Modulation of Erbb2 signaling during development: a threshold level of Erbb2 signaling is required for development

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Summary

We have generated a series of *Erbb2* cDNA knock-in animals to explore the role of signaling pathways coupled to Erbb2 during development. Although this knock-in allele was hypomorphic, expressing tenfold less Erbb2 protein than wild type, the knock-in animals were healthy. However, a further twofold reduction in Erbb2 levels in hemizygous knock-in animals resulted in perinatal lethality with defects in the innervation of the diaphragm. Genetic rescue of this hypomorph was accomplished by expression of the Erbb2-Y1028F mutant in a comparable knock-in allele. Interestingly, hemizygous *Y1028F* animals were viable with normal innervation of the diaphragm. Molecular analyses revealed that the *Y1028F* allele expressed higher levels of Erbb2 and that Y1028 promoted the turnover of the receptor. In addition, ablation of the Shc-binding site in Erbb2 (Y1227) resulted in subtle defects in the sensory nerves not observed in the other mutant erbb2 strains. Thus, we have established how Erbb2 levels may be modulated through development and that a minimum threshold level of Erbb2 is required.

Key words: Erbb2, Tyrosine Phosphorylation, Knock-in, Hypomorph

Introduction

The Erbb2 receptor tyrosine kinase is a member of the epidermal growth factor receptor (Egfr) family, which also includes the Egfr/Erbb1, Erbb3, and Erbb4. Although there is considerable homology amongst the Egfr family members, each Egfr family member can interact with a distinct subset of ligands (Olayioye et al., 2000; Pinkas-Kramarski et al., 1996a; Yarden and Sliwkowski, 2001). For example, Egfr ligands such as Egf or Tgfβ bind directly to the Egfr whereas the heregulins are specific ligands for ErbB3 or ErbB4 (Carraway et al., 1994; Peles et al., 1993). In contrast to these Egfr family members, a direct soluble ligand for Erbb2 has yet to be described. However, there have been several reports suggesting that a member of the mucin family known as Muc4 may act as a membrane-bound ligand for Erbb2 (Carraway et al., 1999).

Despite the lack of a direct soluble ligand for Erbb2, the tyrosine kinase activity of Erbb2 can be stimulated by other Egfr ligands through the formation of specific heterodimers with other members of the Egfr family (Goldman et al., 1990; Karunagaran et al., 1996; Pinkas-Kramarski et al., 1996a; Pinkas-Kramarski et al., 1996b; Tzahar et al., 1996). For example, stimulation of cells with Egf can result in transphosphorylation of Erbb2 through the formation of Egfr-Erbb2 heterodimers (King et al., 1988; Stern and Kamps, 1988). Similarly, stimulation of cells with heregulin can result in the transphosphorylation of Erbb2 through the formation of specific heterodimers of Erbb2 and Erbb4 or heterodimers of Erbb2 and Erbb3 (Goldman et al., 1990; Karunagaran et al., 1996; Pinkas-Kramarski et al., 1998a; Pinkas-Kramarski et al., 1996a; Pinkas-Kramarski et al., 1998b; Pinkas-Kramarski et al., 1996b; Sliwkowski et al., 1994; Stern and Kamps, 1988; Tzahar et al., 1997; Tzahar et al., 1996). Transphosphorylation of Erbb3 is absolutely dependent on its capacity to form specific heterodimers with other members of the Egfr family since it lacks intrinsic tyrosine kinase activity (Guy et al., 1994). Taken together, these observations argue that the formation of specific heterodimers play an important biological role in the function of the Egfr family.

Members of the Egfr family share structural similarities including an extracellular ligand-binding domain, a single-pass transmembrane domain, a highly conserved tyrosine kinase domain, and a regulatory carboxyl-terminal tail containing several tyrosine autophosphorylation sites (Hynes and Stern, 1994). Ligand-mediated activation results in strong mitogenic signals from the receptors, leading to cellular growth and differentiation. Not surprisingly, members of the Egfr family are collectively involved in both development and disease. Whereas amplification and aberrant overexpression of Erbb2 has been implicated in various cancers, most notably in breast cancer (Simpson et al., 1995; Slamon et al., 1987; Slamon et al., 1989), the loss of *Erbb2*, *Erbb3 or Erbb4* expression in knock-out mice has deleterious effects on the developing embryo (Britsch et al., 1998; Gassmann et al., 1995; Lee et al., 1995; Riethmacher et al., 1997). For example, *Erbb2*- and *Erbb3*-deficient animals share similar hypoplastic development of the sympathetic nervous system (Britsch et al., 1998), and

Erbb2- and *Erbb4*-deficient animals exhibit defective formation of cardiac ventricular trabecules (Gassmann et al., 1995; Lee et al., 1995).

Upon receptor activation, specific tyrosine residues in the terminal tail of the receptor are autophosphorylated (Akiyama et al., 1991; Hazan et al., 1990), and then serve as potential binding sites for intracellular signaling proteins harboring phosphotyrosine binding (PTB) (Kavanaugh et al., 1995) or Src homology 2 (SH2) (Pawson, 1995) domains. In a series of studies by Dankort et al. (Dankort et al., 1997), the five major tyrosine autophosphorylation sites (Y1028, Y1144, Y1201, Y1227, Y1253) were evaluated systematically for their roles in constitutively activated Erbb2-mediated transformation of fibroblast cells. Although simultaneous ablation of all five sites in the tyrosine phosphorylation-deficient (*NYPD*) mutant drastically impaired transformation, independent tyrosine-to-phenylalanine mutations at four of five sites only modestly reduced the transforming ability. Substitution at the one remaining site (*Y1028*) resulted in a consistent increase in transformation. Conversely, restoration of individual tyrosine residues to the *NYPD* mutant at one of four sites ('add-back' mutants) was not only able to fully restore the transforming ability, but was also able to transform with a modest increase over the fully functional receptor. The *Y1028* add-back mutant had the opposite effect and completely ablated the residual transforming abilities of the *NYPD* mutant. These observations suggested that while four of the five tyrosine sites positively mediate Erbb2 signaling, Y1028 negatively modulates Erbb2 activity.

We describe here the introduction of wild-type and phosphotyrosine mutant *Erbb2* cDNAs into the endogenous mouse *Erbb2* locus to examine their physiological roles, in vivo. Although mice derived from the different knock-in alleles were viable, examination of the levels of Erbb2 expression revealed that the knock-in strains expressed only 10% of the expected Erbb2 protein. A further reduction of Erbb2 protein, achieved by intercrossing the *Erbb2* knock-in mutants with *Erbb2* null mice, resulted in perinatal lethality. Thus, we established a minimum threshold level of Erbb2 required for viability. In contrast, expression of the Y1028F mutation in knock-in animals genetically rescued this perinatal lethality. Biochemical analyses revealed that these animals expressed higher levels of Erbb2 above the threshold. We further identify that Y1028 mediates the negative regulation of Erbb2 signaling by influencing the turnover rate of the receptor.

Materials and methods

Knock-in animals

cDNAs encoding the non-activated rat Erbb2 (rErbb2) receptor harboring single tyrosine phosphorylation mutations were generated from the corresponding C-terminal domain of the oncogenic *rErbb2* mutants previously described by Dankort et al. (Dankort et al., 1997). The cDNAs were cloned into the targeting vector containing flanking 3′ and 5′ arms of homology as described previously (Chan et al., 2002). These plasmids were electroporated into R1 ES cells and G418 (Geneticin; GIBCO) -resistant colonies were picked and subsequently screened by Southern Blot analysis for correctly targeted mutants. Mutant mice were generated from the positive ES cell clones and were maintained in an SV129/Balb/c background. All animal work was performed with approval and under the strict guidelines of the Canadian Council on Animal Care (CCAC).

Immunoblot analysis of embryo lysates

Embryos were lysed in modified TNE lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 10 mM NaF, 2 mM EDTA) supplemented with 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM sodium orthovanadate. For immunoblotting, the membranes were incubated with an anti-Erbb2 monoclonal (Ab-3, Oncogene Research) or anti-Grb2 polyclonal (1:2500; C-23, Santa Cruz) antibody overnight at 4°C. Subsequently, the membranes were treated with anti-mouseHRP or anti-rabbitHRP secondary antibodies for 1 hour at room temperature, washed three times in TBS and visualized by enhanced chemiluminescence (Amersham), as specified by the manufacturer.

Whole-mount in situ hybridization

For whole-mount in situ hybridization, embryos were fixed in 4% paraformaldehyde/0.2% glutaraldehyde, dehydrated through a graded series of methanol/PBT (PBS+0.1% Tween-20) baths and stored in 100% MeOH at –20°C until needed. Whole-mount in situ hybridization was carried out as previously described (Wilkinson and Nieto, 1993).

