislocalise Erk and its pro-differentiative effects.

Interestingly, the GRB2/MEK pathway has been shown to repress Nanog, in mouse ES cells (Hamazaki et al., 2006). As previously described, Nanog is a homeobox gene and a key intrinsic regulator of ES cells self-renewal. The MEK inhibitor PD98059 as well as GRB2 deficiency prevents the differentiation of mouse ES cells into primitive endoderm induced by Nanog repression. This suggests that Erk activation represses Nanog upon ES cell
differentiation into primitive endoderm and that Grb2 is required for this. Gab1β could take part in the repression of Nanog by potentiating Erk activation. Though not demonstrated, Gab1β is likely to require GRB2 in order to bind to Shp2 and enhance Erk activation. The abundance of Gab1β in ES cells does not mean that its potential role should be associated with self-renewal and pluripotency. ES cells must express proteins that are present in order to regulate their transitions into differentiated cells types. For example, UTF1 (undifferentiated embryonic cell transcription factor), a chromatin-associated protein, is involved in ES cell differentiation, and is not required for the self-renewal of the cells (van den Boom et al., 2007). Under the transcriptional control of Oct4, and despite its high abundance in ES cells, UTF1 is an example of a protein that is involved in ES cell differentiation. Therefore, Gab1β may act as an adaptor protein regulating the transition of ES cells into primitive endoderm by repressing Nanog via Erk phosphorylation. Experiments looking at the potential effect of Gab1β on the repression of Nanog would be very interesting. These could be done using Gab1β-targeted ES cells and checking whether the lack of Gab1β affected the transition into primitive endoderm induced by the repression of Nanog.

Another example of a gene that is highly expressed in ES cells but is involved in their differentiation is the FGF4 gene (Kunath et al., 2007; Wilder et al., 1997). FGF4 null ES cells are not affected in their ability to self-renew (Wilder et al., 1997). Although they are still able to differentiate, the growth and survival of the differentiated progeny is compromised. Furthermore, Erk
activation in response to FGF4 has been shown to trigger the transition of ES cells from self-renewal to lineage commitment (Kunath et al., 2007). In this study the authors demonstrated that Erk acts to promote the transition of ES cells into a state where they are responsive to pro-differentiative cues. Gab1β could participate in this Erk-induced transition by activating it in response to FGF4. However, when FGF4 inductions were performed using Gab1β-targeted ES cells, there was no difference in the levels of Erk activation upon induction (data not shown).

As well as boosting Erk activity, Gab1β may also enhance Akt phosphorylation in response to FBS. The PI3K pathway is important for the maintenance of ES cell self-renewal. For example, a myristoylated, active form of Akt can maintain the undifferentiated phenotype in mouse ES cells, without the addition of LIF (Watanabe et al., 2006). Gab1β -/- cells, though, showed no effect on Akt activation in response to LIF. This suggests that Gab1β does not take part in the self-renewal effects mediated by LIF-induced Akt. This is supported by the fact that Gab1β -/- cells do not have impaired self-renewal (Burdon T., unpublished). Even though BMP has been shown to replace the requirement for FBS in ES cells culture (Ying et al., 2003a), the fact that Gab1β does not affect self-renewal, despite the effect on Akt activation, suggests an alternative role. For example, Akt activation is involved in many other pathways that control the cell proliferation, growth and survival (Jirmanova et al., 2002; Sun et al., 1999; Takahashi et al., 2003). Indeed, Gab1β
-/- cells show a deficit in their growth during long-term competition assays (Burdon T., unpublished).

Additionally, the lack of Gab1β does not seem to have an effect on the phosphorylation of GSK3β at serine 9. This can be attributed to the fact that FBS contains a myriad of growth factors that may have a varying effect on PI3K signalling and therefore any significant difference in GSK3β phosphorylation levels would be masked. Furthermore, this could be attributed to a potential maximum level of GSK3β activation. FBS clearly causes the phosphorylation of GSK3β but any significant difference is masked as the phosphorylation has reached its maximum. However, stimulation with FBS at a lower concentration may allow small differences to appear. Inductions with varying concentrations of FBS and at shorter timepoints may reveal subtle differences in GSK3β activation in the Gab1β-targeted ES cells. Interestingly, however, despite the potential effect of Gab1β on the phosphorylation of serine 9 on Akt in response to FBS, GSK3β remained unaffected in Gab1β-deficient cells. This suggests that the true measure of PI3K activity is not as clear-cut. It may be that, depending on the receptor and the substrate, different branches of the PI3K pathway are activated, whilst others remain unaffected. It may be that the PI3K effectors are differentially activated by substrates in order to have alternative biological outcomes.
In this chapter I also aimed to characterise the potential role that Gab1β has in IGF signalling in ES cells. I showed that Gab1β represses both Akt and Erk phosphorylation in response to insulin. Additionally, as the overexpression of Gab1α also had the same repressive effects, Gab1β does not act in a dominant negative manner to mislocalise Gab1α effectors. Gab1β has an active inhibitory effect on IGF signalling. Furthermore, clonal assays showed that Gab1β does not have an effect on ES cell growth in response to varying amounts of insulin.

Gab1α was originally described as an IRS1-like protein (Holgado-Madruga et al., 1996) and its involvement in insulin/IGF signalling has been demonstrated. For example, liver-specific Gab1α knockout mice show enhanced hepatic insulin sensitivity with reduced glycemia and improved glucose tolerance (Bard-Chapeau et al., 2005). This indicates that Gab1α is a negative modulator of insulin signalling in the mouse liver. Gab1α was shown to repress Akt activation in response to insulin, via activation of Erk. The data presented in this chapter supports this repressive role for both Gab1α and Gab1β. Furthermore, Gab1α has been shown to be tyrosine phosphorylated by the insulin receptor to allow its association with PI3K and Shp2 (Rocchi et al., 1998). Gab1α is also capable of restoring the negative effects of IRS1 deletion in 3T3 fibroblasts (Winnay et al., 2000).

Studies show that insulin and IGF1 stimulate the metabolism and growth of preimplantation embryos, but in ES cells little is known of the connection
between intracellular insulin signalling and cellular growth responses during development (Harvey and Kaye, 1992; Nguyen et al., 2007). In rabbit blastocysts, insulin has a growth effect by activating Erk and regulating GLUT4 transcription (Navarrete Santos et al., 2004). Whether there is a similar mechanism in mice is still unclear. IGF1 and insulin receptor activation leads to Erk phosphorylation in mouse ES cells and that this is associated with increased growth and proliferation (Nguyen et al., 2007; Ying et al., 2008). The fact that Gab1β represses Erk phosphorylation in response to IGF implies that Gab1β would actually act to restrict the growth of ES cells. Our data do not fully support this hypothesis, as Gab1β did not have an obvious effect on the growth of mouse ES cells in response to insulin.

A recent report has suggested that IRS1 may regulate murine ES cell self-renewal and its presence is critical for LIF-mediated self-renewal. Knocking down IRS1 by siRNA resulted in a severe reduction in Oct4 protein expression and that Akt and GSK3β phosphorylation were repressed (Rubin et al., 2007). The knock down also impaired the expression of Id proteins, which are known to inhibit differentiation in ES cells (Ying et al., 2003a). Our data suggests that Gab1β is not involved in this IRS-mediated positive effect on self-renewal, as Gab1β represses Akt activation in response to IGF. This would imply that Gab1β is not involved in the regulation of the self-renewal of ES cells. This is consistent with previous data that Gab1β is not involved in this process (Burdon T., unpublished) and that Gab1β promotes Erk phosphorylation in response to LIF (Figure 13). Though Gab1α has been
shown to be critical for insulin-stimulated Erk activation in the mouse liver, this was accompanied by a reduction in Akt phosphorylation (Bard-Chapeau et al., 2005). Gab1β also had a negative effect on Akt activation in response to IGF in ES cells. This suggests that in the liver, Gab1α acts to sensitize it to insulin and also at the biochemical level, acts to repress Akt activation. A similar role for Gab1β in ES cells is possible.

The PH domain of Gab1α has been reported to be required for the association of the protein with Shp2 (Rocchi et al., 1998), and one would assume for the activation of Erk, after insulin stimulation. This implies that Gab1β is not able to activate Erk in response to insulin. Our data supports this and further suggests that Gab1β actually represses Erk phosphorylation in response to insulin. Therefore, Gab1β acts as a negative regulator of Erk activation in response to insulin. This shows that Gab1β is a multifunctional adaptor protein that, depending on the receptor type, can either act positively or negatively. For example, our data shows that in response to LIF, Gab1β promotes Erk phosphorylation, but in response to insulin, it represses it.

The literature suggests that Gab1 proteins are truly multifunctional adaptor proteins which, depending on the receptor and cell type, act as potentiators or repressors. Our data supports this modularity and indicates that Gab1β may act as an activator and repressor of the same signalling pathways. Gab1β potentiates Erk, and potentially Akt, in response to LIF, but acts as a repressor of Erk and Akt in response to insulin signalling. The potentiation of
ERK activation by Gab1β in response to LIF may translate into Gab1β having a role in either balancing the contradictory signals of Stat3 and Erk, or in promoting the differentiation of ES cells. The repression of Erk activation in response to insulin is likely not to translate into an effect on the growth of the ES cells. Though clear conclusions can't be made about the functional meaning of these contradictory effects of Gab1β, what is clear is that Gab1β adapts the signalling responses of many other receptors and therefore in order to obtain a complete "signalling picture" all the pathways that Gab1β takes part in must be investigated. This, is beyond the scope of this investigation.
CHAPTER 4: CHARACTERISING THE LOCALISATION OF GAB1β IN ES CELLS

4.1 Introduction

Gab1β is ES cell-associated and lacks an important structural component, the PH domain. The PH (Pleckstrin homology) domain was first identified as a 100-120 residue stretch of amino acid-sequence similarity that occurs twice in pleckstrin (the major PKC substrate in platelets) and is found in numerous other proteins with membrane-associated functions (Haslam et al., 1993). PH domains recognise membranes in a target-specific manner (Lemmon and Ferguson, 2000). They recognise a membrane component and then bind to them. They are also the most common domain in the human genome and are best known for their ability to target cellular membranes by binding specifically to phosphoinositides (Lemmon, 2004). There are common structural similarities between the PH domain and lipid-binding proteins, which allow the PH domain to target cellular membranes.

Most PH domains bind to phosphoinositides, and some have been shown to drive membrane translocation of their host proteins through specific, high-affinity recognition of phosphoinositides head groups (Kavran et al., 1998). More specifically, PH domains are well known effectors of the lipid second messengers PtdIns(3,4,5)P3 (PIP3 or phosphatidylinositol trisphosphate) and PtdIns(3,4)P2 (PIP2 or phosphatidylinositol diphosphate), which are generated...
transiently upon activation of almost all cell surface receptors (Cantley, 2002; Lemmon, 2008). Interestingly, a PH domain that recognises only PIP3 will be recruited transiently to the plasma membrane, whilst one that binds to both PIP3 and PIP2 will remain membrane associated for longer (Ferguson et al., 2000). Whether the PH domain of a protein recognises PIP3 only or both PIP3 and PIP2, will therefore have a significant impact on the time course of its response to PI3K activation (Ferguson et al., 2000). The PH domain of Akt specifically recognises one or both of these second messengers with high affinity and specificity (Lemmon, 2004). This takes place in response to receptor-induced recruitment of PI3K to the plasma membrane (Gold et al., 2000). This allows PI3K to phosphorylate phosphoinositides and results in the production of novel phospholipids, which are present at very low levels before receptor engagement (Gold et al., 2000). PI3K phosphorylates PIP2, and this in turn yields PIP3 (Stephens et al., 1991). Both PIP3 and PIP2 function as second messengers by acting as ligands for the PH domains that are found in a number of cytosolic signalling proteins (Toker and Cantley, 1997). PIP3 and PIP2 can therefore recruit PH domain-containing proteins, such as Akt, to regions of the plasma membrane to which PI3K has been recruited (Gold et al., 2000). Akt is a serine threonine kinase, which is defined by an NH₂-terminal regulatory domain of protein-protein interactions that contains the PH domain (Bos, 1995; Downward, 1995). The activity of a protein with a point mutation of Arg to Cys in the Akt PH domain is not significantly increased by PDGF treatment or by expression of an activated form of PI3K (Franke et al., 1997). This establishes the PH domain of Akt as an important structural feature for the function of Akt and its activity in
response to PDGF and PI3K. Furthermore, a PH domain-GFP fusion protein is predominantly cytosolic in unstimulated cells, but undergoes a transient relocalisation to the plasma membrane upon signal-dependent activation of PI3K (Lemmon, 2008; Varnai and Balla, 2006; Venkateswarlu et al., 1998). Another protein whose PH domain has been shown to be crucial for its localisation is Arno, a member of the family of guanine nucleotide exchange factor for ARFs (Adipose Ribosylation Factors) (Donaldson and Klausner, 1994; Venkateswarlu et al., 1998). When Arno is tagged with GFP, it has been shown to localise exclusively in the cytoplasm of 3T3 L1 adipocytes. Stimulation with insulin, however causes a rapid PH domain-dependent translocation to the plasma membrane (Venkateswarlu et al., 1998).

The Gab1α PH domain is located at the NH₂ terminal of the protein and is the most conserved structural element among Gab family members (Nishida and Hirano, 2003). The PH domain of Gab1α has been shown to contain a conserved motif, which has been used to predict high-affinity binding of a subset of PH domains to PI3K products such as PIP2 and PIP3 (Isakoff et al., 1998). This binding was shown in an in vivo assay in yeast. Using a GFP-Gab1α PH domain fusion strategy, the PH domain was shown to specifically bind to PIP3 (Rodrigues et al., 2000), which then leads to the membrane recruitment of Gab1α in response to EGF stimulation. Furthermore, the Gab1α PH domain has been shown to be essential for the targeting of Gab1α to the proximity of the cellular membrane at sites of cell-cell contact in MDCK cells (Maroun et al., 1999a), which is dependent on the provision of
PIP3 by PI3K as an anchor for Gab1α. Interestingly, overexpression of the p110 and p85 subunits of PI3K (Klippel et al., 1994), which causes elevated PI3K activity in the absence of external stimulation, induces the translocation of Gab1α from the cytosol to the membrane in cells maintained at low serum concentrations (Maroun et al., 1999b).

