Gene dosage-dependent functions for phosphotyrosine-Grb2 signaling during mammalian tissue morphogenesis

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Background: The mammalian Grb2 adaptor protein binds pTyr-X-Asn motifs through its SH2 domain, and engages downstream targets such as Sos1 and Gab1 through its SH3 domains. Grb2 thereby couples receptor tyrosine kinases to the Ras-MAP kinase pathway, and potentially to phosphatidylinositol (PI) 3′-kinase. By creating a null (Δ) allele of mouse *Grb2*, we have shown that Grb2 is required for endoderm differentiation at embryonic day 4.0.

Results: Grb2 likely has multiple embryonic and postnatal functions. To address this issue, a hypomorphic mutation, first characterized in the *Caenorhabditis elegans Grb2* ortholog *Sem-5*, was engineered into the mouse *Grb2* gene. This mutation (*E89K*) reduces phosphotyrosine binding by the SH2 domain. Embryos that are compound heterozygous for the null and hypomorphic alleles exhibit defects in placental morphogenesis and in the survival of a subset of migrating neural crest cells required for branchial arch formation. Furthermore, animals homozygous for the hypomorphic mutation die perinatally because of clefting of the palate, a branchial arch-derived structure. Analysis of *E89K*/Δ *Grb2* mutant fibroblasts revealed a marked defect in ERK/MAP kinase activation and Gab1 tyrosine phosphorylation following growth factor stimulation.

Conclusions: We have created an allelic series within mouse *Grb2*, which has revealed distinct functions for phosphotyrosine-Grb2 signaling in tissue morphogenesis and cell viability necessary for mammalian development. The placental defects in *E89K*/Δ mutant embryos are reminiscent of those seen in receptor tyrosine kinase-, Sos1-, and Gab1-deficient embryos, consistent with the finding that endogenous Grb2 is required for efficient RTK signaling to the Ras-MAP kinase and Gab1 pathways.

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Background

The Grb2 adaptor protein has a central SH2 domain and two flanking SH3 domains [1, 2]. Through its ability to bind pTyr-X-Asn motifs on autophosphorylated receptor tyrosine kinases (RTKs) and to simultaneously engage proline-rich motifs in the carboxy-terminal tail of Sos1/Sos2, Grb2 can physically couple growth factor receptors to the activation of the Ras GTPase [3–10]. GTP-bound Ras recognizes c-Raf, which stimulates the MAP kinase pathway as well as other potential effectors such as phosphatidylinositol (PI) 3′-kinase and Ral-GDS [11, 12]. Genetic analysis of tyrosine kinase signaling pathways in invertebrates and in the mouse has indicated that Grb2, and its orthologs in *C. elegans* and *Drosophila*, are critical for the in vivo activation of the Ras-MAP kinase pathway [3, 4, 9, 13]. While the N-terminal Grb2 SH3 domain appears to be critical for Sos1 activation, the C-terminal SH3 domain can engage the Gab1 docking protein; tyrosine-phosphorylated Gab1 binds the SH2 domains of signaling proteins such as PI 3′-kinase, Shp-2, and Crkl, raising the possibility that Grb2 may regulate effectors in addition to Ras [14–17].

RTKs either bind the Grb2 SH2 domain directly, or they can recruit Grb2 through intermediate scaffolding proteins such as ShcA, IRS-1, and FRS2 [18–20]. Indeed, RTKs such as Trk and fibroblast growth factor receptors (FGFRs) are dependent on scaffolding proteins to access Grb2 [21]. The prevalence of such Grb2 binding sites suggests that mammalian phosphotyrosine-Grb2 signaling may direct a variety of biological responses in vivo. Indeed, we have previously found that mouse embryos homozygous for a *Grb2* null (Δ) allele die early in development because of a defect in endoderm differentiation at E4.0 [9]. Furthermore, analysis of chimeric embryos formed by the aggregation of *Grb2*−/− embryonic stem (ES) cells and wild-type cells revealed a subsequent role for Grb2 in the formation of the epiblast at E6.0. The

failure of *Grb2* null cells to contribute to the ectoderm (epiblast) lineage of embryo chimeras precluded investigation of Grb2 functions later in development, however.

One approach toward circumventing the severe effects of the *Grb2* null mutation on early embryogenesis would be to generate a hypomorphic allele of the mouse *Grb2* gene. In this regard, a number of mutations have been identified in the *C. elegans* ortholog of *Grb2*, *Sem-5*. The *Sem-5 n1779* allele causes a defect in sex myoblast migration, but unlike more severe alleles, does not induce larval lethality or a highly penetrant vulvaless phenotype [22]. The *n1779* mutation results in the substitution of a glutamate residue with lysine at the BC1 position of the SH2 domain, within the phosphotyrosine binding loop. This substitution reduces, but does not abrogate, binding of Grb2 to phosphorylated proteins such as epidermal growth factor receptor [13] and ShcA. We have introduced the *n1779* mutation into the mouse *Grb2* gene, and have thereby created a *Grb2* hypomorphic allele. This has allowed the construction of an allelic series that has revealed multiple functions for Grb2 in mammalian development and cell signaling.