Immunohistochemistry

E12.5 embryos were fixed in fresh 4% paraformaldehyde, washed several times with PBS and then post-fixed and bleached in Dent's fixative (methanol:DMSO, 4:1)+5% H_2O_2. After extensive washes, the embryos were incubated in blocking solution (5% goat serum+1% DMSO in TBS) overnight at room temperature. Incubation in anti-NF150 (1:1000, Chemicon) diluted in TBS+1% DMSO was performed for 2 days at room temperature. After 5×1 hour washes in TBS-T (TBS+1% Tween20), the embryos were incubated overnight in goat anti-rabbitHRP (1:1000 in TBS-T). DAB+ kit (DAKO, catalogue no. K3468) was used to visualize the neurofilament staining. Whole-mount immunohistochemistry was performed on diaphragm dissected from E18.5 embryos and fixed in 4% PFA. They were first treated in 0.1 M glycine in PBS, washed several times, then incubated in PBT (PBS+0.5% Triton-X-100) for 5 minutes. Diaphragms were incubated overnight with anti-NF150 diluted 1:1000 in PBT+5% goat serum. After extensive washes in PBT, they were incubated overnight with goat anti-rabbit-Alexa488 (Molecular Probes) diluted 1:1000 in PBT and α-bungarotoxin-Alexa594 (Molecular Probes). Diaphragms were visualized using the Zeiss LSM 510Meta confocal microscope.

Receptor turnover assay

Rat-1 cells stably expressing the oncogenic Erbb2 tyrosine phosphorylation site mutants were seeded (13×10^6 cells) onto 60-mm plates and pulse labeled using 0.1 mCi/ml of ^{35}S-EXPRESS Protein Labeling mix (NEG772, NEN Life Science Products). The cells were then washed and chased with unlabeled medium for the indicated times. Protein lysates from the labeled cells were immunoprecipitated using an anti-Erbb2 antibody (Ab4, Oncogene Science) and resolved on an 8% SDS-PAGE. The gel was dried and exposed to a PhosphoImager screen (Molecular Dynamics) and scanned using the Typhoon8600 scanner (Amersham). Image analyses were performed using ImageQuant software (Molecular Dynamics).

Results

Generation of *Erbb2* cDNA knock-in animals

To assess the relative contribution of the different Erbb2 tyrosine autophosphorylation sites, we employed a targeted knock-in strategy involving replacement of the first coding exon of the mouse *Erbb2* gene (*m-Erbb2*) with the rat *Erbb2* cDNA (*r-Erbb2*) (Fig. 1A). Thus, expression of mouse *Erbb2* is disrupted and replaced by expression of the rat *Erbb2* cDNA under the control of the endogenous promoter. For

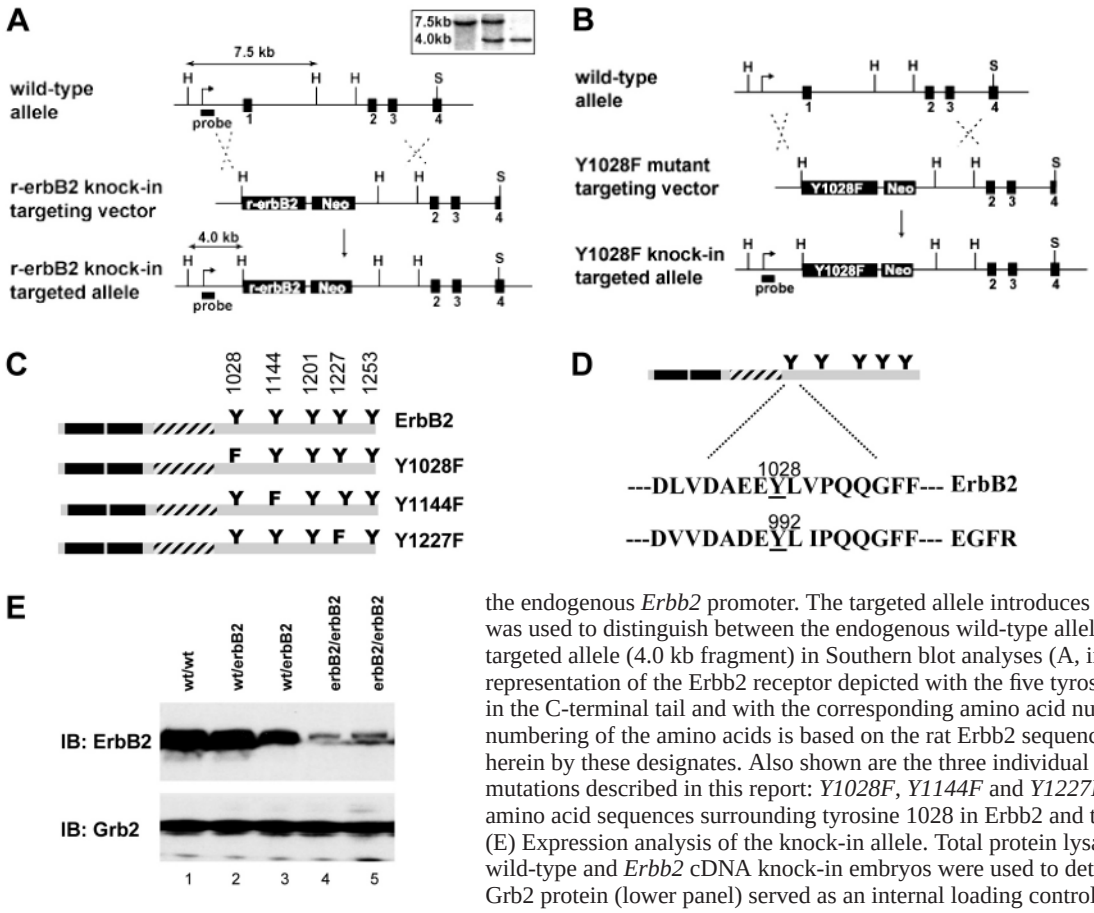

Fig. 1. Generation of knock-in animals expressing Erbb2 tyrosine phosphorylation mutants. The knock-in targeting vector was constructed such that exon 1 of the mouse *Erbb2* gene was replaced with either (A) a rat *Erbb2* cDNA or (B) a cDNA encoding a mutant Erbb2 receptor harboring the *Y1028F* mutation. The cDNA is followed by a PGK-Neomycin/SV40-polyA expression cassette and is targeted to the endogenous *Erbb2* locus by homologous 5′ and 3′ flanking arms, placing it directly under the transcriptional control of the endogenous *Erbb2* promoter. The targeted allele introduces an additional *Hin*dIII site that was used to distinguish between the endogenous wild-type allele (7.5 kb fragment) and the targeted allele (4.0 kb fragment) in Southern blot analyses (A, inset). (C) Schematic representation of the Erbb2 receptor depicted with the five tyrosine autophosphorylation sites in the C-terminal tail and with the corresponding amino acid number. Note that the numbering of the amino acids is based on the rat Erbb2 sequence and will be referred to herein by these designates. Also shown are the three individual tyrosine-to-phenylalanine mutations described in this report: *Y1028F*, *Y1144F* and *Y1227F*. (D) Alignment of the amino acid sequences surrounding tyrosine 1028 in Erbb2 and tyrosine 992 in Egfr. (E) Expression analysis of the knock-in allele. Total protein lysates prepared from E12.5 wild-type and *Erbb2* cDNA knock-in embryos were used to detect Erbb2 levels. Detection of Grb2 protein (lower panel) served as an internal loading control.

genotyping purposes, note that the '*wt*' superscript (e.g. $Erbb2^{wt}$) will be used herein to designate the endogenous mouse *Erbb2* genotype whereas the '*Erbb2*' superscript (e.g. $Erbb2^{Erbb2}$) will be used to designate the knock-in rat *Erbb2* cDNA allele. Following germline transmission of the targeted knock-in allele, transgenic animals homozygous for the *Erbb2* knock-in cDNA ($Erbb2^{Erbb2/Erbb2}$) were viable, appeared healthy, and reproduced at the expected Mendelian frequencies (Table 1A).

To examine the functional roles of the tyrosine autophosphorylation sites in vivo, mutant rat *Erbb2* cDNA harboring single tyrosine-to-phenylalanine substitutions (Fig. 1C) were also expressed in mice using an identical targeting strategy to that described above but substituting the cDNA cassette (Fig. 1B). Specifically, we generated knock-in mice expressing the *Erbb2-Y1028F* mutant, as well as the *Erbb2-Y1144F* mutant (loss of the direct binding site for Grb2) and the *Erbb2-Y1227F* mutant (loss of the direct binding site for Shc). Homozygous $Erbb2^{Y1028F/Y1028F}$ knock-in animals expressing the *Y1028F* mutation were generated from heterozygous matings at the expected Mendelian frequencies (Table 1A) and appeared normal and healthy. Interestingly, homozygous $Erbb2^{Y1144F/Y1144F}$ and $Erbb2^{Y1227F/Y1227F}$ knock-in mice were also normal, healthy and fertile (Table 1A).

