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13. ABSTRACT (Maximum 200 Words)
The ErbB2 receptor tyronsine kinase plays important roles in development and disease. To investigate the developmental roles of ErbB2 mediated signaling in vivo, we have generated a series of erbB2 cDNA knock-in animals expressing different ErbB2 mutants under the transcriptional control of the endogenous promoter. Firstly, we showed that the kinase activity of ErbB2 is essential for embryonic development since this mutant is a phenocopy of the erbB2 null mutants, which die at midgestation with cardiac and peripheral nervous system defects. In our studies, we also established a minimal threshold level of ErbB2 that was required for embryonic development and survival. The tyrosone autophosphorylation site Y1028 mediated a negative regulatory signal that affected the level of ErbB2 proteins expressed. This tyronsine appears to play a role in normal signaling and development. The mechanism of action is known at this point, however, the downregulatory effect is independent of Cbl binding and ubiquitylation of the receptor. In fact, we have also identified the phosphorylation sites in ErbB2 that is required for it's interaction with Cbl, although ErbB2 appears to be refractory to the downregulatory effect of Cbl. The results of my studies will provide important insight into the regulatory mechanisms controlling ErbB2 signaling and may lead to improved therapeutics.

14. SUBJECT TERMS
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Introduction

Amplification and overexpression of the ErbB2/Neu receptor tyrosine kinase result in aberrant activation of the receptor, as exemplified in 20-30% of human breast cancers (Slamon et al., 1987). Conversely, the embryonic lethality and developmental defects observed in animals with a targeted mutation of the receptor clearly demonstrates its critical roles in mammalian development (Britsch et al., 1998; Lee et al., 1995). Thus, due to its important biological functions, it is desirable to understand the diversity and specificity in receptor tyrosine kinase signaling. The ErbB2 receptor is a member of the Epidermal Growth Factor Receptor family, which also includes the EGFR (ErbB1), ErbB3, and ErbB4. These receptors can form different combinations of homodimers and heterodimers, which contribute to this family's complex array of signaling potential (Pinkas-Kramarski et al., 1998; Pinkas-Kramarski et al., 1996). ErbB2 is thought to be the key member of this family particularly because of its potent tyrosine kinase activity. It is the preferred heterodimerization partner for the other ErbB receptors forming stable and strong signaling complexes (Graus-Porta et al., 1997). Previous members of Dr. Muller's lab have characterized five distinct tyrosine autophosphorylation sites in the C-terminal regulatory region of the receptor (Figure 1) (Dankort et al., 1997). These phosphorylated tyrosine residues become sites of interaction with downstream signaling molecules, thereby initiating the signal transduction pathway. My research entailed generating and characterizing different *erbB2* cDNA knock-in mutants to further our understanding of ErbB2 receptor function and signaling.

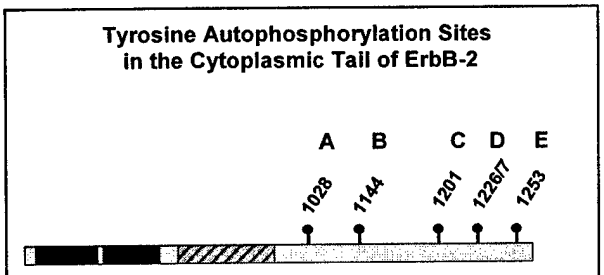

Figure 1. Five tyrosine residues in the cytoplasmic region of ErbB-2 are labeled A through E for simplicity.

Body

The ErbB2 receptor contains an extracellular ligand binding domain, a transmembrane domain, a potent tyrosine kinase domain, and a regulatory C-terminal region harboring several distinct tyrosine autophosphorylation sites (Figure 1) Dankort *et al.* (1997). The functional roles of some of these domains were studied previously in Dr. Muller's lab using *in vitro* transformation assays. In the past, most studies on ErbB2 signaling involved oncogenic activity in transformation or tumorigenic assays, either *in vitro* or *in vivo*. However, as the critical role for ErbB2 signaling in normal mammalian development was realized through gene-targeting studies, this provided an alternate approach to genetically dissect oncogenic ErbB2 signaling. By elucidating the 'normal' ErbB2 signaling, we will be able to better understand what is considered the 'abnormal' state and evaluate how aberrant ErbB2 signaling may result in tumorigenesis. Thus, the aim of my project was to generate and characterize a series of mutant erbB2 cDNA knock-in animals to validate and elucidate the role of different ErbB2 signaling components, *in vivo*.

In animals that were generated previously in Dr. Muller's lab, expression of an erbB2 cDNA in knock-in animals was sufficient to rescue the cardiac defects associated with the targeted disruption (using a PGK-Neo cassette) of the endogenous erbB2 gene. However, these animals unexpectedly expressed only 10% of normal ErbB2 levels, likely due to inefficient mRNA transcript stability and/or processing. Since this was thought to be potentially problematic, the targeting construct was re-designed. In this new construct, the corresponding C-terminal portion of the cDNA was inserted in-frame to exon 4 of the endogenous erbB2 gene at a common Sal I site, followed by a PGK-Neomycin cassette flanked by LoxP sites. The resultant ErbB2 receptor would be a chimera of mouse and rat erbB2 sequences (Figure 2). Note that the

mouse and rat sequences are greater than 93% homologous at both the nucleic acid and protein level. The presumed advantage of this design is that the transcript will follow the normal pathway for transcript processing, which may increase the transport or stability of the transcript. In addition, the floxed PGK-Neo cassette may be removed by the expression of the Cre recombinase, which may also improve the efficiency of expression. The constructs containing a wild type receptor and the tyrosine phosphorylation mutant receptors were completed and electroporated into R1 ES cells and several chimeric mice were generated. However, we did not get successful germline transfer of the knock-in allele after several litters. As a consequence of this misfortune, I decided to focus on the other set of erbB2 cDNA knock-in animals (described above) that were generated previously but had not yet been fully characterized.

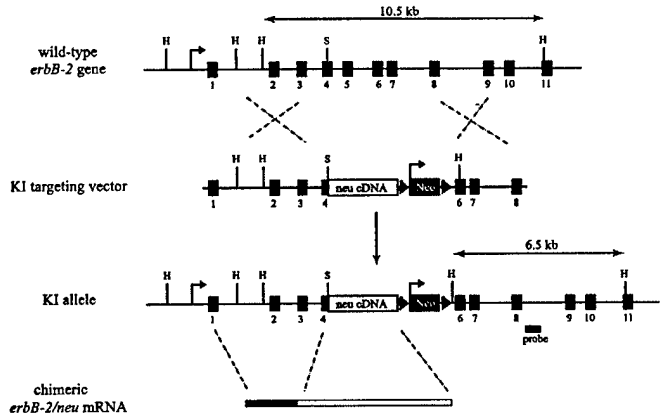

Figure 2. Modified targeted erbB2 knock-in strategy. The final transcribed product is a chimera of the mouse erbB2 exons 1-4 (SalI site) and the rat erbB2 cDNA from a common Sal I site.

The first mutant ErbB2 receptor analyzed addressed the role and importance of the receptor's kinase activity within the array of ErbB receptor heterodimer and homodimer interactions. ErbB2 is thought to be the key player and the preferred dimerization partner for other ErbB receptors. We have addressed the significance of the kinase activity of ErbB2 by generating knock-in mice expressing a kinase-dead ErbB2 (Neu-KD) receptor. This analysis was completed and published in *Molecular and Cellular Biology Volume 22, p. 1073-1078, 2002* (Appendix 1). In summary, we demonstrated that the kinase-dead ErbB2 receptor is a

functionally null receptor *in vivo* and the knock-in mice expressing this mutant die at midgestation due to aberrant heart development and display abnormal peripheral nervous system development. These observations are identical to the erbB2 knock-out animals (Britsch et al., 1998; Lee et al., 1995). Thus, we concluded that the kinase activity of ErbB2 is absolutely required for ErbB-mediated signaling.

Interestingly, in the analysis of knock-in mice expressing the kinase-dead ErbB2 receptor described above, we observed that the control wild type erbB2 cDNA knock-in animal expressed only 10% of normal ErbB2 levels when compared to wild type littermates. This was an unexpected finding but also an intriguing observation from a developmental perspective. The results are described in detail in the appended manuscript (*Modulation of ErbB2 signaling during development: A threshold level of ErbB2 signaling is required for normal development*; Appendix 2) that we recently submitted for publication. Briefly, ErbB2 knock-in animals were phenotypically normal and healthy despite the low 10% expression level. We pushed this limit further by interbreeding erbB2 knock-in mice with heterozygous erbB2 null mice to generate hemizygous animals expressing a single hypomorphic erbB2 knock-in allele. This further two-fold reduction apparently set the ErbB2 protein level below a minimal threshold, which resulted in perinatal lethality. The newborn mutant pups died at birth due to aberrant development of their peripheral nervous system leading to the inability to inflate their lungs. These observations were consistent with the peripheral nervous system defects in cardiac specific rescued erbB2 null animals (Morris et al., 1999; Woldeyesus et al., 1999).

We next asked whether we could genetically rescue this hypomorphic phenotype by introducing a mutant ErbB2 receptor where the intrinsic negative regulatory site was removed. Tyrosine 1028 was identified previously as having negative effects on ErbB2-mediated

transformation of fibroblast cells in culture, whereas mutation of Y1028 resulted in an increase in transformation potential. Using an identical targeted knock-in strategy, mice were generated expressing the ErbB2-Y1028F mutant receptor. These mice were also overtly normal and healthy. However, when the ErbB2-Y1028F knock-in mice were interbred with heterozygous erbB2 null animals, the resultant hemizygous animals were healthy and did not display any acute respitratory distress. Thus, expression of the Y1028F mutation rescued the defects in the peripheral nervous system. These results are consistent with the *in vitro* transformation data showing that Y1028 has negative regulatory effects on ErbB2 signaling.

We then determined the molecular basis for this genetic rescue of the perinatal lethality. Immunoblot analyses of protein lysates from E12.5 embryos revealed that the animals expressing the ErbB2-Y1028F mutation expressed significantly higher levels of protein compared to the control erbB2 knock-in animals. However, this increase did not correspond with any changes in erbB2 transcript levels as determined by ribonuclease protection assays. Therefore, we examined whether Y1028 was affecting the stability or turnover of ErbB2. For this, several independent Rat-1 cell lines stably expressing different ErbB2 mutants were established and then used for ^{35}S-methionine labeled pulse-chase analyses. These experiments revealed that Y1028 affected the downregulation and turnover rate of ErbB2, whereas removal of Y1028 stabilized ErbB2 levels. We conclude that a minimum threshold level for ErbB2 is required for development and survival and that the negative regulatory site may play a role in modulating ErbB2 levels through development.

To further elucidate the mechanism for Y1028 mediated negative regulation of ErbB2 signaling, we investigated whether this effect was mediated by the protein c-Cbl. c-Cbl is a multi-adapter protein that can regulate the activity of receptor tyrosine kinases by recruiting the

ubiquitin machinery and promoting the multi-ubiquitylation of activated receptors (Thien and Langdon, 2001). This targets the receptors for proteosomal degradation and thereby suppresses receptor signaling (Weissman, 2001). Thus, c-Cbl attenuates activated receptor tyrosine kinases by promoting the downregulation and degradation of the receptor. The downregulatory effects of c-Cbl association with other receptor tyrosine kinases such as the EGFR (Levkowitz et al., 1998), MET (Peschard et al., 2001), and PDGFR (Miyake et al., 1999) has been demonstrated previously. The effects of c-Cbl on ErbB2 were unclear at the time we undertook this study. Nonetheless, we co-expressed various ErbB2 tyrosine phosphorylation mutants with Cbl in 293T cells and subsequently demonstrated that Cbl does indeed interact with ErbB2 (Figure 3). However, this association was not dependent on Y1028. Interestingly, we also detected ubiquitylation of ErbB2 that was independent of both Y1028 and c-Cbl ubiquitylation.