Within the PH domain, a putative NLS (Nuclear Localisation Sequence) has been observed in the Gab1α sequence, at amino acids 15-23 (Osawa et al., 2004). An NLS has been classically defined as an amino acid sequence motif that is both necessary and sufficient for transport of the protein to the nucleus (Terry et al., 2007). Eukaryotic cells are characterised by distinct nuclear and cytoplasmic compartments that are separated by the nuclear envelope, a double membrane that is continuous with the endoplasmic reticulum (ER). The nuclear envelope is penetrated by nuclear pore complexes (NPCs), which allow exchange of macromolecules between the two compartments (Gorlich and Kutay, 1999). The NPCs of the nuclear membrane essentially act as stations of intercommunication between the cytoplasm and the nucleus (Sorokin et al., 2007). The NPC consists of about 30 different proteins, called nucleoporins (Rout and Aitchison, 2000), and forms a channel and regulates the nucleocytoplasmic transport of RNA and proteins (King et al., 2006; Suntharalingam and Wente, 2003). As the NPC acts as an entry/exit station in/out of the nucleus (Rout et al., 2000), they allow the passive diffusion of ions and small molecules through aqueous channels with a diameter of 9nm (Paine et al., 1975), whilst they are >60MD macromolecular structures themselves (Cronshaw et al., 2002; Rout et al., 2000).
though passive movement can take place, the translocation of cargoes larger than 40kDa generally requires specific transport receptors (Fried and Kutay, 2003; Pante and Kann, 2002; Terry et al., 2007). Therefore, nucleocytoplasmic transport depends on the interplay between soluble transport factors, their cargoes and the NPC. It is a set of proteins called karyopherins (kaps, also known as importins/exportins) which bind to specific import or export signals and mediate substrate docking to the NPC (Rout and Aitchison, 2000). An example of an import signal is the NLS of Gab1α, which allows the translocation of the protein from the cytoplasm into the nucleus. Not much is known about the role of Gab1α in the nucleus. Data suggests that Gab1α may be responsible for the transport of other proteins that are too large to passively diffuse into the nucleus and lack an NLS of their own. An example of such a protein is the MAPK Erk (Osawa et al., 2004). In this study, the authors hypothesised that Gab1α facilitates the translocation of Erk to the nucleus. Erk nuclear translocation is required in order for it to activate transcription factors such as ETS-like transcription factor-1 and EGR1 (Early Growth response factor 1) (Kaufmann et al., 2001). This takes place in response to endothelial cell migration during wound repair after arterial injury (Osawa et al., 2004) (Khachigian et al., 1996; Santiago et al., 1999). The lack of an NLS as well as the 40-50kDa size of Erk suggested that Erk would require another protein that would mediate its translocation. The potential role of Gab1α in this process is supported by data that suggests that activated Erk interacts with Gab1α (Roshan et al., 1999). In this study, using GST-tagged MBD and Gab1α, the authors demonstrated that the MBD domain of Gab1α associates with
phosphorylated Erk2 and more weakly with Erk1. Therefore, as Gab1α can associate with phosphorylated Erk, it seems plausible that the NLS of Gab1α can act as a nuclear translocator of other proteins that associate with it.

The aim of this chapter is to determine the localisation of Gab1β within ES cells and to see whether the lack of a PH domain and an NLS affects this. This was done by, transfecting EGFP-tagged Gab1α or Gab1β into both fibroblasts and Gab1β-/- cells, and observing the movement of the proteins in question in response to LIF and/or FBS.
4.2 Results

4.2.1 Construction of Gab1-EGFP fusion vectors

The lack of a Gab1β-specific antibody meant that I was unable to specifically stain ES cells for Gab1β without also detecting Gab1α. Therefore, a cloning strategy was devised, whereby Gab1β was specifically tagged with EGFP. The cloning strategy relied upon the availability of an expression vector which contained the Gab1α or Gab1β cDNA under the control of a strong promoter. The vector used to clone EGFP into as a fusion to the Gab1 cDNA, was called pCAGIP-Gab1 (previously generated in the laboratory by T. Burdon and L. Sutherland) (Figure 4.1a). This vector had many advantages. The CAG promoter is a strong and ubiquitous promoter and has been shown to produce high levels of expression both in vivo and in vitro (Alexopoulou et al., 2008; Niwa et al., 2000). The vector also contained a polyoma virus origin of replication, PYori, which, upon interaction with the T antigen, allows efficient replication of incoming plasmids (Jackson et al., 2002). This episomal expression construct contains a polyoma virus origin of replication, which can be transfected into E14/T ES cells. These cells stably express the polyoma large T antigen and the interaction of the large T antigen with the polyoma origin of replication allows efficient episomal propagation of incoming plasmids. This system has high transfection efficiencies (supertransfection), and high levels of expression can be achieved and maintained (Jackson et al., 2002). The SV40ori is similar to the PYori in that it also bypasses cellular
controls on replication and exhibits uncontrolled, high-level replication in permissive cells (Chittenden et al., 1991). Plasmids containing the SV40 origin of replication are rapidly amplified when transfected into COS7 cells (Gluzman, 1981). COS7 cells constitutively express SV40 large T antigen, the only viral protein required for SV40 replication (Chittenden et al., 1991). The IRES (Internal Ribosome Entry Site) is a cis-acting element that recruits the small ribosomal subunits to an internal initiator codon in the mRNA, thereby promoting the internal initiation of translation of RNA (Martinez-Salas, 1999). The use of bicistronic expression vectors in which the first gene is translated in a cap-dependent manner, Gab1 in our case, and the second one, puromycin resistance gene in our case, in an IRES-dependent manner, allows improved efficiency of selection during transgenesis (Martinez-Salas, 1999). This system ensures that over 90% of cells expressing the selectable marker should also express the gene of interest, Gab1 (Martinez-Salas, 1999).

This vector was used as a basic backbone in which EGFP was cloned into as a fusion to Gab1α or Gab1β. Three variations of this EGFP-Gab1 vector were made. In the first simple variation (Figure 4.1b), EGFP was cloned into a Not1 restriction site at the N-terminal of Gab1. In the second variation (Figure 4.1c), EGFP was again cloned into the same Not1 restriction site, with an added polyglycine linker, consisting of 6 consecutive glycines. This glycine linker served to connect EGFP and Gab1 in a flexible manner. The presence of a linker allows for the proper folding of the fusion protein, which in turn is important for the function. Glycine-rich linkers are more flexible than non-glycine linkers, because the absence of a β-carbon permits the
polypeptide backbone to access dihedral angles that are energetically forbidden for other amino acids (Ramachandran and Sasisekharan, 1968). Too much linker flexibility however, has been shown to be detrimental to single-chain protein stability (Robinson and Sauer, 1998). In the third version (Figure 35d), EGFP was cloned into an XhoI restriction site at the C-terminal end of Gab1α or Gab1β with a polyglycine linker separating the two.

Figure 4.1: Gab1-EGFP fusion strategy. a) pYCAGIP backbone vector contained Gab1 cDNA under the control of a strong CAG promoter, along with an IRES element, puromycine resistance gene, SV40ori and PYori. b) pEGFP-GAB1 vector: EGFP was cloned into NotI restriction site, fused to Gab1. c) pEGFP-GLY6-GAB1 vector: EGFP was cloned into the same NotI site as before but with a polyglycine linker separating it and Gab1. d) pGAB1-GLY6-EGFP: EGFP was cloned into an XhoI site at the C-terminal of Gab1 along with a polyglycine linker separating it and Gab1.
4.2.2 Expression of Gab1 fusion proteins in COS7 cells

These six variations of the basic vector (3 for Gab1α and 3 for Gab1β) were transiently transfected initially into COS7 cells, which constitutively express the SV40 large T antigen, the only viral protein required for SV40 replication (Chittenden et al., 1991) which allow high levels of plasmid replication. Once transfected, images of the cells were taken under UV fluorescence to see whether an EGFP-positive protein could be detected (Figure 4.2). Cells were also transfected with non-EGFP myc-tagged Gab1α or Gab1β. These acted as controls once the transfected cells were lysed for protein and run on a Western blot (Figure 4.4). COS7 cells were also transfected with an EGFP control plasmid which was constructed in the same way as the first fusion plasmid (Figure 4.3) but with a couple of extra amino acids between EGFP and Gab1β, which rendered Gab1β cDNA out of frame, and therefore no Gab1β protein was transcribed. The cells were then lysed for protein, which was then analysed on a Western blot probed for Gab1 protein using a Gab1-specific antibody (Figure 4.4). The fluorescent images (Figure 36) show that all six fusion plasmids produce a green fluorescent protein. Furthermore, the Western blot demonstrates that the fusion plasmids also give rise to Gab1α or Gab1β protein, of a larger size than the Gab1α-myc and Gab1β-myc proteins respectively. This is due to the fusion of the proteins with EGFP, which has a size of ~30kDa. Gab1α and Gab1β fluorescent proteins were predominantly restricted in the cytoplasm of the ES cells, unlike the EGFP protein, which was expressed in the cytoplasm and nucleus. This suggests that the presence of Gab1α or Gab1β is restricting the protein in the cytoplasm.
Figure 4.2: EGFP fusion vector transfection of COS7 cells. COS7 cells were transfected with Gab1α or Gab1β EGFP fusion vectors. All vectors gave rise to fluorescent proteins.
Figure 4.3: EGFP control vector transfection of COS7 cells. COS7 cells were transfected with plasmids containing EGFP alone, or myc-tagged Gab1α and Gab1β, containing no EGFP. The control cells were transfected with lipofectamine alone with no plasmid.

Figure 4.4: Expression of fusion proteins in COS7 cells. Transiently transfected COS7 cells were lysed and their protein was analysed on a Western blot probed with a Gab1 antibody, which detects both Gab1α and Gab1β. All vectors gave rise to a fusion protein which was larger than the untagged protein, as indicated by the migration on the blot.
4.2.3 Gab1-EGFP localisation in 10T1/2 fibroblasts

Prior to investigating the localisation of Gab1β in ES cells, I investigated its transient localisation in 10T1/2 fibroblasts. The latter cells have a much larger cytoplasm compared to ES cells, which would potentially allow the visualisation of any change in the localisation of the fluorescent proteins. The vectors chosen to perform this experiment with were the plasmids EGFP-Gab1α and EGFP-Gab1β. They were chosen as they gave rise to the brightest fluorescent fusion proteins out of all 3 variations of the plasmid. The two plasmids were transiently transfected into 10T1/2 fibroblast cells using Lipofectamine 2000 (Invitrogen). The cells were either left to transfect for 4 hours or overnight. Lipofectamine 2000 is recommended to be left on cells for a minimum of 4 hours, but longer should increase the number of positive transfectants according to the manufacturer. Longer periods of transfection with Lipofectamine 2000, however, is known to be toxic to the cells (Corsi et al., 2003; Green et al., 2008). The medium was then changed to normal growth medium for around 1 hour and then the cells were starved of all growth factors and cytokines for 3 hours. Then, the cells were induced with either 1000 units/ml LIF or 1µl/ml FBS. Images were then taken at 10 and 60 minutes in order to observe the localisation of the tagged proteins, and their potential movement within the cells.
Figure 4.5: EGFP-Gab1\(\alpha\) and EGFP-Gab1\(\beta\) transient expression in 10T1/2 fibroblast cells after 4-hour transfection. Cells were induced with 1000 units/ml LIF or 10% FBS for 10 and 60 minutes.

After the 4-hour transfection, the fusion proteins were clearly expressed in the cytoplasm of the fibroblasts (Figure 4.5). Upon induction, however, the expression pattern of neither fusion protein changed. Though EGFP-Gab1\(\beta\) fusion protein does not have a NLS, EGFP-Gab1\(\alpha\) does, and therefore would be expected to enter the nucleus upon stimulation. This suggests that the
proteins may not be fully functional, and therefore cannot react to the stimulation. After the overnight transfection, a similar pattern emerged (Figures 4.6 & 4.7). In uninduced conditions, the fusion proteins were localised in the cytoplasms and some nuclei of the fibroblasts, in contrast to the EGFP protein alone (Figure 4.6c), which was expressed in the whole fibroblast. This suggests that the addition of either Gab1α or Gab1β to EGFP restricts it to the cytoplasm. No change in localisation was observed for either fusion protein after LIF stimulation (Figure 4.6). After 10 and 60 minutes of FBS induction, EGFP-Gab1α expression was observed in the whole fibroblast. This was also the case for EGFP-Gab1β after 60 minutes of FBS stimulation. This is somewhat surprising as Gab1β lacks a NLS, it should not be able to translocate to the nucleus. Furthermore, in the uninduced cells of these transfections, there were cells which also exhibited fluorescence in their nuclei. This suggests that what was observed may have been a result of the harsh toxic effects of Lipofectamine. When the experiment was repeated, no change in localisation was observed for either EGFP-Gab1α, or EGFP-Gab1β (Figure 4.7). These data suggest that the localisation of the fusion Gab1β protein is not affected by the lack of a PH domain or NLS. Whether this is due to EGFP affecting the localisation of the fusion Gab1β, or whether the localisation of Gab1 proteins is not affected by the absence of the PH domain, is still unclear. These observations, however, do not necessarily mean that the fusion proteins are not biochemically functional. They may still be able to partake in the expected signalling pathways in ES cells.
Figure 4.6: EGFP-Gab1α and EGFP-Gab1β transient expression in 10T1/2 fibroblasts. EGFP-Gab1α localised to the whole of the cell in response to LIF after 10 minutes, as well as in response to FBS. EGFP-Gab1β was expressed in the whole fibroblast after 60 minutes of FBS stimulation. EGFP protein alone was expressed in the whole fibroblast under all conditions.
Figure 4.7: EGFP-Gab1α and EGFP-Gab1β transient expression in 10T1/2 fibroblasts. There was no change in the localisation of either fusion proteins in response to LIF or FBS induction.
4.2.4 Gab1 localisation in ES cells

4.2.4.1 Fusion protein expression and localisation in ES cells

The six fusion plasmids (Figure 4.1) were stably transfected into Gab1β -/- ES cells using Lipofectamine 2000. Cells were transfected overnight with lipofectamine in a well of a 6-well dish with 5μg DNA. The next day, the cells were split into 2x10cm² dishes (3/4 and 1/4) and allowed to grow overnight. The day after the medium was changed to a medium containing 150 μg/ml of puromycin. The medium was then changed every 2 days until colonies were big enough to pick and transfer to 24-well plates. Once propagated, transfected cells were then moved to wells of a 6-well dish from which they were frozen down. The expression of the fusion proteins was confirmed by Western blot (Figure 4.8). Cell lysates were obtained from all 6 fusion plasmid transfections and analysed on a Western blot probed with a Gab1-specific antibody.