The use of a rat *Erbb2* cDNA instead of a mouse cDNA was necessary to distinguish expression of the knock-in allele from the endogenous gene and allow for the validation of the knock-in strategy. The mouse and rat *Erbb2* share a greater than 93% sequence similarity at both the nucleic acid and amino acid levels. Importantly, the tyrosine autophosphorylation sites and the sequences surrounding these sites in the carboxyl-terminus are conserved. To confirm that rescue of the embryonic lethality associated with disruption and inactivation of the mouse *Erbb2* gene (Morris et al., 1999) was attributed solely to expression of the knock-in rat *Erbb2* cDNA, RT-PCR was performed on total RNA isolated from wild-type and knock-in animals. Analysis of the RT-PCR product suggested that homozygous animals indeed only expressed the rat *Erbb2* cDNA (data not shown).

Hypomorphic expression of Erbb2 protein resulted in neonatal lethality

Since specific expression of the knock-in allele was validated, we next performed immunoblot analyses on protein lysates prepared from E12.5 wild-type, heterozygous, and homozygous mutant embryos (Fig. 1E) to determine whether the knock-in *Erbb2* allele expressed wild-type levels of Erbb2 protein. Surprisingly, the levels of Erbb2 expressed in the $Erbb2^{Erbb2/Erbb2}$ embryos (Fig. 1E, lanes 4-5) were dramatically reduced relative to the expression of endogenous Erbb2 in wild-type littermates (Fig. 1E, lanes 1 and 2). However, in spite of the considerably reduced levels of Erbb2 protein, homozygous $Erbb2^{Erbb2/Erbb2}$ knock-in animals did not display any obvious phenotype and appeared generally healthy.

Although mice bearing the knock-in rat *Erbb2* cDNA appeared phenotypically normal, we have previously

Table 1. Enumeration of the different genotypes at 3-weeks of age and at birth

A

Parental genotype	Number of animals at 3-weeks of age		
	Wild type	Heterozygous	Mutant
Erbb2$^{wt/Erbb2}$×*Erbb2*$^{wt/Erbb2}$	35	74	31 *(Erbb2/Erbb2)*
Erbb2$^{wt/Y1028F}$×*Erbb2*$^{wt/Y1028F}$	47	103	49 *(Y1028F/Y1028F)*
Erbb2$^{wt/Y1144F}$×*Erbb2*$^{wt/Y1144F}$	40	79	36 *(Y1144F/Y1144F)*
Erbb2$^{wt/Y1227F}$×*Erbb2*$^{wt/Y1227F}$	36	70	33 *(Y1227F/Y1227F)*
Erbb2$^{wt/Erbb2}$×*Erbb2*$^{wt/ko}$	29	60	0 *(Erbb2/ko)*
Erbb2$^{wt/Y1028F}$×*Erbb2*$^{wt/ko}$	31	61	26 *(Y1028F/ko)*

B

Parental genotype	Number of animals at birth/number of survivors	
	Heterozygous	Mutant
Erbb2$^{Erbb2/Erbb2}$×*Erbb2*$^{wt/ko}$	20/20	18/0 *(Erbb2/ko)*
Erbb2$^{Y1144F/Y1144F}$×*Erbb2*$^{wt/ko}$	13/13	13/0 *(Y1144F/ko)*
Erbb2$^{Y1227F/Y1227F}$×*Erbb2*$^{wt/ko}$	12/12	11/0 *(Y1227F/ko)*

demonstrated that they indeed exhibited subtle defects. In particular, our lab showed that rat *Erbb2* cDNA knock-in animals possessed only 10% of the number of muscle spindles found in wild-type animals (Andrechek et al., 2002). Consequently, we investigated the effects of further reducing the expression of Erbb2 by interbreeding heterozygous knock-in (*Erbb2*$^{wt/Erbb2}$) animals with heterozygous *Erbb2* knock-out (*Erbb2*$^{wt/ko}$) animals. This strategy allowed us to express a single *Erbb2* knock-in allele in an *Erbb2*-deficient background to generate hemizygous *Erbb2*$^{Erbb2/ko}$ animals. Significantly, no *Erbb2*$^{Erbb2/ko}$ animals were found at weaning age (three-weeks old) when the animals were genotyped (Table 1A).

To determine the precise reason that the *Erbb2*$^{Erbb2/ko}$ animals were dying, we interbred homozygous *Erbb2*$^{Erbb2/Erbb2}$ knock-in animals with heterozygous *Erbb2*$^{wt/ko}$ animals and assessed whether we could detect viable hemizygous *Erbb2*$^{Erbb2/ko}$ animals at birth. Although the expected number of animals were present at birth, 18 of the 38 newborn pups were either stillborn or started dying immediately after birth (Table 1B). They could not breathe independently, despite the ability to open their mouths, and they became cyanotic and died within a few minutes. Indeed, all of the dead pups were genotyped to be *Erbb2*$^{Erbb2/ko}$ animals. Subsequent postmortem histological analyses of the lungs confirmed that the hemizygous *Erbb2*$^{Erbb2/ko}$ pups were unable to inflate and expand their lungs, despite being vascularized and structurally intact (data not shown). These observations suggest that a critical minimal threshold level of Erbb2 protein is required to maintain viability.

Expression of the *Y1028F* mutation rescues the hypomorph

Previous in vitro analyses of the tyrosine phosphorylation mutants identified tyrosine 1028 as a negative regulator of Erbb2-induced transformation via an undetermined mechanism. Accordingly, we next asked whether we could genetically rescue the perinatal lethality observed with the hemizygous *Erbb2*$^{Erbb2/ko}$ animals, by removing the putative negative regulatory tyrosine residue in Erbb2. This was accomplished by similarly crossing the heterozygous *Erbb2*$^{wt/Y1028F}$ knock-in animals with *Erbb2*$^{wt/ko}$ animals to generate mice expressing a single *Y1028F* cDNA knock-in allele in an *Erbb2*-deficient background. Interestingly, in contrast to the hemizygous *Erbb2*$^{Erbb2/ko}$ pups, no perinatal lethality was observed with the hemizygous *Erbb2*$^{Y1028F/ko}$ animals (Table 1A). In fact, all of the progeny survived and the adult *Erbb2*$^{Y1028F/ko}$ animals appeared normal and healthy and were fertile. To preclude any possibility that the phenotypic differences observed in the *Erbb2-Y1028F* knock-in animals versus the control *Erbb2* knock-in animals were not by chance and that it is specifically due to this particular point mutation, mice expressing either the Y1144F or the Y1227F phosphotyrosine mutant Erbb2 receptor in an *Erbb2*-deficient background were also generated. As indicated in Table 1B, none of the hemizygous *Erbb2*$^{Y1144F/ko}$ or *Erbb2*$^{Y1227F/ko}$ animals survived, and similar to the *Erbb2*$^{wt/ko}$ animals, they died shortly after birth struggling unsuccessfully to breathe.

To further elucidate the nature of the defect resulting in the inability of the animals to inflate their lungs, the neuromuscular junctions in the diaphragm muscles of E18.5 embryos were examined by whole-mount immunostaining with a neurofilament-150 antibody and with α-bungarotoxin. In the control animals, *Erbb2*$^{wt/wt}$ and *Erbb2*$^{Erbb2/Erbb2}$, the central region of the diaphragm muscles was innervated by the relatively large main phrenic nerve with smaller intramuscular branches (Fig. 2A,B). Accordingly, innervation of the diaphragm from the genetically rescued embryos, *Erbb2*$^{Y1028F/ko}$ was similar to that in the control animals (Fig. 2C). In contrast, mutant diaphragms from *Erbb2*$^{Erbb2/ko}$, *Erbb2*$^{Y1144F/ko}$, *Erbb2*$^{Y1227F/ko}$ embryos were very poorly innervated, which probably resulted in the perinatal respiratory failure. As shown in Fig. 2D-F, the innervations were thin, disorganized, and discontinuous or incomplete and they lacked an apparent main nerve trunk from which smaller presynaptic branches originate. However, labeling of postsynaptic acetylcholine receptor (AChR) clusters with α-bungarotoxin did not reveal any significant differences in the number, density or shape of AChR clusters (Fig. 2G-L,G′-L′).