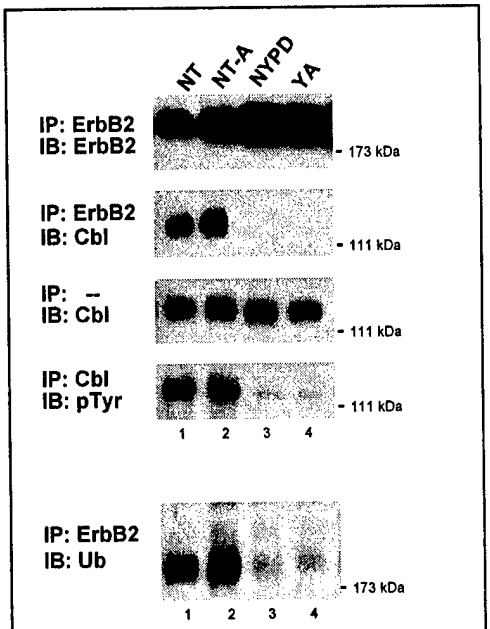

Figure 3. Co-immunoprecipitation analysis was performed on 293T cells expressing the indicated ErbB2 mutants and Cbl. NT=activated ErbB2; NT-A=Y1028F mutant; NYPD=pY deficient mutant; YA= Y1028 add-back mutant

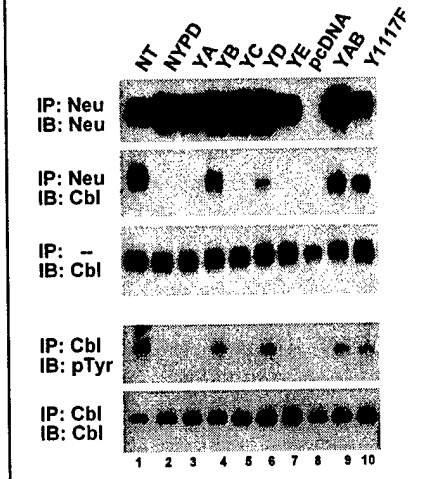

Figure 4. Co-immunoprecipitation experiments revealed that tyrosine 1144 (YB) and 1257 (YD) of ErbB2 interacted with Cbl.

Upon further investigation, we identified that c-Cbl associated with ErbB2 through two other phosphotyrosine residues, Y1144 and Y1227 (Figure 4). These interactions also correlated with the tyrosine phosphorylation of Cbl.

Interestingly however, unlike the EGFR and other receptor tyrosine kinases, the association of Cbl with ErbB2 did not correlate with the ubiquitylation and downregulation of the receptor. In fact, ErbB2 appeared to be refractory to c-Cbl mediated ubiquitylation. We are currently conducting further biochemical experiments to try to understand this ErbB2-Cbl interaction. We have also started to interbreed our MMTV-ErbB2 mouse mammary tumor model with cbl deficient animals to evaluate ErbB2 induced mammary development and tumorigenesis in the absence of Cbl.

Key Research Accomplishments

- Completed analysis of mice expressing a kinase-dead ErbB2 receptor. Results published in Molecular and Cellular Biology (Appendix 1).

- Completed analysis comparing knock-in mice expressing a hypomorphic erbB2 allele versus mice expressing mutant ErbB2-Y1028F receptor. The results are presented in the Manuscript submitted to Developmental Cell August 2003 (Appendix 2).

- Identified c-Cbl association with ErbB2 at two tyrosine autophosphorylation sites (Y1144 and Y1227). Unlike the EGFR, this association did not result in ubiquitylation of ErbB2. This has led us to investigate the role of c-Cbl in ErbB2 mediated mammary tumorigenesis in mice. I have started inter-crossing MMTV-activated ErbB2 mice with the c-cbl knock-out strain. I have also collaborated with new students in our lab to generate a transgenic mouse strain expressing a dominant-negative c-Cbl protein in the mammary gland using the MMTV expression system.

Reportable Outcomes:

- Publication in Molecular and Cellular Biology 22: p1073-1078 (2002): *"The catalytic activity of the ErbB-2 receptor tyrosine kinase is essential for embryonic development"*

- Manuscript submitted to Developmental Cell: *Modulation of ErbB2 signaling during development: A threshold level of ErbB2 signaling is required for normal development.*

- Poster: "Negative regulation of ErbB2 and the role of c-Cbl in mammary tumorigenesis." *Era of Hope, The DOD Breast Cancer research Program Meeting, 2002.* Orlando, Florida, USA.

- Seminar and Poster: "Negative regulation of ErbB2 is mediated through tyrosine 1028." *Growth Factor Receptor Tyrosine Kinases in Mitogenesis, Morphogenesis, and Tumorigenesis. FASEB Summer Research Conference, 2001.* Snowmass Village, Snowmass, Colorado, USA.

- Poster: "Negative regulation of ErbB2 is mediated through tyrosine 1028." *The 5th Conference on Signalling in Normal and Cancer Cells, 2001.* Banff, Alberta, Canada.

- Animal models characterized: knock-in of erbB2 cDNA, erbB2-Y1028F mutant, erbB2-Y1144F mutant, erbB2-Y1227F mutant, erbB2-kinase dead mutant.

- Ph.D. thesis will be submitted in December 2003

- Post-doctoral position offered and accepted with Dr. Benjamin Neel, Division of Hematology-Oncology, Beth Israel-Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

Conclusions

We have generated and characterized an erbB2 cDNA knock-in system to express various mutant ErbB2 receptors *in vivo,* to perform structure-function analyses on the ErbB2 receptor. Not only did this approach directly provide knowledge on ErbB2 receptor signaling in development, extrapolation of the information also provided insight into the function of ErbB2 in cancer. In this study, we established that the ErbB2 kinase activity plays a critical role and it is a requirement for normal development. This may have implications for new or current cancer therapeutic strategies that target the kinase activity of receptor tyrosine kinases. At the same time, we identified a minimal threshold level (10%) of ErbB2 that is required for development and survival. Although further analyses will need to be conducted to examine possible subtle implications in adult animals, this information may establish an upper limit to anti-ErbB2 therapeutic strategies to minimize side effects.

In studying the tyrosine autophosphorylation sites in ErbB2, we have demonstrated that tyrosine 1028 indeed functions in normal signaling providing a negative regulatory effect on ErbB2. Furthermore, the effect of Y1028 is to modulate ErbB2 protein levels by promoting the downregulation and turnover of the receptor. We are currently employing a proteomics strategy to investigate the possible mechanisms and/or mediators of this regulatory effect. Since overexpression of ErbB2 leads to breast cancer, our results may have significant implications for designing a rational drug target that could enhance the natural downregulatory mechanism on ErbB2 signaling. The multi-adapter protein Cbl has been implicated in the ubiquitylation and downregulation of receptor tyrosine kinases such as the EGFR, MET, and PDGFR. Although we have shown an association between ErbB2 and Cbl and have identified the tyrosine phosphorylation sites of interaction, ErbB2 appears to be refractory to Cbl mediated

downregulation. We are currently investigating the mechanism to understand this ErbB2-Cbl interaction. This may be an underlying reason for the potent signaling capacity of ErbB2 and why its overexpression is associated with a more aggressive disease with poor clinical prognosis.

This study has resulted in further insights into ErbB2 signaling in development and has provided a different approach to understanding and dissecting oncogenic ErbB2 signaling in breast cancer. The modulation of receptor tyrosine kinase signaling through downregulation or by other means is an important concept to understand because of its obvious clinical implications in treating cancers. The ability to manipulate the natural mechanisms of downregulating receptor tyrosine kinases may result in more precise therapeutic targets with fewer or minimal side effects.

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The Catalytic Activity of the ErbB-2 Receptor Tyrosine Kinase Is Essential for Embryonic Development

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Activation of the epidermal growth factor receptor (EGFR) family is thought to play a critical role in both embryogenesis and oncogenesis. The diverse biological activities of the EGFR family are achieved through various ligand-receptor and receptor-receptor interactions. One receptor that has been found to play a central role in this signaling network is ErbB-2/Neu, and it is considered the preferred heterodimerization partner for other members of the EGFR family. To assess the importance of the catalytic activity of ErbB-2 in embryonic development, we have generated mice expressing a kinase-dead *erbB-2* cDNA under the transcriptional control of the endogenous promoter. Here, we show that mice homozygous for the kinase-dead *erbB-2* allele die at midgestation and display the same spectrum of embryonic defects seen in *erbB-2* knockout mutants. These observations suggest that the catalytic activity of ErbB-2 is essential for normal embryonic development.

The epidermal growth factor receptor (EGFR) family of growth factor receptor tyrosine kinases, including ErbB-1/EGFR, ErbB-2/Neu, ErbB-3, and ErbB-4, have been implicated in breast cancer as well as several other human cancers (2). Recently, gene targeting studies have demonstrated specific roles for each of the EGFR family members in normal mammalian development. For example, *erbB-2* (12) and *erbB-4* (6) knockout mice die at midgestation due to deficient cardiac function associated with a lack of myocardial ventricular trabeculation and display abnormal development of the peripheral nervous system. Cardiac rescue of the defects seen in *erbB-2* null mice revealed additional roles for ErbB-2 at the developing neuromuscular junction (13, 17). ErbB-3 mutant mice have less severe defects in the heart and consequently are able to survive several days later through embryogenesis. However, sensory and motor neurons in these animals show signs of degeneration due to a lack of proper Schwann cell development (1, 22).

Although the structures of the EGFR family receptors have been described in detail, their individual roles in and contributions to these developmental processes remain to be investigated. To achieve the observed diversity in signaling potential, a coordinate array of ligand-receptor and receptor-receptor interactions are possible. Following activation of the various EGFR family members with one of several EGF family ligands, both homodomeric and heterodimeric combinations of receptors are induced, and their intrinsic catalytic tyrosine kinase activity is stimulated (30). Upon receptor dimerization, specific tyrosine residues residing in the terminal tail of the receptor dimer become phosphorylated and serve as important potential binding sites for various intracellular signaling proteins.

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Although activated ErbB receptors may partake in any particular combination of homodimerization or heterodimerization complexes, it is important to note that a hierarchical order of preference, stability, and signaling potential for each receptor combination is in effect (7). In particular, there is generally a greater preference and likely an advantage for dimerization complexes to include ErbB-2 as the partner because of its potent intrinsic kinase activity. Since no identified ligand is known to bind to and activate ErbB-2 alone, it is considered an orphan receptor. Thus, stimulation of ErbB-2 kinase activity may be mediated through normal ligand activation of another ErbB receptor first, which subsequently engages in a specific heterodimer complex (3, 18, 19). In contrast, ErbB-3, which can bind to and become activated by the ligand neuregulin, is naturally kinase inactive and therefore must depend on a heterodimerization partner for phosphorylation of its tyrosine residues (8, 26). Indeed, the ErbB-2–ErbB-3 complex is very stable and transmits a strong mitogenic signal (11). These observations strongly suggest that ErbB-2 plays a central role in the EGFR family signal transduction.

Although genetic ablation studies demonstrate the importance of a receptor to a biological function, they do not address precisely how and which of the individual functional domains of the receptor contribute to the phenotype. It is also unclear within this complex array of receptor dimerization whether the loss of ErbB-2 results in direct or indirect consequences of a lack of the receptor and its interactions with other proteins.

To assess exactly how ErbB-2 may act as the central mediator of the EGFR family of receptors, we investigated whether the kinase activity of ErbB-2 is indeed essential for its complete biological effects. To accomplish this, we generated mice expressing a kinase-dead *erbB-2* cDNA under the transcriptional control of the endogenous *erbB-2* promoter. Mice homozygous for the kinase-dead *erbB-2* mutation died at embryonic day 10.5 (E10.5) due to a lack of cardiac trabeculation and displayed defects in neural development. These observations argue that the catalytic activity of ErbB-2 is absolutely required

for normal embryonic development and cannot be compensated for by other members of the EGFR family.