One clone from each fusion plasmid transfection was plated and fluorescent images were taken in order to detect the expression of the EGFP fusion protein (Figure 4.9). The fluorescent proteins seem to be restricted in the cytoplasm of the ES cells, whether they are fused to Gab1α or Gab1β (Figure 4.9a). This is in stark contrast to the EGFP protein without a fused Gab1 protein, which is expressed in the whole of the ES cell, including the cytoplasm and the nucleus (Figure 4.9b).
Figure 4.8: Expression of Gab1β fusion proteins in Gab1β -/- cells. Lysates were analysed on Western blots probed with aGab1 and aSHP2 antibodies.

More specifically, the variation of the fusion protein where EGFP was directly fused to the N-terminal of Gab1α or Gab1β, was localised diffusely in the cytoplasm of the cells. EGFP-Gab1β was brighter compared to EGFP-Gab1α. This may be due to differences in amounts of protein rather than intensity of fluorescence. The second variation of the fusion proteins, where EGFP was fused to the N-terminal of the Gab1 proteins linked by a polyglycine linker, also gave a similar pattern. Both EGFP-GLYα-Gab1α and EGFP-GLYα-Gab1β were restricted in the cytoplasm of the cells. Similarly to the first variants, EGFP-GLYα-Gab1β seemed brighter compared to EGFP-GLYα-Gab1α. Interestingly, EGFP-GLYα-Gab1β was also expressed in a body that was found in or near the nucleus of certain cells.
**Figure 4.9a**: Expression of EGFP-tagged Gab1α and Gab1β in Gab1β-/- cells. All six fusion plasmids give rise to fluorescent proteins that are restricted in the cytoplasm.
Figure 4.9b: Expression of EGFP alone in Gab1β-/- cells. The fluorescent EGFP protein is expressed in the whole area of the cells. The control cells were transfected with an empty vector CAG-IP.

In the cells expressing the third variant of the fusion proteins, where EGFP was linked to the C-terminal of the Gab1 proteins via a polyglycine linker, Gab1α-GLY6-EGFP and Gab1β-GLY6-EGFP showed very distinct patterns of expression. Firstly, Gab1α-GLY6-EGFP was not as bright compared to the other Gab1α fusion proteins, but was still retained in the cytoplasm of the cells. Gab1β-GLY6-EGFP, however, had a very different expression pattern compared to the other Gab1β fusion proteins. It seemed restricted to the membranes of the cells, rather than expressed diffusely in the cytoplasm. Furthermore, like EGFP-GLY6-Gab1β, it was also expressed in a small body in or near the nucleus.
Interestingly, unlike EGFP-GLYβ-Gab1β, this body appeared in the majority of cells. In order to investigate the expression of these fusion proteins in this distinct body, GAB1β-GLY6-EGFP, EGFP-GLY6-GAB1β and EGFP-GLY6-GAB1α cell lines were fixed in 4% paraformaldehyde for confocal imaging after induction with FBS and LIF. In unstimulated cells Gab1β-GLY6-EGFP was restricted to the membranes of the cells, as well as the distinct body near the nucleus (Figure 4.10, upper panel). Upon stimulation with LIF+FBS, the expression of the fusion protein was more distinct and slightly brighter at the membranes at points of cell to cell contact. This effect was more pronounced upon stimulation with LIF alone, whereby the fusion protein was distinctly localised at points of cell to cell contact. The EGFP-GLY6-Gab1β fusion protein in unstimulated cells, was expressed diffusely in the cytoplasm of the cells (Figure 4.10, middle panel). There were some cells that also expressed the fusion protein in this distinct body near the nucleus. Upon stimulation with LIF+FBS, the expression of the protein was slightly more distinct. Upon LIF stimulation, this distinct expression, potentially at the membrane, was more pronounced, and potentially at points of cell to cell contact. In comparison to Gab1β-GLY6-EGFP, which was expressed in the distinct body in the majority of cells, EGFP-GLY6-Gab1β was only expressed in the body of some cells. In the case of EGFP-GLY6-Gab1α, in unstimulated cells, the fusion protein was expressed diffusely throughout the cytoplasm (Figure 4.10, lower panel). Upon stimulation with LIF+FBS, EGFP-GLY6-Gab1α expression was more distinct, specifically at the membrane of the cells. Interestingly, EGFP-GLY6-Gab1α was not expressed in this distinct body as the other Gab1β fusion proteins.
Figure 4.10: Expression of Gab1β-GLY<sup>b</sup>-EGFP, EGFP-GLY<sup>b</sup>-Gab1β and EGFP-GLY<sup>b</sup>-Gab1α in Gab1β<sup>−/−</sup> cells stimulated with FBS and/or LIF. Cells were induced with either 100 units/ml LIF combined with 10% FBS, or 100 units/ml LIF alone.
By tagging Gab1 proteins with EGFP, the lack of a PH domain and NLS in Gab1β did not alter the localisation compared to that of Gab1α. The data suggests that the fusion proteins are restricted in the cytoplasm due to the presence of Gab1. However, the three different variants of the fusion plasmids gave rise to proteins that were differentially expressed in transiently and stably transfected cells. Namely, EGFP-GLY⁶-Gab1β and Gab1β-GLY⁶-EGFP were similarly expressed in a distinct body near or perhaps within the nucleus of the cells. Their localisation at the membrane, however, was different. Gab1β-GLY⁶-EGFP was localised distinctly at the membrane, whilst EGFP-GLY⁶-Gab1β was more diffuse along the membrane and the cytoplasm. The third variant, EGFP-Gab1β was not expressed in the perinuclear body, but its expression was still restricted in the cytoplasm of the cells. Therefore, in order to confirm which pattern of expression represents the real Gab1β pattern, immunohistochemical staining was performed on wildtype ES cells (C1 +/+), as well as Gab1β -/- cells overexpressing Gab1β (Gab1β restored cells). 500,000 cells per 1.9 cm² well of a 4-well plate were plated and allowed to grow overnight. The next day cells were fixed in 4% PFA and stained using a Gab1-specific antibody, which detects both Gab1α and Gab1β. After being stained with the appropriate secondary antibody and DAPI, which stains the nucleus, images were taken. Similar patterns of expression emerged when both cell lines were stained for Gab1. Gab1 was expressed distinctly at the cell membrane, in a distinct body near or within the nucleus as well as diffusely in the nucleus itself (Figure 4.11).
Importantly, this antibody detected both Gab1α and Gab1β protein expression, and therefore the nuclear staining may be due to the small amount of Gab1α protein expressed in ES cells. This was confirmed when Gab1 protein expression was compared in Gab1β +/+ and -/- cells (Figure 4.12).

In order to compare the expression pattern of Gab1 to the pattern of the previously generated EGFP-tagged Gab1 expressing cell lines, the same staining protocol was performed using EGFP-Gab1β, EGFP-GLYα-Gab1β and Gab1β-GLYα-EGFP cell lines. EGFP-Gab1β was, as previously shown in Figure 4.9a, expressed diffusely in the cytoplasm of the cell, as seen by both immunohistochemical staining (TRITC), as well as by the fluorescent expression of the EGFP-tagged protein (Figure 4.13, upper panel). A similar pattern was observed with EGFP-GLYα-Gab1β, but this fusion protein was also expressed in this distinct body near the nucleus (Figure 4.13, middle panel). At this point, it is not yet clear whether the fusion protein is expressed in the nucleus. Any nuclear expression however, is likely to be attributed to the little Gab1α expression in the cells. In the case of the third fusion protein, Gab1β-GLYα-EGFP, expression was observed at the cell membrane as well as in the body near the nucleus (Figure 4.13, lower panel). In contrast to the other two fusion protein variants, and as observed previously, Gab1β-GLYα-EGFP is not diffusely expressed in the cytoplasm of the cells.
Figure 4.11: Gab1β wildtype ES cells stained for Gab1. Gab1β wildtype C1 and Gab1β restored cells, using a Gab1-specific antibody, which detects both Gab1α and Gab1β. In both cell lines, Gab1 is expressed at the cell membranes and in a distinct body near the nucleus.
Figure 4.12: Comparison of Gab1 expression in Gab1β +/+ and -/- cells. Gab1β wildtype C1 and Gab1β restored cells, using a Gab1-specific antibody, which detects both Gab1α and Gab1β. In both cell lines, Gab1 is expressed at the cell membranes and in a distinct body near the nucleus.
**Figure 4.13:** Expression of fusion proteins in ES cells. EGFP-Gab1β, EGFP-GLY6-Gab1β and Gab1β-GLY6-EGFP cell lines were stained using a Gab1-specific antibody, which detects both Gab1α and Gab1β. EGFP-Gab1β and EGFP-GLY6-Gab1β are expressed diffusely in the cytoplasm. Gab1β-GLY6-EGFP is expressed more distinctly at the membrane, and in a distinct body near or within the nucleus.
Figure 4.14: Expression of Gab1α in ES cells. Gab1β -/- cells expressing EGFP or Gab1α were stained for Gab1. Gab1α is expressed diffusely in the cytoplasm and nucleus of the cells. Gab1α is clearly expressed in Gab1β -/- cells as well.
EGFP-expressing cells and Gab1β -/- cells overexpressing Gab1α (Gab1α restored) were also stained for Gab1. The results indicate that a Gab1 protein is expressed in Gab1β -/- cells expressing EGFP, as well as Gab1β -/- cells (Figure 4.14). This suggests that ES cells express a small amount of Gab1α protein, which is spread diffusely in both the cytoplasm and nucleus of ES cells. The same expression pattern was observed for Gab1α restored cells. Gab1α expression was detected in the whole of the cell, including the nucleus.

4.2.4.3 Movement of Gab1 proteins in ES cells

What has been demonstrated so far using the EGFP-tagged Gab1 proteins is that Gab1 expression is restricted in the cytoplasm and is not localised in the nucleus of ES cells under normal culture conditions. Furthermore, tagging Gab1β at different termini with EGFP affects the expression pattern. The data demonstrated that the third variant, where EGFP was fused to the C-terminal of Gab1β via a polyglycine linker, exhibited an expression pattern similar to that of wildtype Gab1β in ES cells. Gab1β is expressed distinctly at the membrane of the cell as well as in a distinct body near or potentially within the nucleus. In order to determine the role of the PH domain and NLS in determining Gab1β localisation, induction experiments were performed as previously described in Section 4.2.3. 500,000 cells per 1.9cm² well were plated and allowed to grow overnight. The next day, cells were induced with a combination of 100 units/ml LIF and 10% FBS for 10 minutes. Gab1β
wildtype cells (C1 +/+), Gab1β overexpressing Gab1β knockout cells (Gab1β restored +/+). as well as the three stable cell lines expressing the three variations of the EGFP-tagged Gab1β, were used in this experiment. Gab1β knockout cells expressing either EGFP or Gab1α, were also used. Similar expression patterns were observed in C1 +/+ (Figure 4.15), Gab1β restored +/+ (Figure 4.16) and Gab1β-GLY6-EGFP expressing (Figure 4.17) cells. Although the antibody recognises both Gab1α and Gab1β proteins, the amount of Gab1α expressed in ES cells is minimal. Therefore any staining would be expected to be attributed mainly to Gab1β expression. In uninduced cells, Gab1β was expressed distinctly at the membrane as well as in a distinct body near the nucleus. It is clear from the merged images that this body is not within the nucleus as the Gab1β staining does not match that of DAPI. This suggests that there is a highly specific region near the nucleus of ES cells where Gab1β is localised. Upon induction, the expression within this body does not change. However, Gab1β expression seems much more diffuse within the cytoplasm compared to uninduced cells. The membranes are not so distinctly stained, and some expression is also detected in the nuclei, as indicted by the merged images of all 3 cell lines. This however, could be attributed to the detection of endogenous Gab1α expression within these cells. The change in localisation of Gab1β could either be attributed to the movement of Gab1β or indeed to a change in the shape of the cells. Under serum-starvation conditions, ES cells tend to round up and tighten up. The addition of serum during the induction period may lead to an acute effect on the cell shape and therefore, what we are actually seeing is a redistribution of internal Gab1β during this cell shape change.
In unstimulated EGFP-GLY6-Gab1β expressing cells, Gab1 was expressed diffusely in the cytoplasm, unlike the three aforementioned cell lines (Figure 4.18). The membranes were not distinctly stained, but expression was detected in a similar body near the nucleus. Upon induction the expression pattern did not change significantly, and there was little to no expression in the nuclei. EGFP-Gab1β expressing cells exhibited a similar pattern to that observed previously in Figure 4.9a (Figure 4.19). There was no change in the expression pattern upon stimulation of the cells. Gab1 expression was detected diffusely in the cytoplasm of the cells. In some cells, under both conditions, expression was detected in a body near the nucleus. Gab1α-overexpressing Gab1β knockout cells (Gab1α restored) exhibited a similar change in expression pattern compared to Gab1β (Figure 4.20). Gab1α was localised in the perinuclear body of few cells in both stimulated and unstimulated conditions. Upon stimulation, Gab1α was localised more diffusely in the cytoplasm of the cells, compared to unstimulated cells. The protein was also expressed in the nuclei of some cells upon stimulation. EGFP-expressing Gab1β -/- cells had a distinct pattern of expression compared to all other cell lines. Gab1α expression was detected in the whole cell, including the nucleus (Figure 4.21). In theory, this expression pattern should be similar to that of Gab1α restored cells. This was not the case, and in conjunction with the fact that the pattern is very similar to that of EGFP (Figure 4.14, upper panel), this can probably be attributed to background EGFP fluorescence.
Figure 4.15: Localisation of Gab1 in ES cells. Gab1β wildtype C1 cells were induced for 10 minutes with 100 units/ml LIF combined with 10% FBS, fixed and stained with a Gab1-specific antibody. Upon induction Gab1 expression seemed less distinct and more diffuse within the cytoplasm of the cells.
**Figure 4.16:** Localisation of Gab1 in ES cells. Gab1β restored cells were induced for 10 minutes with 100 units/ml LIF combined with 10% FBS, fixed and stained with a Gab1-specific antibody. Upon induction Gab1 expression was more diffuse within the cytoplasm of the cells.
Figure 4.17: Expression of Gab1β-GLY<sup>6</sup>-EGFP in ES cells. Gab1β-GLY<sup>6</sup>-EGFP cells were induced for 10 minutes with 100 units/ml LIF combined with 10% FBS, fixed and then stained for Gab1 using a Gab1-specific antibody. Upon induction, Gab1 protein expression was more diffuse in the cytoplasm of the cells.
Figure 4.18: Expression of EGFP-GLY<sup>6</sup>-Gab1β in ES cells. EGFP-GLY<sup>6</sup>-Gab1β cells were induced for 10 minutes with 100 units/ml LIF combined with 10% FBS, fixed and then stained for Gab1 using a Gab1-specific antibody. No significant changes were observed in the expression pattern of Gab1β upon induction.
Figure 4.19: Expression of EGFP-Gab1β in ES cells. EGFP-Gab1β cells were induced for 10 minutes with 100 units/ml LIF combined with 10% FBS, fixed and then stained for Gab1 using a Gab1-specific antibody. No significant changes were observed in the expression pattern of Gab1β upon induction. Gab1 expression was observed diffusely in the cytoplasm of the cells, whilst some cells also exhibited expression in a distinct body near the nucleus.
Figure 4.20: Expression of Gab1α in ES cells. Gab1α-overexpressing ES cells were induced for 10 minutes with 100 units/ml LIF combined with 10% FBS, fixed and then stained for Gab1 using a Gab1-specific antibody. Upon induction, Gab1α was expressed more diffusely in the cytoplasm of cells. In some cells, Gab1α expression was detected in the whole cell, including the nucleus.
**Figure 4.21:** Expression of Gab1α in EGFP-expressing ES cells. EGFP-expressing Gab1β -/- cells were induced for 10 minutes with 100 units/ml LIF combined with 10% FBS, fixed and then stained for Gab1 using a Gab1-specific antibody. No significant changes were observed in the expression pattern of Gab1α upon induction. Gab1α expression was detected in the whole cell, including the nucleus.
4.2.4.4 Biochemical function of EGFP-Gab1 fusion proteins

