Essential Role of Gab1 for Signaling by the c-Met Receptor In Vivo

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Abstract. The docking protein Gab1 binds phosphorylated c-Met receptor tyrosine kinase directly and mediates signals of c-Met in cell culture. Gab1 is phosphorylated by c-Met and by other receptor and nonreceptor tyrosine kinases. Here, we report the functional analysis of Gab1 by targeted mutagenesis in the mouse, and compare the phenotypes of the *Gab1* and *c-Met* mutations. Gab1 is essential for several steps in development: migration of myogenic precursor cells into the limb anlage is impaired in *Gab1*−/− embryos. As a consequence, extensor muscle groups of the forelimbs are virtually absent, and the flexor muscles reach less far. Fewer hindlimb muscles exist, which are smaller and disorganized. Muscles in the diaphragm, which also originate from migratory precursors, are missing. Moreover, *Gab1*−/− embryos die in a broad time window between E13.5 and E18.5, and display reduced liver size and placental defects. The labyrinth layer, but not the spongiotrophoblast layer, of the placenta is severely reduced, resulting in impaired communication between maternal and fetal circulation. Thus, extensive similarities between the phenotypes of c-*Met* and *HGF/SF* mutant mice exist, and the muscle migration phenotype is even more pronounced in *Gab1*−/−:*c-Met*+/− embryos. This is genetic evidence that Gab1 is essential for c-Met signaling in vivo. Analogy exists to signal transmission by insulin receptors, which require IRS1 and IRS2 as specific docking proteins.

Key words: hepatocyte growth factor • gene targeting • migration of muscle precursors • placenta development • liver development

Introduction

In the last decade, various receptor tyrosine kinases and their ligands have been molecularly characterized (for reviews see Schlessinger and Ullrich, 1992; van der Geer et al., 1994). In cell culture, the activated receptors elicit specific cellular responses (e.g., proliferation, motility, morphogenesis, differentiation, or survival). In vivo, the receptor tyrosine kinases and their ligands regulate decisive events in development (Birchmeier and Birchmeier, 1993; Lemke, 1996; Pachnis et al., 1998; Holder and Klein, 1999).

Various cellular responses are observed when the c-Met receptor is activated by its specific ligand HGF/SF in cell culture that depend on the cell type used as well as on the exact culture condition. Epithelial cells can respond by scattering, motility or invasiveness, by growth, as well as by formation of branched tubular structures (Stoker et al., 1987; Weidner et al., 1990, 1993; Gherardi and Stoker, 1991; Montesano et al., 1991). The c-Met receptor was initially identified because of its oncogenic potential when mutated (Park et al., 1986), and various evidence implies HGF/SF and c-Met in tumorigenesis and metastasis (Di Renzo et al., 1991; Jeffers et al., 1996, 1997; Sakata et al., 1996; Meiners et al., 1998; Takayama et al., 1997). Activating mutations in the c-*Met* gene are observed in hereditary renal papillary carcinomas in humans (Schmidt et al., 1998; Zhuang et al., 1998).

Two phosphorylated tyrosyl residues in c-Met, Y1349 and Y1356, are essential for its function in vitro and in vivo (Ponzetto et al., 1994; Fixman et al., 1995; Weidner et al., 1995; Maina et al., 1996; Sachs et al., 1996; Giordano et al., 1997). These residues constitute a bivalent docking site that recruits various signaling and adapter proteins like PI(3) kinase, phospholipase Cγ, Src, Shc, Grb2, and Gab1 (Ponzetto et al., 1994; Zhu et al., 1994; Fixman et al., 1995; Pelicci et al., 1995; Weidner et al., 1996). Gab1 requires Y1349 and, to a lesser degree Y1356, for binding to the c-Met receptor (Holgado-Madruga et al., 1996; Weidner et al., 1996). Gab1 is a member of the family of docking proteins that include insulin receptor substrates (IRS-1, IRS2, and IRS-3), FGF receptor substrate (FRS-2/SNT1), the p62dok subfamily, *Drosophila* DOS (daughter of seven-

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less), and linker for activation of T cells (Voliovitch et al., 1995; Herbst et al., 1996; Raabe et al., 1996; Carpino et al., 1997; Kouhara et al., 1997; Yamanashi and Baltimore, 1997; Gu et al., 1998; Zhang et al., 1998). These proteins are characterized by an NH_2-terminal pleckstrin homology (PH)[1] domain or myristilation sequence, a central phosphotyrosyl binding domain (usually PTP) and multiple tyrosyl residues that function as docking sites for SH2 domain–containing molecules. Unique to Gab1 is a novel phosphotyrosyl recognition domain that mediates the binding to phosphorylated c-Met (Weidner et al., 1996; Schaeper et al., 2000). Gab1 is not only phosphorylated by c-Met, but is also indirectly activated by other tyrosine kinases. Extracellular stimuli like EGF, insulin, IL3, IL6, Epo1, or the activation of the B cell receptor result in phosphorylation of Gab1 (Holgado-Madruga et al., 1996; Ingham et al., 1998; Takahashi-Tezuka et al., 1998; Lecoq-Lafon et al., 1999; Rodrigues et al., 2000). PI(3) kinase, Shc, Shp2, and CRKL are direct interaction partners of Gab1 (Holgado-Madruga et al., 1996; Bardelli et al., 1997; Maroun et al. 1999; Schaeper et al., 2000). Association of Gab1 with Shp-2 is essential for the formation of branched tubules by cultured MDCK epithelial cells (Schaeper et al., 2000). Association of Gab1 with PI(3) kinase is important for the prevention of apoptosis (Holgado-Madruga et al., 1997). The PH domain of Gab1 mediates Gab1 translocation to the plasma membrane in response to EGF (Maroun et al., 1999; Rodrigues et al., 2000). Together, these data obtained by in vitro experiments imply Gab1 in the signaling of different tyrosine kinases, which recruit Gab1 either directly or indirectly. Indeed, whether Gab1 plays a functional role in various pathways in vivo is the focus of this work.