MATERIALS AND METHODS

Generation of targeting construct and mutant mice. Oligonucleotide-directed PCR mutagenesis with the following primers was used to create the mutant (K757 M) kinase-dead ErbB-2 receptor: AB11151, GGA AGT ATA CGA TCG CTA GGC; AB10015, CAC CAT GAT AGC CAC GGG GAT TTT CAC; AB 10014, C GTG GCT ATC ATG GTG TTG AGA GAA AAC; and AB 11152, CGA CCT CGG TGT TCT CGG AC. The underlined nucleotides identify the sequence substituted to create the desired mutation. PCR products were subsequently subcloned into a wild-type *erbB-2* cDNA and then cloned into the final targeting vector. This plasmid was electroporated into R1 embryonic stem (ES) cells, and G418- or geneticin (Gibco-BRL)-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 by the method of blastocyst injection into BALB/c-derived blastocysts (10). Subsequent generations were maintained in an SV129/BALB/c background.

RNase protection assays. RNA was extracted and purified from individual embryos by the guanidine isothiocyanate-cesium chloride method as previously described (27). For RNase protection assays, as described previously (24), 30 μg of total RNA was used and hybridized to an antisense *erbB-2* riboprobe (23). The protocol was modified by lowering the overnight hybridization temperature to 45°C and only T_1 RNase (450 U) was used in the digestion reactions for 20 min at 37°C.

Western blot analysis. Fresh or flash-frozen embryos were lysed in TNE lysis buffer (24). Cleared lysates were electrophoresed through sodium dodecyl sulfate (SDS)-polyacrylamide gels, and the proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore). The membranes were blocked in 10% skim milk–Tris-buffered saline (TBS) for 1 h at room temperature and then incubated with the appropriate antibody. For ErbB-2 immunoblots, the membranes were incubated with anti-ErbB-2 antibodies (1:1,000; AB-3, Oncogene Science) overnight at 4°C. Immunoblots for Grb2 were performed with rabbit anti-Grb2 polyclonal sera (1:2,500; C-23, Santa Cruz). After the primary antibody incubations, membranes were subjected to four 15-min washes in TBS–1% Tween 20 (Bio-Rad). Subsequently, horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin secondary antibodies (1:5,000; Jackson Laboratories) were incubated with the membranes for 1 h at room temperature and then washed twice for 15 min each in TBS–1% Tween-20 and twice for 15 min each in TBS alone. Immunoblots were visualized by enhanced chemiluminescence (Amersham) as specified by the manufacturer.

Histology. Embryos from timed matings were dissected free from the placenta and cleared of extraembryonic tissues. A small piece of the visceral yolk sac was retained and placed in tail lysis buffer (100 mM Tris-HCl [pH 8.5], 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 μg of proteinase K per μl), and the DNA was isolated and subsequently used for genotyping the embryos. Dissected embryos were quickly rinsed in ice-cold phosphate-buffered saline (PBS) and transferred directly into 4% paraformaldehyde, fixed overnight at 4°C, washed twice in 70% ethanol, and stored at 4°C in 70% ethanol.

For standard hematoxylin and eosin (Fisher Scientific) staining, samples were embedded in paraffin, and 8-μm serial sections were cut and mounted. For whole-mount in situ hybridizations, embryos were fixed in 4% paraformaldehyde–0.2% glutaraldehyde (Fisher Scientific), dehydrated through a graded series of methanol-PBT (1× PBS, 0.1% Tween 20; Sigma) baths and stored in 100% methanol at −20°C until needed. Whole-mount in situ hybridizations were carried out as previously described (29).

In vitro transcription of Phox2a riboprobes. The Phox2a riboprobe plasmid, pKS903 SSN (25), was digested with *Sst*II and transcribed with the T3 RNA polymerase to generate an antisense riboprobe; the sense riboprobe was generated using the same template but linearized with *Hin*dIII and transcribed with T7 RNA polymerase. The in vitro transcription reactions were carried out in 20-μl reaction volumes containing 14 μl of distilled water (diethyl pyrocarbonate [DEPC] treated), 2 μl of 10× transcription buffer (Boehringer Mannheim), 2 μl of DIG RNA labeling mix (Boehringer Mannheim), 1 μg of template DNA, 30 U of RNAGuard (Pharmacia), and 30 U of RNA polymerase (Boehringer Mannheim or Gibco-BRL). The reactions were incubated at 37°C for 2 h and stopped with the addition of 20 U of DNase I (RNase-free; Boehringer Mannheim). The riboprobes were precipitated and resuspended in 100 μl of DEPC-treated distilled water (≈100 ng/μl).

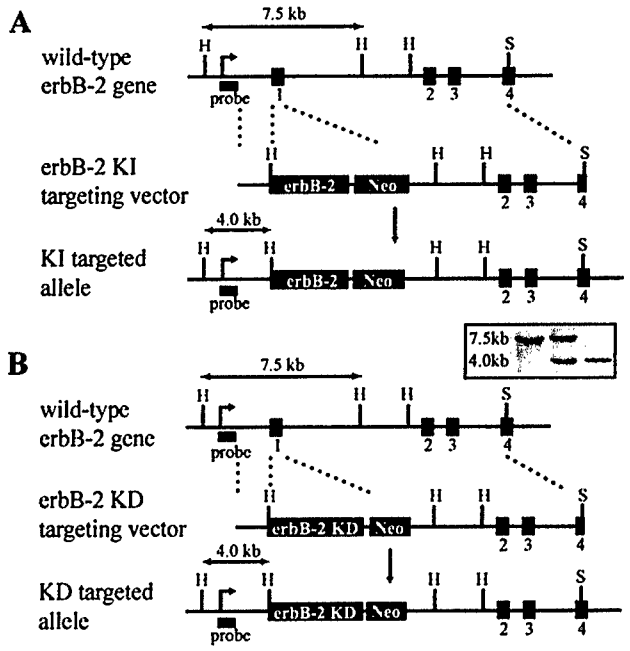

FIG. 1. Targeted *erbB-2* cDNA knock-in strategy by homologous recombination. For germ line expression, a targeting vector was constructed in which exon 1 was replaced by either (A) a wild-type *erbB-2* cDNA or (B) a cDNA encoding the kinase-dead *erbB-2* mutation, followed by a PGK-neomycin (Neo) cassette and targeted to the endogenous *erbB-2* locus by homologous 5′- and 3′-flanking arms. Digestion of genomic DNA with *Hin*dIII (H) and subsequent Southern blot analysis (inset) with an external probe, as indicated, resulted in a 7.5-kb band for the endogenous allele, whereas the knock-in cDNA alleles introduced a *Hin*dIII site and resulted in a 4.0-kb band.

RESULTS AND DISCUSSION

A kinase-dead variant of the ErbB-2/Neu receptor was created by oligonucleotide-directed PCR mutagenesis to generate a point mutation affecting lysine residue 757. This K757M alteration ablates the conserved ATP-binding lysine residue in the tyrosine kinase domain, resulting in its inability to phosphorylate its substrates (20, 21, 28). We have confirmed that disruption of this key amino acid results in ablation of ErbB-2-associated kinase activity and also inactivates the potent transforming activity of an oncogenic *erbB-2* mutant (data not shown).

To determine the functional importance of the ErbB-2 kinase activity in vivo, we generated a targeting vector in which the first coding exon of the endogenous *erbB-2* gene was replaced with either a wild-type *erbB-2* cDNA (*erbB-2* knock-in [KI]) (Fig. 1A) or a cDNA harboring the K747M mutation (*erbB-2* kinase-dead [KD]) (Fig. 1B). To facilitate recovery of targeted recombination events, a PGK-Neo expression cassette was inserted downstream of the inserted cDNA. The constructs were electroporated into R1 ES cells, and independent clonal lines were isolated and subjected to Southern blot analyses with an appropriate external probe to identify successful targeting events (Fig. 1).

After microinjection of several independently targeted ES cell lines into donor blastocysts, chimeric mice were obtained and bred to identify those that transmitted the mutant alleles through the germ line. *erbB-2*$^{wt/KI}$ and *erbB-2*$^{wt/KD}$ mice ap-

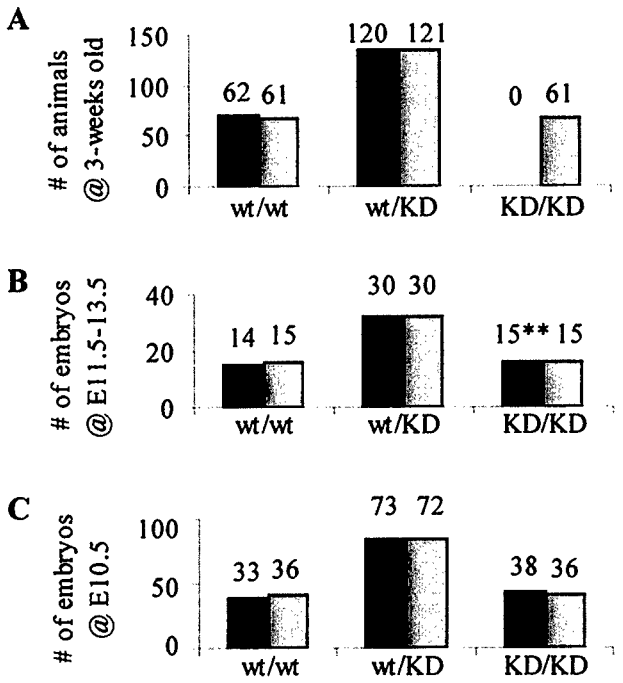

FIG. 2. Embryonic lethality at midgestation in kinase-dead mutants. Mendelian ratios from the progeny of heterozygous matings were determined and compared with the frequency of genotypes observed. (A) No homozygous *erbB-2*$^{KD/KD}$ mutant animals were observed at weaning age (3 weeks old). (B) Observations at E11.5 to E13.5 revealed the expected number of mutant embryos; however, all *erbB-2*$^{KD/KD}$ (**) embryos were being resorbed, and no heartbeat was detected. (C) At E10.5, all the embryos appeared healthy and were present in proportion with Mendelian frequencies. Solid bars, observed; shaded bars, expected.

peared normal and were fertile. *erbB-2*$^{KI/KI}$ mice were also viable and were generated at the expected Mendalian ratios (data not shown). Thus, in contrast to the generation of *erbB-2*$^{-/-}$ mice, in which exon 1 was replaced by a phosphoglycerate kinase (PGK)-neomycin resistance cassette (12), replacement of the first coding exon of *erbB-2* with a wild-type *erbB-2* cDNA rescued the embryonic lethality associated with disruption and inactivation of the *erbB-2* gene. Interestingly, no viable *erbB-2*$^{KD/KD}$ mutant mice were observed in the litters generated from heterozygous matings that were subsequently genotyped at 3 weeks of age (Fig. 2A).

Since *erbB-2*$^{-/-}$ embryos died at midgestation due to defects in heart development (12), we assessed whether we could detect viable embryos at E10.5 for our kinase-dead mutants. To accomplish this, timed matings between heterozygous animals were set up, and embryos were dissected from the uterus and observed. Embryo genotypes were determined by analysis of DNA isolated from their visceral yolk sacs. At E10.5, each possible genotype was present at the expected Mendelian frequencies (Fig. 2C), and all embryos were viable, possessed a heartbeat, and appeared normal in size. However, in mutant embryos the hearts were slightly enlarged and had irregular beats. Further observations at E11.5 to E13.5 (Fig. 2B) revealed that homozygous mutant *erbB-2*$^{KD/KD}$ embryos, although present at the expected frequency, had no heartbeat and showed signs of resorption such as arrested growth, pale color, and soft tissue. Thus, consistent with the embryonic lethality of *erbB-2*$^{-/-}$ mutations, these observations confirmed that mutant embryos expressing the kinase-dead ErbB-2 receptor were dying in utero at midgestation, between E10.5 and E11.5.

To investigate the cause of embryonic lethality, we performed histological analyses of E10.5 embryos. Both *erbB-2*$^{KI/KI}$ (Fig. 3A) and *erbB-2*$^{wt/KD}$ (Fig. 3B) knock-in embryos were completely normal in their development of the heart trabeculae. In contrast, *erbB-2*$^{KD/KD}$ mutant embryos clearly lacked development of ventricular trabeculae (Fig. 3C) that likely resulted in the observed reduction in embryonic blood flow. These results are strikingly consistent with the defects identified previously in *erbB-2*$^{-/-}$ embryos as well as in *neuregulin*$^{-/-}$ and *erbB-4*$^{-/-}$ embryos (6, 12, 15). Thus, the cardiac defects seen in the kinase-dead mutants were directly attributable to a loss of ErbB-2's enzymatic tyrosine kinase activity.

