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TARGETED DISRUPTION OF THE
Grb4 AND Nck LOCI AND GENERATION
OF HOMOZYGOUS NULL nck MICE.

by

Elke Daniela Aippersbach

A thesis submitted in conformity with the requirements
for the degree of Master's of Science
Graduate Department of Molecular and Medical Genetics
University of Toronto

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Abstract

Adaptor proteins are a subset of signaling proteins that lack catalytic function. Adaptor proteins of the Grb2 family have been shown to be critical signaling components downstream of receptor tyrosine kinases (RTKs) that mediate interactions with other signaling molecules through their protein binding domains. Nck and Grb4, the only known mammalian members of the Nck family, are composed of three amino-terminal SH3 domains followed by a carboxy-terminal SH2 domain. Recent biochemical studies and work undertaken in invertebrates have suggested that this family of adaptors may mediate signaling which regulates the actin cytoskeleton and axon guidance in response to RTK stimulation. To better assess the function of these adaptor proteins in mammalian development, a strategy was undertaken to target a mutation into the genomic loci of both Nck and Grb4. Targeting of embryonic stem cells and the generation of chimeric animals was successful for both genes. Furthermore, the targeted Nck locus was transmitted into the murine germline allowing the generation of homozygous null nck mice. This targeting strategy also introduced the β-galactosidase reporter gene into the Nck locus. Analysis of the embryonic expression pattern of β-galactosidase indicates that there are restricted regions of the fetal mouse that highly express nck during embryogenesis. The generation of these mice, antibodies that can discern between Nck and Grb4, and fibroblast cell lines lacking Nck resulting from this work will aid in elucidating the in vivo function of Nck and Grb4 in mammalian development.
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<tbody>
<tr>
<td>Grb</td>
<td>growth receptor bound</td>
</tr>
<tr>
<td>DNA/cDNA</td>
<td>deoxyribonucleic acid/copy DNA</td>
</tr>
<tr>
<td>EB</td>
<td>embryoid bodies</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signal regulated kinase</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem cell</td>
</tr>
<tr>
<td>LIF</td>
<td>leukemia inhibitory factor</td>
</tr>
<tr>
<td>G418</td>
<td>geneticin</td>
</tr>
<tr>
<td>GANC</td>
<td>gancyclovir</td>
</tr>
<tr>
<td>IRES</td>
<td>internal ribosome entry site</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>MAP</td>
<td>multiple antigen peptide</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>Nck</td>
<td>novel cytoplasmic protein</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>RNA/mRNA</td>
<td>ribonucleic acid/messenger RNA</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SAPK</td>
<td>stress-activated protein kinases</td>
</tr>
<tr>
<td>SH2</td>
<td>Src-homology-two</td>
</tr>
<tr>
<td>SH3</td>
<td>Src-homology-three</td>
</tr>
<tr>
<td>5'</td>
<td>five prime</td>
</tr>
<tr>
<td>3'</td>
<td>three prime</td>
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</table>
Introduction

The development of a single cell egg into a complex and patterned organism requires controlled responses to extracellular signals. These signals influence diverse functions including cell migration, differentiation, and inhibition of programmed cell death. The study of cellular signaling mediated by receptor tyrosine kinases (RTKs), has illustrated that the propagation of an external signal is tightly controlled through protein-protein interactions. Consequently, an understanding of how specific proteins interact in a RTK mediated pathway yields insight into signal transduction mechanisms within a cell and their effects on the development of an organism.

Ligand binding to a RTK initiates receptor dimerization followed by receptor trans-autophosphorylation, activation and phosphorylation of downstream molecules (Pawson, 1995). The importance of regulating such downstream signaling was illustrated by the discovery that many oncogenic mutations cause constitutive activation of the cascade, whether by mutations in a RTK itself or in its downstream targets (Hunter, 1997). Study of the diverse array of cytoplasmic signaling proteins has implicated the repeated use of protein modules in mediating signal transduction (Pawson, 1995).

Several conserved protein modules have been found to regulate signal transduction. By mediating specific protein-protein interactions, these modules are critical components in allowing the faithful transmission of a signal from cell membrane to nucleus. Two such domains were first recognized as having homology to regions of Src kinase and were termed the Src-homology-domain-2 (SH2) and the Src-homology-domain-3 (SH3). These domains are found in a variety of proteins and link RTK activation to a broad range of downstream targets. The mode of interaction of these domains with other proteins, therefore, plays a critical regulatory role in signal transduction cascades.
SH2 Domains

SH2 domains are independently folding domains of approximately 100 amino acids (Sadowski et al., 1986). SH2 domains bind tyrosine phosphorylated RTKs and cytoplasmic proteins in vivo. However, the portion of the protein that is recognized by the SH2 domain is small. Thus in vitro, isolated SH2 domains can recapitulate in vivo interactions by binding to short peptide sequences that contain an amino (N) terminal phosphorylated tyrosine (pTyr) with an affinity of Kd 10-100 nM (Panayotou and Waterfield, 1993; Piccione et al., 1993). The ability of SH2 domains to recognize pTyr containing residues is determined by a specific binding pocket of SH2 domains. Structures of several SH2 domains solved by X-ray crystallographic diffraction or Nuclear Magnetic Resonance (NMR) spectroscopy indicate that the pTyr residue contacts a conserved pocket within the SH2 domain as was observed with the prototypic Src SH2 structure (Waksman et al., 1993). The pTyr residue hydrogen bonds with the only invariant residue in the SH2 binding interface, an arginine, deep within this conserved pocket. Thus, the interaction of a SH2 domain with a protein is regulated by the absolute requirement for tyrosine phosphorylation of the ligand.

The binding of a SH2 domain, however, is more complex than simple recognition of a pTyr motif. The SH2 binding interface also contacts peptide residues that are carboxy (C) terminal to the pTyr. This portion of the SH2 domain structure is more variable and is responsible for the specific binding of individual SH2 domains to different pTyr containing sequences (Songyang et al., 1993). Indeed, in vitro selection experiments indicated that there were two major groups of SH2 domains. The first group of SH2 domains binds with high affinity to peptides of the sequence: pTyr-hydrophilic-hydrophilic-isoleucine/proline whereas the second binds: pTyr-hydrophobic-X-hydrophobic peptides, where X denotes any amino acid except tryptophan and cysteine (Songyang et al., 1993).
In addition the Grb2 SH2 domain binds sequences with the consensus pTyr-X-Asparagine, with little preference at the +3 position (Songyang et al., 1993).

The different binding specificity correlates with variations seen in SH2 domain structures. For example, the Src SH2 domain (group 1) interacts directly with two hydrophilic residues of the ligand but then has a small hydrophobic pocket which can accommodate a hydrophobic residue (Waksman et al., 1993). However, the SHP2 SH2 domain (group 2) has an extended hydrophobic groove which can accommodate a peptide with several hydrophobic residues C-terminal to the pTyr. This accounts for the affinity of the Src SH2 domain for the peptide pTyr-Glutamic Acid (Glu)-Glu-Isoleucine (Ile) whereas the SHP2 SH2 selects pTyr-Ile-X-Valine (Val) (Pawson, 1995). Thus the structures of these SH2 domains yield insight into the specificity of SH2 domains in vivo and in vitro. The specificity of SH2 domains for pTyr in context of C-terminal residues ensures that proteins containing SH2 domains interact with specific protein targets rather than promiscuously with all pTyr containing proteins within a cell.

**SH3 Domains**

Another protein interaction domain found in many proteins involved in RTK mediated signaling is the SH3 domain. SH3 domains are independently folding domains of approximately 60 amino acids. Unlike the SH2 domain which recognizes only phosphorylated substrates, the SH3 domain interacts with unphosphorylated sequences of proteins. They bind to proteins containing proline (P) rich stretches. In vitro, recombinant SH3 domains bind short proline rich peptides of the consensus X-P-X-X-P with a dissociation constant of Kd 5-100 μM (Ren et al., 1993; Yu et al., 1994). Thus, SH3 domains commonly recognize proline rich ligands; however, individual SH3 domains only recognize a
specific subset of proteins bearing sequences that match the consensus sequences.

In vitro, recombinant SH3 domains can select peptides from random libraries giving insight into the in vivo binding specificity of a particular SH3 domain (Yu et al., 1994). Structural studies performed by NMR spectroscopy reveal that peptide ligands of SH3 domains adopt a polyproline type II helix and interact with a conserved hydrophobic binding surface within the SH3 domain that is flanked by two variable loops (Feng et al., 1994). The two variable loops are thought to interact with the non-proline regions of the ligand to impart specificity of binding to an individual SH3 domain. Interestingly, there is remarkable flexibility in how the peptide assumes this PPII helix. The Src SH3 domain can bind peptides that assume the PPII helix in either an N to C terminal (class 1) or a C to N terminal (class 2) configuration (Feng et al., 1994). SH3 domains, therefore, mediate high affinity and specific interactions with proline rich sequences on target proteins. Thus, SH3 domains regulate protein interactions and signal transduction.

Adaptor Proteins

SH2 and SH3 domains are a subset of protein interaction domains found in a wide variety of proteins involved in RTK mediated signaling. Many proteins that possess these modules also contain intrinsic enzymatic functions such as kinase, phosphatase, phospholipase, or guanine nucleotide exchange activity. For example, Src contains both SH2 and SH3 domains as well as a protein-tyrosine kinase domain. Activated Src can thus interact with pTyr containing proteins recognized by its SH2 domain and with proline rich proteins recognized by its SH3 domain. Further, down-regulation of Src kinase activity following phosphorylation of a C-terminal Tyr site is mediated by binding of both the SH2
and the SH3 domains to internal ligands through intramolecular interactions (Sicheri et al., 1997; Xu et al., 1997). However, there are subset of proteins involved in RTK signaling that lack endogenous enzymatic function. These proteins are termed adaptor proteins. It is thought that the role of adaptors is to nucleate protein complexes that are responsible for protein relocalization or that allow proteins, held by the adaptor in close proximity, to interact.

**Grb2/Drk/Sem-5 - a link between RTKs and Differentiation/Growth**

The role in cellular signaling and development of one adaptor, Drk, has been genetically and biochemically characterized by study of RTK signaling in the repeated ommatidial units of the *Drosophila* compound eye. There is an absolute requirement for the Sevenless (Sev) RTK in the determination of the fate of one of the photoreceptor (R) cells, R7, from precursor cells (Hafen et al., 1987). In the absence of Sev protein no R7 cells develop, whereas constitutive activation of Sev results in multiple R7 cells per ommatidium (Basler et al., 1991; Tomlinson et al., 1987). Study of this system identified Ras1, a small GTPase, and Sos, a guanine nucleotide exchange factor for Ras1 as genetically downstream components of the Sev signaling pathway (Rogge et al., 1991; Simon et al., 1991). As the *Caenorhabditis elegans* adaptor protein, Sem-5, was implicated in a similar genetic pathway and a highly conserved mammalian orthologue, Grb2, existed, it was reasoned that this adaptor might have been conserved through evolution to function in RTK pathways (Olivier et al., 1993). Drk, a *Drosophila* orthologue of Sem-5/Grb2, was identified as genetically downstream of the Sev RTK and shown to interact with Sos *in vitro* (Olivier et al., 1993; Simon et al., 1993). These data were used to propose a general model of the Grb2/Sem-5/Drk adaptor family in linking RTK activation to downstream
signaling, and to suggest a specific role for the adaptor Drk downstream of Sev in determination of the R7 cell fate.

The Grb2/Sem-5/Drk adaptor proteins are thought to bind through their SH3 domains to Sos. Activation and autophosphorylation of the RTK allows interaction of the adaptor, through its SH2 domain, with the phosphorylated RTK. As Sos is bound to the adaptor, it is concomitantly translocated to the membrane where it can activate membrane bound Ras1. Activated Ras1 stimulates a signaling cascade culminating in MAP kinase nuclear translocation to initiate the transcription of genes involved in growth and differentiation (reviewed in Downward, 1997).

The Nck family of Adaptor Proteins

The roles of other adaptor proteins in RTK mediated signaling is less well defined than that of Grb2/Sem-5/Drk. Likely all adaptors link upstream signaling to downstream targets, but it is surmised that Grb2/Sem-5/Drk may function in growth and differentiation while other adaptors function in other roles. One family of adaptor proteins, the Nck family, has recently been implicated in mediating signaling downstream of RTKs to rearrangements of the actin cytoskeleton. The mammalian members of this family are two closely related proteins, Nck and Grb4. Invertebrate members, Dock and CeNck have been discovered in Drosophila, and C. elegans, respectively.

Mammalian Grb4 and Nck

Nck and Grb4 are adaptor proteins and hence lack any catalytic domains. They are composed of three N-terminal SH3 domains followed by a C-terminal SH2 domain (Figure 1). Nck and Grb4 share an overall identity of 67% and a similarity of 75%. The identity is highest through the SH3 and SH2 domains and
lowest in the intervening linker sequences (Figure 1). The human and murine Nck proteins share 99% identity demonstrating a high degree of conservation of these two genes in evolution (Figure 2).