Targeted mutations of the *HGF/SF* and *c-Met* genes in mice cause identical phenotypes, i.e., embryonal lethality due to a severe deficit in development of the placenta (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995). Such animals also display a reduced liver size and, remarkably, lack particular muscle groups like the muscles of extremities, diaphragm, and tongue. These muscles derive from a migrating precursor population. In c-*Met* or *HGF/SF* mutant mice, migration of myogenic precursor cells is defective: the precursors remain in the dermomyotome, a derivative of the somite, and do not migrate to their targets in the limbs, branchial arches, and the septum transversum (Bladt et al., 1995; Dietrich et al., 1999).

Here, we examined the function of *Gab1* in mice by generating a targeted mutation using embryonic stem (ES) cell technology. A fundamental role of Gab1 for c-Met–specific signaling was found: *Gab1*−/− mutant embryos are characterized by embryonal lethality (because of placental defects), by reduced liver size, and by reduced and delayed migration of myogenic precursor cells. This is reminiscent of the phenotype of *HGF/SF*−/− and *c-Met*−/− mutant embryos. Therefore, our data demonstrate that in vivo Gab1 is an essential mediator of c-Met receptor signals.

[1]*Abbreviations used in this paper:* ES, embryonic stem; PH, pleckstrin homology.

Materials and Methods

Generation of Gab1-deficient Mice

For the construction of the Gab1 targeting vector, genomic fragments isolated from a λFIXII 129/Ola library were introduced into the pTV0 vector (Riethmacher et al., 1995). The Gab1 vector contained at the 5′ end a 1.5-kb genomic sequence ending with codon 26 of Gab1, which was fused in-frame with the β-galactosidase gene that harbors a nuclear localization signal. At the 3′ end, a genomic 10-kb BamHI fragment is present in the vector. Linearized targeting vector was introduced into E14-1 ES cells by electroporation. Homologous recombination events were enriched by selection with G418 and gancyclovir. The structure of the mutant locus and the absence of additional integration events were verified by Southern hybridization. Several independent ES cell clones were used to generate chimeric mice by blastocyst injection as previously described (Riethmacher et al., 1995). Two independent mouse lines with mutated *Gab1* were obtained, and analyzed on a mixed 129/C57Bl6 background.

Mice and embryos were genotyped by β-galactosidase staining of ear tissue as previously described (Hogan et al., 1994) or by PCR using DNA from the tail or visceral yolk sac. PCR primers, PCR1 (CCCTTTGTGGATGGCTTCTTTGT, 300 nM) and PCR2 (TTCTTGGCATGATCGTTTTTGTAA, 300 nM) specific for the wild-type allele, and KO2s (GGATCCCGTCGTTTTACAACG, 240 nM) and KO2as (ACCACAGATGAAACGCGGAGT, 240 nM) specific for the mutated allele were used in a combined reaction in *Taq* buffer (1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1.6 U *Taq* polymerase; GIBCO BRL). Amplification of mutant and wild-type Gab1 alleles generated diagnostic bands of 450 and 336 bp, respectively.

Western Blot Analysis

E14.5 embryos were lysed in Triton buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, and 1 mM PMSF). 10 μg of the lysate was subjected to 7.5% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with PBS-milk (PBS with 5% nonfat dry milk), washed, and incubated overnight with anti–mouse Gab1 antibodies (α-mGab1, 1:500; see below) or anti–human Gab1 (α-hGab1, 1:400, against the COOH-terminal amino acids 664–694; Upstate Biotechnology). HRP-conjugated goat anti–rabbit IgG (1:1,500) and enhanced chemiluminescence substrate (Amersham Pharmacia Biotech) were used for detection of Gab1 protein. The antiserum against mouse Gab1 was produced in rabbits, using the Met binding domain of mouse Gab1 (amino acids 391–541) as the antigen, and affinity-purified.

In Situ Hybridization Analysis and Histological Analysis

Whole mount in situ hybridization using Lbx1 and SHH as probes was performed as previously described (Wilkinson, 1992; Echelard et al., 1993; Brohmann et al., 2000). Placentas of E13.5 embryos were immediately frozen in Tissue Tek and cryo-sectioned (12 μm). In situ hybridization was performed with ^{35}S-labeled probes (Dlx3 or Gcm1) or digoxyginin-labeled probes (Flt1) as previously described (Sonnenberg-Riethmacher et al., 1996). For monitoring development, tissues were fixed in 4% formaldehyde, embedded in paraplast (Oxford Labware), and 7-μm sections were prepared and counterstained with hematoxylin-eosin. Immunohistochemistry of muscle tissue was performed using a 1:2,000 dilution of monoclonal anti–skeletal fast myosin antibody (Sigma M4276) and a 1:100 dilution of the secondary antibody, alkaline phosphatase conjugated anti–mouse IgG (Jackson ImmunoResearch Laboratories). 1% orange G was used as a histological counterstain. The sections were examined by light microscopy (Zeiss Axiovert), scanned by a ProgRes 3012 videocamera (Jenaoptik), and processed using Adobe Photoshop 4.0 software.

Results

Phenotype of Gab1-deficient Mice

We generated a *Gab1* mutation by homologous recombination in ES cells. In the targeting vector, a lacZ cassette was fused in-frame to the exon that encodes the major part of the NH_2-terminal PH domain of Gab1, replacing a 1.5-