In order to determine whether the Gab1 EGFP fusion proteins were functional, their phosphorylation in response to LIF, as well their participation in the Shp2 signalling pathway were investigated. Gab1 proteins were immunoprecipitated from Gab1β-/- cells (transfected with empty CAGIP vector), Gab1β +/+ cells (C1 +/+ ) as well as one clone from each EGFP fusion transfection. 6x10^6 cells were plated in a 10cm dish and allowed to grow overnight. The next day cells were starved of all growth factors and cytokines for 3 hours and were then induced with 1000units/ml LIF for 10 minutes. The cells were lysed and their protein was incubated overnight with an anti-Gab1 antibody and protein sepharose beads. The next day, the beads were repeatedly washed and then resuspended in lysis buffer. The immunoprecipitates were then screened on a Western blot probed for pGab1 Tyr-627, Shp2 and Gab1 (Figure 4.22). The data confirms that all six fusion plasmids give rise to their respective Gab1 proteins in the stably transfected ES cells. Furthermore, all six fusion proteins are phosphorylated at tyrosine 627 in response to LIF, albeit at different levels. The EGFP-GLY⁶-GAB1 proteins seemed to exhibit less phosphorylation than the other tagged proteins. Nevertheless, this suggests that the tagged proteins are functional in that they have the ability to bind to an effector in response to an inducing factor. Therefore, in response to this phosphorylation, the tagged proteins also form a complex with Shp2 in response to LIF. Again, this suggests that they are functional proteins that are able to take part in the signalling pathways that their wildtype counterparts also participate in.
Figure 4.22: Expression and phosphorylation of EGFP-tagged Gab1α and Gab1β proteins stably transfected in Gab1β-/- cells. All six fusion plasmids give rise to their respective Gab1α or Gab1β EGFP fusion proteins, as indicated by the screening of the Western blot with aGab1 antibody which detects both Gab1α and Gab1β.

An important part of Gab1β protein function is its ability to potentiate Erk phosphorylation in response to LIF (Figure 3.7). Therefore, it was crucial to see whether the fusion proteins also did the same. In the next experiment, cells were starved of growth factors for 3 hours and the induced with 1000 units/ml LIF, protein lysates were obtained and screened on Western blots probed for phosphorylated Erk (Figure 4.23). The data suggests that the EGFP-tagged proteins may not have the ability to potentiate Erk phosphorylation in response to LIF, despite their own phosphorylation and association with Shp2. This suggests that although partially functional, the fusion proteins may not behave as wildtype Gab1 proteins.
Figure 4.23: Erk phosphorylation in response to LIF in EGFP-tagged fusion proteins. Similar levels of Erk phosphorylation were observed in all lysates, as well as in CAGIP Gab1β -/- cells. All cells exhibited similar levels of Stat3 phosphorylation compared to C1 wildtype +/- cells.
4.3 Discussion

In this chapter I aimed to characterise the role of the PH domain in the localisation of the Gab1 proteins. Initially, in order to visualise the localisation of the proteins, plasmids containing EGFP-tagged Gab1α and Gab1β were constructed. Three versions for each were constructed, and all plasmids gave rise to fluorescent Gab1 proteins (Figure 4.9), when stably transfected into Gab1β-/- cells. All EGFP-tagged proteins were localised to the cytoplasm of the cells, but distinctions as to whether they were localised at the membranes could not be made. When the fusion proteins were transiently transfected into 10T1/2 fibroblast cells, their expression was retained in the cytoplasm even upon growth factor stimulation with LIF or FBS. All 6 fluorescent proteins were phosphorylated at tyrosine 627 (the binding site for Shp2) in response to LIF in ES cells, and subsequently formed a complex with Shp2. This however did not translate into an effect on the levels of ERK phosphorylation. None of the Gab1β fusion variants potentiated ERK activation in response to LIF, as Gab1β has previously been shown to (Figure 3.7). Therefore, despite the fact that they form a complex with Shp2, they did not enhance Erk activation. This means that somehow, the fusion with EGFP, at either terminal, inhibits Gab1β’s ability to potentiate Erk phosphorylation.

In terms of expression levels, all 6 plasmids gave rise to similar levels of protein expression. In the Gab1-GLY6-EGFP, where Gab1 is tagged at the C-
terminal, the intensity of the fluorescence of the proteins was much lower compared to the other variations of the fusion proteins. Furthermore, the patterns of expression differed amongst the three variants, although they were all restricted to the cytoplasm of the cells. EGFP-Gab1β was expressed diffusely in the cytoplasm, whilst EGFP-GLY6-Gab1β was expressed at the membrane as well as in a distinct body near the nucleus. Gab1β-GLY6-EGFP had a similar pattern, and was localised more clearly at the membrane as well as in the perinuclear body. This was confirmed by confocal microscopy. This expression pattern is similar to the one observed in Gab1β +/+ and Gab1β restored cells. Therefore, this is a representative pattern of Gab1β expression in ES cells. Gab1α was also demonstrated to be restricted in the cytoplasm when overexpressed in Gab1β -/- ES cells. This was also true for the three fusion variants, EGFP-Gab1α, EGFP-GLY6-Gab1α and Gab1α-GLY6-EGFP.

The differences in expression amongst the Gab1β fusion variants suggest that the placement of EGFP on Gab1β significantly affects its expression pattern. When directly tagged at the N-terminal, where the PH domain in the long form of the protein resides, EGFP altered the normal localisation of Gab1β. In wildtype cells, Gab1β is expressed at the membrane as well as in a distinct body near the nucleus. This expression pattern was still not completely restored when EGFP was linked to the N-terminal of Gab1β via a polyglycine linker. The linker was added to allow the fusion protein to form the correct primary structure, which is crucial for its function. EGFP-GLY6-
Gab1β was expressed in the perinuclear body, and less diffusely in the cytoplasm compared to EGF-Gab1β. In the third fusion variant, EGFP was linked to the C-terminal of Gab1β via a polyglycine linker. The expression pattern of Gab1β-GLY₆-EGFP was very similar to that observed in wildtype cells. Its expression was distinct at the membrane as well as in the perinuclear body. These results show that EGFP at the N-terminal acted as a barrier to the proper localisation of Gab1β. Even the addition of the linker did not completely restore the wildtype pattern of expression. Interestingly, all the Gab1α fusion variants had similar patterns of expression. Gab1α was expressed diffusely in the cytoplasm. This is the same pattern as EGFP-Gab1β. Therefore, in the same way EGFP acted as a barrier to the expression pattern of Gab1β, so may the PH domain in Gab1α. This suggests that a novel domain found at the N-terminal of the protein, after the PH domain, may be responsible for the localisation of Gab1β, and fusing EGFP in close proximity alters its localisation. This may explain why fusing Gab1β at the C-terminal did not interfere with the normal localisation of the protein.

The PH domain of Gab1α has been shown to be dispensable for its localisation within a cell (Maroun et al., 1999a). Gab1α has been demonstrated to localise in the cytoplasm following Met activation by HGF stimulation of 18-hour cultures of MDCK cells (Maroun et al., 1999a). After 15 minutes of stimulation, Gab1α translocates from the cytoplasm to the membrane. Interestingly, however, a ΔPH domain mutant protein also translocated to the membrane vicinity in a similar manner to wildtype
Gab1α. This suggests that the PH domain may not be solely responsible for the movement of Gab1α to the membrane, but the association with the receptor may be crucial. While Met activation can result in Gab1α localisation at the membrane, the PH domain may act to stabilise its interactions with membrane-bound phospholipids and enable Gab1α to potentiate and/or compartmentalise the signal downstream from Met (Maroun et al., 1999a).

The PH domain is the only conserved structural element between the multisite docking proteins Gab1, Gab2 and Dos, the *D. melanogaster* isoform. The deletion or mutation of the PH domain in Dos, has been shown not to interfere with the membrane localisation of the protein in photoreceptor cells (Bausenwein et al., 2000). The PH domain of Dos, however, was shown to be required for normal Dos function. In an attempt to rescue the lethal phenotype of Dos mutations, a ΔPH domain Dos mutant was ubiquitously expressed in mutated flies. Most animals died as pharate adults, whilst the few viable flies suffered from defects in eye morphology and wing vein formation. In addition, expression of the ΔPH domain Dos mutant does not restore the loss of R7 cells in the *D. melanogaster* eye.

Recent evidence shows that most PH domains that have been analysed, bind phosphoinositides with only low affinities, if at all, and with little-to-no specificity (Kavran et al., 1998; Yu et al., 2004). A genome-wide analysis of PH domains in *S. cerevisiae* showed that only one of the 33 yeast PH domains
functions as a high-affinity and specific phosphoinositide-binding module (Yu et al., 2004). The authors of this study suggested that, while phosphoinositide binding contributes to membrane targeting of most yeast PH domains, it does not solely control their location on the membrane. This was supported by the data that only 14 out of 33 yeast PH domains showed evidence of membrane localisation, of which only 8 were detectably membrane-targeted as GFP fusion proteins (Lemmon, 2004; Yu et al., 2004).

Collectively, all these data show that the PH domain is not solely responsible for the localisation of Gab1 within a cell. This would suggest Gab1β, despite the lack of a PH domain, may still have the ability to move within a cell in response to growth factor stimulation. In response to LIF+FBS stimulation there was a small change in the expression pattern of Gab1β in ES cells (Figures 4.15-4.17). Some protein was also detected in the nuclei of the cells upon induction. This however, could be attributed to the small amount of Gab1α that cells express which, upon induction, seems to move to the nucleus in Gab1β -/- cells overexpressing EGFP (Figure 4.21). The lack of a NLS would also suggest that Gab1β does not have the ability to translocate to the nucleus. Prior to stimulation, Gab1β was expressed clearly at the cellular membrane of ES cells, as well as in a perinuclear body. Upon stimulation with LIF+FBS, however, Gab1β was more diffusely in the cytoplasm of the cells. This shows that Gab1β, despite the lack of PH domain and a NLS, still has the ability to move within a cell in response to growth factor and cytokine stimulation. It seems unlikely however, that a protein would be
moving away from the membrane in response to growth factor stimulation. One would expect that an adaptor protein, such as Gab1β would move to the membrane proximal region upon stimulation, in order to bind to the receptor and convey downstream signals. The change in localisation that was observed could therefore be attributed to a change in cell shape upon growth factor stimulation. Cells under serum-starved conditions appear more rounded and not adhered to the plastic as flat as they do in normal culture conditions. If this observation is true, then it may be expected that upon stimulation, the shape of the cells may change and they may appear more flattened. Therefore, the change in Gab1β localisation upon stimulation could be attributed to a change in the shape of the cells and the subsequent redistribution of intracellular compartments, and not to its actual movement within the cells.