Results and discussion

Grb2 hypomorphic mutation

We engineered a point mutation in the mouse *Grb2* gene analogous to the *C. elegans n1779 Sem-5* mutation, which results in a substitution of glutamate 89 with lysine (E89K). To achieve this alteration, we replaced the wild-type exon 3 with one encoding the E89K substitution by homologous recombination in ES cells (Figure 1a–c). The mutation is therefore located within an otherwise normal *Grb2* locus; Western blotting of lysates from wild-type and mutant embryos (Figure 1d) confirmed that the *E89K* mutation does not perturb Grb2 expression or stability.

Although most *E89K* homozygous animals developed to term, none survived to postnatal day 1 (P1; Table 1), indicating that the *E89K* mutation creates a hypomorphic allele in the mouse. Homozygous mutant newborns were smaller (2.43 ± 0.15 cm) than their heterozygous (2.80 ± 0.11 cm) or wild-type (2.8 ± 0.12 cm) littermates. Gross autopsy failed to show significant morphological differences between wild-type and mutant newborns, except that *E89K* homozygotes never had milk in their stomachs and failed to fuse the secondary palate (Figure 2b; n = 12). Skeletal preparations (Figure 2c,d) indicated that the ossifying palate had fused at the midline in wild-type newborns, whereas the palatal shelves remained cleft in the *E89K* homozygotes. Thus, the death of homozygous mutant animals may result from failure to separate the nasopharynx and oropharynx [23, 24].

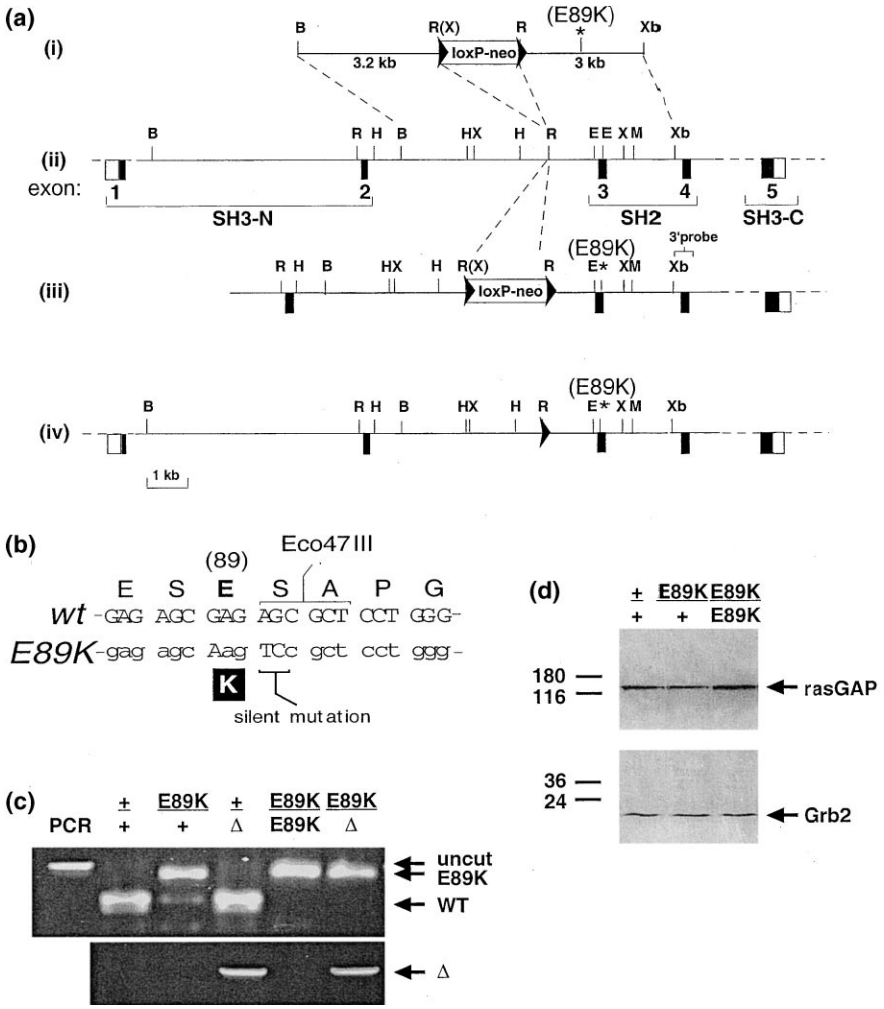
18.6% of the animals at birth were *E89K* homozygotes (Table 1), below the expected 25% Mendelian ratio, suggesting that a fraction of the mutants died in utero. At E13.5–E14.5, 6 out of 22 mutant embryos obtained were hemorrhaging or edemic (Figure 2f), unlike heterozygous (Figure 2e; n = 47) or wild-type (n = 16) littermates. Furthermore, 3 out of 22 of the homozygotes displayed abnormal development of the mandible, or lower jaw (Figure 2f). Some of the hemorrhaging mutant embryos appeared to be already dead at this stage, accounting for the low ratio of *E89K* homozygotes observed at birth.