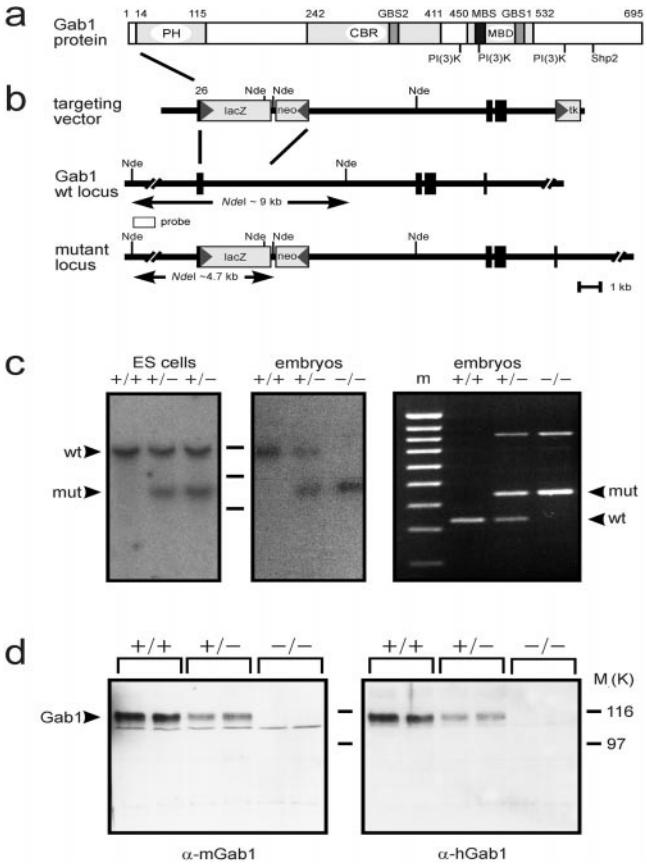

Figure 1. Outline of the strategy used to disrupt the *Gab1* gene. (a) Schematic representation of the Gab1 protein structure. Gab1 contains all of the following: an NH_2-terminal PH domain; a crk binding region (CBR); two SH3 consensus binding sites for Grb2 (GBS1 and GBS2, dark gray); the Met binding domain (MBD), with amino acids essential for c-Met binding (MBS, black box); and consensus binding sites for SH2 domains of PI(3) kinase and Shp2 (Schaeper et al., 2000). (b) Schematic representation of the *Gab1* targeting vector and the wild-type and mutant *Gab1* alleles. Exon sequences are represented by black boxes; the lacZ cassette was fused in-frame to codon 26 of *Gab1*. The neomycin resistance (neo) gene and thymidine kinase (tk) gene from the herpes simplex virus are shown in gray boxes, with arrowheads indicating the direction of transcription. The open box indicates the probe used for Southern hybridization analysis. The sizes of the restriction fragments generated by NdeI in wild-type and mutant DNA are indicated. (c) Southern blot analysis of genomic DNA from wild-type (+/+) and *Gab1* heterozygous (+/−) ES cells and from E12.5 wild-type, heterozygous, and homozygous (−/−) mutant embryos. DNA molecular mass standard: 9.4, 6.6, and 4.4 kb, respectively. A PCR analysis of the *Gab1* locus of wild-type, heterozygous, and homozygous mutant embryos is shown on the right. (d) Western blot analysis from extracts of each of two embryos at E12.5 of the indicated genotypes. Two different antibodies directed against MBD and the COOH terminus of Gab1 (anti–mouse and anti–human Gab1) were used (see Materials and Methods).

kb fragment of genomic DNA (Fig. 1, a and b). Homologous recombination events were identified by Southern blot analysis, yielding a novel fragment of 4.7 kb (Fig. 1 c). Using two lines of mutant ES cells, we generated chimeric and *Gab1* heterozygous mice, which were largely healthy and fertile. Approximately 5% of the Gab1+/− mice of both lines showed spasms after 2–3 mo and died; we did not examine these mice further. Matings between heterozygous animals produced no live *Gab1*−/− offspring, implying that the mutant embryos die during embryogenesis. To determine the time of death, embryos from different developmental stages were dissected and genotyped by Southern blot and PCR (Table I and Fig. 1 c). Up to day 12.5 of embryogenesis, the expected Mendelian ratio of homozygous mutant embryos was observed. At E13.5 to E18.5, the proportion of viable *Gab1*−/− embryos declined, and the surviving embryos showed a gradual delay in growth and development. No *Gab1*−/− embryos were observed at P_0 (Table I). Frequently, *Gab1*−/− embryos were found dead in utero, as judged by the absence of a heartbeat or by signs of resorption. These data indicate that *Gab1*−/−, c-*Met*−/−, or *HGF/SF*−/− mutants die during similar time windows (Schmidt et al., 1995; Uehara et al., 1995; Bladt et al., 1995). To test for Gab1 protein in the mutants, we prepared lysates from E12.5 embryos and analyzed them by immunoblotting using antiserum against the c-Met–binding domain of mouse Gab1 or the COOH terminus of human Gab1. Compared with wild-type embryos, Gab1 protein levels were reduced in *Gab1*+/− mice. Both antisera did not detect Gab1 protein in lysates from *Gab1*−/− embryos (Fig. 1 d). Thus, the introduced mutation in the *Gab1* gene corresponds to a null mutation.

We examined the structure of the placenta of control, *Gab1* and *c-Met* mutant embryos at E13.5. Macroscopically, the placental labyrinth layer of mutant embryos contained less blood than the one of controls (not shown). Cryosections of placentas at E13.5 were analyzed by in situ hybridization with probes for the trophoblast-specific transcription factors Dlx3 and Gcm1, which mark the labyrinth layer (Morasso et al., 1999; Anson-Cartwright et al., 2000). Compared with controls, a reduced thickness and severe disorganization of the placental labyrinth layer is apparent in *Gab1*−/− and *c-Met*−/− embryos (L in Fig. 2, d, e, g, and h, compare with placenta from control in Fig. 2, a and b) The labyrinthine layer of wild-type placenta consists of a dense network of numerous, fine embryonic blood vessels and larger maternal sinuses filled with enucleated maternal blood cells. Sections of placentas were

Table I. Summary of Genotypes Resulting from Gab1 +/− Intercrosses

		Percent genotype of live embryos		
Age*	No. of pups	+/+	+/−	−/−
E9-10.75	131	22	48	22
E12.5	84	25	51	24
E13.5	182	29	55	16
E14.5	105	37	51	12
E15.5	52	29	56	15
E16-17.5	39	26	67	8
E18.5	37	22	73	5
P_0	32	28	72	0
W3	214	32	68	0

*E9-18.5 represent day 9-18.5 of gestation. P_0 embryos were surgically removed after gestation; W3 are 3-wk-old mice.