We also examined whether the same peripheral nervous system abnormalities seen in *erbB-2*$^{-/-}$ mutants were also sim-

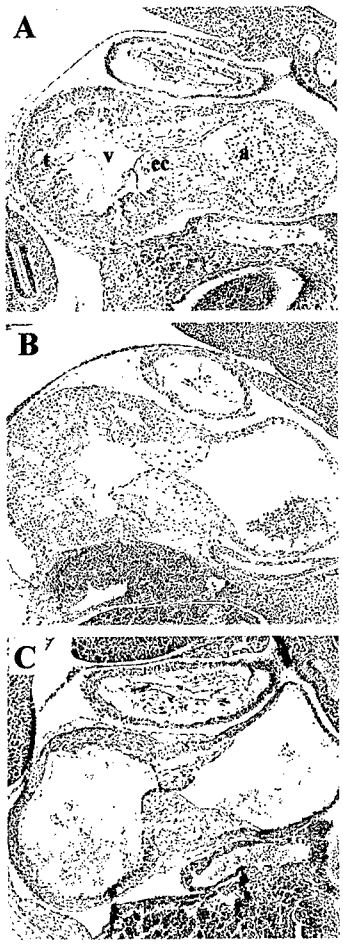

FIG. 3. Defects in heart development in E10.5 mutant embryos. Parasagittal sections of (A) *erbB-2*$^{KI/KI}$, (B) *erbB-2*$^{wt/KD}$, and (C) *erbB-2*$^{KD/KD}$ embryos at E10.5 were stained with hematoxylin and eosin. Although heartbeats were detected at the time of dissection, histological examinations of the hearts revealed a lack of trabeculae in the ventricles of *erbB-2*$^{KD/KD}$ mutants but were present in heterozygous littermates and in age-matched *erbB-2*$^{KI/KI}$ knock-in embryos. t, trabeculae; v, ventricle; ec, endocardial cushion; a, atrium.

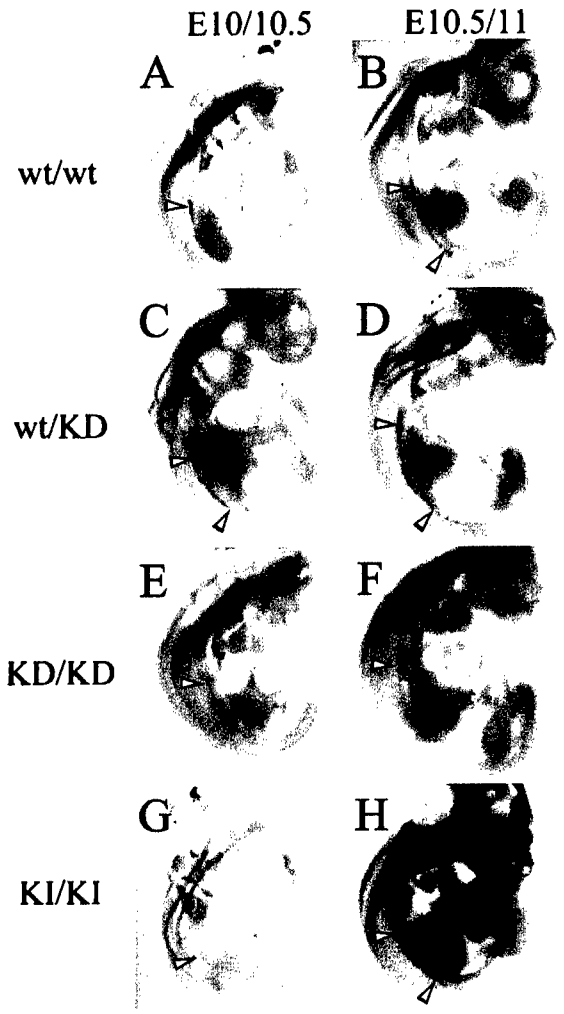

FIG. 4. Lack of sympathetic chain ganglia in kinase-dead mutants. Day E10 to E11 embryos were subjected to whole-mount in situ hybridization analysis using an antisense Phox2a riboprobe. Normal sympathetic chain ganglia development was present in (A and B) *erbB-2*$^{wt/wt}$ embryos and (C and D) *erbB-2*$^{wt/KD}$ heterozygous embryos, whereas (E and F) *erbB-2*$^{KD/KD}$ homozygous mutants lacked proper development or they were delayed in (G and H) *erbB-2*$^{KI/KI}$ embryos. The white arrowheads highlight the developing sympathetic chain ganglia.

ilarly affected by expression of the kinase-dead mutation. One important marker for peripheral neural tissues is the Phox2a transcription factor (16). To explore whether neural development was perturbed in the KD mice, embryos derived from E10.5 and E11.5 were subjected to whole-mount in situ analyses using an antisense Phox2a riboprobe (Fig. 4). In both *erbB-2*$^{wt/wt}$ (Fig. 4A and B) and *erbB-2*$^{wt/KD}$ (Fig. 4C and D) embryos, the sympathetic chain developed normally. However, in the E10.5 to 11 *erbB-2*$^{KD/KD}$ homozygous embryos, only a weak Phox2a signal could be detected in the rostralmost regions of the sympathetic chain, a clear indication that this structure had either failed to initiate development or was incapable of developing in the kinase-dead genetic background (Fig. 4E and F).

Analyses of Phox2a expression in *erbB-2*$^{KD/KD}$ (Fig. 4E) and *erbB-2*$^{KI/KI}$ (Fig. 4G) embryos revealed that the sympathetic chain appeared to be absent at E10 to 10.5 compared to age-matched wild-type and heterozygous embryos (compare Fig. 4G with Fig. 4A and C). However, by E10.5 to 11, a lengthy sympathetic chain had developed in the *erbB-2*$^{KI/KI}$ homozygotes (Fig. 4H) but remained completely absent in the *erbB-2*$^{KD/KD}$ embryos (Fig. 4F). These observations indicate that the catalytic activity of ErbB-2 is also essential for normal development of the primary sympathetic chain ganglia.

To confirm that these knock-in alleles expressed ErbB-2, we performed Western immunoblot (Fig. 5A) and RNase protection (Fig. 5B) analyses. As shown in Fig. 5B, the wild-type *erbB-2* KI allele and the kinase-dead allele expressed similar levels of *erbB-2* transcripts. Similarly, immunoblot detection for ErbB-2 also revealed comparable protein levels between the two knock-in mutants at E10.5 (Fig. 5A). The slightly lower levels of ErbB-2 seen in the *erbB-2*$^{KD/KD}$ embryos relative to *erbB-2*$^{KI/KI}$ embryos is likely due to and consistent with the loss of tissue structures in the kinase-dead mutants (Fig. 2 and 3), which would normally express significant levels of ErbB-2.

Interestingly, both the *erbB-2*$^{KI/KI}$ and the *erbB-2*$^{KD/KD}$ knock-in embryos expressed only 10 to 15% of the expected ErbB-2 protein as detected in *erbB-2*$^{wt/wt}$ embryos (Fig. 5A, compare lanes 1 and 2 to lanes 3 to 6) despite expressing comparable levels of *erbB-2* transcripts (Fig. 5B), which may

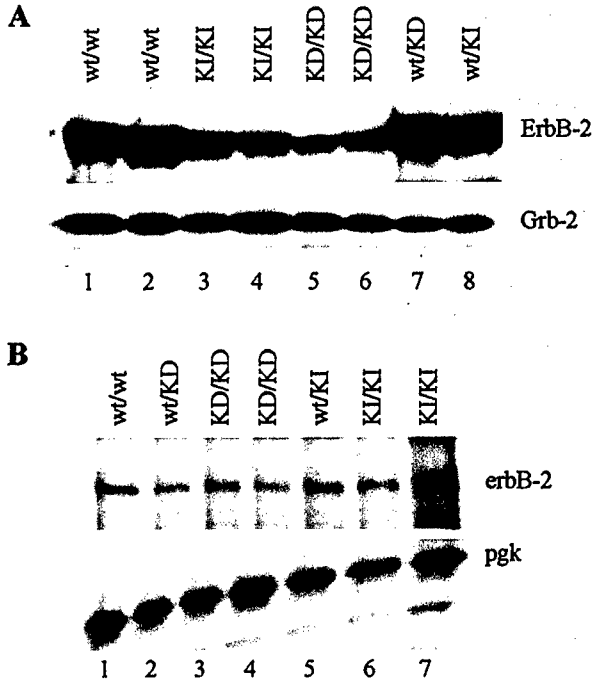

FIG. 5. Detection of ErbB-2 expression in E10.5 embryos. Expression of ErbB-2 generated from the cDNA insert in *erbB-2*$^{KI/KI}$ and *erbB-2*$^{KD/KD}$ embryos was determined. For (A) immunoblot analyses, total protein isolated from E10.5 embryos was subjected to SDS-PAGE and incubated with an anti-ErbB-2 antibody. Detection of Grb-2 (lower panel) protein was used to control for equal protein lysate quantification. (B) RNase protection assays with an antisense *erbB-2* riboprobe were used to detect *erbB-2* transcripts in total RNA isolated from *erbB-2*$^{wt/wt}$ (lane 1), *erbB-2*$^{KD/KD}$ (lanes 3 and 4), and *erbB-2*$^{KI/KI}$ (lanes 6 and 7) embryos. A mouse PGK riboprobe (lower panel) was used as an internal control for equal sample loading in each lane.

reflect a requirement for splicing events for efficient transport and translation of the mRNA. In this regard it should be noted that embryos expressing both copies of the wild-type knock-in *erbB-2* allele display a 24-h delay in development of the sympathetic chain (Fig. 4), suggesting that reduced ErbB-2 levels may have a minor phenotypic consequence.

Our observations have important implications in understanding the role of *erbB-2* in promoting both normal cardiac and neural development. Previous studies have demonstrated that the integrity of ErbB-2, ErbB-4, or neuregulin is essential for the development of the cardiac trabecular extensions. Despite the presence of a functional ErbB-4 protein that could potentially transphosphorylate ErbB-2, our results further suggest that the catalytic activity of ErbB-2 alone is required to recapitulate the necessary signal transduction pathways leading to proper trabeculation.

In contrast to myocardial trabecula formation, ErbB-2 and ErbB-3 are thought to mediate the survival of neural crest cells, contributing to the development of the peripheral nervous system, since similar cranial nerve phenotypes were seen in mutant ErbB-2 and mutant ErbB-3 mice as well as in neuregulin mutants (12, 15, 22). Given that ErbB-3 is kinase defective and is completely dependent on its heterodimerization partners for its activation (8), inactivation of ErbB-2 catalytic activity would be expected to have profound effects on neural development. From these data, we can conclude that ErbB signaling is dependent on the kinase activity of ErbB-2 and that a kinase-dead ErbB-2 mutant acts essentially as a functionally null receptor.

In contrast to the embryonic lethality caused by inactivation of the catalytic activity of ErbB-2, mutants carrying a naturally occurring germ line mutation in the kinase domain of EGFR known as Waved-2 are completely viable and display only epithelial defects, such as a wavy hair phenotype (5, 14). Thus, unlike ErbB-2, other EGFR family members can presumably compensate for the severe impairment in EGFR catalytic activity. The difference between these phenotypes may reflect the hierarchical importance of ErbB-2 within the EGFR family signaling network. Alternatively, the difference in the phenotype in these strains may reflect the fact that the Waved-2 mutation has not completely ablated the catalytic activity of EGFR (14) and thus retains a higher degree of biological function.