Human nck, hnck, was cloned fortuitously through the cross reactivity of Nck to an unrelated antibody (Lehmann et al., 1990). grb4 was cloned from an expression library screen using the phosphorylated tail of the EGF receptor as a probe (Margolis et al., 1992). The chromosomal location of hnck is 3q21, a region involved in neoplasia associated changes (Huebner et al., 1994; Vorobieva et al., 1995). hgrb4 has been localized to chromosome 2q12 (Chen, M. et. al, unpublished data). Other than the cloning of grb4, the literature has not distinguished between Nck and Grb4 although they represent individual members of the Nck family of adaptor proteins. Most of the published biochemical studies used antibodies that were raised to epitopes shared by both proteins. Thus it remains unclear whether the biochemical interactions reported for Nck represent interactions with Nck and Grb4, Nck only, or Grb4 only.

**Binding to the SH3 and SH2 domains of Nck**

Nck is composed of four binding domains the binding partners of which have been investigated *in vivo* and *in vitro* and are listed in Figure 3. *In vitro*, the SH2 domain of Nck selects from a degenerate phosphopeptide library peptides bearing the sequences: pYDE(P/D/V) (Songyang et al., 1993). These motifs are found in a variety of activated RTKs to which Nck has been shown to bind, including the PDGF, EGF and Eph B1 receptors (Li et al., 1992; Meisenhelder and Hunter, 1992; Park and Rhee, 1992; Stein et al., 1998).
Figure 1 Domain diagrams of Nck, Grb4, and Dock.

Schematic representation of Nck, Grb4, and Dock illustrates the modular structure of adaptors within the Nck adaptor family. The protein identities and similarities within the SH3 and SH2 domains of Nck and Grb4 are listed between the appropriate domains. The conserved identity of Nck to Dock is also indicated followed in parentheses by the identity of Grb4 to Dock.
Figure 2 Nck and Grb4 protein sequence alignment.
The alignment of the protein sequences of murine Nck and Grb4 highlight the conserved residues particularly within the SH3 and SH2 domains. The SH3 and SH2 domains are indicated, as are regions of diversity within the two proteins to which antibodies were raised. The alignment also includes human Nck, which has over 99% identity to mouse Nck.
SH3

mouseNck  MAEEVVVKFDYVQAQQEQLDIIKNERLMLDLSDSKSWMRN5MNKTGFVPSNYV  56
humanNck  MAEEVVVKFDYVQAQQEQLDIIKNERLMLDLSDSKSWMRN5MNKTGFVPSNYV
mouseGrb4  MAEEVVVKFDYVQAQQEQLDIIKNERLMLDLSDSKSWMRN5MNKTGFVPSNYV

mouseNck  ERKNSRKAISTVKNLKTQIGVVRKPSVPDIAEPPDSSFVDPGERLYDL  107
humanNck  ERKNSRKAISTVKNLKTQIGVVRKPSVPDIAEPPDSSFVDPGERLYDL
mouseGrb4  ERKNSRKAISTVKNLKTQIGVVRKPSVPDIAEPPDSSFVDPGERLYDL  112

mouseNck  NMPAFVKFNMYAEREDELSSIKGTVVKEKCSGDWRGSYNGCIGWFPSSNYVTEEE  163
humanNck  NMPAFVKFNMYAEREDELSSIKGTVVKEKCSGDWRGSYNGCIGWFPSSNYVTEEE
mouseGrb4  NMPAFVKFNMYAEREDELSSIKGTVVKEKCSGDWRGSYNGCIGWFPSSNYVTEEE  168

************  

mouseNck  GSPLGLDVGSLSKKLAAAVNNLTCVQHVQLPVLYPFSSSNDEEELNFKEKGDVDV  219
humanNck  GSPLGLDVGSLSKKLAAAVNNLTCVQHVQLPVLYPFSSSNDEEELNFKEKGDVDV
mouseGrb4  GSPLGLDVGSLSKKLAAAVNNLTCVQHVQLPVLYPFSSSNDEEELNFKEKGDVDV  224

mouseNck  IEPENPEWKKCKNGMLVPLVPKNYV1LMQNNPLTSILEEPPQCDYRPSL  308
humanNck  IEPENPEWKKCKNGMLVPLVPKNYV1LMQNNPLTSILEEPPQCDYRPSL
mouseGrb4  IEPENPEWKKCKNGMLVPLVPKNYV1LMQNNPLTSILEEPPQCDYRPSL  313

************  

mouseNck  RFAVENWYGYKVRHQAEMALNERGHEGDFLIRDSSSNDVSVLSKAGKNNHKF  331
humanNck  RFAVENWYGYKVRHQAEMALNERGHEGDFLIRDSSSNDVSVLSKAGKNNHKF
mouseGrb4  RFAVENWYGYKVRHQAEMALNERGHEGDFLIRDSSSNDVSVLSKAGKNNHKF  336

mouseNck  VQLKETVYCIQKRFSTMEELVEHYKAPIFTSEQGKLYLVKHL  378
humanNck  VQLKETVYCIQKRFSTMEELVEHYKAPIFTSEQGKLYLVKHL
mouseGrb4  VQLKETVYCIQKRFSTMEELVEHYKAPIFTSEQGKLYLVKHL  383
**Figure 3** Domain diagram of Nck with interacting proteins.

Proteins that interact with various domains of Nck are listed under the appropriate binding domain if the interaction was localized. References for the binding interactions are listed in the text.
3SH3 together bind:
Sos
NIK
HPK-1
Intracellular targets found in association with Nck include IRS-1, p130Cas, and p62dok (Holland et al., 1997; Lee et al., 1993; Schlaepfer et al., 1997).

The preferred recognition sequence for the second Nck SH3 domain was examined in vitro using a phage-displayed random peptide library. The random peptide consensus sequence of PXPPRXSXL was selected (Quilliam et al., 1996). This sequence is found in known serine/threonine (Ser/Thr) kinases which bind the SH3 domain, namely PRK2 and Pak3 (Quilliam et al., 1996). Other Ser/Thr kinases that bind to one or more of the SH3 domains are HPK1 and NIK (Anafi et al., 1997; Su et al., 1997). Similar to Grb2, Nck has also been shown to bind Sos (Hu et al., 1995).

**Nck function in the Ras pathway**

The interaction of Nck with proteins of known signaling cascades prompted examination of whether Nck was involved in these pathways. Initially, binding of Nck to various activated growth factor receptors implicated an involvement of Nck, similar to that of Grb2, in the control of cell growth and proliferation. Indeed, transfection of nck cDNA was found to transform a rat fibroblast cell line (Chou et al., 1992) and overexpression of nck cDNA activates Ras-dependent gene expression (Hu et al., 1995). However, subsequent examination of Ras activation indicated that overexpression of dominant negative nck constructs, unlike similar grb2 constructs, could not block Ras dependent activation of the MAP kinase, Erk1 (Tanaka et al., 1995). Work which may explain the different results seen using different assay systems, suggests that Nck played both a Ras-dependent and independent role in PDGF induced mitogenesis (Roche et al., 1996). Pursuit of Ras independent binding partners for Nck uncovered several proteins linked with distinct Ras superfamily members, namely the Rho family of GTPases.
The Rho family of GTPases

The Rho family of GTPases have been linked to a number of cellular events including membrane trafficking, transcriptional regulation, cell growth control, and development (reviewed in Van Aelst and D'Souza-Schorey, 1997). Most notable is the involvement of the GTPases Cdc42, Rac, and Rho in the regulation of adhesion and the cytoskeleton. The formation of actin cytoskeletal structures has been studied in fibroblasts and a hierarchy of GTPase activation established. Cdc42 can activate Rac which can in turn activate Rho (Nobes and Hall, 1995). Cdc42 activation leads to the formation of filopodia (Nobes and Hall, 1995). Rac activation leads to ruffled or webbed lamellipodia sheets (Ridley et al., 1992). Rho activation leads to production of actin stress fibers important for adhesion due to formation of focal adhesion complexes (Ridley and Hall, 1992). Ectopic expression studies suggested that Rho-family GTPases regulate the motility of the leading edge of axons, the growth cone (Luo et al., 1994). The remodeling of the actin cytoskeleton via rapid polymerization/depolymerization in response to guidance cues is, therefore, thought to be a critical function of the Rho-family of GTPases.

Recent work has begun to unravel the protein interactions through which Rho GTPases exert their cellular effects. Serine/threonine kinases such as the Pak family and PRK2 bind with GTP bound forms of Cdc42 and Rac (Manser et al., 1995; Quilliam et al., 1996). This binding event leads to kinase autophosphorylation and increased enzymatic activity which can stimulate the JNK/SAPK kinase cascades (Brown et al., 1996). Nck has been shown to bind these upstream kinases (Bokoch et al., 1996; Galisteo et al., 1996; Quilliam et al., 1996). Furthermore, Nck was demonstrated to be crucial in activation of cytoplasmic Pak1 by translocating it to the membrane (Lu et al., 1997) and in the
induction of actin cytoskeletal rearrangements in cultured cells by activated Pak1 (Sells et al., 1997).

Non-kinase effectors of the Rho family include a protein, WASP, which was found to cause Wiskott-Aldrich Syndrome (WAS) if mutated (Derry et al., 1994). WASP interacts through a proline rich region with the third SH3 domain of Nck. It was proposed that deficiencies in cellular immune response of WAS patients were due to an underlying defect in organizing the actin cytoskeleton (Symons et al., 1996). To this end, WASP binds to activated Cdc42 and also contains a domain directly involved in actin binding (Aspenstrom et al., 1996; Symons et al., 1996).

As well as downstream kinases and effectors of Rho family GTPases, Nck can interact with Eph receptors, putative upstream RTKs of the Rho family (Holland et al., 1997; Stein et al., 1998). Eph receptors and their ligands are dynamically expressed in the developing nervous system (Gale et al., 1996). The Eph receptors and their transmembrane ligands have been implicated in bidirectional signaling (Holland et al., 1996). Mutations in several Eph receptors lead to defects in murine axon guidance and fasciculation (reviewed in Holland et al., 1998). Recently, Grb4 was shown to bind, in vitro, a ligand of the EphB2 (Nuk) receptor, namely ephrin B1 (Holland, unpublished data). Thus, it is intriguing that Nck and Grb4 have been found downstream of Eph receptors and ephrins, respectively.

Taken together, these data link Nck and Grb4 to many proteins implicated in the Rho GTPase control of the actin cytoskeleton. The growth cone of neuronal cells can transmit environmental cues to the rearrangement of the actin. It has been proposed that Nck/Grb4 may function in the growth cone to link guidance and targeting cues to downstream effectors reminiscent of the role Grb2 plays in linking growth and differentiation cues to downstream effects.
(Garrity et al., 1996). The strongest evidence for this model comes from in vivo studies in Drosophila.

**Drosophila R Cell Guidance and Targeting Requires Dock**

*Drosophila* bearing a homozygous protein null mutation of *dreadlocks*, *dock*, a gene closely related to *nck/grb4* (Figure 1) die at the pupal stage and exhibit profound defects in photoreceptor (R) cell fasciculation and targeting (Garrity et al., 1996). Dock is expressed highly in the growth cone of affected axons (Garrity et al., 1996). In *dock* mutants, many R1-6 axons extend beyond their normal targets, which argues that the outgrowth of the axon is not perturbed; rather their response to appropriate guidance cues is affected (Garrity et al., 1996). Interestingly, experiments expressing mutated *dock* constructs in a *dock* mutant background yield different degrees of rescue in different axons (Rao and Zipursky, 1998). This argues that Dock interacts with a different subset of proteins in different neurons and may function in multiple signaling complexes and pathways.

As discussed, the many biochemical binding partners of Nck and Grb4 have indicated a putative in vivo role of this adaptor family in mediating signaling downstream of guidance cues to rearrangements of the actin cytoskeleton. The role of the evolutionary conserved invertebrate homologue, Dock, in patterning and neural connectivity suggests that study of Grb4 and Nck may reveal a similarly important role in mammalian connectivity. One method of studying the in vivo role of a protein in development is to study the development of an organism in the absence of that protein product. Recent advances in gene targeting technology allow the design and targeting of a mutation into the murine genome to precisely ablate protein production from a gene of interest. This
technique has been employed here to yield insights into the role of the Nck family of adaptor proteins in mammalian development.

**Gene Targeting Strategies**

Saturation mutagenesis screens have proved less tractable in mammalian systems than invertebrate systems. However, the development of murine gene targeting strategies advantageously allows mutations to be designed for specific loci. The creation of mice lacking a gene of interest provides a powerful system for study of the function of that gene *in vivo*. Null mutations of genes are often heterozygous viable, allowing transmission of the mutant allele and intercrossing to obtain homozygous animals. Viable homozygous animals can be directly assessed for phenotypic abnormalities. Many targeted mutations have proven to be homozygous embryonic lethal. Nevertheless, the role of the gene in embryonic development often can be assessed and reagents such as cell lines can be generated to further study cellular events in the absence of the gene of interest.