Additional evidence for the genetic rescue of the hypomorphic *Erbb2* knock-in allele by expression of the Y1028F mutation was observed in the developing sympathetic nervous system, where Erbb2 plays a critical role (Britsch et al., 1998). For comparison, Fig. 3A,C show the normal development of the primary sympathetic chain ganglia in

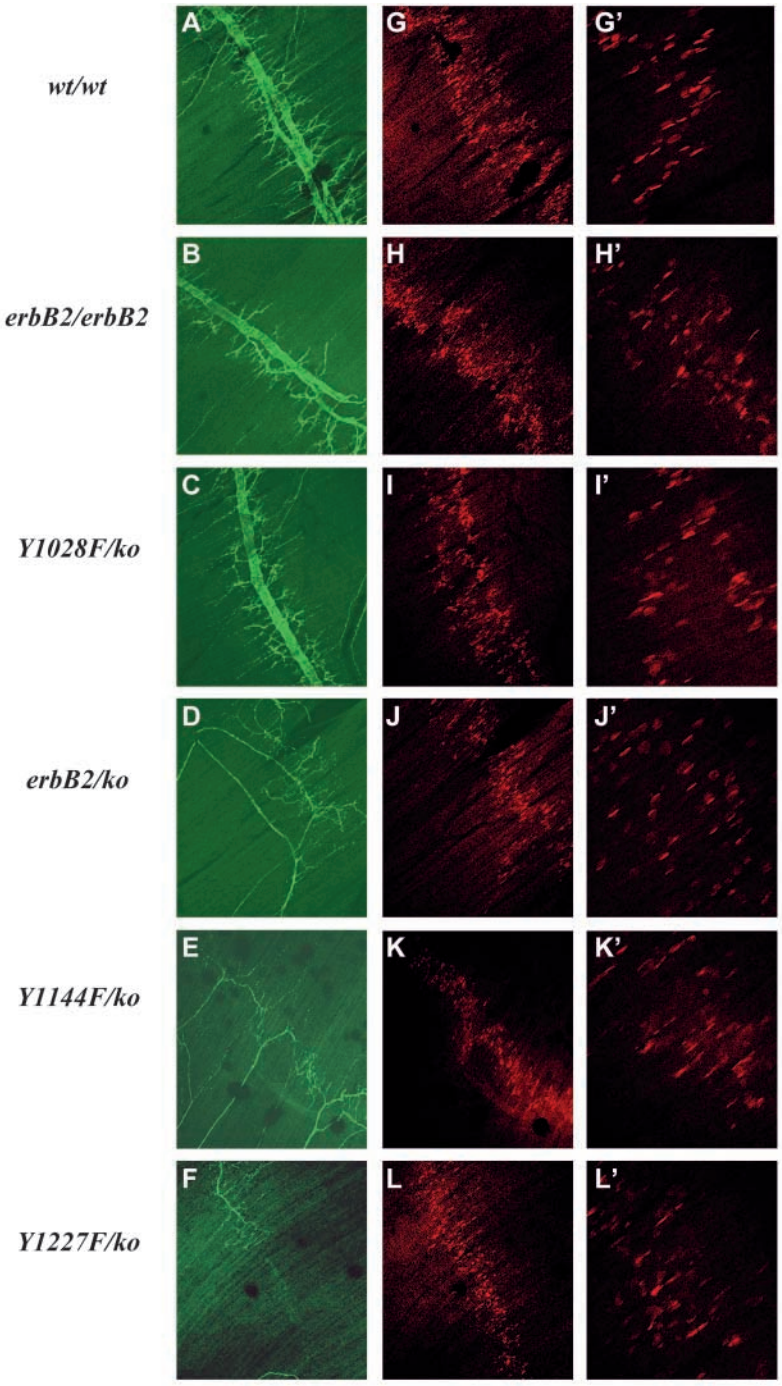

Fig. 2. Expression of the *Y1028F* mutant genetically rescues the hypomorphic knock-in allele. Whole-mount diaphragm muscle from E18.5 embryos was stained with neurofilament antibodies to label presynaptic axons and with ?-bungarotoxin-Alexa594 to label AChRs. (A-C) The main central trunk of the phrenic nerve in diaphragm muscle isolated from (A) $Erbb2^{wt/wt}$ (B) $Erbb2^{Erbb2/Erbb2}$ and (C) $Erbb2^{Y1028F7ko}$ are shown. The clusters of AChR correspond to the path of the presynaptic axon shown at low power magnification and at higher magnification (G′-I′). These particular genotypes are healthy animals and do not exhibit the acute respiratory distress at birth. (D-E) In contrast, the animals that were unable to inflate their lungs (D) $Erbb2^{Erbb2/ko}$ (E) $Erbb2^{Y1144F/ko}$ and (F) $Erbb2^{Y1227F/ko}$, had poorly innervated diaphragms where the phrenic nerves were thinned, defasciculated and fragmented. However, the density and shape of the AChR clusters appeared to be unaffected in these animals (J-L and J′-L′).

heterozygous embryos at E12.5, using Phox2a as a marker. In $Erbb2^{Erbb2/ko}$ embryos, there was only partial development of the thoracic sympathetic ganglia and the defects became more severe in a rostral-caudal gradient (Fig. 3B). In comparison, the sympathetic ganglia of $Erbb2^{Y1028F/ko}$ embryos were similar in size and length to those of heterozygous littermates (Fig. 3D). Take together, these results strongly suggest that Erbb2-Y1028 specifically mediates a negative regulatory effect on the receptor and this plays an important function in vivo to modulate the activity of the receptor throughout development.

The *Y1028F* mutation maintains higher Erbb2 protein levels

Since we hypothesized earlier that a minimal threshold level of Erbb2 is required for normal development, we explored whether this *Y1028F* mutation resulted in increased levels of Erbb2 such that the hemizygous animals survived. To determine the molecular basis for the hypermorphic $Erbb2^{Y1028F}$ allele, immunoblot analyses were performed on protein lysates derived from E12.5 embryos. Expression of the Y1028F receptor mutant in the $Erbb2^{Y1028F}$ knock-in animals consistently resulted in significantly higher levels of Erbb2 (Fig. 4A, lane 2 versus lane 5, lanes 3-4 versus lanes 6-7; Fig. 4B, lane 3 versus lane 4). In the hemizygous embryos, there is a critical difference in Erbb2 levels in the $Erbb2^{Y1028F/ko}$ embryos (Fig. 4A, lanes 6-7) versus the $Erbb2^{Erbb2/ko}$ embryos (Fig. 4A, lanes 3-4), which probably determined the difference as to whether the animals survived or died shortly after birth. Note that the Erbb2 levels in $Erbb2^{Y1028F/ko}$ embryos (Fig. 4A, lanes 6-7) are comparable to the levels in $Erbb2^{Erbb2/Erbb2}$ embryos (Fig. 4A, lane 2), which are essentially healthy animals.

To confirm that the observed increase in Erbb2 protein is specific to expression of the *Y1028F* allele, we also examined the levels of Erbb2 in homozygous E12.5 embryos expressing either the *Erbb2-Y1144F* mutation or the *Erbb2-Y1227F* mutation. The results revealed that only embryos with the *Y1028F* allele expressed elevated levels of Erbb2 when compared to the levels observed in knock-in embryos expressing the *Erbb2* cDNA, the *Y1144F* cDNA, or the *Y1227F* cDNA alleles (Fig. 4B, compare lane 4 versus lanes 3,6,7). In fact, quantitative immunoblotting using ^{125}I-conjugated secondary antibodies and subsequent ImageQuant (Molecular Dynamics) analyses revealed that the levels of Erbb2 in $Erbb2^{Y1028F/Y1028F}$ embryos were at least 3- to 8-fold higher than in $Erbb2^{Erbb2/Erbb2}$, $Erbb2^{Y1144F/Y1144F}$ and $Erbb2^{Y1227F/Y1227F}$ embryos (Fig. 4C). Thus, the quantitative difference in Erbb2 levels alone probably determined the difference between animals surviving or dying at birth.

In order to assess whether the variation in Erbb2 protein levels observed in the *Erbb2* or *Y1028F* knock-in alleles were occurring at a transcriptional or

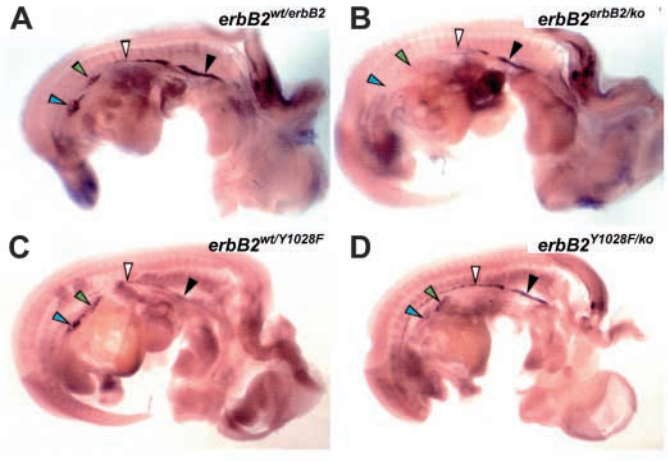

Fig. 3. Development of the primary sympathetic ganglion chain. Whole-mount in situ hybridization staining of the sympathetic nervous system using a Phox2a antisense riboprobe on E12.5 embryos. Mid-sagittal views of (A) *Erbb2*$^{wt/Erbb2}$, (B) *Erbb2*$^{Erbb2/ko}$, (C) *Erbb2*$^{wt/Y1028F}$, (D) *Erbb2*$^{Y1028F/ko}$. Black arrowheads point to the superior cervical ganglia; white arrowheads highlight the thoracic sympathetic chain ganglia; green and blue arrowheads indicate the cells that migrate from the caudal portion of the primary sympathetic chain to the mesentery or the anlage of the adrenal gland.

post-transcriptional level, *Erbb2* transcripts in E12.5 embryos were detected using an RNase protection assay (Fig. 4D). The results showed that samples harboring either the *Erbb2* or the *Y1028F* knock-in allele expressed identical levels of *Erbb2* transcripts (Fig. 4D, lanes 4-5 versus lanes 6-8). Note that either knock-in alleles express significantly lower levels of *Erbb2* transcripts compared to wild-type animals. This phenomenon is probably a result of using a cDNA knock-in strategy and explains the overall lower Erbb2 expression levels in the knock-in animals. Regardless of this, our observations argue that specifically expressing the *Y1028F* knock-in allele results in increased Erbb2 protein levels without affecting transcriptional activity, when compared to the *Erbb2* knock-in allele.