Given the dominant-negative action of the kinase-dead ErbB-2 receptor expressed in vitro (20) it is surprising that mice heterozygous for the *erbB-2* KD allele failed to exhibit any obvious phenotype that might be expected of a *trans*-dominant inhibition of the remaining wild-type allele. One potential explanation for this observation is that the level of kinase-dead ErbB-2 is insufficient to interfere with the remaining endogenous wild-type ErbB-2 receptor. Indeed, the *erbB-2* KD allele produced only 10% of the expected ErbB-2 protein (Fig. 5). To exclude this possibility, we crossed *erbB-2* KI mice with *erbB-2* KD mice because both of these knock-in alleles expressed similar levels of ErbB-2. Phenotypic analyses of these crosses demonstrated that the *erbB-2* KD allele failed to exhibit a dominant-negative effect on the remaining *erbB-2* KI allele (data not shown). Thus, like the Waved-2 EGFR kinase mutation, the *erbB-2* KD allele fails to exhibit any discernible dominant-negative effect on the remaining intact ErbB-2 receptor.

Studies with other receptor tyrosine kinases have concluded that the catalytic activity of receptor tyrosine kinases is dispensable for normal physiological function of the receptor. For example, the Flt-1 (vascular endothelial growth factor receptor) null mutation resulted in early embryonic lethality at E8.5 with disorganized blood vessels (4). However, mice expressing a kinase-deficient Flt-1 survived and showed normal angiogenesis (9), suggesting that other components of the receptor are more important to its functional role. In contrast to these observations, we have found that the catalytic activity of ErbB-2 is essential for embryonic development.

The embryonic lethality associated with the expression of the kinase-dead ErbB-2 is not likely due to inappropriate localization of the receptor, since it has previously been demonstrated that this mutant receptor is efficiently expressed on the cell surface (20). Given that ErbB-2 is the preferred heterodimerization partner for the other EGFR family members (7), the requirement for ErbB-2 catalytic function in vivo suggests that its catalytic activity is critical for EGFR family signaling. Future studies with these strains should allow identification of downstream targets of ErbB-2.

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R.C. and W.R.H contributed equally to this work.

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Modulation of ErbB2 signaling during development: A threshold level of ErbB2 signaling is required for normal development

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Running Title: **Modulation of ErbB2 receptor level**

Summary

We have generated a series of *erbB2* cDNA knock-in animals to explore the *in vivo* role of signaling pathways coupled to receptor tyrosine kinases during development. Although this knock-in allele was hypomorphic expressing 10-fold less ErbB2 protein than in wild type animals, the knock-in animals were healthy. However, a further 2-fold reduction in ErbB2 levels in hemizygous knock-in animals resulted in perinatal lethality with defects in the developing sympathetic nervous system. Genetic rescue of this hypomorphic allele was accomplished by expression of a single tyrosine phosphorylation site mutation (Y1028F) in a comparable knock-in allele. Interestingly, hemizygous Y1028F animals were viable and healthy. Further molecular analyses revealed that the Y1028F allele indeed expressed significantly higher levels of ErbB2 and that Y1028 promotes the turnover of the receptor. Thus, we have established how ErbB2 levels may be modulated through development and that a minimal threshold level of ErbB2 is required for normal development.

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, reviewed in (Olayioye et al., 2000; Pinkas-Kramarski et al., 1996a; Yarden and Sliwkowski, 2001). For example, EGFR ligands such as EGF or TGFα 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 soluable ligand for ErbB2 has yet to be described. However, there have been several reports suggesting that a member of the Mucin family known as Muc-4 may act as a membrane bound ligand for ErbB2 (Carraway et al., 1999).

Despite the lack of a direct soluable 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., 1996a). For example stimulation of cells with EGF can result in transphosphorylation of ErbB2 through the formation of specific 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., 1996b). Transphosphorylation and activation 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 EGFR heterodimers play an important biological role in the function of the EGFR family.

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 C-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 in 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), which 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 known major tyrosine

autophosphorylation sites (Y1028, Y1144, Y1201, Y1227, Y1253) in the regulatory region were evaluated systematically for their roles in constitutively activated ErbB2-mediated transformation of Rat1 or NIH 3T3 fibroblasts. 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 (so-called "add-back" mutants) was not only able to fully restore the transforming ability, but it was also able to transform with a modest increase over the fully functional receptor (Dankort et al., 1997). 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, although possibly redundant in function, positively mediate ErbB2 signaling, tyrosine 1028 negatively modulates ErbB2 activity with respect to transformation.

We describe here the introduction of both wild type and tyrosine phosphorylation site 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 different *erbB2* cDNA mutants with *erbB2* null mice, resulted in perinatal lethality. Thus, we establish a minimal threshold level of ErbB2 expression required to sustain viability. In contrast to these observations, expression of the Y1028F mutation in knock-in animals genetically rescued this perinatal lethality.

Biochemical analyses revealed that these animals indeed expressed higher levels of ErbB2 protein above the threshold, despite expressing similar *erbB2* transcript levels. We further identify that tyrosine 1028 mediates the negative regulation of ErbB2 signaling by influencing the turnover rate of the receptor.

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*) (Figures 1A & C). Thus, expression of *m-erbB2* is disrupted and replaced by expression of the *r-erbB2* cDNA but still under the control of the endogenous promoter. For genotyping purposes, note that the '*wt*' superscript (eg. $erbB2^{wt}$) will be used herein to designate the endogenous mouse *erbB2* genotype whereas the '*erbB2*' superscript (eg. $erbB2^{erbB2}$) 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 (Figure 1B).

To examine the functional roles of the tyrosine autophosphorylation sites *in vivo*, mutant *r-erbB2* cDNA harboring single tyrosine-to-phenylalanine substitutions (Figure 1E) were also expressed in mice using an identical targeting strategy as described above but substituting the cDNA cassette (Figure 1C). Specifically, we generated knock-in mice expressing the ErbB2-Y1028F mutant (site A mutant), as well as the ErbB2-Y1144F

mutant (site B mutant; loss of the direct binding site for Grb2) and the ErbB2-Y1227F mutant (site D 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 (Figure 1D) and appeared normal and healthy. Interestingly, homozygous *erbB2*$^{Y1144F/Y1144F}$ and *erbB2*$^{Y1227F/Y1227F}$ knock-in mice were also normal, healthy and fertile (data not shown).

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* sequences are greater than 93% homologous at both the nucleic acid and amino acid levels and importantly the tyrosine autophosphorylation sites in the carboxyl-terminus are conserved. However, we took advantage of a polymorphic BamHI restriction site that is present only in the endogenous *m-erbB2* gene but not in the *r-erbB2* cDNA sequence (Figure 2A) to confirm that rescue of the embryonic lethality associated with disruption and inactivation of the *m-erbB2* gene (Morris et al., 1999) was attributed solely to expression of the knock-in *r-erbB2* cDNA. Total RNA isolated from wild type, heterozygous, and homozygous knock-in animals were subjected to RT-PCR using the primers indicated in Figure 2A. The RT-PCR product was then subjected to BamHI and the resultant fragments were resolved on an agarose gel. As predicted, BamHI digest of the RT-PCR product (~375bp) from wild type *erbB2*$^{wt/wt}$ animals resulted in two smaller fragments ~245bp and 130bp (Figure 2A, lane 6) whereas the RT-PCR fragment from homozygous *erbB2*$^{erbB2/erbB2}$ knock-in mice was completely resistant to BamHI digestion (Figure 2A, lane 4). BamHI restriction analysis of the RT-PCR product from heterozygous *erbB2*$^{wt/erbB2}$ animals resulted in three fragments

representing expression of the endogenous *m-erbB2* allele (245bp and 130bp) and the *r-erbB2* cDNA knock-in allele (375bp) (Figure 2A, lane 2). These data argue that rescue of the embryonic lethality associated with germline inactivation of *erbB2* is indeed attributed to expression of the knock-in *r-erbB2* cDNA.

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 (Figure 2B) to determine whether the knock-in *erbB2* allele expressed wild type levels of ErbB2 protein. Surprisingly, the levels of ErbB2 expressed in the $erbB2^{erbB2/erbB2}$ (Figure 2B, lanes 4-5) embryos were dramatically reduced relative to the endogenous expression of ErbB2 in wild type littermates (Figure 2B, lanes 1-2). 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 *erbB2* cDNA appeared phenotypically normal, we have previously demonstrated that they indeed exhibit subtle defects. In particular, our lab showed that *erbB2* cDNA knock-in animals possessed only 10% the number of muscle spindles compared to 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 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 (Figure 3A).

Significantly, no *erbB2*$^{erbB2/ko}$ animals were found at weaning age (3-weeks old) when the animals were genotyped.

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 (Figure 3C). Although the expected number animals were present at birth, 18 of the 38 newborn pups were either still born or started dying immediately after birth. They did not attempt to breathe independently despite the ability to open their mouths and they became cyanotic and died within a few minutes. In contrast, their heterozygous *erbB2*$^{wt/erbB2}$ littermates quickly developed a regular breathing pattern. Indeed, all of the dead pups were genotyped to be *erbB2*$^{erbB2/ko}$ animals. Subsequent postmortem histological analyses of the lungs from these animals confirmed that the hemizygous *erbB2*$^{erbB2/ko}$ pups were unable to inflate and expand their lungs despite being vascularized and structurally intact (Figure 3B). 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 *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 (Figure 3B). Interestingly, in contrast to the hemizygous $erbB2^{erbB2/ko}$ pups, no perinatal lethality was observed with the hemizygous $erbB2^{Y1028F/ko}$ animals. In fact, all of the progeny survived and the adult $erbB2^{Y1028F/ko}$ animals appeared normal, 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, hemizygous $erbB2^{Y1144F/ko}$ and $erbB2^{Y1227F/ko}$ mice expressing either the Y1144F or the Y1227F phosphotyrosine mutant ErbB2 receptor in an *erbB2*-deficient background were also generated. Similar to the $erbB2^{erbB2/ko}$ pups, these animals were died shortly after birth struggling unsuccessfully to breathe (data not shown).

Additional evidence for the genetic rescue of the hypomorphic *erbB2* knock-in allele by expression of the ErbB2-Y1028F mutation was observed in the developing sympathetic nervous system. E12.5 embryos were subjected to wholemount *in situ* analysis (Figure 4) using an anti-sense Phox2a riboprobe, which specifically stains tissues of the sympathetic nervous system (Morin et al., 1997; Tiveron et al., 1996). For comparison, Figures 4A and 4C shows the normal development of the primary sympathetic chain ganglia in heterozygous embryos at E12.5. The superior cervical ganglia developed normally in all of the mutant embryos. However, in $erbB2^{erbB2/ko}$ embryos there was only partial development of the thoracic sympathetic ganglia and the defects become more severe along the more posterior extensions of the sympathetic ganglia. These results are similar to the *erbB3*-deficient embryos at E12.5 where a rostral-caudal gradient in the severity of the defects in the sympathetic nervous system were observed (Britsch et al., 1998).

In comparison, the primary sympathetic chain ganglia of hemizygous *erbB2*$^{Y1028F/ko}$ embryos were similar in size and length as its heterozygous littermates (Figures 4D-F). However, at the more caudal region, there was variability in the number of cells that dissociate from the primary ganglion chain and migrate to the mesentery and the anlage of the adrenal gland. In some *erbB2*$^{Y1028F/ko}$ embryos, there was robust Phox2a staining in the posterior region around the mesentery or adrenal gland (Figure 4D), whereas in other *erbB2*$^{Y1028F/ko}$ embryos, there were few Phox2a positive cells in the same region (Figure 4E & F). Therefore, these results strongly suggest that tyrosine 1028 in the carboxyl-terminal tail of ErbB2 specifically mediates a negative regulatory effect on the receptor and this plays an important function *in vivo* to modulate the activity of the ErbB2 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 (Figure 5A, lane 2 vs. 5, lanes 2-3 vs 6-7; Figure 5B, lane 3 vs. 4). In the hemizygous embryos, there is a critical difference in ErbB2 protein levels in the *erbB2*$^{Y1028F/ko}$ embryos (Figure 5A, lanes 6-7) versus the *erbB2*$^{erbB2/ko}$ embryos (Figure 5A, lanes 4-5), which likely determines the difference between the animals surviving or dying at birth. Note that the ErbB2 levels in *erbB2*$^{Y1028F/ko}$ embryos (Figure 5A, lanes 6-7) are

comparable to the levels in *erbB2*$^{erbB2/erbB2}$ embryos (Figure 5A, lane 2), which are essentially normal, healthy animals as described earlier.