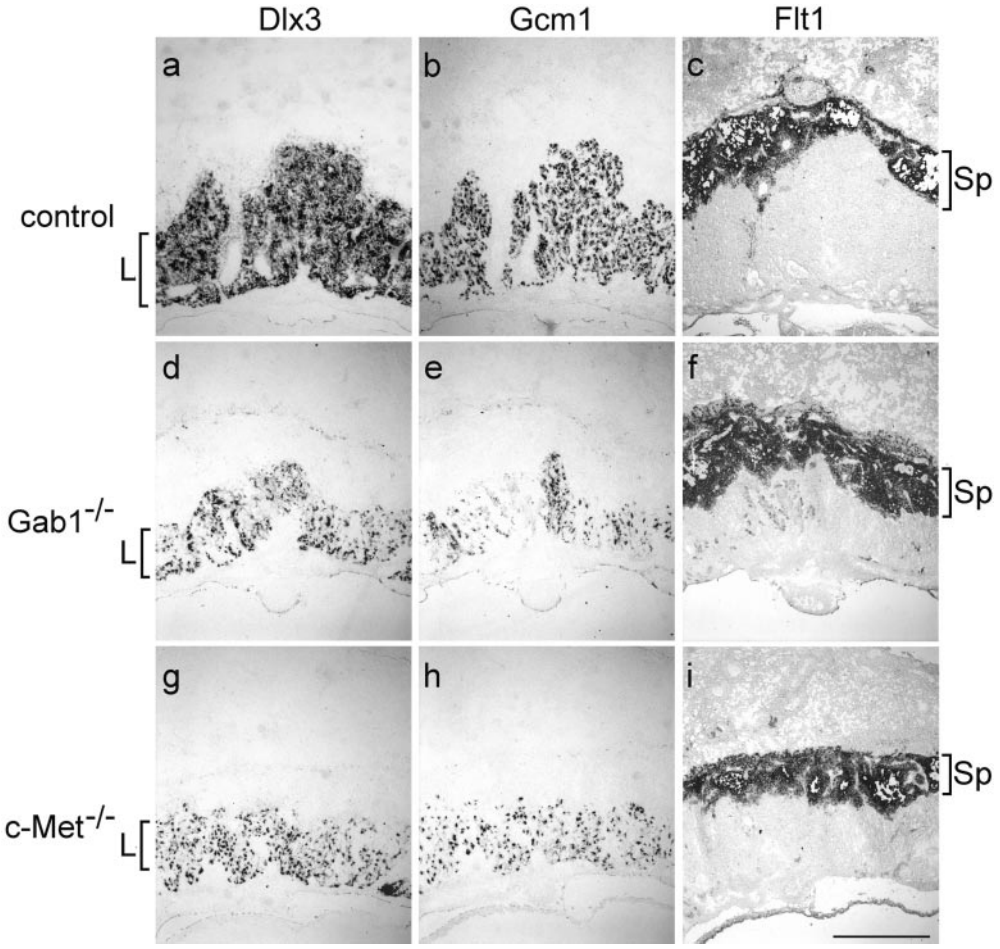

Figure 2. Analysis of the placenta from control (a–c), *Gab1*−/− (d–f), and *c-Met*−/− embryos (g–i). In situ hybridization with probes Dlx3 (a, d, and g) and Gcm1 (b, e, and h) specific for trophoblast cells in the labyrinth layer (L) and probe Flt1 (c, f, and i) specific for the spongiotrophoblast layer (Sp). Note the reduced size of the labyrinth but not the spongiotrophoblast layer and the reduced numbers of trophoblast cells of *Gab1*−/− and *c-Met*−/− placentas. Bar, 1 mm.

also stained with isolectin B_4 from *Bandeiraea simplicifolia*, which visualizes the extracellular matrix surrounding fetal blood vessels that are particularly abundant in the labyrinthine layer (Ohlsson et al., 1999). In the labyrinth layer of *Gab1* mutants, the isolectin B_4–stained matrix around fetal blood vessels is fragmented, trophoblast cells are reduced in number, and fewer blood cells are detectable in the labyrinth layer. Occasionally, fetal blood cells are infiltrating the trophoblast layer of Gab1 mutant placentas, indicating a breakdown of the endothelial lining of embryonic blood vessels (data not shown). In contrast, the normal distribution of Flt-1 mRNA (Breier et al., 1995) indicates that the spongiotrophoblast layer of the placenta is not affected in *Gab1* and *c-Met* mutant embryos (Sp in Fig. 2, c, f, and i). Since the labyrinth layer is required for the exchange of oxygen and nutrients between maternal and fetal circulation, impaired development of the placenta is a likely cause of the embryonic lethality of *Gab1*−/− and *c-Met*−/− embryos (Schmidt et al., 1995; Uehara et al., 1995; Bladt et al., 1995).

We also compared the liver size of control and *Gab1*−/− embryos at E14.5. The ratio of liver to bodyweight was 8.8×10^{-2} in wild-type, 7.8×10^{-2} in *Gab1*+/−, and 5.0×10^{-2} in *Gab1*−/− embryos (each SD $\leq 1.4 \times 10^{-2}$). Thus, the size of the liver is markedly reduced in *Gab1*−/− embryos. A similar reduction in the ratio of liver to bodyweight is observed in c-*Met*−/− embryos at E14.5 (10.6×10^{-2}, 9.8×10^{-2}, and 5.3×10^{-2} in wild-type, c-*Met*+/−, and c-*Met*−/− embryos, respectively; C. Birchmeier, unpublished data).