Grb2 E89K/null compound heterozygotes exhibit defects in chorioallantoic fusion and placental morphogenesis

We reasoned that because the *Grb2* null embryos die at implantation and the homozygous *E89K* animals die during late embryogenesis or at P0, then embryos that are compound heterozygous for these two alleles might display an intermediate phenotype. We therefore intercrossed animals heterozygous for the null and hypomorphic (*E89K*) alleles. At midgestation (E9.5–E10.5), compound heterozygous *E89K*/Δ embryos were present in an approximately Mendelian ratio (23.8%; Table 1); thus, one copy of the hypomorphic allele provides sufficient Grb2 signaling for implantation and epiblast formation. *E89K*/Δ embryos displayed several phenotypes not explored in detail here, including abnormal heart development. Together with the hemorrhaging observed in the *E89K* homozygotes, this suggests a role for Grb2 in cardiovascular integrity.

By E11.5, *E89K*/Δ embryos were no longer viable (Table 1). The chorioallantoic placenta, or labyrinth, becomes essential around this time for sufficient nutrient and waste exchange between the maternal and embryonic circulations. The labyrinth develops in two phases, first with the attachment of the mesodermally derived allantois to a flat plate of chorionic trophoblast cells at E8.5, followed by folding and branching morphogenesis of the chorioallantoic interface and underlying blood vessels. Of the 65 *E89K*/Δ embryos dissected at E9.5–E10.5, 32% (n = 21) showed a failure in chorioallantoic fusion (Figure 3a,b). Histological examination of the placentas from the remaining mutant embryos showed that, although the allantois had attached to the chorionic plate, the *E89K*/Δ mutants had significantly smaller labyrinth structures. At E9.5, the chorioallantoic surface and underlying fetoplacental blood vessels in wild-type placentas showed extensive branching, whereas the mutants had only small folds of the chorioallantoic interface (Figure 3c,d). At E10.5 (Figure 3e–g), the morphogenesis of the *E89K*/Δ placentas was more evident than at E9.5, albeit still significantly less than wild-type, indicating that development is slowed rather than blocked. Interestingly, *E89K/E89K* homozygotes also had reduced labyrinth development, though less severe than that observed in the *E89K*/Δ mutants. Reduced placental function in the *E89K/E89K* homozygotes is a probable explanation for their reduced size at

Figure 1



Generation and characterization of the *E89K* hypomorph allele. **(a)** Strategy for introducing a point mutation affecting the SH2 domain into the *Grb2* gene (ii) in ES cells. The targeting construct (i) contains a neomycin selection cassette flanked by a pair of loxP sites (triangles). The *E89K* mutation (see [b]) was introduced by PCR. Mutant mice derived from targeted ES clones (iii) were crossed with mice transgenic for the *cre* recombinase [46], resulting in the excision of the neomycin cassette and generation of the *E89K* allele (iv). **(b)** DNA sequences of the wild-type and *E89K* allele surrounding the mutation. Mutated residues appear as capital letters, which indicate changes from E to K at position 89 and the silent mutation introduced to destroy the Eco47III site for allele-specific restriction mapping. DNA sequencing of RT-PCR products from mutant embryos confirmed the expected mutations in the mutant allele. **(c)** PCR analysis identifies the five potential genotype classes generated during this study. The top panel represents the PCR used to differentiate the wild-type and *E89K* alleles. The PCR product (far left lane) is digested with Eco47III enzyme; the *E89K* band is of higher molecular weight owing to the destruction of one cut site during the engineering of the allele. The bottom panel shows the same samples genotyped for the null (Δ) allele. **(d)** Western blot analysis of lysate extracted from wild-type (+/+), heterozygous (*E89K*/+), and homozygous mutant (*E89K*/*E89K*) E9.5 embryos. The top panel is probed with anti-p120rasGAP to show protein loading, and the bottom panel is probed with anti-Grb2, showing that expression and stability of the protein is not altered by the substitution at the BC1 position of the SH2 domain.

birth. The failure of placental development, either because of defects in chorioallantoic attachment or labyrinth morphogenesis, likely accounts for the death of the compound heterozygous embryos by E11.5.

The biological activities of the RTK FGFR2 and Grb2 are strikingly similar. Null mutations within Grb2 and a dominant mutation within the *FGFR2* gene result in early embryonic lethality at E4.0 [9, 25], whereas a null mutation in *FGFR2* results in defects in labyrinthine placental development [26] similar to that observed here. Indeed, a number of RTKs in addition to FGFR2, including the