**Embryonic Stem Cell Properties**

The ability to target murine genes requires cells that maintain their totipotency after *in vitro* manipulation (Nagy et al., 1993). Several such Embryonic Stem (ES) cell lines have been established from the inner cell mass of murine blastocysts - the work described here utilized the RI ES cell line (Nagy et al., 1993). Early passages of these cell lines maintain their totipotency if cultured in defined conditions in the presence of myeloid leukemia inhibitory factor (LIF) to prevent differentiation (reviewed in Robertson, 1987). Genetically altered ES cells can be aggregated with wildtype embryos where the ES cells will contribute to the formation of a chimeric embryo (Wood et al., 1993). ES cell
contribution to the germline of a chimeric animal allows the genetic information of the ES cell to be transmitted to the progeny of the chimera.

**Homologous Recombination**

In order to target specific alleles, the property of DNA to undergo homologous recombination has been exploited (reviewed in Capecchi, 1989). Targeting vectors are often designed such that there are two arms with homology to the locus of interest. Homologous recombination through both arms results in the integration of any vector components flanked by the arms into the locus; any sequence of the endogenous locus flanked by sequence homologous to the vector arms is concomitantly deleted. The use of isogenic DNA in the arms decreases the amount of polymorphic variation between the vector and the chromosome and results in increased frequency of recombination (te Riele et al., 1992). The length of homology in the vector also influences the recombination frequency and should be over 5 kb for efficient recombination events (Thomas and Capecchi, 1987). Even with these considerations accounted for, the integration of DNA into the ES cell genome is a rare event and homologous versus random integration is an even rarer event (Joyner and Wurst, 1993).

**Positive and Negative Selection Strategies**

Strategies have been devised to select for rare recombination events. The two targeting vectors used in this work, plasmid PNT (pPNT) and pPNT loxP, utilize both a positive and negative selection cassette (Tybulewicz et al., 1991; Shalaby et al., 1995). Both the positive and negative markers are driven by the promoter of the gene which encodes the ubiquitously expressed phosphoglycerate kinase-1 (PGK-1) protein. The bacterial gene *neomycin* (*neo*) codes for an aminoglycoside phosphotransferase which confers antibiotic
resistance to the drug geneticin (G418). As well as often acting as a mutagen, the integration of the neo gene allows for selection in G418 of the rare transfected cells that have integrated the vector. This is the positive selection step as it positively selects for integration events.

Integration of the targeting vector, however, is often a random event rather than a homologous recombination event. To increase the selection for the latter event, a negative selection cassette is included in the targeting vector. The Herpes simplex viral gene product, thymidine kinase, produces toxic products in the presence of the drug gancyclovir (GANC). The negative selection cassette resides at one end of the linearized vector. Random integration of the vector will often integrate this negative selection cassette and result in cell lethality in the presence of the selectable agent (Mansour et al., 1988). Negative selection therefore affords some selective pressure against clones that have randomly integrated the vector. Thus, use of negative selection decreases the number of cell lines screened as it decreases the pool of viable cells that have randomly integrated the vector.

**Screening for Homologous Recombination Events**

After Positive/Negative selection, surviving cell lines must be screened to confirm that they have undergone homologous recombination through both arms of the targeting vector with the locus of interest. As the genomic locus is characterized prior to vector construction, the restriction map is known. Vectors are designed so that integration into the endogenous locus will result in a loss or gain of restriction sites in the locus. These variations can be assessed with restriction digests of genomic DNA of putative clones. Southern blotting of this DNA and hybridization with diagnostic probes will reveal the novel restriction pattern upon integration. As integration of the vector through homologous
recombination with only one arm is possible, most screening protocols confirm recombination with two probes that are 5 and 3 prime to the arms chosen. Internal probes or PCR are also used to confirm integration events.

**Insertions at the targeted locus - LacZ**

The *Escherichia coli* LacZ gene encodes a β-galactosidase (β-gal) molecule which is a widely used enzymatic reporter of gene expression. Most forms of LacZ encode a cytoplasmic or nuclear-targeted form of β-gal. Insertion of a promoterless LacZ into a gene leads to expression of β-gal under control of neighboring transcriptional enhancers and promoters. Many experiments have indicated that such a technique allows expression of the reporter to reflect a portion or all of the transcriptional activity of the nearby gene. However, to elaborate neuronal pathfinding and connectivity, a fusion product of tau, a microtubule associated protein and β-gal can be used to overcome the lack of diffusion into axons of traditional β-gal proteins (Callahan and Thomas, 1994; Mombaerts et al., 1996).

**Insertions at the targeted locus - IRES LacZ**

The LacZ reporter has been fused downstream of the Internal Ribosome Entry Site (IRES) sequence to widen its applications. Translation of eukaryotic mRNA is usually dependent on 5'-cap-mediated ribosome binding and scanning of the transcript for a suitable translational start site (reviewed in Mountford and Smith, 1995). However, the viral IRES sequence assumes a secondary structure which allows cap-independent recognition of the IRES mRNA by the ribosome and consequent dicistronic mRNA translation when a second gene such as LacZ is put downstream of the initial translation start site (Kim et al., 1992). If the insertion of the IRES LacZ sequence is early in a gene and is preceded by stop
codons in three frames, it is expected that translation from the initiation site will be prematurely truncated. Hence only the β-gal protein is expected to be translated which leads to a protein null mutation. This, therefore, allows insertion of the β-gal reporter into an exon without any deletion of genomic enhancer elements. As transcription is under the control of the endogenous promoter, translation of the reporter protein is expected to reflect the endogenous gene's expression. Minimizing the deletion of genomic sequence will minimize the chance of losing intronic enhancer elements.

**Insertions at the targeted locus - Floxed Neo**

Though one can minimize the amount of deletion in a locus upon insertion of a reporter element and selectable marker, arguably the locus has been altered by these new insertions. Insertion of neo into an endogenous locus can alter gene expression from that locus (Moens et al., 1992; Nagy et al., 1998). In order to minimize the amount of sequence inserted into the endogenous locus, one can remove inserted elements after targeting if they have been flanked by specific 34 base pair repeats, loxP sites. Cre is a bacteriophage recombinase that recognizes and catalyzes recombination between loxP sites (reviewed in Jiang and Gridley, 1997). In the presence of the Cre recombinase, sequence flanked by two loxP sites ('floxed' sequence) is deleted. The vector pPNT loxP has the neomycin cassette ‘floxed’ and one can therefore remove this cassette after targeting ES cells *in vitro* by transient Cre expression or *in vivo* through breeding mice derived from targeted ES cells with transgenic Cre recombinase expressing mice (Jiang and Gridley, 1997).
Redundancy in gene families

A final consideration in gene targeting strategies is whether a mutation in a gene of interest is expected to yield a phenotype. Remarkable conservation of gene function has been demonstrated across diverse species, for example Hox genes defining positional information. Thus, known mutations in orthologues can offer insight into possible phenotypes and scoring thereof in other species. However, many gene families are larger in mammals versus invertebrates. Functional compensation of one family member for another can lead to mild or masked phenotypes. For example, the severity of the axon guidance defect observed in mice lacking the EphB2 (Nuk) or EphB3 (Sek4) mutation was more severe in double mutants of these two family members (Orioli et al., 1996).

Experimental Rationale

In order to understand the role that the mammalian adaptors Grb4 and Nck play in murine development, a targeting strategy was devised. As mutational analysis can be complicated in the presence of truncated protein products, targeted mutations into the loci were designed to favour the creation of protein null mutations. To address the issue of redundancy, a targeting strategy for all known mammalian members of the adaptor family, namely Nck and Grb4, was devised. Cell lines lacking endogenous Nck were also created to assess in vitro whether the pathways and cascades these adaptors are implicated to function in were perturbed in the absence of Nck.

In order to create targeting vectors for Nck and Grb4, their genomic loci were cloned and characterized. Targeting vectors were designed and constructed such that integration events could be screened. Three targeted ES cell lines were obtained: two for Nck and one for Grb4. These lines were used to create aggregation chimeras. The chimeras were tested for germline
transmission. To date, the Grb4 chimeric animals have not produced germline transmission of the targeted allele. One ES cell line in which the Nck locus had been disrupted and a reporter construct inserted, contributed to the germline of chimeric animals and allowed transmission of the mutation. Animals homozygous for the mutant allele are viable. Phenotypic analysis of these animals is currently underway to determine whether any aspects of murine development, especially axon guidance, are dependent on Nck.

Homozygous null fibroblast lines and ES cell lines have been generated to further assess, in vitro, how the biochemical pathways that Nck has been implicated in function in its absence. The production of specific antibodies to Nck and Grb4 was undertaken. The production of Nck and Grb4 specific antisera is crucial to test whether the biochemical interactions formerly attributed to Nck are in fact due to Nck alone, Grb4 alone, or Nck and Grb4. These antibodies may allow the expression pattern of these genes to be further refined and contrasted.

Ultimately separating the expression patterns of these genes will aid in understanding whether these closely related adaptor proteins have separable functions or solely redundant roles. The creation of a strain of mice that expresses β-gal under the control of the Nck promoter was exploited to augment the adult Nck expression studies with reporter expression studies at various embryonic stages. The homozygous null animals and the tools generated for in vitro studies will provide invaluable resources for further understanding how the Nck family of adaptor proteins functions in signaling downstream of RTKs and the effects of these adaptors on mammalian development.
Material and Methods

RECOMBINANT DNA METHODS

Genomic Library Screen

A library of murine strain 129SvJ genomic kidney DNA inserted in Lambda Dash II (Stratagene) created by Dr. Andrew Reaume and provided by Dr. J. Rossant was screened. The library was titred and 20 (150 mm) plates were plated with $6 \times 10^4$ plaque forming units (pfu's) resulting in $1.2 \times 10^6$ pfu's being screened. Bacteriophage were processed by standard techniques to purity (Sambrook et al., 1989). All bacteriophage plating used the *Escherichia coli* strain ER1647 (Novagen). Plaques were lifted in duplicate onto Optitran Supported Nitrocellulose Membranes (Schleicher & Schull) and processed by standard protocols (Sambrook et al., 1989). Rather than baking, however, DNA was fixed to the membrane by crosslinking in the UV 1800 Stratalinker (Stratagene).

Library Screening

Library screening followed standard protocols (Sambrook et al., 1989). However formamide was omitted from the hybridization solution. Consequently, the hybridization temperature was raised to 58°C. The hybridization solution comprised: 5x Denhardt's solution, 5x SSPE, 0.1% SDS, 100 μg/ml sheared and denatured salmon sperm DNA.

Probe Labeling

Probes were gel purified and electro-eluted or obtained by Quiax purification (Qiagen). Probes larger than 0.15 kilo basepairs (kb) were primed with random nonamers using the T7 Quick Prime Kit (Pharmacia). Oligo-
nucleotide probes used in mapping the bacteriophage inserts were end labeled using standard techniques (Sambrook et al., 1989).

**Large Scale Phage DNA Preparations (Genomic Library)**

Bacteriophage DNA was prepared as described (Sambrook et al., 1989). DNA for restriction digests was resuspended in 0.2x TE buffer to minimize amount of salt in digest buffer.

**Southern Blotting and Hybridization**

For bacteriophage insert mapping, 0.4μg digested DNA per lane was separated by gel electrophoresis and blotted onto GeneScreen (Dupont) Nylon based membranes using standard capillary blotting techniques (Southern, 1975). The pre-hybridization solution consisted of 50% formamide, 5x SSPE, 5x Denhardt's solution, 0.2% SDS, 100 μg/ml sheared and denatured salmon sperm DNA. The hybridization solution consisted of 50% formamide, 5x SSPE, 1x Denhardt's solution, 9%w/v Dextran Sulphate, 0.2% SDS, 100 μg/ml sheared and denatured salmon sperm DNA. Pre-hybridization of membranes typically occurred for 3-6 hours and hybridization occurred overnight, both at 42°C. Standard hybridization buffers for membranes to be probed with oligonucleotide probes were used (Sambrook et al., 1989).

**High Stringency Washing**

High stringency washing (for Southerns and Library Screens) was based on the following conditions with alterations in length of washes dependent on experimental variation. Membranes were washed twice at room temperature in 0.2x SSC/0.1% SDS followed by two 60°C washes in 0.2x SSC/0.1% SDS. For
oligonucleotide probes, high stringency washing was 5x SSC/0.1% SDS at room temperature followed by 42°C washes. All washes were typically 10-30 minutes.

**Subcloning and Sequencing**

All DNA subclones were subcloned into plasmid BlueScript (pBS) (Stratagene). Products from PCR reactions were cloned into pCRII (Invitrogen). Targeting vectors were constructed from pPNT or pPNT loxP (Tybulewicz et al., 1991; Shalaby et al., 1995).

DNA to be sequenced was subcloned into pBS and prepared with fluorescein labeled primers from the AutoRead™ kit (Pharmacia) and sequenced on the ALF sequencer (Pharmacia).

**ES CELL TISSUE CULTURE AND MANIPULATIONS**

**ES Tissue Culture**

The RI ES cells were maintained in Dulbecco's modified Eagles medium with high glucose and glutamine. The media was supplemented with additives previously described (Joyner and Wurst, 1993). Cells were maintained on 0.1% gelatinized plates or on mitomycin C (Sigma) treated primary embryonic fibroblasts (EMFI) cells.