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 (Figure 5B, compare lane 4 vs. lanes 3, 6, and 7). In fact, quantification of immunoblots using I^{125}-conjugated secondary antibodies and subsequent ImageQuant (Molecular Dynamics) analyses revealed that the levels of ErbB2 in *erbB2*$^{Y1028F2/Y1028F}$ embryos were at least 3-8-fold higher than in *erbB2*$^{erbB2/erbB2}$, *erbB2*$^{Y1144F/Y1144F}$ and *erbB2*$^{Y1227F/Y1227F}$ embryos (Figure 5C). Note that the range of ErbB2 levels detected in animals expressing the Y1028F allele is consistent with the variability in the extent of development of the caudal portion of the sympathetic ganglia in *erbB2*$^{Y1028F/ko}$ embryos, as described above. Thus, the quantitative difference in ErbB2 levels alone likely 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 post-transcriptional level, *erbB2* transcripts in E12.5 embryos were detected using an RNase protection assay (Figure 5D). The results showed that samples harboring either the *erbB2* or the *Y1028F* knock-in allele expressed identical levels of *erbB2* transcripts (Figure 5D, lanes 4-5 vs. lanes 6-8). Note that either knock-in alleles express significantly lower levels of *erbB2* transcripts compared to wild type animals. This phenomenon is likely a result of using a

cDNA knock-in strategy and explains the overall lower ErbB2 expression levels in the knock-in animals. Regardless, taken together, 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.

To explore whether reduced levels of ErbB2 affected the levels of its heterodimer partners ErbB3 and ErbB4, we measured ErbB3 and ErbB4 protein levels in the E12.5 knock-in embryos (Figure 5E). Interestingly, lower levels of both ErbB3 and ErbB4 receptors correlated with the marked reductions in the level of ErbB2 in the various knock-in embryos. Thus, the effects of the hypomorphic expression levels of ErbB2 throughout development may be accentuated because of a corresponding attenuation in the levels of ErbB3 and ErbB4 receptors.

Y1028 influences ErbB2 receptor turnover rate

Since many receptor tyrosine kinases are downregulated by endocytosis and subsequently targeted for lysosomal or proteosomal degradation (Katzmann 2002), we examined whether tyrosine 1028 affects ErbB2 receptor turnover. Rat-1 cell lines stably expressing the oncogenic ErbB2 (V664E mutation) or its mutant tyrosine phosphorylation site derivatives were established. Specifically, we compared the turnover rate of the oncogenic ErbB2 receptor versus the Y1028F mutant receptor (Figure 6A-B). 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 (Figure 6C-D). 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 phosphorylation sites.

Rat-1 cell lines were pulse labeled with the ^{35}S-protein labeling mix and chased with media containing excess non-labeled methionine and cysteine for the indicated times. Point mutation of tyrosine 1028 in ErbB2 significantly stabilized the receptor compared to the intact ErbB2 receptor (Figure 6A-B). Alternatively, when tyrosine 1028 was restored to the Y1144 add-back mutant receptor, the turnover rate of ErbB2 decreased (Figure 6C-D). Endocytosis and turnover of ErbB2 receptor from the plasma membrane, as determined by biotin-labeling surface receptor (data not shown), was consistent with the ^{35}S pulse-chase results. Based on these results, we examined whether the E3-ubiquitin ligase, c-Cbl, was responsible for mediating the negative regulatory effect of phosphotyrosine Y1028 on ErbB2 activity since c-Cbl has been shown to mediate the downregulation of the EGFR and other receptor tyrosine kinases. Although our data suggest that c-Cbl is able to associate with ErbB2 through other specific phosphotyrosine residues, tyrosine 1028 is not responsible for this recruitment of c-Cbl to ErbB2 (data not shown). Taken together, these observations suggest that tyrosine 1028 modulates ErbB2 protein levels through a c-Cbl and ubiquitylation independent manner.

Discussion

We have presented evidence identifying a minimal threshold level of ErbB2 (above ~5% of wild type levels) that is required to sustain normal physiological functions. A series of *erbB2* cDNA knock-in animals encoding ErbB2 receptors with different tyrosine autophosphorylation site mutations were generated to explore their developmental roles and functions, *in vivo*. Inadvertently, this knock-in allele turned out to be a hypomorph expressing only ~10% the level of ErbB2 expressed in wild type littermates. Despite the

low levels of ErbB2 receptors, homozygous knock-in animals were viable and overtly normal and healthy. However, when the *erbB2* knock-in animals were intercrossed with *erbB2* deficient animals to generate hemizygous knock-in animals, this further 2-fold reduction in ErbB2 levels fell below the threshold level and resulted in perinatal lethality due to acute respiratory distress. The inability of newborn $erbB2^{erbB2/ko}$ pups to inflate their lungs resulting in their perinatal death is remarkably similar in phenotype to the cardiac specific rescue of *erbB2* deficient mutants (Morris et al., 1999; Woldeyesus et al., 1999). These mice by-passed the cardiac defects and the midgestation lethality seen in homozygous *erbB2* deficient embryos, however newborn pups were unable to inflate their lungs due to aberrant innervations of their diaphragms resulting in the newborn animals not being able to breathe independently.

Previous studies in our lab have suggested that the reduced ErbB2 protein observed in the *erbB2* knock-in mice is associated with a 10-fold reduction in the number of muscle spindle cells without any obvious phenotypic consequence (Andrechek et al., 2002). Therefore, it is conceivable that the reduced ErbB2 levels may be associated with other specific cell types. In this regard, the $erbB2^{erbB2/ko}$ embryos displayed severe defects in the development of the sympathetic nervous system at E12.5 (Figure 4), strikingly similar to the neural phenotype in *erbB3* deficient mutants (Britsch et al., 1998). In contrast, these mice exhibited normal heart trabeculation suggesting that the nervous system is more sensitive to the low levels of ErbB2 than the cardiac system.

Furthermore, the reduction in ErbB2 levels resulted in a concomitant attenuation of ErbB3 and ErbB4 receptor levels, suggesting that ErbB3 and ErbB4 are dependent on heterodimerization complexes with ErbB2 to escape receptor turnover. These results are

consistent with the report that heterodimer complexes involving ErbB2 as a partner are very stable and are a more potent signaling unit (Graus-Porta et al., 1997). Thus, the developmental defects observed in the hypomorphic ErbB2 knock-in animals are likely the result of a negative synergistic effect with ErbB3 and ErbB4 on normal development.

ErbB2 knock-in mice bearing the phosphotyrosine mutant *Y1028F* allele expressed much higher levels of ErbB2 protein than in the *erbB2* knock-in mice. In fact, introduction of the *Y1028F* knock-in allele into an *erbB2* deficient background ($erbB2^{Y1028F2/ko}$) rescued the perinatal lethality associated with the corresponding hemizygous $erbB2^{erbB2/ko}$ allele. This genetic manipulation affecting the specific phosphotyrosine site in the carboxyl-terminus of ErbB2 resulted in an increase in ErbB2 levels above the minimal threshold levels (Figure 5A), thus these hemizygous animals survived and were phenotypically healthy. In contrast, similar experiments performed with the *Y1144F* or *Y1227F* knock-in alleles did not result in an increase in ErbB2 protein levels above the threshold and these animals also died shortly after birth.

Although previous *in vitro* experiments concluded that tyrosine 1028 negatively regulated oncogenic ErbB2-mediated transformation, we were also interested in determining if 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 acts to modulate ErbB2 protein levels by promoting the downregulation and turnover of the receptor. Conversely, loss of Y1028 resulted in an increase in ErbB2 protein stability. Thus,

stabilization of ErbB2 receptor levels above the minimal threshold in the hemizygous Y1028F knock-in animals allowed these particular strains to develop normally.

Interestingly, sequence alignment of ErbB2 with the Epidermal Growth Factor Receptor shows that ErbB2 Y1028 and its surrounding sequences are homologous to the region surrounding the EGFR-Y992 (Figure 7). Despite the identity and function of EGFR-Y992 in 1989 (Chen et al., 1989), the mechanism of its function has not been clearly elucidated. Carboxyl-terminal truncation analyses of the EGFR have suggested that an 18 amino acid region surrounding Y992 conforms to an "internalization" domain and is required for EGF-dependent receptor internalization and downregulation. However, a subsequent study showed that point mutation of Y992 did not affect EGFR internalization (Sorkin et al., 1992). In contrast, mutation of tyrosine 992 to phenylalanine in another study actually increased the rate of EGFR internalization and they 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 ErbB2 receptor from the cell surface should lead to clarification of these conflicting reports concerning the role of Y992 in the EGFR.

Knock-in animals expressing ErbB2 phosphotyrosine mutations where the Grb2 site (Y1144) or the Shc binding site (Y1227) were removed had little impact on the normal development of mice, even at 10-fold less expression. These results are consistent with the previous *in vitro* data concluding that morphological transformation occurs through

multiple functionally redundant signaling pathways (Dankort et al., 2001; Dankort et al., 1997). Similar to transgenic mice bearing the wild type *erbB2* cDNA, expression of the Y1144F or the Y1227F ErbB2 mutants did not result in any discernable phenotype. These specific phosphotyrosine docking site mutants uncouple ErbB2 from the Grb2 and the Shc intracellular adaptor proteins, respectively. In contrast, uncoupling of Grb2 from the Met receptor in a knock-in model resulted in severe defects in muscle development (Maina et al., 1996). The $erbB2^{Y1144F/Y1144F}$ and $erbB2^{Y1227F/Y1227F}$ knock-in mutants exhibited the same hypomorphic effects as the control $erbB2^{erbB2/erbB2}$ cDNA knock-in model. Taken together, these observations suggest that unlike Met, the remaining ErbB2 autophosphorylation sites are able to functionally substitute for the inability of these ErbB2 mutants to recruit either Grb2 or Shc. Alternatively, it is possible that the ErbB2 heterodimerization partners such as EGFR, ErbB3 or ErbB4 can compensate for the lack of Grb2 or Shc 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 more subtle developmental defects in different tissues in the $erbB2^{Y1144F}$ or $erbB2^{Y1227F}$ 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, we have demonstrated using a series of unique *erbB2* knock-in animals and genetic manipulation that a minimal threshold level of ErbB2 receptor expression is required during development. This critical level is only 5-10% of normal

ErbB2 levels in wild type embryos. We have also identified an important *in vivo* function of an intrinsic negative regulatory site in ErbB2 (Y1028) to modulate the stability/turnover rate of the receptor throughout development.

Experimental Procedures

Knock-in animals

cDNAs encoding the rat ErbB2 (rErbB2) receptor harboring single tyrosine phosphorylation mutations were generated from the corresponding C-terminal domain of the oncogenic ErbB2 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.

RNA analyses

Total RNA from fresh or flash frozen tissue/embryos were extracted and purified by the Guanidine Isothyiocyanate-Cesium Chloride (GiT-CsCl) method (Webster et al., 1998). For RT-PCR, the RNA was treated with DNaseI (RNAse-Free, Roche) prior to the reverse-trancriptase reaction using Superscript II (Invitrogen). PCR amplification of the RT product was performed using the following primers: AB11851 (forward) CCCAGATCTCCACTGGCTCC; AB11852 (reverse) TTCAGGGTTCTCCACAGCACC. 10μl of the PCR reaction was subsequently digested with BamHI and the products resolved

on a 1.5% agarose gel. Ribonuclease protection assays were performed as described previously (Siegel et al., 1994) with minor modifications. 30 μg of total RNA was used and hybridized to an anti-sense *erbB2* riboprobe at 45°C. T1 RNase (450 units) was used in the ribonuclease digestion reactions for 20 minutes at 37°C.