Long-range Migration of Muscle Precursor Cells in Gab1 Mutant Embryos

Muscles of limbs, diaphragm, and the hypoglossal cord are generated by migrating precursor cells that delaminate from lateral dermomyotome, a derivative of the somite (Chevallier et al., 1977; Christ et al., 1977). The *Lbx1* gene encodes a homeobox-containing transcription factor that is strongly expressed in migrating muscle precursor cells. These cells form distinct streams in the wild-type E10.25 embryo (Fig. 3, a and b, the upper arrowhead marks the hypoglossal stream, the lower arrowhead, the forelimb, and the arrow mark muscle precursors cells retained in the dermomyotome; Jagla et al., 1995; Dietrich et al., 1998). In c-*Met* or *HGF/SF* mutant mice, the muscle precursor cells remain in the dermomyotome and do not take up long-range migration (Fig. 3 d; Bladt et al., 1995). In *Gab1*−/− embryos, some delamination of myogenic precursor cells occurs, but is strongly reduced in efficiency (Fig. 3 c). Compared with control embryos, less cells have left the occipitally located somites in *Gab1* mutants, and the precursor stream headed towards the floor of the branchial arches contains a reduced number of cells. Moreover, the

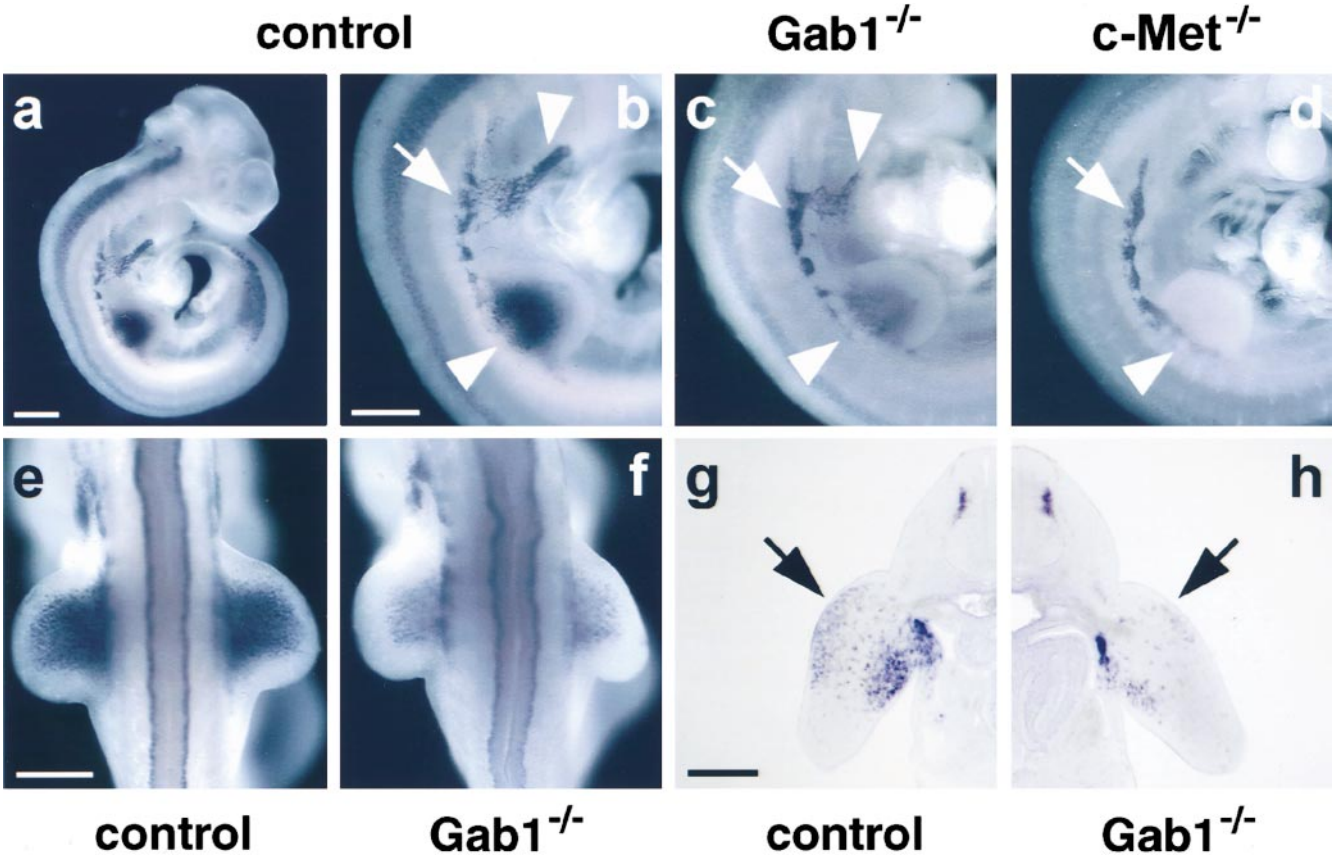

Figure 3. Whole-mount in situ hybridization of muscle precursor cells in the control (a, b, e, and g), *Gab1*−/− (c, f, and h) and c-*Met*−/− (d) embryos at stage E10.25, as visualized by in situ hybridization with an Lbx1-specific probe. (a–d) Lateral views and (e–g) dorsal views; (g and h) vibratome cross-sections at the forelimb level. Arrowheads in b–d point towards muscle precursor cells migrating into the hypoglossal cord and into the forelimbs; arrows mark remaining precursor cells in the dermomyotome. Arrows in g and h mark muscle precursor cells in the dorsal forelimb. Note that, in *Gab1*−/− embryos, less muscle precursor cells emigrate, and that migration is less progressed compared with control embryos. In c-*Met*−/− embryos, no emigration of muscle precursor cells occurs. Bars: (a) 500 μm; (b–f) 400 μm; (g and h) 250 μm.

occipital stream does not extend as far distally as in control embryos (Fig. 3 c, upper arrowhead). Similarly, much less precursor cells have reached the forelimb bud (Fig. 3 c, lower arrowhead). Impaired migration of muscle precursor cells into the forelimbs of *Gab1*−/− embryos is also evident in a dorsal view of the embryos (Fig. 3 f, compare with control embryos in e). Sections reveal a particularly pronounced reduction of precursor cells in the dorsal forelimb (Fig. 3, g and h, arrow).