**Electroporation**

Electroporation followed protocol 8 in *Gene targeting: a practical approach* (Joyner and Wurst, 1993). The following modifications were made to the protocol. ES cells were not pre-plated. ES cell concentration was adjusted to 7.5 x 10⁶ cells/ml. 15μg of linearized DNA was used per cuvette. The voltage for electroporation was 0.25 mA.
Selection, Expansion and Freezing of Colonies

Colonies were subjected to selection 48 hours after electroporation in the presence of 2μM GANC and 150 μg/ml G418 as described (Joyner and Wurst, 1993). After 10-12 days, colonies were picked into 96 well plates and subsequently split into four populations. Two master plates were frozen either by the quick freeze method, protocol 13 in Gene targeting: a practical approach (Joyner and Wurst, 1993). Alternately, cells were frozen by first trypsinizing cells, neutralizing the trypsin with ES media, chilling on ice for five minutes and adding an equal volume of ice-cold 2x freezing media from the aforementioned protocol.

High G418 selection

High G418 selection was performed by plating heterozygous ES cells at three concentrations (1x10³, 1x10⁴, and 1x10⁵ cells/10 cm plate) and treating with varied G418 concentrations ranging from 500 μg/ml to 1800 μg/ml. High G418 concentrations selected for cells lines in which a double stand break occurred at or near the wildtype allele. Subsequent gap formation and repair using the mutated allele as a template resulted in cell lines possessing two copies of the neomycin gene and concomitant higher resistance to G418 selection. Colonies were screened as described previously and lines which had lost their wildtype alleles were chosen from the lowest concentration of G418 to induce homozygousity (1000 μg/ml).

ES cell DNA extraction

ES cell DNA was extracted as described in protocol 11 in Gene targeting: a practical approach (Joyner and Wurst, 1993) with the following modifications. Extractions were performed in 96 well plates so volumes were adjusted accordingly, for example, 50μl of lysis buffer. 100-120μl of absolute ethanol was
added after lysis and plates were left undisturbed for two or more hours to allow DNA to settle and stick to the bottom of wells. All subsequent steps could be performed in the wells without loss of DNA. DNA was resuspended overnight in 25μl of TE buffer before digestion in a total volume of 40μl using all of the isolated DNA. One third to one half of the digested DNA was sufficient for Southern blotting.

In *vitro* differentiation of ES cells

Differentiation of ES cells was performed in suspension or adherent cultures. In suspension cultures, approximately 1x10^6 cells ES cells were dispersed as single cells in 15 ml of LIF-free media in plastic bacterial plates. Cells were cultured for 8-12 days. Embryoid body (EB) formation was scored by visual inspection. EBs were washed with PBS prior to photography.

Adherent *in vitro* differentiation was performed by plating 200-700 dispersed ES cells onto gelatinized tissue culture plates in 15 ml of LIF-containing media. Cells were incubated for 8-10 days at which point LIF was removed from the media and cells were cultured for a further 2-4 days. Plates were washed with PBS prior to photography of colonies.

ANTIBODY PRODUCTION

Synthetic peptide sequences were chosen from linker regions of Grb4 and Nck based on hydrophobicity, antigenicity and uniqueness (Figure 1). The multiple antigen peptide (MAP) system was used as the core for peptide synthesis (Tam, 1988). The MAP approach is based on a core of eight amino terminals from which peptides are synthesized (Tam, 1988). The resultant macromolecule has a high density of peptide antigens and can be used directly for challenging animals. New Zealand White rabbits were immunized as
described (Tam, 1988) with the exception that one ml of MAP: Freund's adjuvant was used per rabbit and the schedule for injections was at 0, 14, 28, 42, 72, and 122 days with bleeds at 38, 52, 82, and 132 days. Crude sera were frozen at -80 °C or affinity purified against the antigens used to immunize the rabbits. Antiserum was purified by linking 10 mg of the MAP peptide to an activated Sepharose 4B column (Pharmacia) and following manufacturer's directions for serum purification.

WHOLE MOUNT β-GALACTOSIDASE STAINING OF EMBRYOS

β-galactosidase staining of embryos was performed as described (Puri et al., 1995). For sectioning, embryos were washed overnight in the wash buffer, fixed, and embedded; sections were cut at 5 μm, mounted, dewaxed and stained with nuclear fast red (Puri et al., 1995).

WESTERN BLOT ANALYSIS

Cells or tissues were rinsed in ice-cold PBSA and lysed in PLC lysis buffer (1% Triton X-100; Henkemeyer et al., 1994). Where indicated, protein concentrations of cleared lysates were determined using a BCA assay (Pierce). Denatured lysates were resolved on 10% SDS-polyacrylamide gels and transferred to Immobilon-P membrane (Millipore). Membranes were blocked in 5% skimmed milk powder in TBST (0.05% Tween-20). Western blots were performed with anti-Nck (3x3, or peptide-specific) or anti-Grb4 polyclonal antibodies 1:500, followed by 1:10000 protein A-horseradish peroxidase (prA-HRP) (Biorad) and chemiluminescence (ECL, Amersham).
Isolation and targeting of the *nck* and *grb4* genes

Isolation of the *Nck* genomic locus

In order to screen a murine genomic library, a murine *nck* probe was required. Based on the human *nck* sequence, oligonucleotide primers were designed and used to amplify a 393 base pair (bp) probe by the polymerase chain reaction (PCR) using CD-1 murine spleen cDNA as a template (Figure 4D). This murine cDNA fragment spanned part of the first and second SH3 domains and the intervening linker sequence and was used to probe a murine 129SvJ female kidney genomic DNA library. Six positive clones were isolated and four (lambda clones 4, 9, 11, and 18) were characterized.

Isolation of the *Grb4* genomic locus

Based on the human *grb4* sequence, oligonucleotide primers were designed and used to amplify a 409 bp murine product as was done with *nck* (Figure 5D). This cDNA fragment, which also spanned part of the first and second SH3 domains and the intervening linker sequence of *grb4*, was used to probe the aforementioned murine genomic DNA library. Three positive clones (lambda clones 1, 2, and 3) were isolated and characterized.

Characterization of the *Nck* genomic locus

Lambda phage clones containing murine genomic sequences were isolated and DNA prepared. Initial characterization of the clones involved complete restriction digests by several enzymes followed by Southern blotting with the following probes: T7 (5' TAATACGACTCACTATAGGG), T3 (5' ATTACCCCTCACTAAAGGGA), and the 393 bp *nck* probe. These data allowed
**Figure 4** *nck* genomic map

A) Restriction digest map of *Nck* genomic locus derived from lambda clones 4 and 18. Key in kb, listed above locus. Dark filled boxes represent coding exons one and two. B) Non-repetitive intronic probes depicted with open boxes were derived from digests of appropriate subclones with restriction enzymes flanking the probe. C) The coding region of two exons is depicted on a representation of *nck* cDNA. D) Region of cDNA used for 393 probe as depicted on a representation of *nck* cDNA. (BglIII, *Bgl* II; RV, *EcoR* V; PmlII, *Pml* I; H, *Hind* III; E47, *Eco47* III; MunI, *Mun* I; Bam, *BamH* I; Xhol, *Xho* I; X, *Xba* I)
A. Restriction Map
B. Intronic probes
C. Exon Structure
D. cDNA probe

- cDNA 393 probe
Figure 5 *grb4* genomic map

A) Restriction digest map of *grb4* genomic locus derived from lambda clone 3. T7 and T3 denote the orientation of the clone within the Lambda Dash II vector (Stratagene). Key in kb listed above the locus. Dark filled box corresponds to coding exon one.  

B) Non-repetitive intronic probes depicted by open boxes were derived from digests of appropriate subclones with the flanking restriction enzymes.  

C) The coding region of the first exon is depicted on a representation of *grb4* cDNA.  

gross mapping of restriction enzyme sites within the inserts and orientation of the inserts with respect to the lambda phage arms. Two of the clones (lambda 9 and 11) were found to contain pseudogenes by subcloning and sequencing and were, therefore, not pursued. Lambda clones 4 and 18, however, were overlapping clones spanning approximately 23 kilo bases (kb) of the Nck genomic locus.

Further subcloning, sequencing, and Southern blotting were used for finer mapping of the locus. The nck probe recognized two exons in the genomic locus isolated (Figure 4A). The first coding exon contained the ATG codon and continued through the first SH3 domain to the subsequent linker region (Figure 4C). The second coding exon was 12 kb downstream of the first and encoded a large exon which spanned the second and third SH3 domains as well as the majority of the SH2 domain (Figure 4C).

**Characterization of the Grb4 genomic locus**

The Grb4 locus was characterized in a similar fashion to the Nck locus using T7, T3, and grb4 specific probes. Two of the isolated clones (lambda 1 and 2) were found to contain pseudogenes and were not pursued. Lambda 3, however, spanned 16.5 kb of the Grb4 locus and contained the first coding exon (Figure 5A). The intron/exon boundaries of the first coding exon were conserved between nck and grb4 (Figure 5C).

**Isolation of non repetitive intronic probes in the Nck and Grb4 loci**

Prior to designing targeting vectors, appropriate non-repetitive probes were obtained from each locus. Convenient restriction sites uncovered in the mapping process were used to isolate small fragments of DNA. These fragments were used to probe Southern blots of digested genomic DNA to determine if they hybridized to single DNA fragments or to many fragments which would indicate
that they contained repetitive sequences. For the Nck locus two probes were obtained (Figure 4B). Both were 3' to the first coding exon. For the Grb4 locus, two probes that were 5' and 3' to the exon were found (Figure 5B). The existence and location of appropriate probes was used in subsequent design of the targeting vectors.

**Design of the nck deletion targeting vector**

To favour the creation of a null mutation, the nck deletion vector was designed such that correct integration of the vector would insert the neomycin resistance cassette into the Nck locus and result in a deletion of 15 kb of genomic sequence (Figure 6A). This deletion would result in a loss of the coding sequences from amino acids (aa) 23-313 (Figure 6B). At best, any translation from the locus would yield partial portions of the first SH3 domain (aa 1-23) or the SH2 domain (aa 313-377). These fragments, if translated, would not be expected to fold into stable domains and likely would be degraded.

To detect integration of the deletion vector into the Nck locus, two approaches were used (Figure 6C). As integration of the vector would abolish all endogenous Hind III sites, loss of these sites would indicate targeting of the Nck locus. Thus, the 3' probe would recognize a Hind III restriction fragment of at least 2.7 kb larger upon vector integration (Figure 6D). To assess correct 5' integration, an internal probe, neo, was expected to recognize no bands in the endogenous locus but a band of approximately 11 kb upon targeting of the Nck locus (Figure 6C).

**Construction of the nck deletion targeting vector**
**Figure 6** Gene targeting of the murine *Nck* locus.

**A)** Targeting strategy. The homologous recombination of the Nck deletion vector into the *Nck* locus replaces the intervening sequence with *neo*. Homology of locus to Nck deletion vector denoted with thin dark line. Exons denoted with thick dark boxes. 3' probe indicated with open box. Fragment of locus recognized with 3' probe after Hind III digestion of genomic DNA is underlined.

**B)** Targeted *Nck* locus. Restriction map of *Nck* locus after homologous integration of Nck deletion targeting vector. Arrows denote diagnostic sites and open boxes the probes used to recognize DNA fragments after restriction digests. **C)** Mapping of targeted events. Expected length of diagnostic restriction fragments summarized in table. (BglI, Bgl II; RV, EcoR V; PmlII, Pml I; H, Hind III; E47, Eco47 III; MunI, Mun I; B, BamH I; X Xho I)
A.

HindIII digest

Nco deletion targeting vector

B.

BamHI digest

C.

<table>
<thead>
<tr>
<th>neo probe + BamHI digest</th>
<th>3'probe + HindIII digest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Targeted integration</td>
<td>11.5 kb 8.7 + X kb</td>
</tr>
<tr>
<td>Endogenous locus</td>
<td>-          6.0</td>
</tr>
</tbody>
</table>
The vector contained a total of 6 kb of sequence homologous to the locus (Figure 6A). The restriction digest of genomic DNA with Not I and Eco RV resulted in a 5' arm. To clone this and other fragments that lacked compatible sticky ends, DNA overhangs were filled with T4 polymerase to yield blunt ends which were cloned by blunt ended ligation. Thus, the 5' arm was cloned into the unique Xho I site of pPNT which had been digested by Xho I and end-filled. The 3' arm was subsequently end filled and cloned into the blunt ended unique BamHI site of the pPNT+5' arm plasmid (Figure 6A). Correct orientation of ligation products were assessed by diagnostic restriction digests. Large scale DNA preparations were linearized with Not I prior to electroporation.

**Design of the grb4 deletion targeting vector**

To favour the creation of a null mutation, the grb4 deletion vector was designed such that correct integration of the vector would insert the neomycin resistance cassette into the Grb4 locus and result in a deletion of 3 kb of genomic sequence (Figure 7A). This targeting event would delete the first coding exon including the initiation methionine codon.

Homologous recombination of the targeting vector with the Grb4 locus would alter the endogenous restriction sites (Figure 7C). Using these variations, one could assess integration of the vector through its two homologous arms into the endogenous locus (Figure 7D). An integration event would be detected as an increase in digest size by the 3' probe as opposed to a decrease by the 5' probe (Figure 7D).