Immunoblot Analysis of Embryo Lysates

Embryos were lysed in modified TNE lysis buffer (50mM Tris- HCl pH 7.6, 150mM NaCl, 1% NP-40, 10mM NaF, 2mM EDTA) supplemented with 10μg/ml leupeptin, 10μg/ml aprotinin and 1mM sodium orthovanadate. Cleared lysates were electrophoresed through SDS-polyacrylamide gels and the proteins transferred to PVDF membranes (Immobilon-P; Millipore). For immunoblotting, the membranes were incubated with an anti-ErbB2 (Ab-3, Oncogene Science), anti-ErbB3 (C-17, Santa Cruz), or anti-ErbB4 (C-18, Santa Cruz) antibody overnight at 4°C. Immunoblots for Grb2 were performed with rabbit anti-Grb2 polyclonal sera (1:2500; C-23, Santa Cruz). After the primary antibody incubations, membranes were subjected to four 10-minute washes in TBS-1% Tween-20 (Bio-Rad). Subsequently, horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:5000; Jackson Laboratories) were incubated with the membranes for 1 hour at room temperature then washed twice for 10-minutes in TBS-1% Tween-20 and once for 10-minutes in TBS. Immunoblots were visualized by enhanced chemiluminescence (Amersham) as specified by the manufacturer.

Wholemount in situ

Embryos from timed-matings were dissected free from the placenta and cleared of extraembryonic tissues. For genotyping embryos, a small piece of the visceral yolk sac was placed in tail lysis buffer (100 mM Tris-HCl, pH 8.5; 5 mM EDTA; 0.2% SDS; 200 mM

NaCl; 100 μg/μl Proteinase K) and incubated overnight at 55°C. The DNA was extracted by phenol:chloroform extraction and ethanol precipitation. For whole-mount *in situ* hybridizations, embryos were fixed in 4% paraformaldehyde/0.2% glutaraldehyde (Fisher Scientific), dehydrated through a graded series of methanol/PBT (1X PBS/0.1% Tween-20, Sigma) baths and stored in 100% MeOH at -20°C until needed. Whole-mount *in situ* hybridizations were carried out as previously described (Wilkinson and Nieto, 1993). To generate the Phox2a riboprobe, the Phox2a plasmid, pKS903 SSN (Valarche et al., 1993), was digested with SstII and transcribed with the T3 RNA polymerase to generate an antisense riboprobe; the sense riboprobe was generated using the same template, but linearized with HindIII and transcribed with T7 RNA polymerase. The *in vitro* transcription reactions were carried out in 20μl reaction volumes containing 14 μl dH_2O (DEPC), 2 μl 10X transcription buffer (Roche), 2 μl DIG RNA Labeling Mix (Roche), 1 μg of template DNA, 30 units RNAGuard (Amersham) and 30 units of RNA polymerase (Roche). The reactions were incubated at 37°C for 2 hours and stopped with the addition of 20 units of DNaseI (RNAse-Free, Roche). The riboprobes were precipitated and resuspended in 100 μl of DEPC dH_2O (~100 ng/μl).

Receptor turnover assay

Rat-1 cells were co-transfected (FuGene6; Roche) with pJ4-based plasmids encoding the oncogenic ErbB2 tyrosine phosphorylation site mutants (Dankort et al., 1997) along with a PGK-Puromycin resistant plasmid. Puromycin (3μg/ml) resistant colonies were isolated after 12-14 days and stable cell lines expressing similar levels of ErbB2 were selected. 1 x 10^6 Rat-1 derived cell lines were seeded onto 60mm plates and pulse labeled using 0.1 mCi/ml of ^{35}S-Express Protein Labelling mix (NEG772, NEN Life Science

Products). The cells were then washed and chased with unlabelled 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 (Kodak) and scanned using the Typhoon8600 scanner (Amersham). Image analyses were performed using ImageQuant software (Molecular Dynamics).

Acknowledgements

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Figure 1. Generation of knock-in animals expressing ErbB2 tyrosine phosphorylation mutants.

For expression of an *erbB2* cDNA by the endogenous mouse *erbB2* promoter, a knock-in targeting vector was constructed where exon 1 of the mouse *erbB2* gene was replaced with either (**A**) a rat *r-erbB2* cDNA or (**C**) 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. Following correct homologous recombination, the cDNA is placed directly under the transcriptional control of the endogenous *erbB2* promoter. The targeted allele introduces an additional HindIII site, which was used to distinguish between the endogenous wild type (wt) allele (7.5kb fragment) and the targeted allele (4.0kb fragment) in Southern Blot analyses (inset). Positive homologous recombinant clones were used in blastocyst injections to generate mutant animals. Mendelian ratios for the progeny of (**B**) $erbB2^{wt/erbB2}$ or (**D**) $erbB2^{wt/Y1028F}$ heterozygous matings were calculated and compared with the frequency of the genotypes observed at weaning age (3-weeks old). (**E**) Schematic representation of the ErbB2 receptor depicted with the five tyrosine autophosphorylation sites in the C-terminal tail and conveniently labeled A through E with the corresponding amino acid number. Note that the numbering of the amino acids is based on the sequences of the rat homologue of ErbB2 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.

Figure 2. Expression analysis of the knock-in allele

(A) To ensure specific expression of the knock-in *erbB2* allele versus the endogenous wild type allele, total RNA was prepared from adult kidneys and subjected to RT-PCR using the two primers indicated, P1 and P2. Within the ~375bp fragment amplified from the C-terminal region of the receptor, a polymorphic BamHI site, which is present only in the endogenous wild type gene but not in the knock-in rat *erbB2* cDNA, was used to distinguish between the two transcripts. Following PCR, the products were digested with BamHI (lanes 2, 4 and 6) and resolved on a 1.5% agarose gel. The first lane of each set (lanes 1, 3, and 5) is the uncut PCR product (~375bp) prior to BamHI digestion. Lane 6 shows the two smaller fragments (~245bp and ~130bp) from complete BamHI digestion of the wild type PCR product. **(B)** Protein lysates prepared from E12.5 wild type and *erbB2* cDNA knock-in embryos were used to detect ErbB2 levels by immunoblotting with an anti-ErbB2 antibody (Ab-3, Oncogene Science). Detection of Grb2 protein (lower panel) using an anti-Grb2 antibody (C-23, Santa Cruz) served as an internal loading control.

Figure 3. Expression of the Y1028F mutation genetically rescues the hypomorphic knock-in allele.

The progeny of animals from (**A**) $erbB2^{wt/erbB2}$ x $erbB2^{wt/ko}$ and (**B**) $erbB2^{wt/Y102F}$ x $erbB2^{wt/ko}$ crosses were genotyped at weaning age (3 wks.) and the results were compared to the expected Mendelian frequencies. The "hets" or heterozygous genotype is either $erbB2^{wt/erbB2}$ or $erbB2^{wt/ko}$ since we did not distinguish between the two. No $erbB2^{erbB2/ko}$ animals were found at 3-weeks. Observation of newborn pups at birth from (**C**) $erbB2^{erbB2/erbB2}$ x $erbB2^{wt/ko}$ crosses revealed that 18 of the 38 pups (47%) were either still born or died shortly after birth. **All of the pups that died were $erbB2^{erbB2/ko}$ animals. (**D**) The lungs from newborn $erbB2^{wt/erbB2}$ or $erbB2^{erbB2/ko}$ animals were collected within a few minutes after birth and fixed in 10% formalin. Histological sections were stained with hematoxylin and eosin. Magnification = 25X (upper panels) and 50X (lower panels).

Figure 4. Development of the primary sympathetic ganglion chain.

Specific staining of the sympathetic nervous system using a Phox2a antisense riboprobe on E12.5 embryos in whole-mount in situ hybridization analyses. Mid-saggital views of (**A**) $erbB2^{wt/erbB2}$, (**B**) $erbB2^{erbB2/ko}$, (**C**) $erbB2^{wt/Y1028F}$, (**D-F**) $erbB2^{Y1028F/ko}$. 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. Original magnification of A through F is 16X.

Figure 5. Comparison of ErbB2 protein and transcript levels in knock-in embryos.

For immunoblot analyses, total protein was isolated from the different E12.5 embryos 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. 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**) Detection of ErbB2, ErbB3 and ErbB4 protein levels in individual blots but from the same set of protein lysates derived from E12.5 knock-in embryos. A representative Grb2 immunoblot is shown to demonstrate consistent sample quantitation.

Figure 6. Tyrosine 1028 promotes the downregulation of ErbB2 in Rat-1 cells.

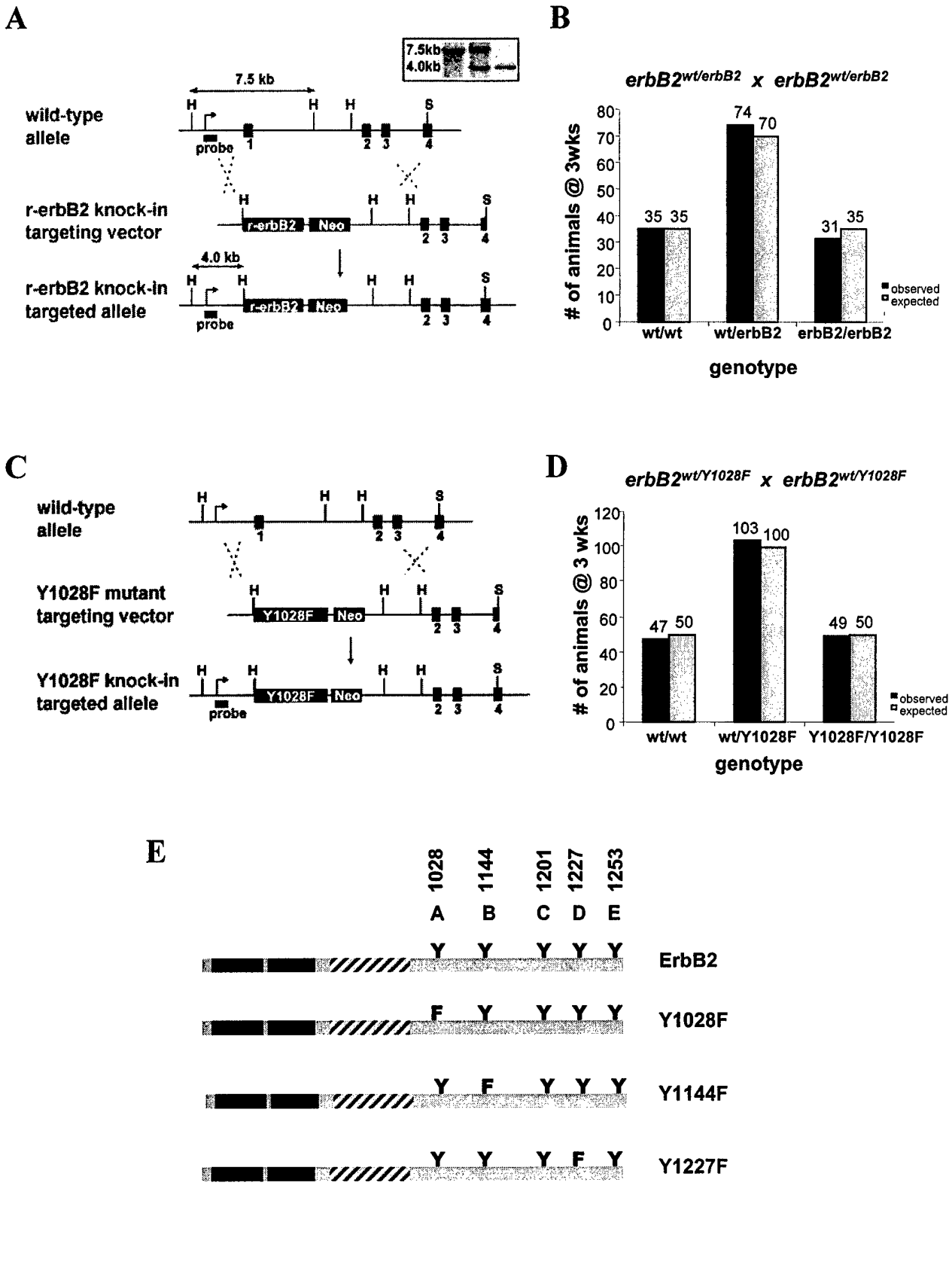
The stability of mutant ErbB2 receptors in the absence or presence of Y1028 was examined by receptor turnover assays. Pulse-chase analysis was performed using ^{35}S-methionine labelled Rat-1 stable cell lines expressing the constitutively active oncogenic ErbB2 mutants. (**A**) ErbB2 versus ErbB2-Y1028F phosphotyrosine mutant. (**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.