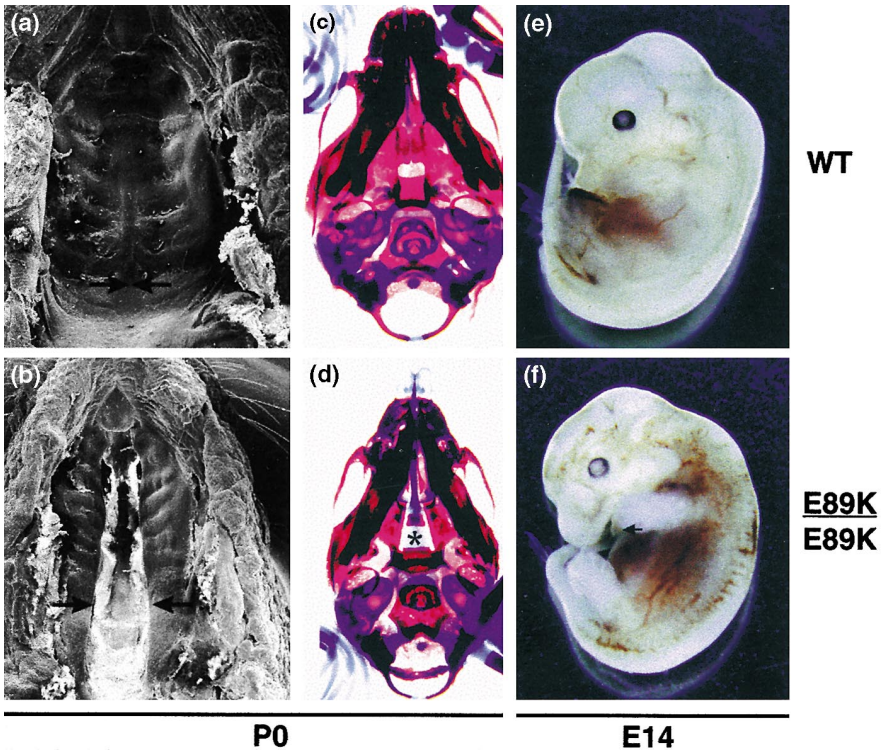
Table 1

Summary of Grb2 heterozygous null and hypomorph allele intercrosses.

	Stage analyzed	Wild-type	Heterozygous[a]	Mutant[b]
E89K/+	E10.5	22.0% (30)	49.0% (70)	29.0% (41)
intercrosses	E13.5–14.5	18.4% (16)	54.0% (47)	27.6% (24)
	E18.5/P0	35.4% (23)	46.1% (30)	18.6% (12)[c]
	P1	39.0% (12)	61.0% (19)	0
E89K/+: Δ/+	E9.5–10.5	22.7% (62)	53.5% (146)	23.8% (65)
intercrosses	E11.5	29.6% (8)	70.4% (19)	0[d]

[a] Heterozygous specimens were either E89K/+ or Δ/+.
[b] Mutant specimens were either E89K/E89K homozygotes or E89K/Δ compound heterozygotes.
[c] These embryos often survived longer than 6 hr after birth.
[d] Eight resorptions were observed, which likely represent the mutant progeny class; the material was too degraded for genotype analysis.

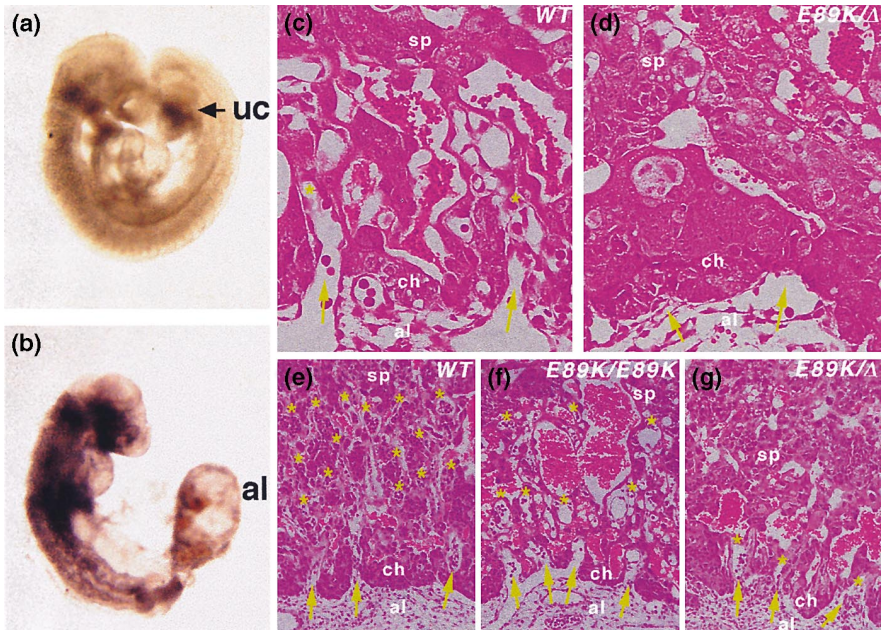
Figure 2



Grb2 hypomorph embryos exhibit defects in secondary palate fusion. **(a,b)** Scanning electron micrographs [48] of the palates of wild-type (a) and homozygous mutant (b) animals at P0 indicate that the palatal shelves have failed to fuse in the homozygous mutant embryos (arrows), a process normally completed by E15 of development. **(c,d)** Alizarian red/alician blue staining [49] of P0 skeletons indicates that the neural crest cell-derived bone that divides the nasopharynx and oropharynx does not fuse in the homozygous mutant embryo (asterisk). **(e,f)** Whole-mount photos of wild-type (e) and homozygous mutant (f) embryos at E14.0 of development shows the hemorrhaging that was observed in 27.3% (n = 22) of homozygous mutant embryos dissected at E13.5–E14.5. The arrow points to the lower jaw, or mandible, a neural crest cell/b2-derived structure that does not develop normally in all *E89K* homozygotes.