**Construction of the grb4 deletion targeting vector**

The construction of the vector utilized a 2.6 kb 5' arm and a 4.0 kb 3' arm to result in a total of 6.6 kb of homology to the Grb4 locus (Figure 8A). Both the
Figure 7  Gene targeting of the murine Grb4 locus.

A) Targeting strategy. The homologous recombination of the grb4 targeting vector into the Grb4 locus replaces the intervening sequence with neo. Endogenous locus is shown above Grb4 deletion vector. Regions of homology between vector and locus denoted by thick arrows. 5' probe noted with striped box. 3' probe indicated with shaded box. B) Targeted Grb4 locus. Restriction map of Grb4 locus upon integration of Grb4 targeting vector. Novel restriction sites noted in bold type. C) Mapping of targeted events in Grb4 locus. Expected length of diagnostic restriction fragments summarized in table. D) Southern blot results of grb4 5' probe [above] and 3' probe [below] of targeted ES line E1 and untargeted lines. (B, Bgl II; A, Asp 718; RV, EcoR V; H, Hind III; Bam, BamH I; X, Xba I)
A.

B.

C.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>INTEGRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' probe</td>
<td>13</td>
<td>10.0</td>
</tr>
<tr>
<td>+ BamH1 digest</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3' probe</td>
<td>8.9</td>
<td>10.7</td>
</tr>
<tr>
<td>+ HindIII digest</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D.

E1

13kb-
10kb-
10.7kb-
8.9kb-
Figure 8 Cloning of *grb4* targeting vector arms.

A) 5' and 3' arms of lambda 3 were digested at restriction sites noted in bold. Fragments were subcloned into pBS (Stratagene). B) The plasmids with the fragments in the desired orientation are diagrammed; the ligation products with the incorrect orientations are not diagrammed. C) Expected products from diagnostic restriction digests for the correct and the incorrect orientation of ligation products from A are listed under the appropriate enzyme. D) The subclones with the arm fragments integrated in the correct orientation were further digested to yield fragments with newly engineered restriction sites (denoted in bold). The resulting fragments represent the 5' and 3' arms that were later cloned into the targeting vector (pPNT) backbone. See figure legend for *Grb4* genomic locus (Figure 5) for abbreviations.
A. Grb4 lambda3

- 5' Arm
  - Digest RglII [2.6 kb]
  - Blunt end
  - Ligate to pBS
  - Check orientation

- 3' Arm
  - Digest HindIII XbaI [4.1 kb]
  - Blunt end
  - Ligate to pBS
  - Check orientation

B. Digest Smal Blunt

B. Digest NotI/XhoI for 3' arm

C. Digest NotI/XhoI for 3' arm

- RV Digest if correct
  - 1.8 kb
  - 0.7 kb
  - 1.9 kb
  - 4.9 kb

- RV Digest if incorrect
  - 3.7 kb
  - 2.3 kb

D. Digest XbaI/Asp718 [KpnI] for 5' arm

- BamHI Digest if correct
  - 1.8 kb
  - 5.3 kb

- BamHI Digest if incorrect
  - 2.3 kb
  - 4.8 kb

D. NotI

- RV
  - A
  - H
  - 5' ARM
    - 2.7 kb

D. XhoI

- RV
  - RV
  - Bam
  - 3' ARM
    - 4.1 kb
5' and 3' arms were independently cloned into the plasmid Bluescript (pBS) to generate additional restriction sites (Figure 8B). After orientation in pBS was confirmed (Figure 8C), the two arms were removed from pBS, using two sites for each arm that are unique in pPNT (Figure 8D). Digestion of unique pPNT sites, thus, allowed the construction of the targeting vector in a four-way ligation step (two arms and two fragments of pPNT) using directional cloning (Figure 9A) to generate the Grb4 deletion targeting vector (Figure 9B). The targeting vector was linearized with Not I prior to electroporation.

Electroporation of Targeting Vectors

Electroporation of ES cells with the targeting vectors was undertaken as described in Materials and Methods. ES cells were selected in the presence of geneticin (G418) and gancyclovir (GANC). One plate per experiment was selected in the presence of only G418 to determine what proportion of colonies were sensitive to GANC indicating integration of the entire targeting vector. Typically, there were 5-10 fold more colonies in the absence of GANC indicating a 5-10 fold enrichment against non-homologous integration events in its presence.

Screening of ES Colonies for integration of the nck and grb4 targeting vectors

Colonies surviving positive and negative selection were isolated and expanded into four populations. Two of these populations were frozen separately and the other two were used to isolate DNA. Extracted DNA was digested by the appropriate diagnostic restriction enzyme, Southern blotted, and probed with the appropriate diagnostic probe. The results are summarized in Table 1.
Figure 9 Cloning of the *grb4* deletion targeting vector.

A) Fragments generated from digestion of pPNT were ligated with the 5' and 3' arm fragments.  B) The *grb4* deletion targeting vector was generated from a four way ligation of the fragments listed above. See figure legend for *Grb4* genomic locus (Figure 5) for abbreviations.
A.

5' Grb4 ARM
2.7 kb

3' Grb4 ARM
4.1 kb

B.

Grb4 deletion vector
14.1 kb
Table 1. Targeting Frequency of Nck and Grb4 Vectors

<table>
<thead>
<tr>
<th>Vector Type</th>
<th># of colonies screened</th>
<th>Targeted events</th>
<th>Targeting frequency</th>
<th>ES cell lines</th>
<th>Germline Transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nck Deletion Vector</td>
<td>&gt;550</td>
<td>0</td>
<td>&lt; 1/550</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>Nck LacZ Vector</td>
<td>565</td>
<td>2</td>
<td>1/282</td>
<td>F3 &amp; G12</td>
<td>F3</td>
</tr>
<tr>
<td>Grb4 Deletion Vector</td>
<td>267</td>
<td>1</td>
<td>1/267</td>
<td>E1</td>
<td></td>
</tr>
</tbody>
</table>
Over 550 colonies from ten experiments with the *nck* deletion targeting vector survived electroporation, double selection, expansion and analysis. However, no integration events were detected with the 3' probe.

267 colonies from six experiments with the *grb4* deletion vector were screened. One colony exhibited the diagnostic changes in restriction sites within the endogenous *Grb4* locus as assessed by both the *grb4* 5' and 3' probes. This targeted ES line was termed Grb4 E1 and it represented a targeting frequency of approximately 1/267.

**Design of the *nck* LacZ Insertion Targeting Vector**

As no integration was observed with *nck* targeting vector, a new targeting vector was designed and constructed. Rather than a deletion vector, this vector was designed to result in the integration of a reporter gene, *IRES LacZ*, and the selectable gene, *neo*, downstream of the *nck* initiation methionine without any deletion of genomic sequence.

Integration of the Nck LacZ insertion targeting vector into the endogenous locus would result in an insertion of *IRES LacZ* into the first coding exon thereby terminating any translation from the endogenous locus (Figure 10A). Insertion of novel restriction sites into the locus by targeting with the insertion vector would result in a diagnostic decrease in size of the *Hind* III fragment recognized by the 3' probe (Figure 10C). Verification of homologous integration through the 5' arm can be confirmed by PCR or internal probes (Figure 10C). In an integration event, a 1.8 kb PCR product is expected by using PCR primers to genomic sequence 5' to the short arm and primers within the targeting vector.
**Figure 10** Gene targeting of the murine *Nck* locus with the *nck* LacZ targeting vector.

A) Targeting strategy. The homologous recombination of the Nck LacZ targeting vector into the *Nck* locus inserts both the *IRES LacZ* and *neo* sequence into the first coding exon. The *Nck* locus is depicted above the Nck LacZ targeting vector. The shaded box indicates the sequence used for the 5′ arm and the stippled box indicates the sequence used as the 3′ arm. The 3′ probe is indicated as a dark thick box. B) Targeted *Nck* Locus. The targeted locus with the altered restriction sites is depicted. C) The expected diagnostic products for assessing homologous recombination of the targeting vector. D) Southern blot of ES cell lines including the targeted line, F3. E) Southern blot of DNA from progeny of founder Nck F3 mouse. See figure legend for *Nck* genomic locus for abbreviations.
A. INTEGRATION

B. BamHI digest

C. | WT | INTEGRATION |
   |    |            |
   | 3' probe | 13 | 6.0 |
   | + BamHI digest | none | 1.8 |
   | PCR Product | none | 1.8 |

D. 13kb-

E. 6 kb-

F3
Construction of the LacZ insertion *nck* targeting vector

Construction of the Nck LacZ targeting vector involved several steps. Firstly, the 3' arm was blunt end cloned into pPNTloxp (Figure 11A). One clone assessed for correct orientation was denoted pPNTloxp+3' arm (Figure 11B). A similar short arm to that used in the *nck* deletion vector was constructed; however, PCR primers were used to shorten the 5' borders of the arm (Figure 12A). This left available some 5' genomic sequence from which PCR primers were designed to assess integration of the 5' arm (Figure 10C). The 5' arm PCR product was subcloned into pCRII; subsequently, unique sites in the vector and the arm were used to directionally clone the 5' arm into PBS to yield PBS+5' arm (Figure 12A). A fragment containing the IRES LacZ sequence was then blunt end cloned into the vector PBS+5' arm to yield PBS+5' arm+LacZ (Figure 12B). Correct orientation of ligation products was assessed by diagnostic restriction digests (Figure 12C). Unique sites were used to excise the short arm and the reporter tag, together, and to subclone this fragment into pPNTloxp+3' arm (Figure 13A) to generate the Nck LacZ insertion targeting vector. Large scale DNA preparations were linearized with *Not* I prior to electroporation.

Screening of ES colonies for integration of the *nck* LacZ targeting vector

Colonies were screened as described in Materials and Methods. Over 565 colonies from six experiments with the *nck* LacZ insertion vector survived electroporation, double selection, expansion, and analysis. Two independent ES cell lines with the targeted integration events were detected with the 3' probe (Figure 10D and data not shown). These lines were called Nck F3 and G12 and represented a targeting frequency of 1/282 (Table 1).
Figure 11  Subcloning 3' Arm of Nck LacZ insertion targeting vector

A) pPNT manipulated as shown was ligated with a genomic fragment of the Nck locus that had been digested with EcoRV [noted in bold type]. B) The ligation products were assessed for correct orientation of the insert by Xba I [noted in bold type] and one clone of the correct orientation was used for further construction. See figure legend for Nck genomic locus for abbreviations.
A. Kpn1 digest, blunt end

B. Xba Digest

Correct:
1.9
2.5
6.5
10.9

Incorrect:
1.1
1.9
7.9
10.9

-XhoI/NotI digest
-ligate with 5' Arm+ires
-NotI/XhoI fragment

TPNT loxP +3' ARM
10.9 kb

Xba digest

EcoRV digest

nck locus

5400 kb

1800 kb

H3

3' ARM

X

B

nck exon 1

H3

H3

H3

T7

BglII

BglII

Xba digest

- - -

10.9 10.9

-RV digest

Ligate
Figure 12 Subcloning the 5' Arm and LacZ of the nck targeting vector

A) The Nck 5' arm was produced by PCR of a genomic fragment and subcloned into the vector pCRII (Invitrogen) which results in a 5' EcoRI site. This site and the EcoRV site in the exon were used to remove the fragment and clone it directionally into pBS (Stratagene), resulting in a subclone termed pBS + 5' Arm. 

B) A fragment containing IRES LacZ was removed from the pBS IRES LacZ vector with the enzymes noted in bold and blunt end cloned into pBS + 5' Arm at the EcoRV site. 

C) Correct orientation of the ligation products was assessed by HindIII digests (data not shown). 

D) The resulting subclone was termed pBS+5'Arm+IRES and was used in subsequent cloning. See figure legend for Nck genomic locus for abbreviations.
A. HindIII: 4.2 & 5.2 kb
Correct HindIII: 4.2 & 5.2 kb
Incorrect HindIII: 0.3 & 9.0 kb

B. pBS + PARM

C. IRES LacZ

D. pBS + 5' Arm + LacZ
9.2 kb

EcoRI and EcoRV digestion

1.8 kb PCR product done in PCR1
remove by PstI digest followed by RV to
result in 1.8 kb Pst-IV arm

NotI
SmaI
BamHI
EcoRV

SmaI 4501
BamHI 4300

Correct HindIII:
4.2 & 5.2 kb
Incorrect HindIII:
0.3 & 9.0 kb
Figure 13  Construction of the nck LacZ targeting vector

A) The subclone pPNT + 3' Arm was manipulated as noted. A fragment containing the 5' arm and IRES LacZ sequence that resulted from a Not I/Xho I double digest of pBS+5'Arm+IRES was subcloned into pPNT + 3' Arm. This directional cloning resulted in the Nck LacZ targeting vector (B). See figure legend for Nck genomic locus for abbreviations.
-NotI/Xhol digest, clone in [5'Arm+IRES] fragment
-NotI/Xhol digest NotI/Xhol [5'Arm+IRES] clone into pPNT + 3' ARM

B.
Aggregation of Targeted ES Cells and Generation of Chimeric Mice

The three ES cell lines with targeted mutations in the Nck or the Grb4 locus were aggregated with eight cell random-bred (CD-1 or ICR) embryos in the transgenic facility of Mt. Sinai Hospital by S. Kulkarni. CD-1 or ICR mice are albino and carry the pink-eyed dilution allele whereas the ES cells were derived from mice with allelic variations in those marker genes. Thus, ES cell derived pups are dark-eyed and agouti in coat colour. In aggregation experiments, all three targeted ES cell lines contributed at a low to moderate degree to the chimeric animals as assessed by the degree of agouti coat colour in an otherwise albino coat coloured animal.