Gab1β expression was also detected in a body near or within the nucleus of ES cells. Data shows that this body is not contained in the nucleus, as it was only stained for Gab1 and not DAPI (Figures 4.15-4.18). Therefore, this distinct body is found near the nucleus of the cell, but not within it. As an adaptor molecule, Gab1β takes part in signalling downstream from receptors, such as the EGF receptor. A major event in the function of receptors is their internalisation and recycling back to the surface in endosomes, in response to growth factor binding (Oksvold et al., 2001). Endosomes are intracellular organelles, which consist of a network of vesicles and tubules extending from the cell surface to the perinuclear area.
As well as being involved in the uptake of nutrients, hormones and growth factors, they also take part in the recycling of receptors and ligands (Enrich et al., 1999). Such endosomes are called recycling endosomes, which are dispersed within the cell. Interestingly, in a study attempting to identify EGFR-dependent signalling abnormalities, Gab1α-associated ERK activation was shown to be regulated by EGF in early endosomes of mouse 3T3 cells (Kostenko et al., 2006). In another study Grb2 was shown to be localised in late endosomes of HeLa cells, in response to EGF activation (Oksvold et al., 2001). Whilst EGFR colocalised with Grb, pERK1 and pERK2, the endosomes were negative for transferrin, a classical marker of recycling endosomes (Daro et al., 1996). Co-precipiation results showed that internalised EGFR remained in complex with Grb2, Shc and Cbl at a timepoint where the receptor had reached the late endosomes. Collectively, these studies suggest that Gab1 proteins could be localised at endosomes. This suggestion, along with the fact that Gab1β was shown to be expressed in a distinct perinuclear body, may mean that Gab1β is indeed found at endosomes of ES cells. By screening for co-localisation of Gab1β with endosome markers, such as transferrin (recycling endosomes) or EEA1 (early endosomes) (Mu et al., 1995), the exact location of Gab1β and its potential role in the recycling of receptors within endosomes could be investigated.

In this chapter, I have attempted to demonstrate the potential role of the PH domain and NLS in the localisation of Gab1 proteins. In doing so, I have shown that the PH domain is not the only domain that is important for
localising Gab1 proteins to the membrane. I have hypothesised that there may be another domain at the N-terminal of Gab1β that is also crucial. Furthermore, I have demonstrated that Gab1β is expressed in a distinct body near the nucleus of ES cells, and I have hypothesised that this may be an endosomal compartment, where receptors are recycled. Future experiments investigating the colocalisation of Gab1β with endosomal proteins such as EGFR, transferrin and EEA1 will prove informative for the function of Gab1β in ES cells.
5.1 Introduction

The abundance of Gab1β in ES cells, suggests that it is involved in pathways that regulate ES cell growth and/or self-renewal. My experiments, however, demonstrate that Gab1β may actually potentiate the pro-differentiative factor Erk (Burdon et al., 1999b) in response to LIF (Figure 3.7). This poses a dilemma as to what the function of Gab1β is in ES cells. Its abundance would suggest that it is involved in the maintenance of ES cell identity. However, the potentiation of Erk that it takes part in suggests that it may be involved in the early differentiation of ES cells. The stimulation of Erk by FGF4 was recently shown to trigger the transition of pluripotent ES cells from self-renewal to lineage commitment (Kunath et al., 2007). Although the authors suggest that the provision of LIF to self-renewing cultures may intervene downstream of Erk via Stat3 to override the autoinductive capacity of FGF4, this has not been proven. Therefore, the activation of Erk via Gab1β in response to LIF may also stimulate this transition. Furthermore, Gab1β has also been shown to have no effect on the self-renewal of ES cells (Burdon T., unpublished). Therefore, it is possible that Gab1β is involved in the differentiation of ES cells.
Gab1β is expressed in various areas of the adult mouse *in vivo* (Burdon T., unpublished). The activity of the Gab1β promoter was reported by inserting a βgeo resistance gene into Gab1β 5’ exon in E14Tg2a ES cells. Targeted cell lines were then injected into blastocysts and mice where the transgene was transmitted into the germline were sectioned and stained for LacZ. Along with staining in the heart, testes, kidney and pancreas, Gab1β expression was also observed in the dentate gyrus of the brain. The dentate gyrus is the primary afferent pathway into the hippocampus and it has an important role in learning and memory (Galichet et al., 2008). The dentate gyrus of the hippocampus, along with the subventricular zone of the lateral ventricle wall, is the area of the mouse brain where neural stem cells reside (Gage et al., 1995). These neural stem cells can self-renew and give rise to new neurons, astrocytes and oligodendrocytes (Gage, 2002; Pierret et al., 2007). These data, combined with the evidence that Gab1β is expressed in the dentate gyrus, suggest that Gab1β may have a role in the biology of neural stem cells.

Mouse ES cells are pluripotent cell lines that, in addition to being able to participate fully in foetal development upon re-introduction into blastocysts, they also have the capacity to differentiate into various cell types *in vitro*. In order for an ES cell to differentiate, the self-renewing stimulus LIF needs to be removed. ES cells then differentiate in suspension into multicellular aggregates called embryoid bodies (EBs) (Martin et al., 1977). EBs exhibit multilineage developmental potential similar to that in normal mouse
embryogenesis and give rise to all three embryonic germ layers (Doetschman et al., 1985; Stavridis and Smith, 2003). Neuron-like cells are only a small percentage of the total cells and are intermingled amongst other cell types, such as skeletal muscle-, cardiac-like cells (Bain et al., 1995). Interestingly, EBs were recently shown to demonstrate a certain degree of organisation, similar to that of normal embryonic gastrulation (ten Berge et al., 2008). The rarity of neuron-like cells has sparked many studies on increasing this small proportion of cells in order to better study neural specification. For example, retinoic acid (RA) treatment of ES cell–derived EBs causes a high proportion of the cells to express multiple neuronal properties, such as class III β-tubulin and neurofilament M subunit. This study showed that these cells were capable of generating action potentials and also expressed TTX-sensitive sodium channels, as well as voltage-gated potassium and calcium channels (Bain et al., 1995). RA-induced differentiated cells seem to be a mixture of neuronal–like cells such as oligodendrocytes, astrocytes, neurons, as indicated by the expression of cell-specific antigens (Fraichard et al., 1995).

Though EB studies have proved useful in the study of neuronal specification, it is difficult to manipulate and better understand the process by which an ES cell differentiates to a neuron, because of the multitude of cell types within an EB. In attempting to solve this problem, Ying et al demonstrated that ES cells could convert to neural fates in monolayer adherent culture in defined conditions without the need for RA (Ying et al., 2003b). Here, the authors demonstrated that, by eliminating inductive signals for alternative fates with
the addition of FGF4 to cells at clonal density, ES cells reproducibly commit to a neural fate.

The aim of this chapter is to investigate whether Gab1β is involved in the neural differentiation of ES cells. At what stage in this differentiation process Gab1β may be involved in is an important question as well. Whether or not it is involved in the differentiation of ES cells into neural stem cells, or of neural stem cells into neural precursors is of great interest. *In vivo* data indicate that Gab1β may be involved in the determination of neural precursors from neural stem cells in the adult mouse brain. Therefore, I aim to investigate the role of Gab1β during the whole process of ES cell differentiation into neural precursors. I plan to do this by firstly establishing the protocol of neural differentiation based on previous publications. Using Gab1β-targeted ES cells, I will test the hypothesis that Gab1β has a role in the process of neural differentiation of ES cells.
5.2 Results

5.2.1 Establishing a defined neural monolayer differentiation protocol

To avoid the use of EBs, Ying et al. developed an adherent monoculture system where, with the addition of FGF, ES cells could be converted to neural precursors in defined conditions (Ying and Smith, 2003; Ying et al., 2003b). It was found empirically that a mixed formulation of basal media and supplements provided optimum cell viability and efficient neural differentiation (Ying and Smith, 2003). This formulation called N2B27 contained DMEM/F12 medium supplemented with modified N2, which contained insulin, apo-transferin, progesterone, putrescine and sodium selenite. This mixture was then supplemented with BSA combined with Neurobasal (Gibco) medium with added B27 (Gibco).

In this section, I aimed to establish a reliable neural differentiation protocol based on the above formulation. The optimal number of cells plated was tested in order to maximise the number of neural precursors obtained during the differentiation process. In addition, to assay the effects of FGF4 on the emergence of neural precursors, cells were plated in the presence and absence of 1µg/ml heparin, the carrier that is required for the efficient absorption of FGF4 by the cells (Mummery et al., 1993). The binding of FGFs to heparin is required for their binding to the high affinity FGF receptors (Yayon et al., 1991). Briefly, cells were plated in 90mm wells at varying densities in N2B27 plus 100 units/ml LIF and allowed to grow overnight.
Cells were then transferred to N2B27 medium alone or containing 2ng/ml FGF4 and 1µg/m heparin. The medium supplemented with FGF4 and heparin was then changed every two days. There were two crucial conditions that needed to be tested, the presence of heparin and the number of cells plated. Firstly, though heparin has been shown to be required as a carrier for FGF4 (Mummery et al., 1993), Ying et al did not use it when testing the effects of FGF4 on neural specification (Ying and Smith, 2003; Ying et al., 2003b). In a more recent publication however, where FGF4-induced Erk stimulation was shown to be an autoinductive stimulus for ES cells to exit the self-renewal programme, heparin was added to FGF4-supplemented N2B27 medium (Kunath et al., 2007). The other condition that needed to be tested was the number of cells required for the optimum neural precursor generation. These two conditions were tested within the same experiment. Gab1β-targeted cells (C1 +/+, 26 -/-, Gab1β -/- with α overexpression and Gab1β -/- with β overexpression) were plated for 24 hours at 0.7x10⁵ cells or at 1.0x10⁵ cells per 9.4 cm² well in N2B27 supplemented with LIF and then transferred to N2B27 plus FGF4 with or without heparin the next day. The medium was changed every two days thereafter. One day after plating (day 1) and before LIF was removed and FGF4 was added, the plating efficiency was examined by taking brightfield pictures using a Nikon microscope (Figure 5.1). The medium was then changed to N2B27 supplemented with 2ng/ml FGF4 with or without 1µg/ml heparin.
Figure 5.1: The effect of cell plating on the emergence of neural precursor-like cells. Cells were plated in N2B27 medium supplemented with 100 units/ml LIF at 0.7x10^5 and 1.0x10^5 cells per 9.4cm^2 well and left to grow overnight. Pictures were taken the next day before the medium was changed to N2B27 without LIF but supplemented with FGF4 (with or without heparin). Four Gab1β-targeted cell lines were used: C1 (+/+), HT (+/+), 26 (-/-) and 55 (-/-).
On day 3, images were taken of all cell lines under the new conditions in order to see whether heparin had an effect or not on the emergence of neural precursor-like cells. The addition of heparin did enhance the “spiky” appearance of the cells, which is characteristic of neural precursors (Figures 5.2 & 5.3). The number of cells plated did not affect the emergence of neural precursor-like cells, which were identified by their spiky and elongated appearance. On day 5 of the experiment, the effect of FGF4 on the differentiation of the ES cells was more pronounced (Figures 5.4 & 5.5). Cells appeared elongated with protrusions, signs of early neural differentiation (Kunath et al., 2007). Interestingly, these characteristics were more pronounced in the cells that had been supplemented with heparin. This was especially evident in cell line 55 (-/-), where in the absence of heparin, there were no live cells visible. This suggests that as ES cells take on their new neural fate, they require the efficient presence of FGF4, and without the addition of heparin, this is not possible. By day 8 of the experiment, all cells grown in N2B27 and FGF4 without heparin had died (images not shown). Therefore, the addition of heparin along with the supplementation with FGF4 is important for the emergence as well as the survival of differentiated neural-like cells. It may be that as neural precursors develop, the localised autocrine effects of FGF4 are minimised and therefore the external addition of FGF4, which requires heparin, is crucial. At this stage of the experiment it seemed that plating cells at the lower density seemed to be more advantageous for the emergence of neural precursor-like cells (Figure 5.6). Cells plated at the higher density gave rise to a flattened cell type, and fewer neural-like cells.
Figure 5.2: The effect of heparin on the emergence of neural precursor-like cells on day 3 ($0.7 \times 10^5$). The addition of 1µg/ml heparin did not have an effect on the emergence of neural-like “spiky” cells.
Figure 5.3: The effect of heparin on the emergence of neural precursor-like cells on day 3 (1.0×10⁵). The addition of heparin did seem to give the cells an elongated spiky appearance, which is similar to that of early neural precursors.
Figure 5.4: The effect heparin on the generation of neural precursor-like cells on day 5 (0.7x10^5). The addition of heparin seemed to enhance the differentiation of ES cells into neural precursor-like cells.
**Figure 5.5**: The effect heparin on the generation of neural precursor-like cells on day 5 ($1.0 \times 10^5$). The addition of heparin seems to enhance the differentiation of ES cells into neural precursor-like cells.
Figure 5.6: The effect cell density on the generation of neural precursor-like cells on day 8. Cells grown in the absence of heparin did not survive (image not included). Apart from cell line 26 (-/-), more neural precursor-like cells arose when $0.7 \times 10^5$ cells were originally plated. Cells plated at the higher density gave rise to a flattened cell type that does not resemble neural-like cells, which exhibit elongated protrusions/axons.
On day 10 of the experiment it seemed that apart from cell line C1 (+/+), all three other cells lines successfully differentiated into neural precursors when plated at $0.7 \times 10^5$ cells/well, as seen by the characteristic axons connecting one cell to another (Figure 5.5). In these three cell lines, the emergence of differentiated neural cells was more efficient when plated at a lower density, as there were more neural-like cells. It was concluded that the favourable conditions for maximum neural precursor cell-like generation included supplementation of N2B27 with heparin as a cofactor for FGF4. Cells should be plated at the lower density of $0.7 \times 10^5$. Apart from establishing an efficient neural differentiation protocol, these results also suggest that Gab1β is not involved in the differentiation of ES cells into neural precursors as Gab1β-/- cells gave rise to comparable levels of neural-like precursor cells to wildtype cells.
Figure 5.7: The effect cell density on the generation of neural precursor-like cells on day 10. Apart from cell line C1 (+/+), the other three cell lines gave rise to neural precursors at the lower density.
5.2.2 Effect of Gab1β on neural stem cell differentiation

The results in the previous section suggested that Gab1β is not involved in the differentiation of ES cells into neural precursors as Gab1β -/- cells gave rise to them very efficiently and there was no difference between them and Gab1β +/+ cells. These observations however, were only based on the morphology of cells. Although Gab1β did not have an obvious effect on the neural differentiation of ES cells, based on the fact that it potentiated Erk phosphorylation in response to LIF (Figure 3.7), it may have a subtle role in predisposing cells to a differentiated state. In order to establish this, Gab1β-targeted ES cells were differentiated under the same conditions as before using N2B27 and FGF4 and then immunostained for an early-mid neural marker α-tubulin (TUJI).