We analyzed differentiated muscle groups in the limbs of E14.5 embryos; at this stage, skeletal muscle cells in the limbs express muscle-specific proteins such as fast myosin heavy chain, which was visualized by immunohistochemistry. The extensor muscle groups of the proximal lower forelimbs are either absent or very small in *Gab1*−/− mice compared with control mice; flexor muscles are present (Fig. 4, a and c, arrows mark extensors, arrowhead, flexors). In the distal lower forelimb, the phenotype is more pronounced, and only traces of muscle cells can be detected at this site in *Gab1*−/− embryos; both extensor and flexor muscles groups are strongly affected (Fig. 4, b and d). In the proximal lower hindlimb, some muscle groups are present but reduced in size; distally, the size reduction is again more pronounced (Fig. 4, a′–d′). We also set up cross-breedings of *Gab1* and *c-Met* (Bladt et al. 1995) mutant mice to obtain compound *Gab1*−/−:*c-Met*+/− embryos. Remarkably, virtual absence of all muscles was observed in the lower limbs of these embryos (Fig. 4, e–f′). The diaphragm muscle, which is also colonized by migrating muscle precursor cells, was examined. Sagittal sections of wild-type embryos at E13.5 reveal the diaphragm muscle (Fig. 5 a), which is split by the esophagus in this particular section plane. In *Gab1* mutant embryos, the diaphragm muscle is strongly reduced in size (Fig. 5 b). No change was observed in the internal tongue muscle also generated by migrating cells (not shown). Note that other muscle groups that do not develop from migrating cells, like intercostal or body wall muscle, are well developed in the *Gab1* mutants (Fig. 4 and data not shown). Thus, specific muscle groups that derive from migrating precursor cells are severely impaired in their development in *Gab1* mutants. In *HGF/SF*−/−, c-*Met*−/−, and *Gab1*−/−:*c-Met*+/− embryos, these muscle groups are completely absent (Bladt et al., 1995; Dietrich et al., 1998). Interest-

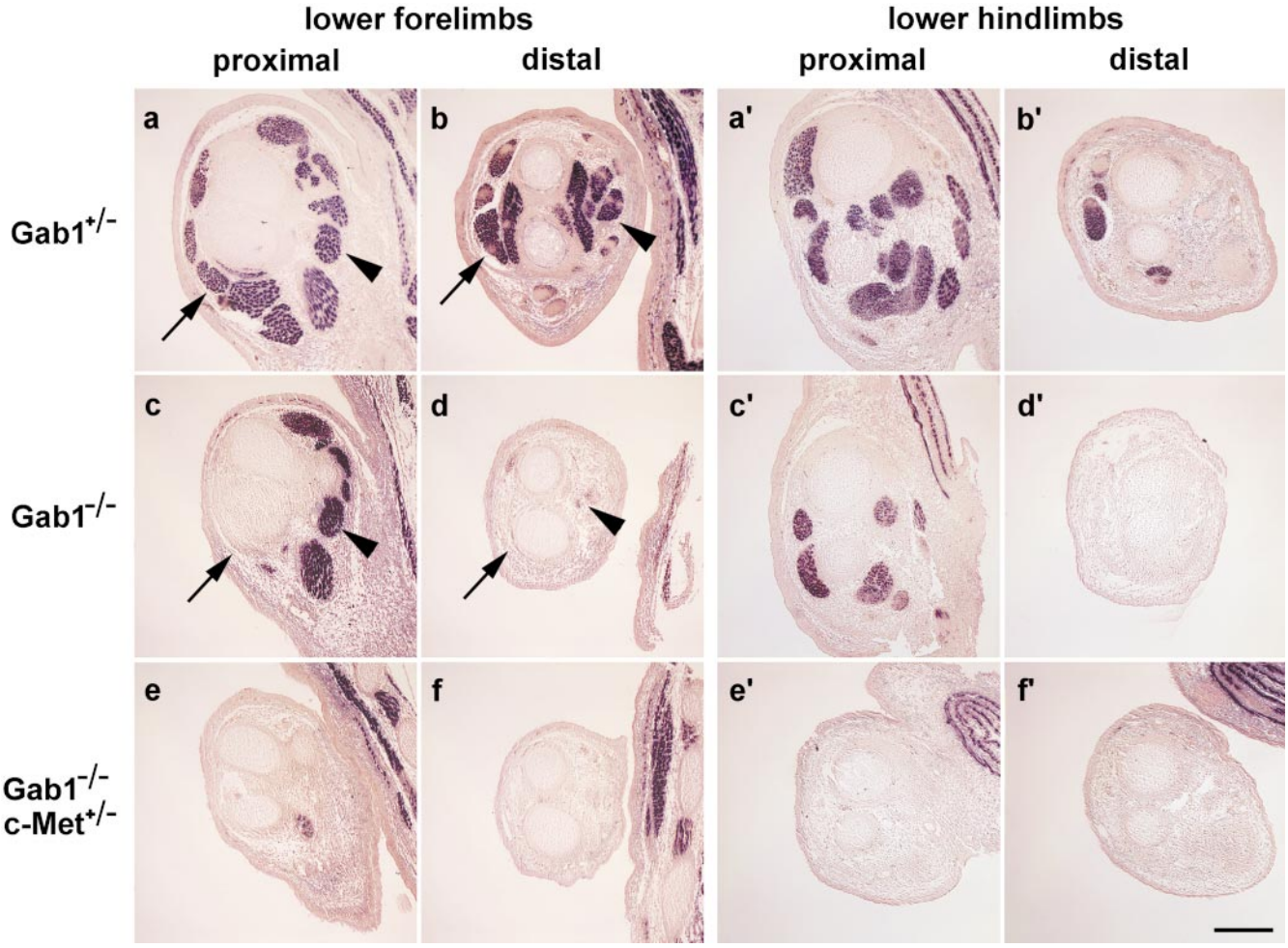

Figure 4. (e–f′), Immunohistological analysis of muscle groups in control (a–b′), *Gab1*−/− (c–d′) and *Gab1*−/−:*c-Met*+/− embryos (e–f′) as visualized by staining with anti–skeletal fast myosin heavy chain antibodies and HRP. Cross-sections of the proximal (a, c, and e) and distal (b, d, and f) lower forelimbs and proximal (a′, c′, and e′) and distal (b′, d′, and f′) lower hindlimbs of E14.5 embryos are shown. Note the strong reduction of muscles in the lower fore- and hindlimbs in *Gab1*−/− embryos, and the virtual absence of muscles in compound *Gab1/c-Met* mutant embryos. Bar, 250 μm.

ingly, the reduced colonization of limbs and diaphragm with muscle precursor cells in *Gab1*−/− embryos is not markedly compensated at later stages in development.