Contribution of the targeted ES cells to the germline of the chimeric animals was assessed by test-mating of male chimeras to ICR females. Birth of dark-eyed pups indicated germline transmission of the targeted ES cell line, whereas pups derived from host blastocyst gametes had pink eyes. One of the three aggregated ES cell lines, Nck F3, gave germline transmission from two chimeric males. One male exhibited a transmission frequency of less than 1% while the other was 100%. The 100% germline transmitter was termed the founder and was used for generating the line of Nck F3 mice.

Genotyping and Mendelian Transmission of the nck Targeted Mutation

F1 offspring of the founder and ICR females were initially genotyped by PCR or Southern blot analysis of digested tail DNA probed with the 3' probe as previously described (Figure 10E). However, as tails of 2-6 week old pups that carried the targeted mutation expressed β-galactosidase, subsequent identification of heterozygous progeny was undertaken by a X-Gal staining protocol.
Half of the F1 animals tested carried the targeted allele, indicating proper Mendelian segregation of the allele (data not shown). Furthermore, these animals had no obvious phenotype compared to their wildtype litter mates indicating that disruption of the *Nck* locus did not result in haplo-insufficiency. These heterozygous animals were fertile and were interbred to assess if homozygous mutant animals were viable.

Southern blot genotyping of progeny from heterozygous intercrosses indicated that 28/106 (26%) offspring were homozygous for the targeted disruption in the *Nck* locus, 47/106 (44%) were heterozygous, and 31/106 (29%) were wildtype. This indicates the mutation was transmitted with expected Mendelian ratios of 1:2:1. Furthermore, analysis of embryos from E9.5 to E18.5 indicated no resorptions as would be expected if the homozygous mutation was embryonic lethal with a variable penetrance. Homozygous animals were recently interbred to establish if they were fertile. 10/10 progeny from one cross were homozygous mutant indicating that the homozygous animals were fertile.

**Determination of Targeted Disruption of *Nck* locus as a Protein Null**

To assess whether the targeted disruption of the *Nck* locus resulted in a protein null mutation, Western blot analysis was undertaken of fibroblast cell lines derived from E14.5 embryos from a heterozygous intercross. As expected, the amount of Nck detected in the heterozygous fibroblasts was approximately half of that seen in fibroblasts from wildtype litter mates (Figure 14). The homozygous mutant lines had no protein of the expected size (47 kilodaltons), although a slightly larger faint band was seen on some exposures. As the antibody used recognized Nck and Grb4 (discussed later) the blot was probed with anti-Grb4 specific antibodies (discussed later) to assess whether this band represented Grb4 protein levels. These Grb4 antibodies, however, failed to
Figure 14 Expression of Nck in fibroblast cell lines derived from a Nck +/- F1 intercross.

Embryonic fibroblasts derived at E13.5 were genotyped by PCR (data not shown) (+/+, wildtype; +/- heterozygote; +/- homozygote). Protein extracts were standardized and separated by SDS-PAGE and immunoblotted with anti-Nck (3x3) antibody to detect levels of Nck/Grb4.
recognize the band (data not shown). As the design of the targeted insertion precluded translation products except of a truncated nature, this band likely represented a protein that the sera recognized non-specifically or Grb4 protein that was below the level of detection of the Grb4 specific anti-sera. No truncated products were observed indicating that this targeted mutation indeed results in a protein null mutation.

**Obtaining Homozygous Targeted ES Cells**

Several homozygous mutant nck ES cells were obtained by treatment of heterozygous Nck ES cell lines with high concentration of G418 (data not shown). Nck protein levels were assessed and, as with fibroblast cell lines above, were not detectable in the homozygous cell lines (data not shown). *In vitro* differentiation experiments were undertaken with these cell lines to assess the differentiation potential of the cell lines *in vitro*. When ES cells are allowed to grow without LIF they differentiate into endoderm-like cells (Williams et al., 1988; reviewed in Dushnik-Levinson and Benvenisty, 1995). ES cells that aggregate in the absence of LIF form spheroid balls of cells that have an outer layer of both visceral and parietal endoderm cells (reviewed in Dushnik-Levinson and Benvenisty, 1995). They further develop into cystic bodies with an inner layer of ectoderm-like cells (reviewed in Dushnik-Levinson and Benvenisty, 1995). ES cells homozygous for the targeted nck allele had the same, uncompromised, differentiation potential as their heterozygous counterparts to form cystic embryoid bodies in suspension cultures (Figure 15A). The production of endoderm cells in adherent cultures, as scored visually by the production of differentiated endoderm-like cells at the edges of undifferentiated colonies, was indistinguishable between homozygous mutant and heterozygous ES cell lines (Figure 15B).
**Figure 15** *In vitro* differentiation of heterozygous and homozygous *nck* targeted ES cells

**A)** Embryoid bodies derived from heterozygous (+/-) and homozygous (-/-) targeted ES cells cavitate and become cystic. **B)** *In vitro* differentiation of adherent ES colonies in the absence of LIF, as described in Material and Methods, leads to the presence of visually scored endoderm-like differentiated cells at the edges of the colony in both +/- and -/- derived colonies.
Expression of \textit{nck} and \textit{grb4}

\textbf{Generation of Nck and Grb4 specific antisera.}

To determine whether the rabbit polyclonal antisera raised previously in our laboratory against the three SH3 domains of human Nck (Nck 3x3) cross-reacted with the closely related Grb4, Myc-tagged \textit{grb4} cDNA was transfected into Cos-1 cells. Cell lysates from the transient transfection were immunoprecipitated with Myc, Nck 3x3, or Grb4 specific (discussed later) antisera and western blot analysis was performed with the 3x3 anti-sera (Figure 16). The anti-Myc sera immunoprecipitated the Myc-Grb4 only in lysates from transfected cells. The 3x3 sera immunoprecipitated endogenous Nck from both transfected and untransfected lysates. However, the 3x3 sera immunoprecipitated the slightly larger Myc tagged Grb4 from transfected cells. In contrast, anti-sera raised to a peptide specific for Grb4 only immunoprecipitated the larger Myc tagged Grb4 and not the endogenous Nck from transfected cell lysates indicating it was specific for Grb4 (Figure 16A). Comparison of the immunoprecipitates illustrated that the 3x3 sera recognized both the endogenous Nck and the Myc tagged Grb4 found in transfected Cos-1 cells (Figure 16A).

To distinguish between Nck and Grb4, therefore, new antibodies were raised to linker region peptides. Though the homology between the SH2 and SH3 domains of Grb4 and Nck is very high, divergence in the intervening or linker sequences allows creation of peptides specific to only one of the adaptor proteins (Figure 2). Both anti-sera were able to immunoprecipitate Nck or Grb4 specifically from lysates containing Nck or Grb4, respectively (Figure 16A and data not shown). After affinity purification, the Grb4 sera was able to detect Grb4 specifically by Western blot analysis (Figure 16B).
Figure 16 Analysis of antisera specificity

A) Cos-1 cells were transiently transfected (Tf) with 5 µg Myc tagged Grb4 cDNA or left untransfected (Un). Cells extracts were harvested and immunoprecipitated with antisera raised against: Myc; the three SH3 domains of Nck, 3X3; or peptides specific for Grb4. The immunoprecipitates were analyzed by Western blot with the polyclonal Nck (3x3) antisera. B) Standardized Cos-1 cells extracts were separated by SDS-PAGE, and analyzed by Western blot with crude (Cr) or affinity purified (Af) Grb4 antisera.
A.

| Un | Tf | Un | Tf | Un | Tf |

**IP:** Anti Myc | Anti 3X3 | Anti Grb4

**Blot** Nck 3X3

B.

**Blot** Cr Af
Expression of Nck and Grb4 in adult mouse tissue

Expression of Nck and Grb4 in adult murine tissue was assessed with the Nck 3x3 antiserum. The serum recognized a widely expressed band of approximately 47 kilodaltons, the expected size of both Nck and Grb4 (Figure 17). Slightly higher expression was observed in the testis, brain and spleen as was previously observed (Park, 1997) and also intestine. Tissue samples were similarly blotted with affinity purified Grb4 antiserum to define Grb4 expression in adult mice. No protein expression, however, was observed (data not shown). This result indicates that either there was no adult murine Grb4 expression or that the antibody, though specific for Grb4, had too low an avidity to recognize Grb4 levels that were present.

Expression of Nck during embryonic mouse development

As targeting led to insertion of the LacZ reporter gene into the Nck locus, expression of β-galactosidase was examined at several embryonic stages ranging from 9.5 to 13.5 days post coitum (E9.5 to E13.5). At all stages examined, strong expression was observed in neural tube, somites, dorsal root ganglia, facial ganglia and eye. Continued incubation of embryos led to low levels of β-galactosidase activity that appeared, at the whole mount level, throughout the embryo. Sections of highly stained embryos were not uniformly blue as the whole mount appearance might have suggested. Rather, sectioning recapitulated the initial expression pattern indicating that these regions had the highest levels of β-galactosidase with lower levels throughout other regions of the embryo.
Figure 17 Expression levels of Nck in adult murine tissue samples.
A) Tissue protein lysates were standardized as described in Material and Methods and 10μg of protein was separated by SDS-PAGE and Western blotted with Nck (3x3) antisera (Ht, heart; In, intestine; Kd, kidney; Li, liver; Lu, lung; Sp, spleen; BS, brain stem; CE cerebellum; Ne, nerve). B) Standardized tissue protein lysates were separated by SDS-PAGE and Western blotted with Nck (3x3) antisera (Th, thymus; Br, brain; Te, testis; In, intestine).
Blot: anti-Nck 3x3
Wholemount staining

Wholemount β-galactosidase staining of embryos was undertaken at various embryonic stages and representative embryos from E10.5, E11.25, and E11.75 are shown (Figure 18). Initial specific staining (Figure 18A, C and D) was hidden by later, more extensive staining (Figure 18B). Dorsal views of the embryo highlighted expression in the neural tube, epithelia of the brain, and dorsal root ganglia (Figure 19A). Lateral views illustrated neural tube as well as segmented expression. Strong expression of β-galactosidase was observed in the facial region particularly the neural epithelium of the mid and hind brain, the eye and the trigeminal (V) ganglion (Figure 19B and C). Expression was also associated with mesenchymal condensations of the developing limbs (Figure 19E).

Sectioned Embryos

Sectioning of the embryos allowed more definitive analysis of β-galactosidase expression patterns. Intriguingly, the wholemount expression observed in both the cephalic region and in the neural tube was found to be restricted. The non-ventricular side of the neuroepithelium exhibited the strongest staining (Figure 20). The reporter activity in the neural tube was also observed to be non-ventricular and further restricted to the ventral region of the tube (Figure 22 and 23A). As proliferating cells are restricted to the ventricular layer adjacent to the lumen in the neural tube, this expression pattern appears consistent with non-proliferating cells (Kaufman, 1992).

The β-galactosidase reporter used in this study does not elaborate axonal tracts (Callahan and Thomas, 1994). As expected, no axonal process staining was observed although the ganglia, which are composed of neuron cell bodies, showed strong expression. Staining was observed in the facio-
Figure 18  Wholemount β-galactosidase expression levels driven by the Nck promoter

A) Initial expression of β-galactosidase at E10.5, E11.25 (B), and E11.75 (C) is limited to specific regions of the +/NcklacZ heterozygous embryos. Further staining produces less widespread expression - contrast B with C. D) Three embryos photographed together illustrate that the expression pattern is maintained as embryogenesis continues.
Figure 19  Wholemount β-galactosidase expression levels driven by the Nck promoter

A) Nck expression as assessed by β-galactosidase activity is detected in the dorsal root ganglia, DRG, the neuroepithelium, NE, of the neural tube and the cephalic region in a dorsal view of an E11.75 +/NcklacZ heterozygous embryo.

B) Lateral view of an E10.5 +/NcklacZ heterozygous embryo highlights segmented somite-like expression as well as neural tube expression. C and D) Lateral views of a head of an E11.25 +/NcklacZ heterozygous embryo exhibit staining in neuroepithelium (NE), eye (E) and trigeminal ganglion (TGG). E) Expression of β-galactosidase is associated with hindlimb mesenchymal condensation (ME) of an E11.75 +/NcklacZ heterozygous embryo.
Figure 20 Expression of β-galactosidase in E10.5 -/- Nck embryo

A) Sagittal sections through embryo reveal restriction of expression to the non-ventricular neuroepithelium (H, heart; BA, brachial arch; HP, hepatic primordium; LB, lung bud; MG, midgut; S, somite). Restricted expression is also observed in somites (S). A more medial section of the embryo (B) confirms that the staining is also observed in the dorsal root ganglia (DRG). (C) Higher magnification view of the cephalic neuroepithelium (NE) from (B) highlights the restricted staining in the NE. (D) The turning of the caudal regions of this embryo offers a unique view of both the restricted somite expression and the variegated DRG expression (E)

Higher magnification view of the somite and DRG.
acoustic ganglion (FAG), the trigeminal (V) ganglion, and the glossopharyngeal (IX) ganglion (Figure 20, 21B, and 22). These facial ganglia are largely cephalic neural crest derived structures (Kaufman, 1992; Le Lievre and Le Douarin, 1975). Neural crest cells are a transient population of cells that form from the dorsal neural tube where neuroepithelium and surface ectoderm are juxtaposed (Hogan et al., 1994). Their derivatives are widespread and include bone and cartilage, several types of neurons, and portions of dorsal root ganglia (DRG) (Hogan et al., 1994). Variegated blue was observed in the DRG (Figure 21C and 21D). The β-galactosidase expressing cells were not further examined to determine whether they were neural crest derived.