Before determining whether Gab1β is involved in the determination of neural cells, Gab1β +/+ and -/- cells were immunostained for Oct4, which is required for ES cells to maintain their pluripotent identity (Chambers and Smith, 2004; Niwa et al., 2000) and upon withdrawal of LIF, its expression is downregulated (Chambers and Smith, 2004; Nichols et al., 1998). This established whether the absence of Gab1β had an effect on the levels of Oct4, which could ultimately have an effect on the differentiation of the cells. 0.5x10⁶ cells were plated in a 9.6 cm² well in normal ES medium supplemented with LIF and FBS and allowed to grow overnight. The next day, cells were fixed and stained overnight using an anti-Oct4 antibody, and
were then stained with the appropriate secondary antibody. Images of the stained cells suggest that Gab1β +/+ cells exhibit slightly lower levels of Oct4 compared to Gab1β -/- cells (Figure 5.8 upper panel).

**Figure 5.8:** The expression of Oct4 in Gab1β +/+ and -/- cells. Although there are not any differences in the number of cells stained, the staining of Gab1β -/- (KO26) cells seems to be more intense than that of the Gab1β +/+ (C1) cells in both experiments. In the lower panel, cells were stained with the same Oct4 antibody, but of a different batch.
The experiment was repeated using the same cells stained with the same Oct4 antibody, but of a different batch, in order to avoid the possibility that the reduced Oct4 staining was due to a weak antibody. The same results were observed when this was done (Figure 5.8 lower panel). Gab1β +/+ exhibited slightly lower levels of Oct4. This suggests that Gab1β may be downregulating Oct4 expression. These results would have to be confirmed by immunostaining other Gab1β +/+ and -/- cells for Oct4. Furthermore, it would be interesting to see whether there were differences on the levels of Oct4 protein expression by Western blot.

The intensity of the Oct4 staining in the two different cell lines using the two different antibodies was measured using the programme ImageJ (Abramoff, 2004). Using this software, the mean grey value of each stain (TRITC for Oct4 and BLUE for DAPI) was measured. Then, the measurements for Oct4 were normalised to DAPI (Oct4/DAPI) and plotted on a graph (Figure 5.9). It seems that this clone of Gab1β -/- cells (KO 26 -/-) exhibit more Oct4 staining compared to Gab1β +/+ (C1 +/+ ) cells according to the ImageJ measurements. This confirms the visual data in Figures 5.8 & 5.9. Repeating this experiment using other Gab1β-targeted cells lines would confirm these observations, that Gab1β expression may indeed have a negative effect on Oct4 expression.
Figure 5.9: Measurement of Oct4 staining in Gab1β +/+ and +/- cells using ImageJ software. When normalised to total DAPI staining, using either antibodies, Gab1β KO26 +/- cells exhibit less Oct4 staining than Gab1β Cl +/- cells.

In order to determine whether Gab1β has a role in the determination of neural progenitors, Gab1β-targeted ES cells were differentiated using the N2B27 plus FGF4 method described in the previous section. Briefly, 0.7x10⁵ cells were plated in a 9.6cm² well in N2B27 medium plus 100 units/ml LIF and allowed to grow overnight. The next day cells were transferred to N2B27 medium supplemented with 2ng/ml FGF4 and 1µg/ml heparin. Pictures of the cells were then taken on alternate days and/or were stained with DAPI, anti-tubulin (TUJI) and/or -Oct4 antibodies. The experiment was repeated 3 times (Experiments A, B and C). A table summarising the data can be found in Figure 5.10. Images of the cells undergoing neural differentiation can be found in: Figures 5.11-5.13 (Experiment A), Figures 5.14-5.20 (Experiment B) and Figures 5.21-5.25 (Experiment C). During the early days of all three
experiments (days 1-4), there were no differences in the number of TUJI-positive cells. Furthermore, no obvious differences in the morphology or emergence of neural TUJI-positive axons were observed either. All cell lines gave rise to neuronal precursor-like cells which stained for TUJI. On day 5 however, some differences started to emerge between the Gab1β-targeted cells. Gab1β +/+ (C1) had fewer TUJI-positive cells compared to all other cell lines, even Gab1β restored +/+ cells. In 2 out of 3 experiments, Gab1β restored +/+ cells gave rise to fewer TUJI-positive cells compared to Gab1β -/- and Gab1α restored cells. Furthermore, Gab1β restored +/+ cells gave rise to cells with fewer axons compared to the other two cell lines in the majority of the experiments. These data suggest that there may be slight differences in the rate at which Gab1β-targeted ES cells differentiate into neural precursors. On days 6/7 these differences were not as pronounced. Gab1β +/+ (C1) cells still gave rise to fewer TUJI-positive cells than all the other cell lines. Gab1β restored +/+ cells had similar levels of TUJI-positive cells as Gab1β -/- cells in the majority of the experiments. What was evident on days 6/7 was that Gab1α restored cells gave rise to slightly more TUJI-positive cells with more protruding axons. This observation became clearer on the latter days of the neural differentiation process, where Gab1α restored cells gave rise to TUJI-positive cells with long, thick axons. This pattern was distinct and the other cell lines did not adopt it. During the later days in the neural differentiation process (days 8-12), Gab1β restored +/+ and Gab1β -/- cells had similar patterns of TUJI expression. Both cell lines gave rise to similar numbers of TUJI-positive cells of similar intensity, but neither cell line gave rise to cells with thick elongated axons, as observed with the Gab1α restored cells.
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<th>DAYS 1-4</th>
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<tr>
<td><strong>DAY 5</strong></td>
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<tr>
<td>Gab1β +/+ (C1)</td>
<td>Fewer neural-like cells compared to 3 other cell lines</td>
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<td>Gab1β restored +/-</td>
<td>Similar number of neural-like cells compared to -/- and α restored</td>
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<td>Gab1β -/- (26)</td>
<td>More axons, but a similar number of neural-like cells compared to β restored cells</td>
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<td>Gab1α restored</td>
<td></td>
</tr>
<tr>
<td><strong>DAY 6/7</strong></td>
<td><strong>A</strong></td>
</tr>
<tr>
<td>Gab1β +/+ (C1)</td>
<td>Fewer neural-like cells compared to 3 other cell lines</td>
</tr>
<tr>
<td>Gab1β restored +/-</td>
<td>Similar number of neural-like cells</td>
</tr>
<tr>
<td>Gab1β -/- (26)</td>
<td>Similar number of TUJI-positive cells</td>
</tr>
<tr>
<td>Gab1α restored</td>
<td></td>
</tr>
<tr>
<td><strong>DAYS 8-12</strong></td>
<td>Gab1β restored +/- and Gab1β -/- have similar patterns of TUJI staining; Gab1α restored cells have many more axons, which are thick and elongated, compared to the other two cell lines</td>
</tr>
</tbody>
</table>

*Figure 5.10: Comparison of the neural differentiation of Gab1β-targeted cells. A, B and C represent separate experiments. (TUJI: ; Oct4: ).*
Figure 5.11: The differentiation of Gab1β-targeted ES cells into neural precursors on days 1 and 3. Similar numbers of cells were plated on day 1 of the differentiation process. On day 3, neural outgrowths were observed in all cell lines, apart from C1 +/-.
Figure 5.1: The differentiation of Gab1β-targeted ES cells into neural precursors on days 5 and 7. On day 5, all cell lines exhibit neural outgrowths, with C1 +/+ having very few. On day 7, C1 +/+ had very few cells, whilst β +/+ and 26 -/- had similar amounts of neural precursors. Cell line α-/- had fewer neural precursors.

Figure 5.12: The differentiation of Gab1β-targeted ES cells into neural precursors on days 5 and 7. On day 5, all cell lines exhibit neural outgrowths, with C1 +/+ having very few. On day 7, C1 +/+ had very few cells, whilst β +/+ and 26 -/- had similar amounts of neural precursors. Cell line α-/- had fewer neural precursors.
Figure 5.13: The differentiation of Gab1β-targeted ES cells into neural precursors on day 12. Cells were stained for the neural marker tubulin. All cells gave rise to neural precursors. Cell line α−/− gave rise to dense colonies of cells with numerous neural outgrowths.
Figure 5.14: The differentiation of Gab1β-targeted ES cells into neural precursors on day 1. All cell lines express Oct4. Cells are also stained for tubulin.
Figure 5.15: The differentiation of Gab1β-targeted ES cells into neural precursors on day 4. Cells are stained for both Oct4 and tubulin.
Figure 5.16: The differentiation of Gab1\(\beta\)-targeted ES cells into neural precursors on day 5. The levels of Oct4 have diminished to give rise to morphologically-characteristic tubulin-positive neurons.
Figure 5.17: The differentiation of Gab1β-targeted ES cells into neural precursors on day 6. Most cells were tubulin-positive, whilst Oct4 expression was not observed.
Figure 5.18: The differentiation of Gab1β-targeted ES cells into neural precursors on day 8. Cell line 26 -/- has fewer neurons than the other cell lines, whilst α-/ - has more dense colonies of neuronal-like cells.
Figure 5.19: The differentiation of Gab1β-targeted ES cells into neural precursors on day 9. The number of neuronal precursors is the same between β +/+ and 26 -/- . Cell line α-/ has dense, thicker neuronal outgrowths.
Figure 5.20: Measuring the intensity of TUJI staining during the differentiation of Gab1β-targeted ES cells into neural precursors. Values are corrected to DAPI intensity. On day 4, Gab1β -/- and Gab1α restored cells have more tubulin expression per cell compared to Gab1β restored cells. On day 9, Gab1α restored cells exhibit higher levels of TUJI expression compared to Gab1β -/- cells.
Figure 5.21: The differentiation of Gab1β-targeted ES cells into neural precursors over 7 days. On day 1 similar numbers of cells were plated and evenly distributed. On day 3, all cell lines, apart from C1 +/+, started to change morphologically, giving rise to cells with neuronal outgrowths.
Figure 5.22: The differentiation of Gab1β-targeted ES cells into neural precursors over 7 days. On day 5, C1 +/+ and β +/+ had similarly less neuronal cells compared to 26 -/- and α -/- . On day 7, all cells had characteristic neuronal morphologies.
Figure 5.23: The differentiation of Gab1β-targeted ES cells into neural precursors on day 5. C1 +/+ was less efficient at giving rise to tubulin-positive neuronal precursors compared to the other three cell lines.
Figure 5.24: The differentiation of Gab1β-targeted ES cells into neural precursors on day 7. All cell lines gave rise to tubulin-positive cells. Cell line $\alpha -/-$ was most efficient at giving rise to tubulin-positive neuronal precursors compared to the other three cell lines.
Figure 5.25: The differentiation of Gab1β-targeted ES cells into neural precursors on day 8. All cell lines gave rise to tubulin-positive cells. C1 +/+ gave rise to the fewest tubulin-positive neurons.
5.2.3 The effect of insulin on the neural differentiation of Gab1β-targeted ES cells

Insulin has been shown to be the main component of N2 which is required for the efficient proliferation and survival of dissociated postnatal mouse cerebellar neurons (Huck, 1983). IGF1 activation of PI3K has been shown to be responsible for this survival effect of cerebellar neurons, which is mediated by the activation of the serine-threonine kinase downstream of PI3K, Akt (Dudek et al., 1997). This is not surprising, as the IGFs and their receptors are widely expressed and distributed in the forebrain zones of neurogenesis (Baron-Van Evercooren et al., 1991; Hopkins and Williams, 1997). In dorsal root ganglia, IGF1 has been shown to prevent apoptosis of neurons by regulating PI3K/Akt pathway effectors, such as CREB (cyclic AMP response element binding protein), GSK3β and FKHR (forkhead), as well as by blocking caspase activation (Leinninger et al., 2004). Insulin has also been shown to provide trophic support and aid survival for retinal neurons through a PI3K/Akt-dependent pathway (Barber et al., 2001). More recently, using oxygen deprivation as a cause of hypoxia, Zhao et al demonstrated that after 24 hours of hypoxia, 41% of neural stem cells are apoptotic and that insulin treatments reduces the frequency of hypoxia-induced apoptosis by 33% (Zhao et al., 2007). The authors also showed that Akt phosphorylation increased upon this insulin treatment and that this increase was completely blocked with pharmacological inhibitors against PI3K. The use of the PI3K-specific inhibitor also reversed the effect of insulin-induced Erk inhibition, which suggests that insulin inhibits Erk in a PI3K-
dependent manner. This is contradicted, however, by the suggestion that insulin acts to sensitise cells to differentiation-inducing signals (Ying et al., 2008). In this study, the authors demonstrated that by adding inhibitors to Erk, FGF and Gsk, ES cells could be maintained in culture without the addition of LIF or FBS. When insulin was omitted from the medium, cells were more sensitive to the Erk and FGF inhibitors, and although they remained undifferentiated over 4 weeks, their propagation rate decreased over time.