Since the function and activity of the ErbB family of receptors are closely related, it is plausible that other family members may be affected by the low Erbb2 levels or may be elevated to compensate for the loss Erbb2 activity. In this regard, the expression level of other ErbB receptor family members was measured to determine if there were corresponding differences in expression. Interestingly, Egfr levels were modestly affected depending on the level of Erbb2 in the knock-in animals, which may augment the defects observed. No significant differences in ErbB3 or ErbB4 levels were noted in the wild-type and knock-in animals that would suggest any kind of compensatory role for these receptors.

Y1028 influences Erbb2 receptor turnover rate

Since many receptor tyrosine kinases are downregulated by endocytosis and subsequently targeted for degradation (Katzmann et al., 2002), we examined whether Y1028 affected Erbb2 receptor turnover rate (Fig. 5A,B). Rat-1 cell lines stably expressing oncogenic Erbb2 (V664E mutation) or its mutant tyrosine phosphorylation site derivatives were established. Specifically, we compared the turnover rate of the oncogenic

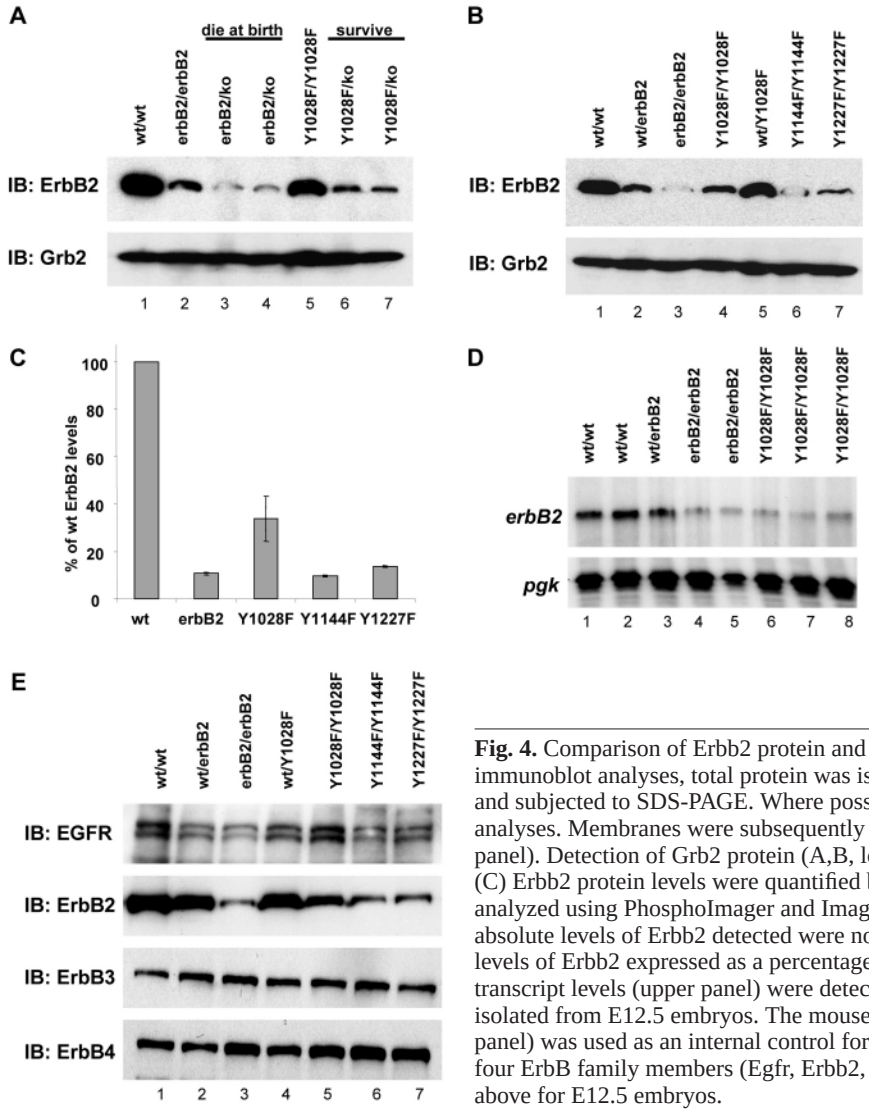

Fig. 4. Comparison of Erbb2 protein and transcript levels in knock-in embryos. For immunoblot analyses, total protein was isolated from the different E12.5 embryos as indicated and subjected to SDS-PAGE. Where possible, control littermates were used for comparison analyses. Membranes were subsequently incubated with an anti-Erbb2 antibody (A,B, upper panel). Detection of Grb2 protein (A,B, lower panel) was used as a sample loading control. (C) Erbb2 protein levels were quantified by using ^{125}I-conjugated secondary antibodies and analyzed using PhosphoImager and ImageQuant software (Amersham Biosciences). The absolute levels of Erbb2 detected were normalized to Grb2 levels. The graph depicts relative levels of Erbb2 expressed as a percentage of Erbb2 levels in wild type embryos. (D) *Erbb2* transcript levels (upper panel) were detected by RNase protection assays on total RNA isolated from E12.5 embryos. The mouse phosphoglycerate kinase (*pgk*) riboprobe (lower panel) was used as an internal control for equal sample loading. (E) The protein levels of all four ErbB family members (Egfr, Erbb2, ErbB3, and ErbB4) were also detected as described above for E12.5 embryos.

Erbb2 receptor versus the *Y1028F* mutant receptor. Conversely, we also assessed the effects of restoring *Y1028* to the *Y1144* add-back mutant to generate the *Y1028/Y1144* double add-back mutant Erbb2 receptor. Add-back mutants are derived from an Erbb2 receptor stripped of the five major tyrosine autophosphorylation sites by tyrosine-to-phenylalanine mutations and then individual mutant sites are 'added-back' or reverted to tyrosine residues.

Pulse-chase analyses using Rat-1-derived stable cell lines revealed that the *Y1028F* mutation significantly stabilized the Erbb2 receptor compared to the wild-type receptor (Fig. 5A). Alternatively, when Y1028 was restored to the *Y1144* add-back mutant, the receptor turnover rate of Erbb2-Y1028/Y1144 increased (Fig. 5B). Endocytosis and turnover of the Erbb2 receptor at the plasma membrane, as determined by monitoring biotin-labeled surface receptor (data not shown), was consistent with the ^{35}S-labeled pulse-chase data. The subcellular localization of Erbb2 or the Y1028F mutant receptor was subsequently determined in transiently transfected Rat-1 cells. Consistent with the retention of the Y1028F receptor at the cell surface, there was a significantly higher proportion of Y1028F receptor localized at the cell surface as determined by immunofluorescent detection when compared to the wild-type Erbb2 receptor (Fig. 5C).

Based on the above results, we next examined whether the E3-ubiquitin ligase, c-Cbl, was responsible for mediating the negative regulatory effect of Y1028 on Erbb2 activity since c-Cbl has been shown to mediate the downregulation of the Egfr and other receptor tyrosine kinases (Thien and Langdon, 2001). Our data suggest that c-Cbl is able to associate with Erbb2 through other specific phosphotyrosine residues, and that Y1028 is not responsible for the recruitment of c-Cbl to Erbb2, nor is it required for the ubiquitylation of Erbb2 (Fig. 5D). Taken together, these observations suggest that Y1028 modulates Erbb2 protein levels in a c-Cbl- and ubiquitylation-independent manner.