Mesoderm derivatives such as the notochord and somites also exhibited β-galactosidase activity (Figure 22 and 23). Somites form from sequential epithelialization of mesenchymal segmental plates (Stern et al., 1988). They further differentiate into dermatome, myotome and sclerotome, which give rise, respectively, to various structures such as trunk dermis and muscle cells, axial skeletal muscles, and the vertebral column (Stern et al., 1988). Sagittal sections indicate apparent segmental somite staining which is restricted to a thin strip of cells (Figure 20A and 20E). Transverse sections confirm somite staining which appears within the somite as medial or closer to the neural tube (Figure 22C).

The strong expression pattern observed in the eye was examined at several stages of embryonic development. All days examined showed expression associated with both neuronal cell types such as the neural layer of the retina as well as the non-neuronal future pigmented cell of the retina (Figure 23D and 23E). The ganglia cells from which nerve fibers form at E12.5-E13.5 exhibit expression as does the lens (Figure 23D and 23E).
Figure 21 Expression of β-galactosidase in E11.5 +/- Nck embryo.

A) Sagittal section through the embryo reveals expression of β-galactosidase in the facio-acoustic ganglion (FAG), the trigeminal ganglion (TGG) as well as the eye (E) and neuroepithelium (NE). B) A higher magnification of (A) highlights cephalic expression. C) A more medial section of the same embryo exhibits expression in the NE as well as the dorsal root ganglia (DRG). D) A higher magnification of C reveals variegated expression in the DRG.
**Figure 22** Transverse sections of Nck E10.5/- embryo.

A) Transverse sectioning reveals the restricted non-ventricular and ventral β-galactosidase expression in the neural tube.  B) Higher magnification views of the rostral region exhibit glossopharyngeal ganglia (GPG) staining and notochord (NC).  C) Higher magnification views of the caudal region reveal variegated expression in the dorsal root ganglia (DRG) and restricted medial somite expression. The urogenital ridge, particularly the mesonephric duct (MD) and the lining of the midline dorsal aorta (DA) have pronounced reporter activity. The region of the neural tube where motor neurons are born show the highest level of β-galactosidase and are denoted with a *. Note that low levels of expression is also detectable in cells adjacent to structures with high levels of expression.
**Figure 23** β-galactosidase expression in dorsal 10.5 -/- embryo and in eye.

**A**) This dorsal transverse section in its extremities is at the level of the neural tube, thus, the neuroepithelium of the neural tube (NE) and the neural lumen (NL) are exposed, yet the center portion of the section is below the level of the neural tube. **B** and **C**) Higher magnifications, respectively, of the more rostral and more caudal neural tube indicate that the expression is predominantly restricted to the ventral neural tube. Note the change in expression of β-galactosidase where the section is level with the neural tube compared to that below the neural tube. Level with the neural tube, variegated dorsal root ganglia (DRG) staining is observed as opposed to the restricted non-variegated somite staining below the neural tube. **D**) A sagittal section indicates the plane from which the higher magnification view (E) of the eye was taken. The mesenchymal condensations of the developing limb have prominent reporter activity. **E**) Expression is observed throughout the eye in non-neuronal derivatives such as the future pigment layer of the retina (FPL) as well as in the inner neural layer (INL) of the retina. Note that low levels of expression is also detectable in cells adjacent to structures with high levels of expression.
Other regions that exhibited high expression of β-galactosidase included the mesenchymal condensations in the limb (Figure 23A and 23D). Low levels of expression were observed throughout the internal organs including the heart, the liver, the kidneys, and the spleen in agreement with expression previously defined with Nck antisera in adult rats (Park, 1997). These data indicated that Nck has a dynamic expression pattern in fetal development. Expression of β-galactosidase was found in both cells of neuronal decent and in other structures that were likely derived from mesodermal cells. Although there appeared to be low levels of expression throughout the embryo, the regions of high expression were quite restricted as exemplified by in the cephalic neuroepithelium. These data provide the first insight into Nck expression during murine development.
Discussion

Recent studies have begun to decipher the crucial role that adaptor proteins play in linking RTK activation to downstream signaling. As adaptor proteins can specifically interact with different subsets of proteins through protein binding modules such as SH2 and SH3 domains, it is possible that each adaptor may mediate a distinct signaling pathway. A clear role for the Grb2/Sem-5/Drk family in mediating cell growth and differentiation has been delineated in a number of organisms. Nevertheless, the role of the Nck/Grb4/Dock family of adaptor proteins has until recently remained unclear. An increasing number of biochemical interactions of Nck and Grb4 with proteins involved in axon guidance, and in mediating changes in cellular actin architecture has recently been observed. These interactions implicate Nck/Grb4 in signaling complexes downstream of RTKs which respond to guidance cues and mediate changes in the actin cytoskeleton. In vivo support for this model has come from a protein null mutation in a Drosophila homolog of mammalian Nck/Grb4 which causes severe defects in photoreceptor fasciculation and targeting (Garrity et al., 1996).

Targeting a mutation into the murine genome is a powerful technique that allows the precise ablation of a gene of interest. Examination of embryonic development in the absence of a specific protein can yield insights into its role in vivo. Furthermore, the generation of cell lines lacking endogenous protein can foster in vitro biochemical studies of signaling pathways that also give insight into the in vivo function of that gene product. For these reasons, to better assess the role that Nck and Grb4 play in mammalian development, a gene targeting approach was designed. Redundancy of function between two related genes can potentially mask some or all of the defects that might otherwise result from mutation of a single gene. Due to the similarity of Nck and Grb4, targeting of
both genes was undertaken to overcome possible functional redundancy between the two genes.

**Targeting the Nck and Grb4 Loci**

As sequence homology between the targeting vector and the genomic locus is required for a successful targeting strategy, genomic DNAs from the Nck and Grb4 loci were cloned. The restriction maps and exon/intron boundaries of the 5' portions of the Grb4 and Nck loci were characterized prior to construction of the targeting vectors since deletions in 5' exons favour the creation of protein null mutations (Joyner and Wurst, 1993). R1 ES cells were electroporated with the targeting vectors. To select against the majority of cells that did not incorporate the targeting vector into their genome or those that had incorporated it randomly, positive and negative selection was used (Mansour et al., 1988; Joyner and Wurst, 1993). Genomic DNA of surviving cell lines was subjected to restriction digests and probing with diagnostic probes to determine whether the loci had undergone homologous recombination events with the targeting vector.

The Grb4 vector was designed to delete the first coding exon with its translational start site. One ES cell line, E1, was found to have undergone the targeted mutation in the Grb4 locus. The initial Nck targeting vector was designed such that integration of the vector would result in a deletion of the first two coding exons and a concomitant deletion of approximately 15 kb of genomic sequence. As targeting ES cells with this vector was unsuccessful, a second vector was designed. As described, integration of this vector inserts a reporter gene encoding β-galactosidase into the locus under the transcriptional control of the Nck promoter. Two ES lines, F3 and G12, bearing the targeted mutation were isolated.
All three targeted ES cell lines were aggregated with staged diploid morulae which were re-implanted into pseudopregnant mice. These aggregations produced chimeric animals. No germline transmission of the E1 Grb4 ES cell line or the G12 Nck ES cell line was obtained. However, the F3 Nck ES cell line was transmitted through the germline of two chimeric animals. The mutation was transmitted with Mendelian ratios to homozygosity. This targeting event apparently created a null mutation as no Nck polypeptides were detected in homozygous mutant cells. The homozygous mutant animals were viable, fertile and exhibited no obvious phenotype. This is in contrast to the pupal lethality seen in *Drosophila* lacking the Nck/Grb4-like adaptor protein, Dock. These results indicate that Nck is not required for survival during mammalian development. Furthermore, as the targeted mutation in Grb2 adaptor protein has recently been shown to result in early embryonic lethality, this work clearly differentiates the role of Nck in cellular signaling from other adaptor proteins such as Grb2 (Cheng, A., unpublished results). Furthermore, the viability of the Nck deficient mice indicates that contrary to the suggestion from the biochemical data, Nck does not play a crucial role in Ras signaling upstream of MAP kinase. It should be noted that the possible redundancy of Nck and Grb4 might mask a role in Ras signaling.

**Expression of Nck and Grb4 in Adult Tissue**

The inability of the commonly used 3X3 antisera raised against Nck to discern between Grb4 and Nck was verified by immunoprecipitation and Western blotting of cells expressing Myc-tagged Grb4. Therefore, peptide sequences unique to Grb4 or Nck were used to create antisera that could immunoprecipitate either Grb4 or Nck. Following affinity purification, the Grb4 serum was shown to specifically recognize high levels of Grb4 in Cos cells transfected with tagged
Standardized protein lysates from various murine tissues were blotted to detect the amount of adaptor protein recognized by either the 3X3 sera or the Grb4 sera. The expression pattern observed with the 3X3 sera corresponds to a similar published study (Park, 1997). The Grb4 affinity purified sera did not detect any protein in adult murine tissue samples. This indicates that either there is no Grb4 present in the adult animals or that the avidity of the antibody is too low to detect the Grb4 present. The latter interpretation is likely to be correct since another laboratory has indicated that their antisera raised against peptides specific for Grb4 recognize a widely distributed 47 kd protein in murine tissue lysates (Li et al., unpublished data).

**Embryonic Expression of Nck**

The expression of β-galactosidase was assayed in mice carrying the targeted Nck locus. In both the heterozygous and homozygous transgenic but not wildtype animals, β-galactosidase expression was observed confirming that this activity was due to the targeting event. Hitherto, expression of Nck has only been defined in adult homogenized tissue samples. Specific and restricted expression was observed in several neural structures which had not been noted before. Staining was also observed in several non-neuronal structures such as the notochord, somites and non-neural (as well as neural derived) portions of the eye. Eyes were examined in some detail at several embryonic stages due to the profound defects observed in the pathfinding of R cells in larvae lacking Dock. However, on a morphological level, eyes were normal and more detailed studies await the generation of Grb4 deficient mice.

The cephalic neuroepithelium and neural tube exhibited high levels of β-galactosidase activity. Expression was strongest on the non-ventricular sides and was further restricted, in the neural tube, to the basal or ventral half.
region is associated with differentiated cells supporting the possible role of Nck signaling in post-mitotic cells such as during pathfinding of neurons. As this region is also associated with the birthplace of motor neurons, the observed expression patterns indicate that the pathfinding of motor neurons may be affected in the absence of the Nck/Grb4 family of adaptor proteins (Hogan et al., 1994). This indicates axonal pathfinding should be examined in detail during further phenotypic analysis.

The neural crest, a transient population of cells derived from the dorsal part of the neural tube contributes to several structures, including ganglia, which are encapsulated clusters of neurons (Nauta and Feirtag, 1986). High expression of the reporter of Nck was observed in the trigeminal (V), facio-acoustic (VII-VIII), and glossopharyngeal (IX) ganglia. Variegated expression observed in the dorsal root ganglia (DRG) may also have been due to neural crest cells and should be ascertained. As marking of murine neural crest cells with antibodies is difficult, vital dye labeling of embryos followed by further culturing can be undertaken along with labeling cells of the DRG for Nck expression (Serbedziji et al., 1990). The apparent high levels of Nck expression in neural crest cells raises the intriguing possibility that Nck may mediate signal transduction involved with the highly dynamic migrations of this group of cells.

Although neural crest cells migrate through somites, the somite is largely derived from mesenchymal cells of the segmental plates (Stern et al., 1988). Restriction of β-galactosidase expression appears to be medial between the rostral and caudal portions of the somite. It remains unclear whether this represents neural crest cells or a region of somatic mesoderm. Hence, in conjunction with Nck expression, examination of neural crest markers and metameric markers of the somite such as Dil1 should be undertaken (Tajbakhsh and Sporle, 1998). Intriguingly, the Eph and ephrin families have recently been
implicated in neural crest migration through somites. Higher vertebrates exhibit neural crest migration and motor axon growth that is restricted to the rostral compartment of somites (Keynes and Stern, 1984; Rickmann et al., 1985). In avian somites, neural crest cells, which express EphB3 mRNA were shown to migrate through the rostral half of the somite which also contained EphB3 mRNA expressing cells (Krull et al., 1997). Neural crest cells were excluded from the caudal half-sclerotome of the somite which expressed ephrin-B1; this exclusion was lost in vivo and in vitro in the presence of ephrin-B1 treatment indicating a functional significance to the asymmetric expression of receptor and ligand (Krull et al., 1997; Wang and Anderson, 1997). Furthermore, in vitro studies suggest that neural crest cells collapse and retract in the presence of ephrin-B1 much like neuronal cells (Krull et al., 1997; Wang and Anderson, 1997). These data suggest that Eph receptors and ligands mediate neuronal growth cone collapse through a similar mechanism in both axon pathfinding and neural crest migration. This evidence combined with the high levels of β-galactosidase in neural crest derived structures suggests a hitherto unexplored role for Nck mediated signaling in neural crest migration.