In chapter 3, I showed that Gab1β represses both Akt and Erk activation in response to IGF. The biological effect of this repression is still not clear. As insulin has been shown to inhibit ERK and is also an important additive to N2B27 medium for the maintenance of neural stem cells and early neural precursors, it would be interesting to see whether Gab1β is involved in the process of ES cells differentiating to neural stem cells and in turn their differentiation into neural precursors. At what stage Gab1β may be involved in this pathway is still unclear. The fact, however, that Gab1β expression is detected in the dentate gyrus of the adult mouse brain (Burdon T., unpublished), suggests that it may be involved in the latter stages of neural differentiation, i.e. in the determination of neural precursors. In this section I aim to establish the role that Gab1β has in the generation of neural precursors in response to varying levels of insulin.
The same differentiation protocol as before was used. Briefly, Gab1β-targeted ES cells were plated in N2B27 plus 100 units/ml LIF, supplemented with varying levels of insulin: 0 μg/ml (zero), 0.25 μg/ml (low) and 25 μg/ml (high). The next day (day 1), LIF was removed and the cells were replenished with N2B27 medium supplemented with 2ng/ml FGF4 and 1μg/ml heparin. The medium was then replenished on days 3 and 5. Brightfield images were taken on days 1 (Figure 5.26), 3 (Figure 5.27) and 4 (Figure 5.28) and on day 5 the cells were fixed and stained with aOct4 and aTUJI antibodies (Figures 5.29-5.33). On day 1, all four cell lines plated equally well in the varying concentrations of insulin (Figure 5.26). Though the effect of insulin was subtle, it was clear that in the low and high concentrations, cells appeared flatter and more adherent than they did in the absence of insulin. By day 3 there were more adherent cells at the high concentration of insulin (Figure 5.27). This could be attributed to either the faster growth of the cells or more death of the cells at the lower concentrations of insulin. Interestingly on both days 1 and 3, there were no significant differences between Gab1β +/+ and -/- cells. On day 4, the effect of insulin was clearer. There was a gradual increase in the size of the colonies of neural precursor-like cells as the concentration of insulin increased (Figure 5.28). Furthermore, both Gab1β -/- cells seemed to produce more neural precursor-like cells as distinguished by the long axons connecting the cells, compared to +/+ cells. Interestingly, in the absence of insulin, more Gab1β +/+ and Gab1β restored +/+ cells were seen compared to Gab1β -/- and Gab1α restored cells. it is not clear whether these cells were neural-like or not.
Figure 5.26: The effect of insulin on the differentiation of Gab1β-targeted ES cells into neural precursors. Cells were differentiated in response to FGF4 in varying amounts of insulin over 4 days. On day 1, there was no difference between cell lines.
Figure 5.27: The effect of insulin on the differentiation of Gab1β-targeted ES cells into neural precursors. Cells were differentiated in response to FGF4 in varying amounts of insulin over 4 days. More cells are present at high concentrations of insulin.
Figure 5.28: The effect of insulin on the differentiation of Gab1β-targeted ES cells into neural precursors. Cells were differentiated in response to FGF4 in varying amounts of insulin over 4 days. On day 4, cells in no insulin, though differentiating, were doing so at a much slower rate. As the concentration of insulin increased, so did the size of the neural precursor-like colonies.
When the cells were fixed and stained on day 5, it was clear that insulin provided a survival and growth advantage. In the absence of insulin, the colonies of neural precursors were very small and there were quite a few Oct4 positive cells (Figure 5.29). In low insulin, though there were still some Oct4-positive cells, the colonies of tubulin-positive cells were much larger (Figure 5.30). Furthermore, at both zero and low insulin, there seemed to be no significant differences in the levels of TUJI or Oct4 staining between Gab1β +/+ and -/- cells. At the higher insulin concentration (Figure 5.31), Gab1β -/- cells seemed to give rise to larger colonies of tubulin-positive cells, with less Oct4-positive cells, compared to +/+ cells. When a larger field was inspected, however, the differences were not as pronounced, with all cell lines having Oct4-positive cells (Figure 5.32). There were, however, more Gab1β -/- TUJI-positive cells. This suggests that in the absence of Gab1β, cells may be more sensitive to the potential inhibition of ERK in response to high levels of insulin concentration. These results suggest that Gab1β may have a slight effect on the generation of neural precursors at high levels of insulin. In the case of α -/- cells, the overexpression of Gab1α did not have a great effect on the emergence of neural precursors (Figure 5.33). The cells had a similar pattern to that of Gab1β 26 -/- cells. The addition of increasing amounts of insulin benefited the emergence of neural precursors.

When comparing the levels of tubulin and Oct4 separately for the different cells line under the different insulin concentrations, a pattern seems to emerge. The mean grey values of TUJI and Oct4 were measured using
ImageJ software, and were then normalised to DAPI. When comparing the levels of TUJI intensity at different insulin concentrations, what is clear is that insulin has a negative effect on the expression of tubulin in neuronal precursors (Figure 5.34). The addition of insulin has a negative effect on the amount of TUJI expressed per cell, as values have been normalised to DAPI. Furthermore, Gab1β−/− cells exhibit more TUJI expression at all three insulin concentrations. This suggests that the presence of Gab1β somehow represses the expression of TUJI. When the levels of Oct4 were measured at the differing concentrations of insulin for the different cell lines, a similar pattern emerged (Figure 5.35). Apart from the Gab1β restored +/+ cell line, the increasing amounts of insulin had a negative effect on the intensity of Oct4 staining. Interestingly, in accordance with was observed in Figures 5.8 & 5.9, Gab1β−/− cells exhibit more Oct4 expression compared to +/+ cells, irrespective of the insulin concentration. This suggests that Gab1β has a repressive effect on the neural differentiation of ES cells. Although these data stem from one experiment only, it would be interesting to find out the mechanisms by which Gab1β exerts these effects on both tubulin and Oct4.
Figure 5.29: The effect of insulin on the differentiation of Gab1β-targeted ES cells into neural precursors on day 5. In the absence of insulin, colonies were small and stained for both Oct4 and TUJI.
Figure 5.30: The effect of insulin on the differentiation of Gab1β-targeted ES cells into neural precursors on day 5. At low levels of insulin, there is an increase in the number of TUJI-positive cells and a decrease in Oct4-positive cells.
Figure 5.31: The effect of insulin on the differentiation of Gab1β-targeted ES cells into neural precursors on day 5. Upon the addition of high levels of insulin, Gab1β−/− cells seem to have fewer Oct4-positive cells and larger TUJI-positive neural colonies.
Figure 5.32: The effect of high levels of insulin on the differentiation of Gab1β-targeted ES cells into neural precursors on day 5. In the larger field, all four cell lines had similar numbers of Oct4-positive cells, whilst the Gab1β -/- cells had more TUJI-positive cells.
Figure 5.33: The effect of insulin on the neural differentiation of Gab1α-overexpressing Gab1β -/- ES cells on day 5. The addition of insulin had a positive effect on the emergence of TUJI-positive cells, and a negative effect on Oct4-positive cells.
Figure 5.34: The effect insulin on the expression of TUJI in Gab1β-targeted ES cells on day 5 of neuronal differentiation. Insulin seems to have a repressive effect on the expression of TUJI in all 4 cells lines. Gab1β also seems to inhibit the expression of TUJI in neuronal precursor-like cells. (n=3 different fields of view in 25µg/ml insulin).

Figure 5.35: The effect insulin on the expression of Oct4 in Gab1β-targeted ES cells on day 5 of neuronal differentiation. Insulin has a repressive effect on the expression of Oct4 in all 4 cells lines. Gab1β also seems to inhibit the expression of Oct4 in neuronal precursors. (n=3 different fields of view in 25µg/ml insulin).
5.3 Discussion

The aim of this chapter was to establish the neural differentiation potential of Gab1β -/- cells. Before attempting to do this, a reproducible neural differentiation protocol was established using N2B27 and FGF4. After several pilot experiments, it was concluded that the number of cells to be plated for the maximum yield of neural progenitors was $0.7 \times 10^5$ cells/9.6 cm$^2$ well and that FGF4 required the addition of heparin to act as its carrier. Using these conditions, Gab1β-targeted ES cells were differentiated down the neural pathway and results showed that Gab1β -/- cells could indeed differentiate and give rise to neural precursors. In light of previous results, whereby Gab1β potentiated the activation of Erk in response to LIF (Figure 3.7), one might expect Gab1β +/+ cells to differentiate faster, as activation of Erk in response to LIF has been shown to act as a pro-differentiative signal, rather than contributing to self-renewal (Burdon et al., 1999a; Burdon et al., 1999b). The experiments performed here, however, indicate that this is not the case. Gab1β +/+ cells did not give rise to neural precursors at a faster rate compared to -/- cells. This suggests that the increased LIF-induced Erk activation observed in Gab1β +/+ cells may have a different effect unrelated to differentiation, such as growth during normal culture. Gab1α has been shown to enhance epidermal cell proliferation by promoting MAPK signalling (Cai et al., 2002), and Gab1β may also have a similar role in ES cells via LIF-induced Erk activation.
Surprisingly, on day 5 Gab1β restored +/+ cells exhibited fewer TUJI-positive cells and fewer axons compared to Gab1β -/- cells. This indicates that the presence of Gab1β may inhibit the neural differentiation process of ES cells. Furthermore, Gab1α-overexpressing Gab1β -/- cells progressed through the differentiation process much faster and gave rise to more precursors compared to all other cell lines. When the intensity of TUJI expression was measured on day 9 neuronal precursors of this cell line, there was in fact no difference in the level of TUJI expression per cell, compared to Gab1β restored +/+ cells (Figure 5.21). In fact, it was merely the number of precursors that was much greater when Gab1α was overexpressed instead of Gab1β. This suggests that Gab1α promotes the differentiation of Gab1β -/- ES cells. Gab1α has been shown to be essential for EGF-dependent proliferation of Olig2(+) progenitor cells of the murine spinal cord, where glial cells originate (Hayakawa-Yano et al., 2007). Furthermore, Gab1α-overexpressing Gab1β -/- cells consistently gave rise to neural precursors with long, thick axons. This indicates that Gab1α promotes the neural outgrowths of the TUJI-positive neural-like cells. Interestingly, Gab1α has been shown to promote Neural Growth Factor (NGF) – induced cell survival by binding to PI3K (Holgado-Madruga et al., 1997). Furthermore, adenovirus-expressed Gab1α has been demonstrated to promote neurite outgrowth of PC12 cells (Korhonen et al., 1999). Using MAPK and PI3K inhibitors, Korhonen et al showed that Gab1α-induced neurite outgrowth is suppressed. The recruitment of Shp2 by Frs2 and the catalytic activity of Shp2 have been demonstrated to be important for FGF-induced differentiation and neurite outgrowths of PC12 cells (Hadari et al., 1998). The
downstream activation of the MAPK pathway in response to Shp2 recruitment at the FGF receptor 1, was suggested to be crucial for the differentiation of the cells. The recruitment of Shp2 may indeed require the presence of Gab1α. In the case of the neural differentiation of ES cells, the presence of Gab1α promoted the outgrowth of neurites as well as the differentiation of the cells, a process dependent on FGF4 (Kunath et al., 2007). Therefore, one could hypothesise about the pathway: FGF4 induces the recruitment of Shp2 via Gab1α, which in turn promotes the neural differentiation, as well as the neurite outgrowths. Interestingly, Gab1β did not promote either of these processes. Gab1β may in fact inhibit this process. This suggests that in neurons, Gab1β may act as a dominant negative adaptor protein by somehow inhibiting the differentiation pathway. For example it may bind and mislocalise Shp2. Alternatively, due to the lack of a PH domain, it may just not have the ability to maintain the contact with the membrane for long enough, in order to induce the sustained activation of Erk which has been shown to be required for the efficient differentiation of PC12 cells (Cowley et al., 1994; Traverse et al., 1994).

Insulin has been shown to promote the survival of neurons by activating the PI3K signalling pathway and its downstream effector Akt (Dudek et al., 1997; Leinninger et al., 2004; Zhao et al., 2007). As insulin is one of the most abundant components of the differentiation medium N2B27, I was interested in seeing whether insulin had an effect on the differentiation of Gab1β -/- cells. What was clear from the results was that, regardless of their Gab1β
genotype, cells survived better and differentiated much faster at higher levels of insulin. Data indicated that the number of neural precursors increased in response to higher levels of insulin, which suggests that insulin may potentiate the growth of differentiated cells. Although the same cells have been shown to repress Akt in response to IGF (Figure 3.20), they also repress Erk (Figure 3.24), which could give cells a survival advantage. This is supported by the fact that the suppression of Erk has been shown to promote the survival of neural stem cells (Zhao et al., 2007). The suppression of Erk and Akt by Gab1β in response to insulin, however, was demonstrated in ES cells. The effect of Gab1β on Akt and Erk in neural cells may be completely unrelated to that. Interestingly, both IGF1 and insulin have been shown to enhance cell survival and formation of neurites in sympathetic and sensory neurons (Recio-Pinto et al., 1986). Furthermore, this promotion of neurite outgrowth in response to insulin has been demonstrated to require the activation of MAPK (Kim et al., 1997). The data presented here, suggests that Gab1β suppresses the neural differentiation of ES cells at high levels of insulin. Interestingly, the overexpression of Gab1α in Gab1β -/- cells does not have the same effect. At higher levels of insulin, Gab1α restored cells exhibited similar levels of TUJI expression per cell, but had more TUJI-positive cells compared to Gab1β restored cells. This suggests that Gab1α promotes the neural differentiation of ES cells in response to insulin. It further suggests that Gab1β may either act as a dominant negative effector, or it may indeed be unable to promote neural differentiation and neurite outgrowth in response to high levels of insulin as it cannot induce sustained Erk activation.
It would be interesting to see whether Gab1β had an effect on nestin and/or Ki-67 expression. Both these proteins have been shown to be expressed in the subventricular zone of mice (Tanaka et al., 2007), where Gab1β has been shown to be expressed (Burdon T. et al, unpublished). Nestin is a neuroepithelial stem cell marker (Lendahl et al., 1990), whilst Ki-67 is a marker of proliferating cells (Kim et al., 2002). Furthermore, it would be very interesting to determine the levels of Akt and Erk phosphorylation during the process of neural differentiation. These data would provide an important insight into the biochemical changes that take place in the presence of Gab1α and Gab1β.
Gab1 is a multifunctional adaptor protein that takes part in a myriad of signalling pathways. It has been shown to be involved in many biological processes ranging from cell survival (Holgado-Madruga et al., 1997; Holgado-Madruga and Wong, 2003) to cytoskeletal reorganisation and chemotaxis (Kallin et al., 2004) and glucose tolerance and hepatic insulin action (Bard-Chapeau et al., 2005). As they lack any enzymatic activity, their structure is what gives them this ability to multitask and to participate in the transmission of signals from various receptors ranging from the EGFR (Holgado-Madruga et al., 1996) to the gp130/LIFR (Takahashi-Tezuka et al., 1998). The presence of tyrosine phosphorylation sites as well as specific binding sites for other adaptors and proteins are what give Gab1 this multifunctionality. Therefore, its structure is pivotal to its function, and allows Gab1 to regulate signalling cascades in a dynamic manner. For example, whether an emanating signal is transient or sustained may depend on the affinity of Gab1 proteins to a specific receptor. The PH domain acts to bring Gab1 to points of cell-to-cell contact near the cellular membrane. It anchors Gab1 near the membrane and hence sustains the signalling cascade. It was therefore interesting to find that ES cells express a variant that lacks this critical structural component. In spite of this, I found that not only is Gab1β phosphorylated, it also forms active complexes with Shp2 and the p85 subunit of PI3K.
Most importantly, I showed that Gab1β promotes Erk and potentially Akt activation in response to LIF. The biological effect of Erk activation in response to LIF is still unclear. This effect is likely to be unrelated to self-renewal, as Gab1β -/- cells are still able to self-renew (Burdon T. unpublished). The kinetics of Erk activation, however, have been shown to influence the biological outcome. For example, transient phosphorylation of Erk has been shown to be associated with a proliferative response, whilst sustained Erk activation is thought to be associated with differentiation (Sharrocks, 2006). It would be interesting to see whether Gab1β promotes transient-proliferative or sustained-differentiating Erk. The lack of PH domain would potentially restrict Gab1β from anchoring to the membrane following receptor activation. Therefore, one might predict that Gab1β may favour transient bursts of Erk activation due to its inability to maintain sustained anchoring to the membrane. This would suggest that the potentiation of Erk in response to Gab1β recruitment to the LIFR/gp130 receptor complex was associated with a proliferative effect. Although there are little differences between the growth rates of wildtype and Gab1β-deficient cells, Gab1β -/- cells exhibit a deficit in their long-term growth, as observed in long-term growth competition assays (Burdon T., unpublished). As well as boosting Erk activity, Gab1β may also potentiate Akt activation in response to LIF. Although Akt activation has mainly been demonstrated to be associated with the maintenance of self-renewal, the PI3K pathway has also been implicated in the regulation of ES cell growth. ES cells with a mutation of PTEN, a lipid phosphatase that normally inhibits PI3K activity,
exhibit enhanced viability and increased rate of proliferation (Sun et al., 1999).