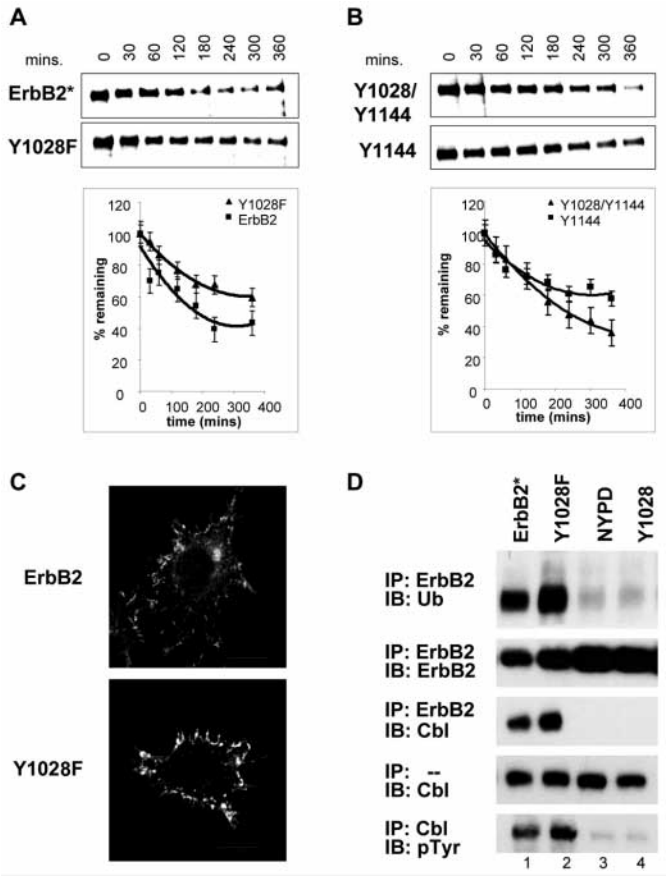

Fig. 5. Tyrosine 1028 promotes the downregulation of Erbb2 in Rat-1 cells. The stability of mutant Erbb2 receptors in the presence or absence of Y1028 was examined by pulse-chase analyses using ^{35}S-methionine labeled Rat-1 stable cell lines expressing the oncogenic versions (*V664E* mutation) of the *Erbb2** phosphorylation mutants. (A) Erbb2* versus Erbb2*-Y1028F. (B) Erbb2*-Y1144 add-back mutant versus Erbb2*-Y1028/Y1144 double add-back mutant. Representative gels are shown for each and the average of multiple experiments is depicted graphically as a percentage of the original Erbb2 levels remaining. (C) The subcellular localization of Erbb2 was determined by immunofluorescent staining using an anti-Erbb2 antibody (Ab4, Oncogene Science) on Rat-1 cells expressing the non-oncogenic versions of Erbb2. The image was taken on a Zeiss LSM510 confocal microscope and is representative of comparable *z*-plane sections through the cell. Scale bar: 10 μm. (D) The ubiquitylation status of *Erbb2** mutants expressed transiently in 293T cells were examined by immunoprecipitation using an anti-Erbb2 antibody and then blotting the top half of the membrane with an anti-ubiquitin antibody (Santa Cruz). The blots were then stripped and blotted with an anti-Erbb2 antibody to check for equal levels of Erbb2. The bottom half of the membrane was incubated with anti-Cbl antibodies (Santa Cruz) to examine Erbb2-Cbl association. The phosphorylation status of c-Cbl when co-expressed with different Erbb2* phosphorylation site mutants was examined by immunoprecipitation of c-Cbl followed by blotting with an anti-phosphotyrosine antibody (PY20, Transduction Labs). Erbb2*, constitutively activated (oncogenic) Erbb2; Y1028F is the point mutation; NYPD is the tyrosine-deficient *Erbb2* mutant; Y1028 is the *Y1028* add-back mutant.

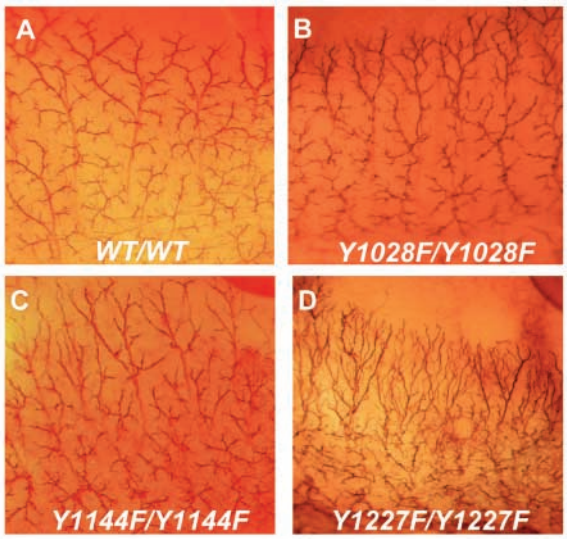

Fig. 6. Sensory nerve defects in Y1227F knock-in mutants. Homozygous E12.5 knock-in embryos were subjected to whole-mount immunostaining using anti-neurofilament antibodies. The morphologic appearance of the sensory cutaneous nerves in the thoracic cavity wall region is shown for (A) *Erbb2*$^{wt/wt}$ (B) *Erbb2*$^{Y1028F/Y1028F}$ (C) *Erbb2*$^{Y1144F/Y1144F}$ and (D) *Erbb2*$^{Y1227F/Y1227F}$. Note that the nerves in D are disorganized and defasciculated.

Differential Erbb2 signaling requirements in development

Although knock-in animals expressing a single Erbb2 receptor tyrosine phosphorylation site mutation were grossly normal, it was conceivable that there may be subtle defects as a result of ablating a particular Erbb2-initiated signaling pathway. Thus, whole embryos (E12.5) were immunostained with anti-neurofilament (Fig. 6) to examine the appearance of sensory cutaneous nerves, which were abnormally defasciculated in the cardiac rescued *Erbb2* mutants and in *Erbb3*-deficient mutants (Woldeyesus et al., 1999). Interestingly, in comparison with $Erbb2^{wt/wt}$ embryos (Fig. 6A), there was a striking defect in the morphology of the developing cutaneous nerves in the thoracic body wall of $Erbb2^{Y1227F/Y1227F}$ embryos (Fig. 6D) that was not observed in $Erbb2^{Y1028F/Y1028F}$ or $Erbb2^{Y1144F/Y1144F}$ knock-in embryos (Fig. 6B,C). The trajectories of the sensory nerves in $Erbb2^{Y1227F/Y1227F}$ mutants appeared intact but were highly disorganized and severely defasciculated, similar to the defects in the cardiac-rescued *Erbb2* mutants (Woldeyesus et al., 1999). These results suggest that the signal transduction pathways initiated specifically by Erbb2-Y1227 are absolutely required for the proper development of the cutaneous sensory nerves in the thoracic body wall.

Discussion

Using a series of *Erbb2* cDNA knock-in animals encoding Erbb2 receptors with different tyrosine autophosphorylation site mutations, we have identified a minimum threshold level of Erbb2 (above ~5% of wild-type levels) that is required to sustain viability and physiological function. We have also presented evidence suggesting that the individual Erbb2 tyrosine phosphorylation sites have specific and unique roles in development. Inadvertently, the cDNA knock-in allele turned out to be a hypomorph expressing only ~10% of the level of Erbb2 expressed in wild-type littermates. Despite the low levels of Erbb2, homozygous *Erbb2* knock-in animals were viable and overtly normal and healthy without any compensatory effect from other ErbB receptor family members. We cannot however exclude the possibility that there may be more subtle defects that are undetectable or have not been identified. Indeed, we have recently demonstrated that similar hypomorphic knock-in animals displayed no phenotypic differences compared to their wild-type siblings despite having tenfold fewer spindle cells (Andrechek et al., 2002).

Nonetheless, it is intriguing that the animals express significantly higher levels of Erbb2 but only require about 10% for development. Since Erbb2 acts primarily as a co-receptor and its activation is dependent on the ligand-mediated activation of its ErbB counterparts, the high levels of Erbb2 may suggest its excess availability to engage in a dimer signaling unit. Thus, the threshold that we have identified may also reflect the threshold activity of the other ErbB receptors. When the *Erbb2* knock-in animals were inter-crossed with *Erbb2*-deficient animals to generate hemizygous knock-in animals ($Erbb2^{Erbb2/ko}$), this additional twofold reduction in Erbb2 levels fell below the threshold level required and resulted in perinatal lethality due to acute respiratory distress. The inability of newborn $Erbb2^{Erbb2/ko}$ pups to inflate their lungs is remarkably similar in phenotype to the cardiac-specific rescue of *Erbb2*-deficient mutants (Morris et al., 1999; Woldeyesus et al., 1999).

Evaluation of the diaphragm muscles of E18.5 embryos revealed an obvious defect in the phrenic nerve of mutant embryos. In the cardiac-rescued *Erbb2* mutants, Woldeyesus et al. (Woldeyesus et al., 1999) reported that the phrenic nerve was thin and poorly fasciculated at E14.5 but only remnants were detected at later stages. Observations by Morris et al. (Morris et al., 1999) showed that diaphragms of E18.5 mutant embryos were completely devoid of innervations, and AChR clusters were more dispersed and rounded in shape. Interestingly, the diaphragm muscles of our $Erbb2^{Erbb2/ko}$ embryos at E18.5 (Fig. 2) were innervated, although a main nerve trunk was absent and any nerves that were present were very thin, highly disorganized and fragmented. We also did not observe any differences in the density or shape of AChR receptor clusters compared to those of wild-type embryos. These results support the notion of progressive threshold sensitivities to the level of Erbb2 signaling in the developing peripheral nervous system. Although the low levels of Erbb2 prevented or impeded the complete degeneration of motor neurons in the diaphragm muscle and maintained apparently normal clustering of AChR at the neuromuscular junctions, this was not sufficient for the diaphragm to respond and function. Also note that the low levels of Erbb2 were sufficient to bypass the cardiac trabeculation defects in *Erbb2* null embryos, suggesting that the cardiac system is not as sensitive as the peripheral nervous system to this threshold level of Erbb2.