The expression of β-galactosidase is expected to represent the endogenous Nck expression pattern and thus represents the first insight into the regions of the embryo that express Nck at high levels. The neural staining is consistent with the putative role of Nck in signaling downstream from RTKs in response to axon guidance cues. The mammalian data that support this role are largely in vitro; the in vivo expression data presented here substantiates this model.

**Assessing the role of Nck/Grb4 downstream of RTKs - Future Work**
Generation of mice lacking Nck is a critical step in better understanding the role that the Nck/Grb4 family of adaptor proteins play in mammalian development. These mice, and the -/- Nck fibroblast cell lines will be critical reagents in assessing whether this family of proteins functions downstream of RTKs in signaling that controls cellular responses to guidance cues or in signaling responsible for actin cytoskeletal rearrangements. Much of the work here represents the generation of reagents that will allow specific experiments to address the in vivo role of these adaptor proteins. Furthermore, logical experiments to follow this work can now be realistically proposed with the reagents in hand.

**Confirmation of Reporter Construct Expression**

As previously discussed the expression of the reporter construct from the targeted Nck allele is thought to mimic endogenous Nck expression patterns. To confirm this hypothesis, several lines of experimentation could be undertaken. Not all antibodies function in immunohistochemistry experiments making the 3X3 antiserum, successfully used in wholemount *Drosophila* staining, an attractive choice for murine immunohistochemistry (Garrity et al., 1996). Thus, using this antiserum on wholemount or sectioned embryo samples would offer a simple means to confirm the expression pattern of β-galactosidase seen at E9.5-13.5. However, as the antiserum recognizes Grb4 as well, the expression domain may exceed that seen with β-galactosidase. In that case, further boosting and affinity purification of the antisera from rabbits immunized with peptides specific to Nck should be undertaken. As well, more peptide antibodies could be raised. Alternately, the untranslated region of *nck* cDNA can be used to generate an antisense RNA probe for in situ hybridization detection of the accumulation pattern of *nck* RNA. As the UTR differs significantly with that of Grb4, cross-
reactivity would not be expected as might be the case for a probe from the coding region (Aippersbach, E., unpublished data). As the Nck expression pattern defined by the reporter construct gives insight into the role of Nck in mammalian development, validating the use of the reporter construct is paramount.

In the event that the observed β-galactosidase staining differs significantly with that of endogenous Nck, the neo cassette can be removed from the nck locus. It has been demonstrated that neo can alter transcription from a locus (Moens et al., 1992; Nagy et al., 1998). Exposure of either the Nck F3 ES cells or the mutant line of mice to Cre recombinase will result in the excision of the floxed neo sequence (Jiang and Gridley, 1997). The expression of β-galactosidase in mice bearing this mutant allele would then more likely represent endogenous Nck expression as the only alteration at the locus would be the insertion of IRES-LacZ, as opposed to the additional insertion of neo.

Assessing regions of Nck or Grb4 specific expression

Assessing Nck expression will assist in the search for a phenotype in the mice lacking endogenous Nck. If a detectable phenotype exists, it will provide the first insight into the in vivo role of this family of adaptor proteins, in mammals. As no obvious phenotype has yet been detected, the embryonic domains where Nck is expressed highlight regions that deserve more scrutiny. As Nck is an intracellular protein, lack of expression would most likely result in a cell autonomous phenotype. However a non-autonomous role for Nck could be imagined if it were involved in a pathway that culminated in a signal being sent to other cells. As the ephrin transmembrane ligands are implicated in bi-directional signaling, interaction of Nck in this pathway could cause a non-autonomous defect. However, if the lack of Nck is compensated for by the presence of Grb4,
the phenotype will be masked. Thus, the obvious area to concentrate on when investigating a phenotype due to lack of Nck is any region wherein expression of Grb4 does not overlap with Nck. This requires that the developmental expression pattern of Grb4 be assessed.

To assess the Grb4 expression pattern two lines of experimentation could be pursued. The first would be in situ hybridization to detect RNA levels using the Grb4 UTR which differs significantly from the Nck UTR (Aippersbach, E., unpublished data). Secondly, the Grb4 specific antisera described in this work could be used for whole mount antibody staining if the avidity of the rabbit sera was shown to increase over subsequent boosts. Careful comparison of the expression pattern of Grb4 to that of Nck would then reveal which, if any, regions of the developing embryo expressed only one of these closely related adaptors, Nck and Grb4.

**Generation of Grb4-/- Mice**

The most direct method for dealing with the putative redundancy between Grb4 and Nck is by assessing the Nck mutation in a Grb4 mutant background and vice-versa. As the genomic locus of Grb4 has already been cloned, a targeting vector designed, and one line of targeted ES cells exists, future work should focus on the generation of a germline mutation in Grb4. The most obvious experiment is to use the existing targeted ES cell line to perform another aggregation. The lack of strong chimeras from the last aggregation may indicate that the ES cell line is compromised in its developmental potential. The work required to generate a successfully targeted ES cell line, however, warrants another aggregation to ensure germline contribution potential of this line has been adequately tested. Concomitantly, the vector should be electroporated again. The 1/267 targeting efficiency of the last round of electroporations
indicates the amount of homology between the vector and the Grb4 locus is sufficient to support a reasonable frequency of homologous recombination, in order to obtain more targeted ES cell lines.

Though the generation of Grb4 null mice from the existing targeting vector would be sufficient to address the issue of redundancy between Nck and Grb4, future work should include the construction of a new targeting vector. Based on the success of the β-galactosidase reporter in establishing a developmental expression pattern of Nck, a new Grb4 targeting strategy employing a reporter for Grb4 expression might prove useful. The high β-galactosidase expression driven by the Nck promoter was restricted to the cell bodies of neurons as β-galactosidase is not transported into axonal processes, unlike tau-β-galactosidase (Callahan and Thomas, 1994). Recent work in the murine olfactory system has indicated that tau-β-galactosidase can be used to highlight axon targeting (Mombaerts et al., 1996). As data suggest that axon targeting may be affected in the absence of Nck/Grb4, it would beneficial to stain β-galactosidase expressing cell bodies and axonal processes of neurons to observe directly targeting or fasciculation defects. Redesigning and constructing a targeting vector that inserts a tau-β-galactosidase reporter into the Grb4 locus would be useful in determining whether axon guidance is perturbed in Grb4 deficient mice.

Identification of phenotype in Grb4, Nck or Grb4/Nck deficient mice

Once Grb4 deficient mice are obtained, they can be assessed for a phenotype in a manner similar to Nck. However, unless there are specific domains of Grb4 expression wherein Nck is not expressed, a similar lack of phenotype might be observed. Production of doubly homozygous mutant mice
will be the critical experiment to address redundancy if no phenotype can be observed in Nck deficient mice.

Though the possibility exists that there will still be no observable phenotype in Grb4;Nck deficient mice, two lines of reasoning argue the contrary. Firstly, severe defects are observed in Drosophila lacking Dock expression. To date there has only been one Nck family member identified in both Drosophila and C. elegans. The adaptor family has been tightly conserved in evolution which indicates that function may also have been conserved. The pupal lethality and profound defects in axon guidance observed in the fly would likely be recapitulated in some manner in vertebrates. Secondly, redundancy in function has been addressed in several mammalian gene families by breeding compound mutant animals (Orioli et al., 1996). If the role of these adaptors in neuronal connectivity and viability is as profound as the Drosophila data suggests, then an effect should be observed when both Grb4 and Nck, the only known mammalian family members of this adaptor family, are mutated.

In the event of embryonic lethality, the block in development will be assessed. Dissections performed at various embryonic stages will assess the gestational stage at which the embryos no longer are viable. Gross morphological examination to determine cause of death will be undertaken. The stage of embryonic lethality will, therefore, guide in interpretation of the phenotype. In the event of a less severe phenotype, the compound homozygous mutant animals will be assessed in a similar fashion to the animals which are homozygous mutant for either Nck or Grb4.

Biochemical interactions have been shown for Eph receptors and their ephrins ligands for both Nck and Grb4 respectively (Holland et al., 1997; Stein et al., 1996; Holland, unpublished data). Mice deficient in two Eph receptors, Nuk/EphB2 and Sek4/EphB3 exhibit defects in the formation of commissures that
connect the two cerebral hemispheres (Henkemeyer et al., 1996; Orioli et al., 1996). If Nck or Grb4 function in signaling pathways downstream of these RTKs as the biochemical data suggests, axon pathfinding defects may be observed in the corpus callosum, the anterior commissure or the diencephalic axon tract which are respectively defective in Sek4, Nuk, or Sek4; Nuk deficient mice (Henkemeyer et al., 1996; Orioli et al., 1996). Sectioning brains of Nck, Grb4 or Nck; Grb4 deficient mice to analyze these three axon tracts can be complemented by dye tracing experiments or antibody staining to visualize the axon projections. If this is not successful, analysis should focus on any areas that express only one of the adaptor proteins, Grb4 or Nck. Further sectioning of the entire brain complemented with dye tracking and axon staining can be undertaken if no defects are observed, however, this will require more effort than a focused analysis.

Loss of adaptor function has strengthened phenotypes of hypomorphic receptor mutations in genetic pathway such as the Sevenless RTK pathway (Olivier et al., 1993). Crossbreeding the Nck and Nuk mutant mice may reveal a genetic interaction between the adaptor and the receptor. The redundancy between Eph family members leads to a more pronounced defect in Sek4 and Nuk double mutants than observed with single mutants and can be exploited in a manner similar to hypomorphic receptor mutations. Nck cannot mediate all signaling downstream of Sek4 and Nuk as Nck deficient mice would then be expected to phenocopy the double homozygous mutant Nuk; Sek4 animals, which largely die from malformations in the palate (Orioli et al., 1996). A small imbalance in signaling downstream of RTKs like Sek4 due to lack of Nck, however, might reduce the functional compensation of Sek4 for Nuk. If the observed Nuk phenotype is more pronounced when combined with a lack of Nck,
these data would argue a genetic role of Nck in signaling downstream of Eph receptors and lend \textit{in vivo} support to the biochemical interaction studies.

\textbf{Biochemical Pathway Experiments}

The focus of this work has been the creation of mice that lack Nck and Grb4 to better define the role of the Nck/Grb4 adaptor family in mammalian development. The generation of Nck or Grb4 specific antisera and fibroblast cell lines that lack Nck, are also useful reagents for biochemical studies. To date, little or no distinction has been made between Nck and Grb4. Thus, the antibodies raised that can immunoprecipitate either Nck or Grb4 allow confirmation of previous interaction data. Nck has been shown to interact with the EphB2/Nuk receptor (Holland et al., 1997; Stein et al., 1998). Grb4 has been shown to interact with the conserved carboxy tail of the Eph receptor ligand, ephrin-B1 (Henkemeyer, M., and Holland, S., unpublished data). Adaptor binding to either the receptor or ligand may be a functional demarcation between Grb4 and Nck, and would be of interest to pursue with the peptide-specific antibodies.

The creation of primary fibroblast cell lines offers another avenue to test the \textit{in vitro} based predictions of signaling roles of Nck. As experimental variation can be a problem with primary fibroblasts, stable cell lines should also be established from these fibroblasts. If a Pak binding portion of Nck is localized to the cell membrane, Pak is activated (Lu et al., 1997). This implies that Nck mediated relocalization of Pak to the membrane is required for Pak activation (Lu et al., 1997). Hence, assaying the activation of Pak in Nck deficient fibroblasts would assess the prediction that Nck is required for this activation. The actin cytoskeletal rearrangements mediated by the Rho family of small GTPases have been described in fibroblast cell lines (Nobes and Hall, 1995). Thus, the Nck
deficient fibroblasts would provide an ideal environment to assess whether lack of Nck interferes with the formation of actin architecture such as filopodia and lamellipodia. The resulting projections from fibroblasts micro-injected with constitutively active GTPases constructs or proteins would be expected to be perturbed if Nck is the main adaptor through which these GTPases function to bring about their cellular effects. Thus, the work carried out in vivo targeting of the Nck gene has provided important reagents that will offer insight into the biochemical signaling pathways Nck functions in.

In vitro biochemical studies and work utilizing the invertebrate system have linked Nck/Grb4 to signaling pathways involved in remodeling cellular actin structures and responses to guidance cues mediated by RTK activation. To further address the role of these two adaptor proteins in mammalian development, a gene targeting strategy was undertaken. These efforts have characterized the genetic loci of both genes, produced viable homozygous Nck mutant mice, identified an expression pattern of a reporter for Nck, and created invaluable biochemical reagents. Future biochemistry and identification of a phenotype in mice lacking Nck and/or Grb4 can now build on this work to better elucidate the role of these adaptors in mammalian development.
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