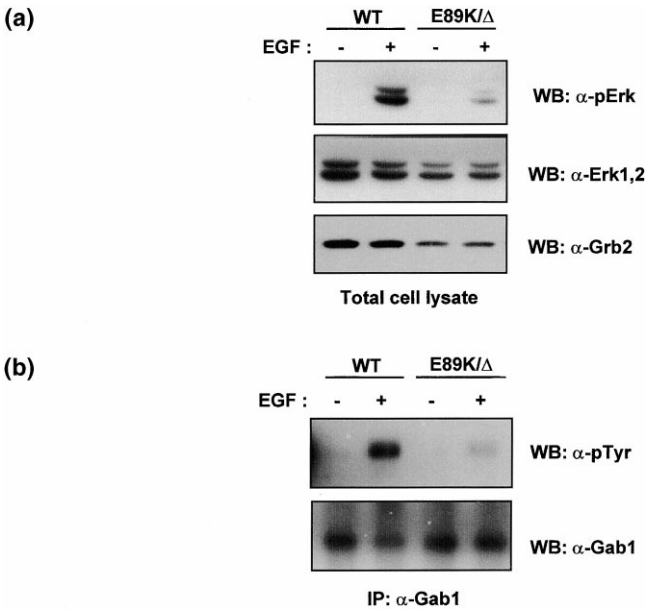
epidermal growth factor receptor (EGFR) [27] and Met [28], are required for normal development of the placental labyrinth. Given that a major biochemical function for Grb2 is to activate the Ras-MAPK cascade, it is significant that mutations in the *Sos1* [29] and *MEK1* [30] genes also lead to defective labyrinth development. Collectively,

Figure 3



E89K/Δ compound heterozygous embryos exhibit defects in placental development. **(a,b)** Whole-mount views of wild-type (a) and *E89K*/Δ (b) embryos at E9.5 illustrate the failure of the allantois to fuse with the chorion in many of the compound heterozygous embryos. **(c–g)** Hematoxylin- and eosin-stained histological sections of placentas at E9.5 (c,d) and E10.5 (e–g). The asterisks indicate the positions of fetoplacental blood vessels. The arrows indicate the sites of intrusion of stromal cells into the chorionic plate. Note that at E9.5, the chorioallantoic interface in *E89K*/Δ mutants is relatively flat in contrast to the wild-type placenta in which extensive interdigitations of the chorionic trophoblast, stroma, and underlying blood vessels are present. By E10.5, the chorioallantoic interface has undergone some morphogenesis in *E89K*/Δ embryos, but is severely reduced compared to wild-type and is comparable to a normal E9.5 placenta. Note that the *E89K*/*E89K* placentas show an intermediate phenotype. al, allantois; uc, umbilical cord; ch, chorionic trophoblast cells; sp, spongiotrophoblast.

Figure 4



MAP kinase activation and Gab1 tyrosine phosphorylation are compromised in *E89K*/Δ MEFs. MEFs derived from wild-type or *E89K*/Δ embryos were quiesced and stimulated with 5 ng/ml of EGF for 5 min at 37°C (+) or PBS control (−). **(a)** Twenty μg of total cell lysate was resolved by SDS-PAGE and then analyzed by immunoblotting with anti-phospho-ERK, anti-ERK1,2, or anti-Grb2 antibodies. **(b)** Lysates from the same preparation were immunoprecipitated with anti-Gab1 antibodies. Resolved immunoprecipitates were immunoblotted with anti-phosphotyrosine or anti-Gab1 antibodies.

these data begin to outline a signaling pathway that regulates chorioallantoic placental morphogenesis, most likely by mediating RTK signaling in the labyrinthine spongiotrophoblast layer.

If the *E89K*/Δ genotype causes a defect in MAP kinase activation, this should be detectable biochemically. To explore this point, we isolated early passage mouse embryo fibroblasts (MEFs) from *E89K*/Δ and wild-type E9.5 embryos, and compared the activation of the ERK1 and ERK2 MAP kinases in these cells following EGF stimulation. Quiescent, early passage MEFs were treated with EGF (5 ng/ml, 5 min, 37°C), or were left unstimulated, and were then analyzed for MAP kinase activation by immunoblotting of a whole cell lysate with phospho-specific ERK antibodies (Figure 4a). EGF stimulation strongly activated ERK1 and ERK2 in wild-type MEFs, but ERK phosphorylation was severely compromised in EGF-treated *E89K*/Δ mutant cells. Immunoblotting with anti-Grb2 antibodies showed that the mutant cells express approximately half as much Grb2 as do wild-type cells, as would be expected from the presence of the protein null allele combined with normal expression of the *E89K* mutant allele. Similar results were obtained from multiple early passage cultures isolated from different embryos, indicating that the biochemical defect observed in the mutant fibroblasts is indeed due to reduced Grb2 signaling. These data show that MAP kinase activation is defective in the *E89K*/Δ mutant MEFs.