Conversely, we were able to genetically rescue the perinatal lethality in hemizygous $Erbb2^{Erbb2/ko}$ embryos by similarly introducing the *Y1028F* knock-in allele into an *Erbb2*-deficient background to generate $Erbb2^{Y1028F2/ko}$ animals. Tyrosine 1028 is a negative regulatory signal that affects the stability of the receptor (discussed below). All *Erbb2* knock-in mice bearing the *Y1028F* allele expressed higher levels of Erbb2 protein than in the *Erbb2* knock-in mice. Thus, this genetic manipulation ablating a negative regulatory phosphotyrosine site in the carboxyl-terminus increased the level of Erbb2 above the minimal threshold required for the phrenic nerve in the diaphragm to develop normally. $Erbb2^{Y1028F2/ko}$ animals survived and were phenotypically healthy. In contrast, similar experiments performed with the *Y1144F* or the *Y1227F* knock-in alleles also resulted in perinatal lethality with similar defects in the innervation of the diaphragm muscle. These two mutations, of course, did not affect the level of Erbb2 expressed in the knock-in animals.

Although previous in vitro experiments concluded that Y1028 negatively regulated oncogenic Erbb2-mediated transformation, we were also interested in determining whether Y1028 would also have this suppressive effect in vivo in the context of a non-oncogenic Erbb2 receptor. As described above, the results of the in vivo experiments with the *Y1028F* knock-in animals are consistent with a negative regulatory effect of Y1028 on Erbb2 activity. Moreover, here we have also identified the biochemical basis for this effect, which was previously unknown. Phosphotyrosine 1028 modulates Erbb2 protein levels by promoting the downregulation and turnover of the receptor in a c-Cbl- and ubiquitin-independent manner. Conversely, loss of Y1028 resulted in an increase in Erbb2 protein stability. Thus, the stabilization of Erbb2 levels above the minimum threshold in

the hemizygous *Erbb2*$^{Y1028F2/ko}$ animals allowed these particular strains to develop normally.

Sequence alignment of Erbb2 with the Egfr shows that Erbb2-Y1028 and its surrounding sequences share a high degree of similarity to the region surrounding the Egfr-Y992 (Fig. 1D). Despite the identity and function of Egfr-Y992 suggested in 1989 (Chen et al., 1989), the mechanism of its function has not been clearly elucidated. Carboxyl-terminal truncation of the Egfr has suggested that an 18 amino acid region surrounding Y992 conforms to an 'internalization' domain and is required for Egf-dependent receptor internalization. However, a subsequent study showed that point mutation of *Y992* did not affect Egfr internalization (Sorkin et al., 1992). In another study, the *Y992F* mutation actually increased the rate of Egfr internalization and it was suggested that the increase in negative charge associated with phosphorylation of Y992 would reduce the rate of ligand-induced endocytosis (Holbrook et al., 1999). The nature of these discrepancies may be due to artefactual differences in cell types, the level of ectopic Egfr expression in the cells, or the nature of the mutation used in their analyses. Our results showing the effect of Y1028, in a physiologically relevant context, to promote the turnover rate of the Erbb2 receptor from the cell surface should lead to clarification of these conflicting reports concerning the role of Y992 in the Egfr.

Homozygous knock-in animals expressing *Erbb2* phosphotyrosine mutations where the Grb2 site (Y1144) or the Shc-binding site (Y1227) were ablated had little impact on the gross development of mice, even at tenfold less expression. As discussed above, previous studies in our lab revealed a significant reduction in the number of muscle spindle cells in *Erbb2*$^{Erbb2/Erbb2}$ animals without any obvious phenotypic consequences (Andrechek et al., 2002). Therefore, it is conceivable that other specific cell or tissue types may be more sensitive to the altered Erbb2 signaling. Indeed, the cutaneous sensory nerves in the thoracic body wall of *Erbb2*$^{Y1227F/1227F}$ mutants were thin and defasciculated, whereas *Erbb2*$^{Y1144F/Y1144F}$ embryos were similar to wild type. Since Erbb2-Y1227 binds to the Shc adapter protein, these results suggest that the Shc signaling pathway may play an important and unique role downstream of Erbb2 in the development of these sensory nerves. Furthermore, it is interesting to note that Shc can recruit phosphatidylinositol 3-kinase (PI3K) (Gu et al., 2000). PI3K is a major signaling pathway downstream of ErbB3, which also display a strikingly similar abnormal sensory nerve phenotype in *Erbb3*-deficient animals (Woldeyesus et al., 1999). Thus, it is conceivable that the Erbb2 and ErbB3 signaling pathways converge downstream at the level of PI3K, such that PI3K plays a key role in the development of sensory nerves. In general, this raises the idea that signaling pathways downstream of the individual receptor phosphotyrosine sites can function differentially and independently in the development of specific tissues.

Unlike with Erbb2, where loss of the Grb2 binding site (Y1144) did not result in any discernable phenotype, uncoupling of Grb2 from the Met receptor in a knock-in model resulted in severe defects in muscle development (Maina et al., 1996). Taken together, these observations suggest that the remaining Erbb2 autophosphorylation sites are able to functionally substitute for the inability of these *Erbb2* mutants to recruit Grb2. Alternatively, it is possible that the Erbb2 heterodimerization partners such as Egfr, ErbB3 or ErbB4 can compensate for the lack of Grb2-binding sites on Erbb2. Indeed, it has been demonstrated that Egfr or ErbB3 can independently bind Shc and Grb2 (Batzer et al., 1994; Carraway and Cantley, 1994; Okutani et al., 1994; Prigent and Gullick, 1994). It is also possible that there may be other more subtle developmental defects in different tissues of the *Erbb2*^{Y1144F} knock-in mutants. In this regard, transgenic mice expressing constitutively activated *Erbb2* mutants coupled to either Grb2 or Shc specifically in the mammary epithelium develop morphologically distinct mammary tumors that possess inherently different metastatic properties (Dankort et al., 2001).

In summary, using a series of unique *Erbb2* knock-in animals and genetic manipulation, we have established that a minimum threshold level of Erbb2 receptor expression is required during proper development. This critical threshold is only 5-10% of normal Erbb2 levels in wild-type embryos. We have also identified an important in vivo function of the intrinsic negative regulatory site in Erbb2 (Y1028) to modulate the stability/turnover rate of the receptor throughout development. In addition, our data suggest that individual Erbb2 signaling pathways are not redundant, but rather play unique roles in specific tissues throughout development.

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References

Akiyama, T., Matsuda, S., Namba, Y., Saito, T., Toyoshima, K. and Yamamoto, T. (1991). The transforming potential of the c-erbB-2 protein is regulated by its autophosphorylation at the carboxyl-terminal domain. *Mol. Cell. Biol.* **11**, 833-842.

Andrechek, E. R., Hardy, W. R., Girgis-Gabardo, A. A., Perry, R. L., Butler, R., Graham, F. L., Kahn, R. C., Rudnicki, M. A. and Muller, W. J. (2002). ErbB2 is required for muscle spindle and myoblast cell survival. *Mol. Cell. Biol.* **22**, 4714-4722.

Batzer, A. G., Rotin, D., Urena, J. M., Skolnik, E. Y. and Schlessinger, J. (1994). Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor. *Mol. Cell. Biol.* **14**, 5192-5201.

Britsch, S., Li, L., Kirchhoff, S., Theuring, F., Brinkmann, V., Birchmeier, C. and Riethmacher, D. (1998). The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. *Genes Dev.* **12**, 1825-1836.

Carraway, K. L., 3rd and Cantley, L. C. (1994). A neu acquaintance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling. *Cell* **78**, 5-8.

Carraway, K. L., 3rd, Sliwkowski, M. X., Akita, R., Platko, J. V., Guy, P. M., Nuijens, A., Diamonti, A. J., Vandlen, R. L., Cantley, L. C. and Cerione, R. A. (1994). The erbB3 gene product is a receptor for heregulin. *J. Biol. Chem.* **269**, 14303-14306.

Carraway, K. L., 3rd, Rossi, E. A., Komatsu, M., Price-Schiavi, S. A., Huang, D., Guy, P. M., Carvajal, M. E., Fregien, N., Carraway, C. A. and Carraway, K. L. (1999). An intramembrane modulator of the ErbB2 receptor tyrosine kinase that potentiates neuregulin signaling. *J. Biol. Chem.* **274**, 5263-5266.

Chan, R., Hardy, W. R., Laing, M. A., Hardy, S. E. and Muller, W. J.