HGF/SF and c-Met have also been suggested to be involved in the formation of hair follicles (Jindo et al., 1998; Lindner et al., 2000). No difference was seen in the initiation of hair follicle morphogenesis between wild-type and *Gab1*−/− embryos at day E14.5 (Fig. 6, a–d): whole mount in situ hybridization using a sonic hedgehog probe revealed an identical staining pattern in the forming epithelial placodes during stage 1 of hair follicle morphogenesis (Karlsson et al., 1999). At E17.5, *Gab1* mutant embryos displayed a retardation of hair follicle outgrowth (Fig. 6, e and f) as well as a reduction of the total number of hair follicles, which may reflect the smaller size of the mutant embryos. In addition, epidermal thickness and keratinization was reduced in mutant embryos.

Discussion

We identified Gab1 originally in a yeast two-hybrid screen as a direct binding partner of c-Met (Weidner et al., 1996); accumulated evidence indicates an important role of Gab1 in c-Met signaling in cultured cells (Weidner et al., 1996; Bardelli et al., 1997; Maroun et al., 1999; Schaeper et al., 2000). Here, we present evidence for an essential role of Gab1 in the transmission of c-Met signals in vivo. Ablation of the *Gab1* gene in mice results in phenotypes that resemble those of mice harboring mutations in the *c-Met* and *HGF/SF* genes (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995; Maina et al., 1996). Placenta and liver development, but also the migration of muscle precursor cells from the dermomyotome to distant targets are affected in all three mutants. Thus, the genetic data demonstrate that Gab1 is an essential and specific mediator of signaling by a particular receptor tyrosine kinase, c-Met.

Our work places *Gab1* into the genetic hierarchy that controls the development of muscle derived from migratory precursors. The transcription factor Pax3 is essential for the formation and specification of migratory muscle precursors in the dermomyotome. It also induces expression of the *Lbx1* and the *c-Met* genes. The precursor cells

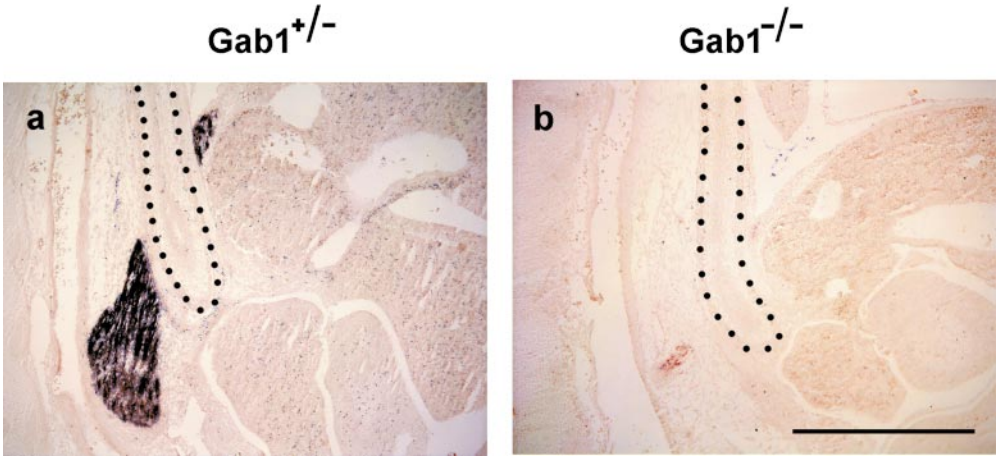

Figure 5. Immunohistological analysis of muscle groups in *Gab*+/− (a) and *Gab1*−/− embryos (b), as visualized by staining with anti–skeletal fast myosin heavy chain antibodies and HRP. Sagittal sections of E13.5 embryos show the muscle of the diaphragm, which is split by the esophagus (dotted line). Note the strong reduction of the size of diaphragm muscles in *Gab1*−/− embryos. Bar, 250 μm.

are primed to receive the HGF/SF signal, which is provided by mesenchymal cells close to the somites and along the migration routes (Cossu et al., 1996; Daston et al., 1996; Epstein et al., 1996; Yang et al., 1996; Mennerich et al., 1998; Dietrich et al., 1999). Our data suggest that the transmission of the c-Met signals that induces delamination of hypaxial muscle precursors and long-range migration requires the docking protein Gab1. The multiadaptor Gab1 binds directly and specifically to c-Met (Weidner et al., 1996) and, thus, is well suited for mediating such a characteristic biological response.

Upon phosphorylation by c-Met, Gab1 associates with several signaling proteins, like PI(3) kinase, Shc, CRKL, and Shp2 (Holgado-Madruga et al., 1996; Maroun et al., 1999; Sakkab et al., 2000; Schaeper et al., 2000). Which substrates are required downstream of Gab1 for the migration of muscle precursor cells in vivo is currently unknown. In vitro studies showed that the association of Gab1 with Shp2 and PI(3) kinase is required for c-Met–induced branching morphogenesis and dissociation/scattering of epithelial cells, respectively (Khwaja et al. 1998; Schaeper et al., 2000). In addition, the ras MAPK pathway is involved in cell scattering (Hartmann et al., 1994; Ridley et al., 1995; Khwaja et al., 1998). Shp2, a tyrosine phosphatase, positively regulates the MAPK cascade and cell migration (Bennett et al., 1996; Saxton et al., 1997; Yu et