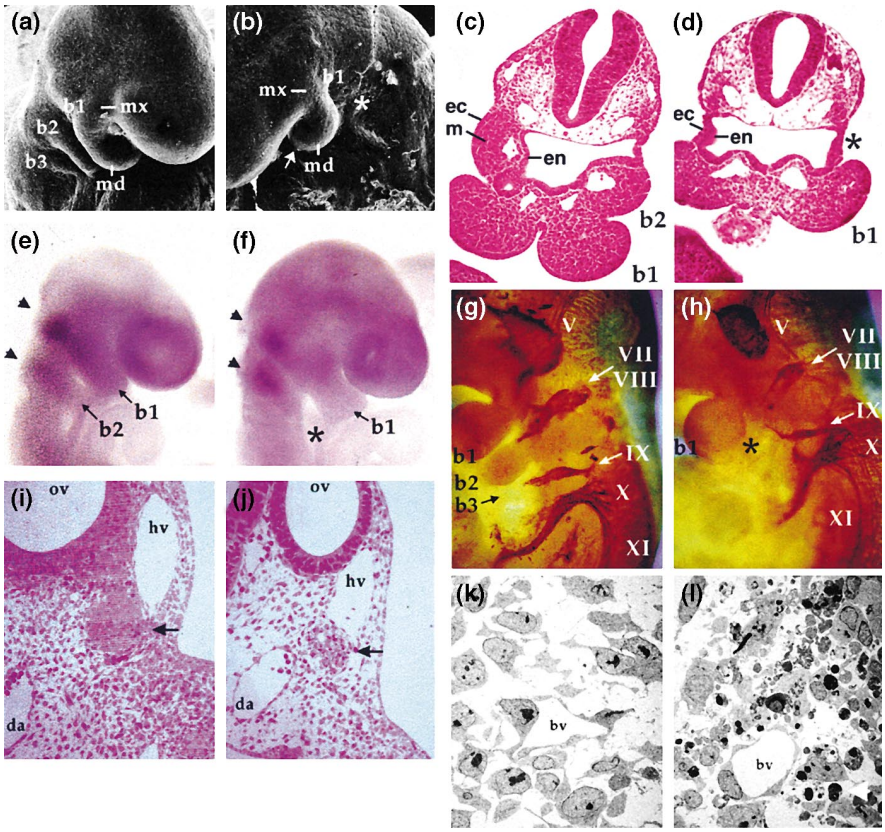
As noted above, the C-terminal SH3 domain of Grb2 binds the scaffolding protein Gab1 in a fashion that appears to recruit Gab1 to RTKs, resulting in Gab1 phosphorylation and its subsequent binding to effectors such as PI 3′-kinase and Shp-2. PI-(3,4,5)-P_3 generated by PI 3′-kinase activates targets involved in cell survival such as the Akt protein kinase, while Shp-2 can potentiate MAP kinase signaling [16]. Interestingly, *Gab1* mutant embryos display a placental defect, suggesting that the Grb2-Gab1 interaction may be of physiological relevance [31, 32]. Given that recent data suggest that Grb2 can link the EGFR to Gab1 [33], we examined Gab1 tyrosine phosphorylation in wild-type and *E89K*/Δ mutant cells stimulated with EGF (Figure 4b). Gab1 was heavily tyrosine phosphorylated in response to EGF stimulation of wild-type cells, but this was barely detectable in the *E89K*/Δ mutant MEFs. These data provide direct genetic evidence that Grb2 is required for efficient Gab1 tyrosine phosphorylation, and raise the possibility that Grb2 signaling to Gab1, as well as to Sos1, may be involved in placental morphogenesis.

Grb2 is required for branchial arch development

Intriguingly, the branchial arches beyond arch 1 (b1) failed to fully form in the *E89K*/Δ mutant embryos (Figure 5a,b). Histological sections demonstrated that branchial arch 2 (b2) is not present in mutant embryos observed at E9–E10 (Figure 5c,d). The ectoderm covering and endoderm lining were present, but the mesenchymal interior that comprises most of the branchial arch tissue was absent. The severe branchial arch defects in the *E89K*/Δ embryos are especially interesting, given that the *E89K*/*E89K* embryos exhibited dysmorphogenesis of branchial arch-derived skull elements such as the mandible and palate. Thus, the phenotype observed in the compound heterozygotes may reflect a more severe manifestation of the subtle phenotypes observed in the hypomorph homozygotes.

The majority of the mesenchymal cells of the branchial arches are descendants of neural crest (NC) cells. NC cells delaminate from the dorsal neuroepithelium and migrate throughout the embryo to form a wide range of derivatives, including most of the peripheral nervous system, all epithelial pigment cells, and many of the skull elements [34, 35]. Cad6 is a marker for the NC cells that will eventually populate the branchial arches [36]; in both wild-type and mutant embryos (Figure 5e,f), Cad6 expression defined two streams of cells extending from the dorsal neural tube, identifying cells that will colonize b1 and b2, respectively. Expression of AP-2, another marker for the