I also demonstrated that Gab1β inhibits Erk and Akt in response to IGF. The role of the IGF-insulin signalling pathway in ES cells is still unclear and of great interest. Published data on this point is, however, contradictory. For example, the activation of the PI3K signalling pathway in response to insulin has been suggested to be involved in the maintenance of ES cell proliferation, through the activation of Akt-dependent inactivation of Gsk3β (Paling et al., 2004; Welham et al., 2007). More recently, the differentiation of ES cells has been suggested to be associated with a reduction in IRS1 expression, as well as a decrease in Akt and Gsk3 phosphorylation (Rubin et al., 2007). Recent data, however, suggests that insulin acts to sensitise cells to differentiation-inducing signals, such as FGF4 (Ying et al., 2008).

The data presented here demonstrates that Gab1β is an adaptor protein whose function is dependent on the environment the cells are surrounded by. For example, Erk activation is potentiated by Gab1β in response to LIF, but it is also inhibited by Gab1β in response to insulin. Which outcome is achieved, is likely to depend on the instructions that a cell receives from its surroundings. This suggests that the function of Gab1β is context and receptor-dependent. Literature suggests that the context of a cell limits the possible responses to growth factor signalling and the magnitude and duration of RTK activation (Hopper, 2006). Its possible that the context of the
cell determines the specific response. As an adaptor protein, Gab1β may act to potentiate or inhibit a pathway, depending on the specific signals and the biological outcomes that a cell requires. For example, the data obtained here indicate that Gab1β may in fact inhibit the neural differentiation of ES cells, and that Gab1α actually promotes it. When insulin is present at high levels, the effect is more pronounced. Gab1β-deficient cells exhibit more neural-like precursor cells compared to Gab1β wild type cells. But I also demonstrated that Gab1β potentiates the activation of Akt in response to LIF, an effect that would be expected to promote the self-renewal of the cells. It would be very interesting to determine the signals that lie behind the differentiating effects, and to see whether Gab1 has an effect on Erk and/or Akt signalling during the neural differentiation process. The data surrounding the role of Gab1α and differentiation are varied and reflect the ability of this protein to convey signals from to the many receptors it binds to. Gab1α has been shown to oppose differentiation by activating the MAPK signalling pathway in epidermal cells (Cai et al., 2002). Recently, the Gab1α-Shp2 complex has been shown to negatively regulate myogenic differentiation by activating the MAPK pathway (Koyama et al., 2008). In contrast, the Gab1α-Shp2 complex has also been shown to promote colon cancer cell enterocytic differentiation in response to HGF (Kermorgant et al., 2001). Therefore, it may be that in ES cells, Gab1α can have a role in their differentiation into neural precursors, as long as its present at a high enough concentration, i.e. when it is artificially overexpressed. This is interesting and suggests that although Gab1β is able to activate signalling pathways in response to growth factors and cytokines, this does result in a biological effect, whilst introducing high levels of Gab1α
into ES cells does. It would be interesting to see whether deleting Gab1α from the Gab1β-/- cells will have a more profound effect on their ability to convey signals and to differentiate into neural precursors.

The ability of Gab1β to potentiate and inhibit Erk in response to LIF or insulin respectively, suggests that it may take part in a regulatory mechanism. ES cells are cultured in an environment where self-renewal is aimed for, but they still have the ability to differentiate. ES cells are in a state of self-renewal and once they receive the appropriate signal, they can for example differentiate into neural cells. In vivo this is reflected by the process of delayed implantation, diapause. During this state of delayed implantation, embryos develop to the hatched blastocyst stage, but then cease development and remain unimplanted in the uterus (Holmes and Dickson, 1975). During diapause, proliferation and metabolism is arrested and the blastocyst cells are in a state of metabolic and proliferative quiescence (Holmes and Dickson, 1975; Spindler et al., 1996). Once diapause is interrupted, the blastocyst regains an active metabolism, cell proliferation is initiated, the blastocyst implants in the uterus and development continues (Hondo and Stewart, 2005). Diapause is therefore a reversible mechanism that can halt the proliferation of early embryonic cells, which in fact have the natural ability to proliferate very rapidly. Interestingly, LIF is required so that the uterus is induced to become receptive to the blastocyst but is dispensable for normal embryonic development (Stewart et al., 1992). If blastocysts undergo diapause, however, gp130 signalling is required to sustain ICM cells in conjunction with the arrest of ICM proliferation (Hondo and Stewart, 2005;
Nichols et al., 2001). In *C. elegans*, the process of dauer resembles in some respects the blastocyst dormancy state, diapause. The process of dauer is regulated by a gene called daf-2 (Kimura et al., 1997). Interestingly, daf-2 encodes IRS1 in *C. elegans*, which acts to sense the nutritional status of the worm to repress dauer formation (Kimura et al., 1997; Morris et al., 1996), and Gab1 proteins were initially discovered as IRS1-like proteins (Holgado-Madruga et al., 1996). Furthermore, Soc1, the *C. elegans* Gab1 homologue, has been shown to negatively regulate dauer formation. IRS1 has also been identified as a upregulated gene in dormant blastocysts in mice, in a global gene expression microarray study (Hamatani et al., 2004). Therefore, IRS1 may take part in the regulation of diapause by initiating growth and metabolic activity when delayed implantation ceases. Gab1β could therefore, also be expressed in blastocysts during delayed implantation. It may have a similar role as IRS1 in promoting an increase in metabolic rate when diapause ceases, and proliferation and differentiation are initiated. The maintenance of this quiescent state of homeostasis may in fact be the reason it is so highly expressed in ES cells, which are the *in vitro* counterpart of ICM cells in the blastocyst. Interestingly, human ES cells express Gab1α, but do not express a Gab1β homologue which lacks the PH domain. Furthermore, human blastocysts are not known to undergo delayed implantation (Tarin and Cano, 1999). It may be that there is no requirement for the expression of a short form of Gab1α as blastocysts do not undergo diapause and therefore do not have the necessary signalling proteins in order to initiate the exit out of this inactive metabolic state during embryogenesis.
Most published data suggests that the lack of a PH domain in Gab1 inhibits its ability to act as an adaptor protein. PH domain mutants have been shown to either fail to form complexes downstream of receptor activation, or indeed fail to potentiate the appropriate effectors. For example, the PH domain of Gab1α has been shown to be essential for the ability of Gab1α to support branching morphogenesis downstream from the Met receptor tyrosine kinase in epithelial cells, in concert with PI3K activity (Maroun et al., 1999a). Expression of a Gab1α mutant lacking the PH domain in fibroblasts, however, has been shown not to interfere with EGF-induced Erk activation. Furthermore, a “naturally occurring” Gab1β variant is tyrosine phosphorylated in response to EGF in transformed SHE (Syrian Hamster Embryo) cells. Therefore, whether the PH domain would also affect the localisation of the protein and its ability to move to the plasma membrane is still unclear. Even if the short form of Gab1α is biochemically active, the localisation may be different due to the lack of a PH domain and a NLS. I demonstrated that depending on which terminal Gab1β was tagged with EGFP, a different localisation pattern was obtained. When Gab1β was tagged at the C-terminal end, a localisation pattern similar to that of Gab1β wild type ES cells was obtained. However, when the protein was directly tagged at the N-terminal, where the PH domain would normally reside, this was not the case. The tagged protein did not localise to the perinuclear foci as the wild type protein did. Furthermore, when the N-terminal tag and the Gab1β protein were separated by a polyglycine linker, a more wild type pattern of localisation was exhibited. This suggests that another domain following the PH domain, which is present in both Gab1α and Gab1β, may be important.
for the localisation of the protein, as well its function potentially. It would be interesting to investigate whether the published PH domain mutants that have been demonstrated to interfere with the function of Gab1α, had a similar pattern of sequence deletion. It may be that in the cases where the loss of the PH domain interfered with the localisation of the protein, a extra domain following the PH domain was also deleted. Interestingly, the recruitment of Gab1α to the plasma membrane was recently shown to be regulated by a separate domain other than the PH domain (Eulenfeld and Schaper, 2008). The authors demonstrated that when the Gab1α PH domain alone was transiently expressed in HEK393T cells, it was located at the plasma membrane, unlike the full-length protein, which was found in the cytoplasm. The authors therefore speculated that the full length Gab1α protein has properties that prevent the protein’s translocation to the plasma membrane. It was then demonstrated that the membrane targeting of the full length Gab1 protein is in fact blocked by a protein region found after the PH domain. The data presented in this thesis suggest that Gab1β does not include this novel domain, as Gab1β is membrane-targeted in Gab1β +/+ ES cells. The membrane-targeting of Gab1β was also demonstrated to be prevented by the fusion of EGFP to the N-terminal. The presence of the EGFP tag at the N-terminal of Gab1β may in fact act in the same manner as this novel domain characterised by Eulenfeld & Schaper (Eulenfeld and Schaper, 2008), in that it also inhibits the membrane localisation of the tagged Gab1β. Progressively truncated constructs engineered to include different sequences post-PH domain, would allow the investigation of this potential novel domain.
The localisation data demonstrated here raise more questions as to the requirement for the high expression of Gab1\(\beta\) in ES cells. Gab1\(\beta\) was shown to be highly expressed in a distinct perinuclear body. An important event during the lifecycle of a receptor is the internalisation and recycling back to the surface in endosomes, in response to growth factor binding (Oksvold et al., 2001). Recycling endosomes are in charge of the recycling of receptors and ligands (Enrich et al., 1999). Grb2 in complex with the EGFR has been shown to be localised in endosomes (Oksvold et al., 2001) and therefore one could hypothesise that Gab1\(\beta\) may also be found in this complex in endosomes as well. In attempting to relate this observation with the fact that Gab1\(\beta\) may have a role in maintaining a state of homeostasis in ES cells, one could hypothesise that Gab1\(\beta\) is recycled in endosomes in complex with the receptors it conveys signals from.

What controls the decision to transcribe Gab1\(\beta\) instead of Gab1\(\alpha\) in ES cells is of great interest. Is this controlled by ES cell-specific transcription factors? Or is there a mechanism of silencing in place, whereby cues emanating from the surrounding environment control this process? It is likely that transcription factors that are important for ES cell biology may in fact instruct the transcription of Gab1\(\beta\), rather than that of Gab1\(\alpha\). Deciphering the potential factors that regulate the maintenance of ES cells during diapause would be of great help in understanding the regulation of Gab1\(\beta\) protein expression. One of the most exciting advances in ES cell biology is the discovery of induced pluripotent stem cells, or iPS cells (Takahashi et al., 2007; Takahashi and
Yamanaka, 2006). These cells are pluripotent and are artificially derived from non-pluripotent fibroblasts by forcing the expression of four genes: Oct4, Sox2, cMyc and Klf4. It would be interesting to see whether Gab1β is expressed in iPS cells. This would indicate that one of these four factors may potentially be responsible for the decision to transcribe Gab1β in ES cells instead of Gab1α, which is normally expressed in differentiated cell types, such as fibroblasts.

Although the exact role of Gab1β in ES cells is not clear, what is evident is that the exact biological identity of an ES cell is also of great interest. ES cells represent the in vitro counterpart of epiblast cells (Smith et al., 2001), but whether they truly reflect what happens in the epiblast is still unknown. One might expect that the signals that regulate ES cells, such as gp130 signalling, would also be important in vivo. During normal development, however, LIF is not required for the maintenance of the ICM in mouse epiblasts. The only time where the epiblast is dependent on gp130 signalling is during the process of delayed implantation, or diapause (Nichols et al., 2001). This is an example of the disparity that exists about what ES cells truly represent. Future work on identifying the signals involved in the in vivo regulation of ES cells may indeed further our knowledge and more importantly the potential clinical use of them as a therapeutic tool.


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