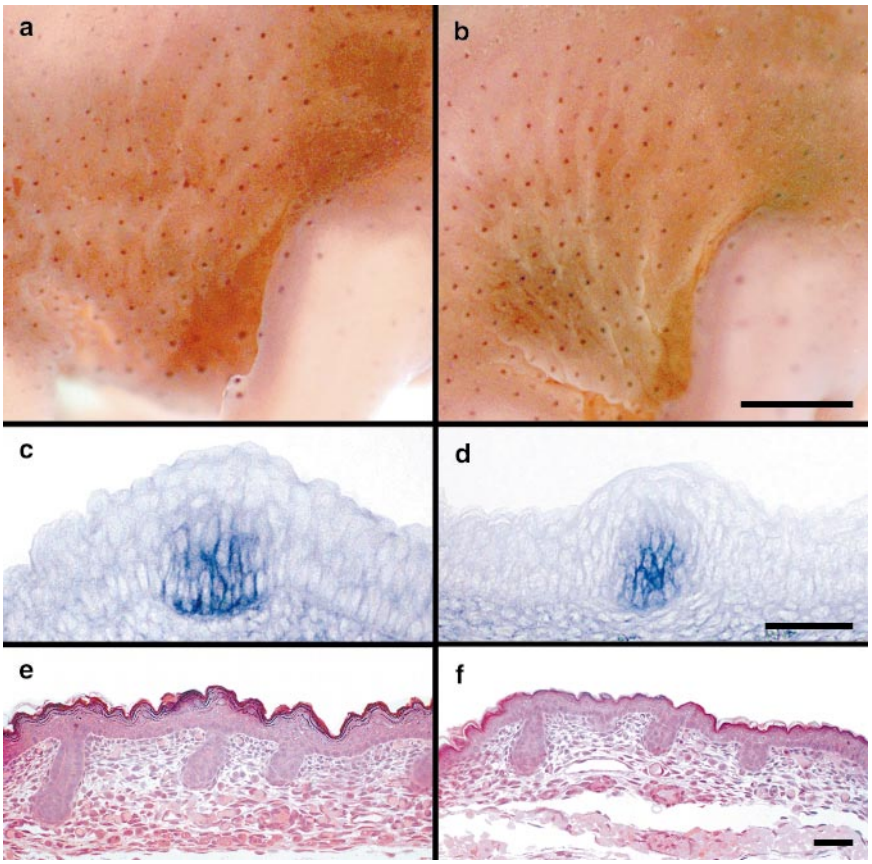

Figure 6. Initiation of hair follicle formation in *Gab1*−/− embryos. Whole-mount in situ hybridizations of wild-type (a and c) and mutant embryos (b and d) with a sonic hedgehog–specific probe at E14.5. (a and b) Side views of the embryos, (c and d) transversal sections of skin demonstrating the correct localization of sonic hedgehog–specific signals in the developing epithelial placodes. Transversal sections of hematoxylin-eosin–stained skin of E17.5 wild-type (e) and mutant (f) embryos. Note the delayed development of the skin of *Gab1*−/− embryos. Bars: (a and b) 500 μm; (c–f) 50 μm.

al., 1998). Gene ablation of *Shp2* demonstrates its role in many developmental processes, including morphogenic movements at gastrulation (Saxton et al., 1997; Qu et al., 1998; Saxton and Pawson 1999). However, in *Shp2*−/− mice, somite differentiation is disturbed (Saxton et al., 1997), which interferes with the analysis of a *Shp2* function in migration of muscle precursors. Thus, Shp2 is a candidate substrate, which is downstream of Gab1, for the HGF/SF/c-Met signaling cascade that controls migration in vivo.

In *Gab1*−/− embryos, the migration of muscle precursor cells into limbs and the diaphragm is strongly reduced, but not completely blocked as it is in *HGF/SF*−/−, c-*Met*−/−, and compound *Gab1*−/−:*c-Met*+/− embryos, or in embryos that carry homozygous mutations in the bivalent docking site of c-Met (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995; Maina et al., 1996). Apparently, c-Met can transmit some signals in the absence of Gab1. The multiple docking site of c-Met binds not only Gab1, but can also directly associate with signaling molecules like p85 PI(3) kinase, Shc, phospholipase Cγ, and Shp2, which are also recruited by Gab1 (Ponzetto et al., 1994; Zhu et al., 1994; Fixman et al., 1995; Pelicci et al., 1995; Schaeper et al., 2000). Moreover, it is possible that additional docking proteins contribute to the transmission of the c-Met signal that controls migration of muscle precursor cells. A Gab1 homologue, p97/Gab2, has been identified recently (Gu et al., 1998). p97/Gab2 does not associate directly with c-Met and would have to be recruited indirectly, possibly via the Grb2 adapter (Schaeper et al., 2000).

In contrast, embryonic death of *Gab1*−/− mice occurs during a very similar time window as the death of *HGF/SF*−/−, c-*Met*−/−, and c-*Met* point mutant mice, that lack the multiple docking site, Y1349F and Y1356F (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995; Maina et al., 1996). Also, we detected no differences in the placental and liver phenotypes between *Gab1*−/− and c-*Met*−/− mice. In the placenta, the labyrinth layer, but no other layer, is severely affected, which may cause the death of the mutant embryos. Thus, Gab1 is the essential substrate of c-Met in the placenta and liver. Selective mutation of the Grb2 binding site in c-Met, $Y^{1356}VNV \rightarrow Y^{1356}VHV$, affects the formation of skeletal muscles derived from migratory precursor cells, but does not cause embryonic lethality and has no effect on liver development (Maina et al., 1996). This suggests that the coupling of c-Met to Gab1 via Y1349, the major Gab1 binding site of c-Met (Weidner et al., 1996), is sufficient for c-Met–induced proliferation and survival, whereas the coupling of c-Met to both Gab1 and Grb2 is required for efficient migration of myogenic precursor cells.

A number of genetic experiments show the importance of specific docking proteins in the signaling of receptor tyrosine kinases. *DOS* (*daughter of sevenless*), which encodes a Gab1 homologue in *Drosophila* (the *Drosophila* genome does not contain a *c-Met* gene), was identified in a genetic screen for downstream signaling components of the receptor tyrosine kinase sevenless (Herbst et al., 1996; Raabe et al., 1996). Here, we showed that Gab1 plays an essential role in c-Met signaling in the mouse. Mice with targeted disruption of the *IRS-2* gene, which encodes a protein with a similar domain structure as Gab1, develop diabetes, demonstrating a role for this docking protein in the signaling of the insulin and insulin-like growth factor receptors (Withers et al., 1998, 1999). A disruption of the *LAT* (*linker for activation of T cells*) gene in mice, which encodes a transmembrane docking protein, demonstrates its essential role in T cell activation and development (Zhang et al., 1998). Thus, evidence obtained by genetic experiments in mammals supports the notion that specific tyrosine kinases use specific docking proteins.

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