Figure 5



Compound heterozygous embryos exhibit defects in branchial arch development. **(a,b)** A comparison of SEM images [48] of an E9.5 wild-type (a) and an E10.0 *E89K/*Δ (b) embryo highlights the failure of the arches beyond b1 to develop in the compound heterozygotes. Furthermore, in the compound heterozygous embryos, b1 is not as highly developed as in the wild-type embryo, showing reduction in both the maxillary and mandibular components (white arrow). **(c,d)** Hematoxylin- and eosin-stained transverse histological sections just below the otic vesicle highlight the failure of b2 to develop in the *E89K/*Δ mutant (d) compared to wild-type embryos (c). **(e,f)** Whole-mount RNA in situ [50] probing for expression of *Cad6*. Two streams of neural crest cells are born at the dorsal neural tube and migrate towards b1 and b2 on the ventral side of the embryo (arrowheads). Expression was comparable between the wild-type ([e]; n = 5) and the *E89K/*Δ mutant ([f]; n = 5/6) embryos. **(g,h)** Whole-mount staining for anti-neurofilament (NF160, Sigma) in wild-type (g) and mutant (h) E10.5 embryos indicated that the neural crest-derived cranial ganglia develop normally in the compound heterozygous embryos, but the cranial nerves wander at the dorsal body wall because their targets do not form. **(i,j)** Toluidine blue-stained transverse plastic sections [47] of wild-type (i) and *E89K/*Δ mutant (j) embryos at the level of the otic vesicle show the pycnotic nuclei that cluster at this level in the mutant embryos. **(k,l)** Transmission electron micrographs [38, 39, 47] of the pycnotic nuclei indicate that the mesenchyme cells of the wild-type specimen (k) are healthy, whereas the mesenchyme of the compound heterozygote (l) are dying, as indicated by the apoptotic bodies (white arrowhead). b, branchial arch; mx, maxillary component of b1; md, mandibular component of b1; ec, ectoderm; m, mesenchyme; en, endoderm; ov, otic vesicle; hv, head vein; da, dorsal aorta; bv, blood vessel; *, b2 region in the mutant embryos.

NC cells of the craniofacial mesenchyme [23, 37], was also maintained in the *E89K/*Δ embryos (n = 3; data not shown). NC cells that originate at rhombomere 4 (r4) of the hindbrain give rise to the mesenchyme of b2 and contribute to cranial nerves VII/VIII. Staining for neurofilament indicated that all the cranial nerves and ganglia formed in the *E89K/*Δ embryos (Figure 5g,h), although cranial nerve X appears to be disorganized. Strikingly, cranial nerve VII, which normally projects into b2, wanders at the dorsal body edge in the compound heterozygotes, likely because its intended target, b2, does not develop normally. Together, these data show that the b2-destined NC population is born and begins its migration path normally, and that other r4-derived NC descendants develop normally in the compound heterozygous embryos.

Examination of transverse serial sections showed that large clusters of pycnotic nuclei were present in mutant embryos at the level of the otic vesicle (n = 15; Figure 5j) that were not observed in wild-type embryos (n = 11; Figure 5i). This is significant for two reasons. First, this location is in the migration path of NC cells, which are born at r4 and migrate toward the ventral body to b2; second, pycnotic nuclei indicate the presence of dying cells [38, 39]. Transmission electron microscopy showed that while there were few dying cells in the mesenchyme of wild-type embryos (n = 3; Figure 5k), there were numerous apoptotic bodies in the *E89K/*Δ specimens (n = 3; Figure 5l), which is a hallmark of programmed cell death [38, 39]. Furthermore, only mesenchymal cells were dying, as cells of other origins that reside in this region, such as endothelial cells (Figure 5l) or mesenchymal cells of b1 (data not shown), were healthy. This indicates that in the absence of appropriate Grb2 signaling, cells in this embryonic location, potentially NC cells en route to b2, die by apoptosis.

These results suggest that Grb2 may be required to promote the survival of a subset of cranial NC cells. Such cells require survival factors that are often transmitted by RTKs, including Trk for neurogenic cells [40], Kit for

melanocytes [41], and Ret for enteric ganglion precursors [42]. Of particular interest, animals lacking the EGF receptor have an increased incidence of cleft palate [24] and embryos that are deficient for α5 integrin show increased apoptosis of cranial NC cells [43], indicating that phosphotyrosine signaling is a requisite for craniofacial morphogenesis. Furthermore, MAP kinases [44] and PI 3′-kinase/Akt activated by Ras and Gab1 [45] can transmit cell survival signals. We cannot be certain whether Grb2 is acting in a cell-autonomous fashion to promote the survival of the cells that die in Grb2-deficient embryos, or whether this is a secondary phenotype resulting from a primary defect within the surrounding cells, tissues, or environment. Nonetheless, the phenotype of compound heterozygous embryos strongly suggests that Grb2 links phosphotyrosine signals to a survival pathway during branchial arch formation.

Null mutations can be very revealing; however, they suffer the disadvantage of only identifying the earliest stage at which a gene has a critical role. Strategies involving the generation of conditional alleles can potentially circumvent these problems, but depend upon efficient inactivation of the mutated gene. An approach that has been especially valuable in the analysis of gene function in invertebrates is the isolation of alleles with reduced biological function. Although hypomorphic alleles of mammalian genes have been obtained in the past, these usually have been created serendipitously. Here, we have described a rational approach toward creating a hypomorphic mutation in the mouse *Grb2* gene, based on an impaired allele of the orthologous *sem-5* gene in *C. elegans*, which compromises the ability of the SH2 domain to bind phosphorylated sites. By analyzing embryos homozygous for this hypomorph allele or compound heterozygous with the null allele, we have identified novel functions for Grb2 signaling during placental and craniofacial morphogenesis.

In the placenta, Grb2 appears to be important for the expansion and branching of the chorioallantoic interface to form the labyrinth cells, whereas Grb2 is essential for the survival of specific NC cells within the embryo proper. These results indicate that the same core phosphotyrosine-Grb2 signaling pathway has multiple successive and distinct functions during the development of the embryo. Furthermore, responses such as endoderm differentiation, placental morphogenesis, NC survival, and palate formation show different sensitivities to the level of Grb2. This suggests that molecules that regulate the potency of signaling through the core Grb2 pathway may determine its biological output.