(2002). The catalytic activity of the ErbB-2 receptor tyrosine kinase is essential for embryonic development. *Mol. Cell. Biol.* **22**, 1073-1078.

Chen, W. S., Lazar, C. S., Lund, K. A., Welsh, J. B., Chang, C. P., Walton, G. M., Der, C. J., Wiley, H. S., Gill, G. N. and Rosenfeld, M. G. (1989). Functional independence of the epidermal growth factor receptor from a domain required for ligand-induced internalization and calcium regulation. *Cell* **59**, 33-43.

Dankort, D. L., Wang, Z., Blackmore, V., Moran, M. F. and Muller, W. J. (1997). Distinct tyrosine autophosphorylation sites negatively and positively modulate neu-mediated transformation. *Mol. Cell. Biol.* **17**, 5410-5425.

Dankort, D., Jeyabalan, N., Jones, N., Dumont, D. J. and Muller, W. J. (2001). Multiple ErbB-2/Neu Phosphorylation Sites Mediate Transformation through Distinct Effector Proteins. *J. Biol. Chem.* **276**, 38921-38928.

Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R. and Lemke, G. (1995). Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature* **378**, 390-394.

Goldman, R., Levy, R. B., Peles, E. and Yarden, Y. (1990). Heterodimerization of the erbB-1 and erbB-2 receptors in human breast carcinoma cells: a mechanism for receptor transregulation. *Biochemistry* **29**, 11024-11028.

Gu, H., Maeda, H., Moon, J. J., Lord, J. D., Yoakim, M., Nelson, B. H. and Neel, B. G. (2000). New role for Shc in activation of the phosphatidylinositol 3-kinase/Akt pathway. *Mol. Cell. Biol.* **20**, 7109-7120.

Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A. and Carraway, K. L., 3rd (1994). Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. *Proc. Natl. Acad. Sci. USA* **91**, 8132-8136.

Hazan, R., Margolis, B., Dombalagian, M., Ullrich, A., Zilberstein, A. and Schlessinger, J. (1990). Identification of autophosphorylation sites of HER2/neu. *Cell Growth Differ.* **1**, 3-7.

Holbrook, M. R., O'Donnell, J. B., Jr, Slakey, L. L. and Gross, D. J. (1999). Epidermal growth factor receptor internalization rate is regulated by negative charges near the SH2 binding site Tyr992. *Biochemistry* **38**, 9348-9356.

Hynes, N. E. and Stern, D. F. (1994). The biology of erbB-2/neu/HER-2 and its role in cancer. *Biochim. Biophys. Acta* **1198**, 165-184.

Karunagaran, D., Tzahar, E., Beerli, R. R., Chen, X., Graus-Porta, D., Ratzkin, B. J., Seger, R., Hynes, N. E. and Yarden, Y. (1996). ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. *EMBO J.* **15**, 254-264.

Katzmann, D. J., Odorizzi, G. and Emr, S. D. (2002). Receptor downregulation and multivesicular-body sorting. *Nat. Rev. Mol. Cell Biol.* **3**, 893-905.

Kavanaugh, W. M., Turck, C. W. and Williams, L. T. (1995). PTB domain binding to signaling proteins through a sequence motif containing phosphotyrosine. *Science* **268**, 1177-1179.

King, C. R., Borrello, I., Bellot, F., Comoglio, P. and Schlessinger, J. (1988). Egf binding to its receptor triggers a rapid tyrosine phosphorylation of the erbB-2 protein in the mammary tumor cell line SK-BR-3. *EMBO J.* **7**, 1647-1651.

Lee, K. F., Simon, H., Chen, H., Bates, B., Hung, M. C. and Hauser, C. (1995). Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* **378**, 394-398.

Maina, F., Casagranda, F., Audero, E., Simeone, A., Comoglio, P. M., Klein, R. and Ponzetto, C. (1996). Uncoupling of Grb2 from the Met receptor in vivo reveals complex roles in muscle development. *Cell* **87**, 531-542.

Morris, J. K., Lin, W., Hauser, C., Marchuk, Y., Getman, D. and Lee, K. F. (1999). Rescue of the cardiac defect in ErbB2 mutant mice reveals essential roles of ErbB2 in peripheral nervous system development. *Neuron* **23**, 273-283.

Okutani, T., Okabayashi, Y., Kido, Y., Sugimoto, Y., Sakaguchi, K., Matuoka, K., Takenawa, T. and Kasuga, M. (1994). Grb2/Ash binds directly to tyrosines 1068 and 1086 and indirectly to tyrosine 1148 of activated human epidermal growth factor receptors in intact cells. *J. Biol. Chem.* **269**, 31310-31314.

Olayioye, M. A., Neve, R. M., Lane, H. A. and Hynes, N. E. (2000). The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J.* **19**, 3159-3167.

Pawson, T. (1995). Protein modules and signalling networks. *Nature* **373**, 573-580.

Peles, E., Ben-Levy, R., Tzahar, E., Liu, N., Wen, D. and Yarden, Y. (1993). Cell-type specific interaction of Neu differentiation factor (NDF/heregulin) with Neu/HER-2 suggests complex ligand-receptor relationships. *EMBO J.* **12**, 961-971.

Pinkas-Kramarski, R., Shelly, M., Glathe, S., Ratzkin, B. J. and Yarden, Y. (1996a). Neu differentiation factor/neuregulin isoforms activate distinct receptor combinations. *J. Biol. Chem.* **271**, 19029-19032.

Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B. J., Sela, M. et al. (1996b). Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J.* **15**, 2452-2467.

Pinkas-Kramarski, R., Lenferink, A. E., Bacus, S. S., Lyass, L., van de Poll, M. L., Klapper, L. N., Tzahar, E., Sela, M., van Zoelen, E. J. and Yarden, Y. (1998a). The oncogenic ErbB-2/ErbB-3 heterodimer is a surrogate receptor of the epidermal growth factor and betacellulin. *Oncogene* **16**, 1249-1258.

Pinkas-Kramarski, R., Shelly, M., Guarino, B. C., Wang, L. M., Lyass, L., Alroy, I., Alimandi, M., Kuo, A., Moyer, J. D., Lavi, S. et al. (1998b). ErbB tyrosine kinases and the two neuregulin families constitute a ligand-receptor network. *Mol. Cell. Biol.* **18**, 6090-6101.

Prigent, S. A. and Gullick, W. J. (1994). Identification of c-erbB-3 binding sites for phosphatidylinositol 3′-kinase and SHC using an EGF receptor/c-erbB-3 chimera. *EMBO J.* **13**, 2831-2841.

Riethmacher, D., Sonnenberg-Riethmacher, E., Brinkmann, V., Yamaai, T., Lewin, G. R. and Birchmeier, C. (1997). Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature* **389**, 725-730.

Simpson, B. J., Phillips, H. A., Lessells, A. M., Langdon, S. P. and Miller, W. R. (1995). c-erbB growth-factor-receptor proteins in ovarian tumours. *Int. J. Cancer* **64**, 202-206.

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A. and McGuire, W. L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**, 177-182.

Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A. et al. (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **244**, 707-712.

Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L. and Carraway, K. L., 3rd (1994). Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. *J. Biol. Chem.* **269**, 14661-14665.

Sorkin, A., Helin, K., Waters, C. M., Carpenter, G. and Beguinot, L. (1992). Multiple autophosphorylation sites of the epidermal growth factor receptor are essential for receptor kinase activity and internalization. Contrasting significance of tyrosine 992 in the native and truncated receptors. *J. Biol. Chem.* **267**, 8672-8678.

Stern, D. F. and Kamps, M. P. (1988). EGF-stimulated tyrosine phosphorylation of p185neu: a potential model for receptor interactions. *EMBO J.* **7**, 995-1001.

Thien, C. B. and Langdon, W. Y. (2001). Cbl: many adaptations to regulate protein tyrosine kinases. *Nat. Rev. Mol. Cell Biol.* **2**, 294-307.

Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunagaran, D., Lavi, S., Ratzkin, B. J. and Yarden, Y. (1996). A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol. Cell. Biol.* **16**, 5276-5287.

Tzahar, E., Pinkas-Kramarski, R., Moyer, J. D., Klapper, L. N., Alroy, I., Levkowitz, G., Shelly, M., Henis, S., Eisenstein, M., Ratzkin, B. J. et al. (1997). Bivalence of EGF-like ligands drives the ErbB signaling network. *EMBO J.* **16**, 4938-4950.

Wilkinson, D. G. and Nieto, M. A. (1993). Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. *Methods Enzymol.* **225**, 361-373.

Woldeyesus, M. T., Britsch, S., Riethmacher, D., Xu, L., Sonnenberg-Riethmacher, E., Abou-Rebyeh, F., Harvey, R., Caroni, P. and Birchmeier, C. (1999). Peripheral nervous system defects in erbB2 mutants following genetic rescue of heart development. *Genes Dev.* **13**, 2538-2548.

Yarden, Y. and Sliwkowski, M. X. (2001). Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell Biol.* **2**, 127-137.