Analysis of mutant embryos and newborn animals has revealed that Grb2 has a variety of biological functions that are critical for mammalian development. It is intriguing that mice that are deficient in specific signaling genes show defects that are very similar to those observed in *Grb2* mutants. In particular, mutations in RTKs [26] as well as in Sos1 [29] and the MAP kinase MEK1 [30] all cause abnormalities in placental labyrinth development reminiscent of those seen in *Grb2 E89K*/Δ mutant embryos. These findings raise the possibility that RTK signaling through the Grb2-Sos1 complex activates the Ras-MAP kinase pathway in a fashion that is critical for placental morphogenesis. Consistent with this scheme, *E89K*/Δ mutant cells are severely impaired in RTK-induced ERK activation. In addition, the Gab1 scaffolding protein has been implicated both as a binding partner for the C-terminal Grb2 SH3 domain and in placental development, and we find that RTK-stimulated Gab1 tyrosine phosphorylation is very markedly reduced in *E89K*/Δ MEFs. These data provide definitive genetic evidence that Grb2 mediates Gab1 phosphorylation, and suggest that a Grb2-Gab1 complex may participate in the development of the placental labyrinth. By comparing mouse mutant phenotypes and the signaling properties of mutant cells, we have begun to define the biochemical pathways through which Grb2 may exert its effects during the later stages of mammalian development.

Materials and methods

E89K mutation

Glutamate residue 89 lies in a 121 bp exon that codes for the amino-terminal region of the SH2 domain. To introduce the *E89K* mutation, a mutating primer (5′-gGA ctT gct ctc tcg gat cag gaa-3′; capital letters indicate the mutated nucleotides) was used together with E47-80T (5′-cag agc cag gta aga gcc cca-3′) in a polymerase chain reaction to generate a 280 bp fragment. This fragment carried the *E89K* mutation, with nucleotides changed from GAG (glutamate) to AAG (lysine), immediately followed by a silent mutation at the serine residue, with nucleotides changed from AGC to TCC to destroy the Eco47III restriction site (Figure 1b). The PCR product was digested with Eco47III and subcloned into the corresponding Eco47III sites in the 3 kb EcoRI-XbaI genomic fragment, which was subsequently used as the targeting right arm; the 3.2 kb BamHI-EcoRI fragment served as the left arm (Figure 1a [i]). One clone, C5, yielded germline transmission of the *E89K* (neomycin+) allele (iii). To excise the neomycin cassette from the germline DNA, mutant mice were bred to a mouse strain transgenic for the *cre* recombinase [46] whose expression was directed by the CMV promotor. Littermates derived from these crosses were analyzed for excision of the neomycin cassette (by Southern blotting and PCR) and the presence of the *E89K* substitution (by PCR and restriction analysis; [iv]). To further confirm the *E89K* mutation and the DNA sequence of the mutated 121 bp exon, RT-PCR and sequence analysis were performed on total RNA isolated from mutant embryos.

Genotype analysis

Isolated DNA samples amplified with PCRXhoB (5′-ttg ggt cca ggt gaa cac cag ga-3′) and E47-80T (above) oligonucleotides. PCR products were purified and digested with Eco47III to differentiate the wild-type and *E89K* alleles. In a separate polymerase chain reaction, PCRXhoB and Neo1 (5′-cct tct atc gcc ttc ttg acg cg-3′) were used to amplify the null allele. Products were then subjected to agarose gel electrophoresis and genotype determined on the basis of fragment size (Figure 1c).

Western blot analysis

Litters from *E89K*/+ intercrosses were dissected at E9.5; the embryos were immediately frozen on dry ice and the yolk sacs were used for

genotyping. +/+, *E89K*/+, and *E89K*/*E89K* embryos were lysed in Tx-100 lysis buffer and the protein concentration was determined by BCA assay (Pierce). Equivalent amounts of lysates were analyzed by Western blotting with anti-Grb2 (Transduction Laboratories) and anti-p120ras-GAP [47] antibodies as previously described [48]. For analysis of MEFs, cells were quiesced by growing in a serum-free medium for 20 hr, and were then treated with EGF (5 ng/ml for 5 min at 37°C) or PBS alone, and then lysed. Twenty μg of total cell lysates was resolved by SDS-PAGE and then analyzed by immunoblotting with anti-phospho-ERK (New England Biolabs), anti-ERK1,2 (Santa Cruz Biotechnology), or anti-Grb2 antibodies. Alternatively, 200 μg of cell lysate was immunoprecipitated with anti-Gab1 antibodies, followed by SDS-PAGE and Western blotting with anti-phosphotyrosine or anti-Gab1 antibodies.

Isolation of MEFs

E9.5 mouse embryos were dissected and incubated in cell dissociation solution (Sigma) and then cultured in DMEM supplemented with 20% FBS and 100 units/ml each of penicillin and streptomycin. DNA extracted from the yolk sacs and resulting fibroblasts was used for genotyping by PCR. For experiments, MEFs from passage numbers 2 and 3 were used.

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