Figure 7. Amino acid sequences surrounding Y1028

Alignment of the amino acid sequences surrounding tyrosine 1028 in ErbB2 and tyrosine 992 in EGFR

Figure

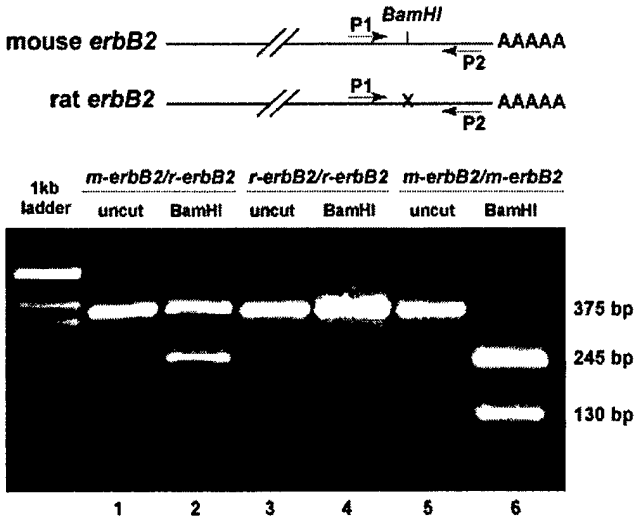
Figure 1, *Chan et al.*



Figure

Figure 2, *Chan et al.*

A



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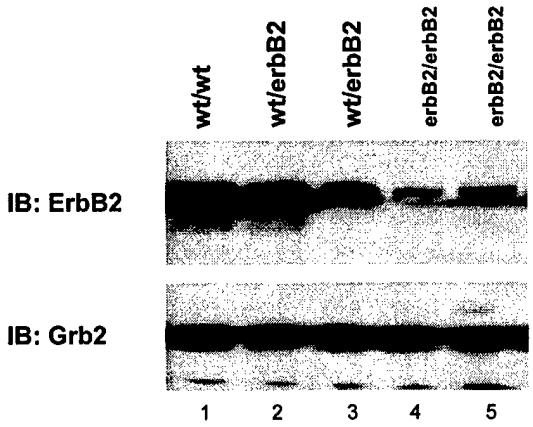

Figure

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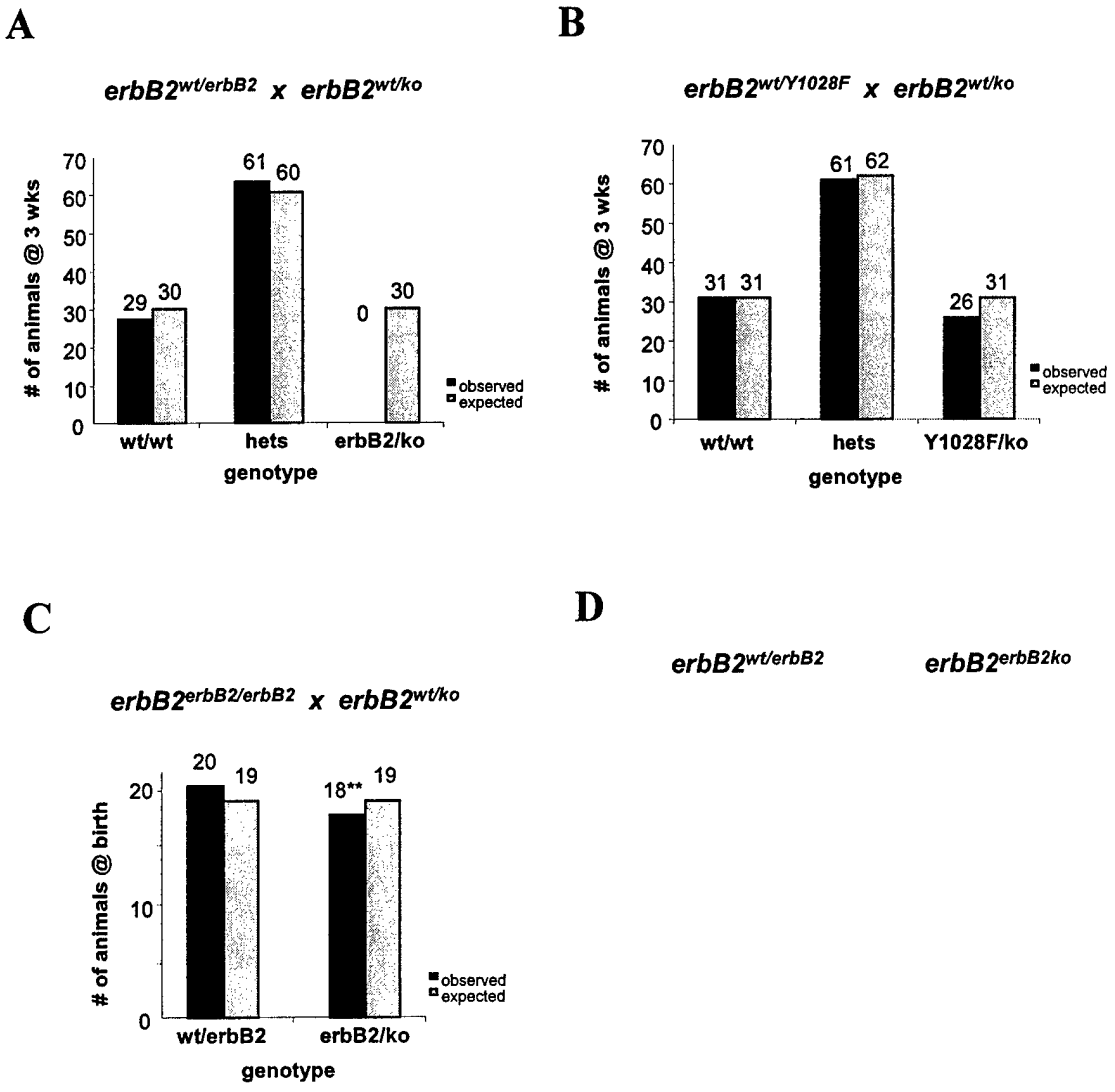

Figure

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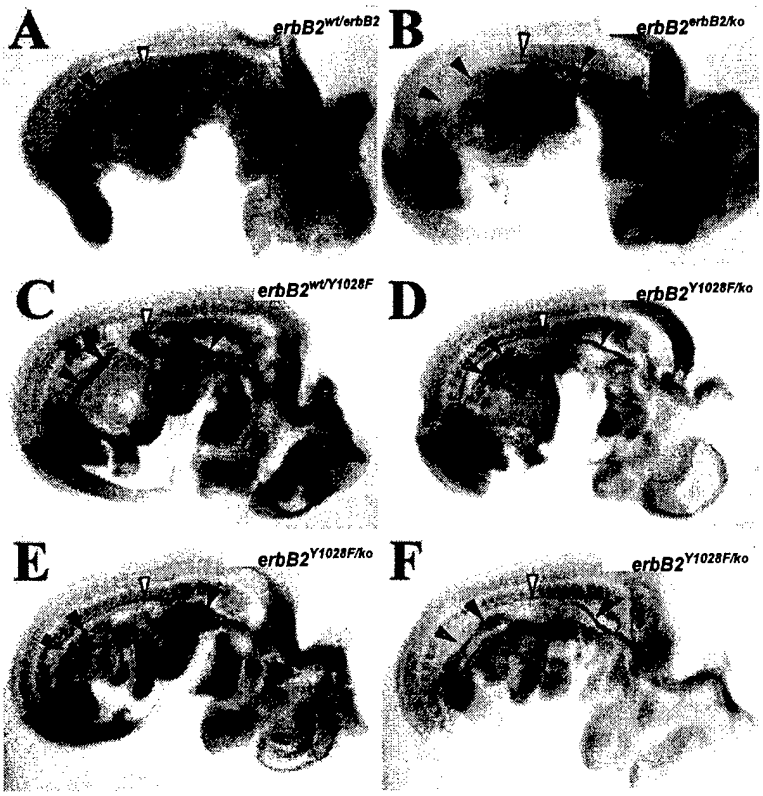
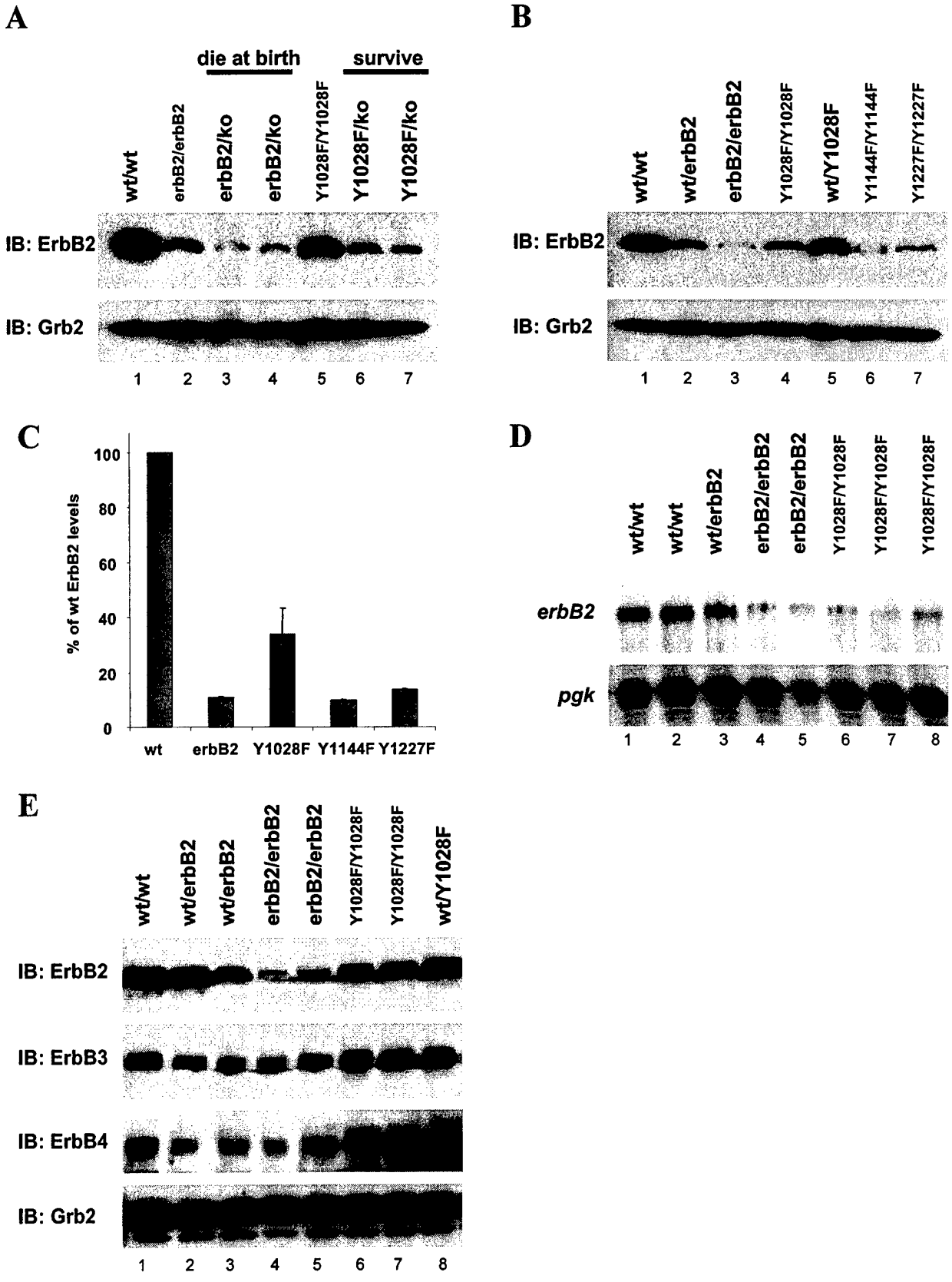


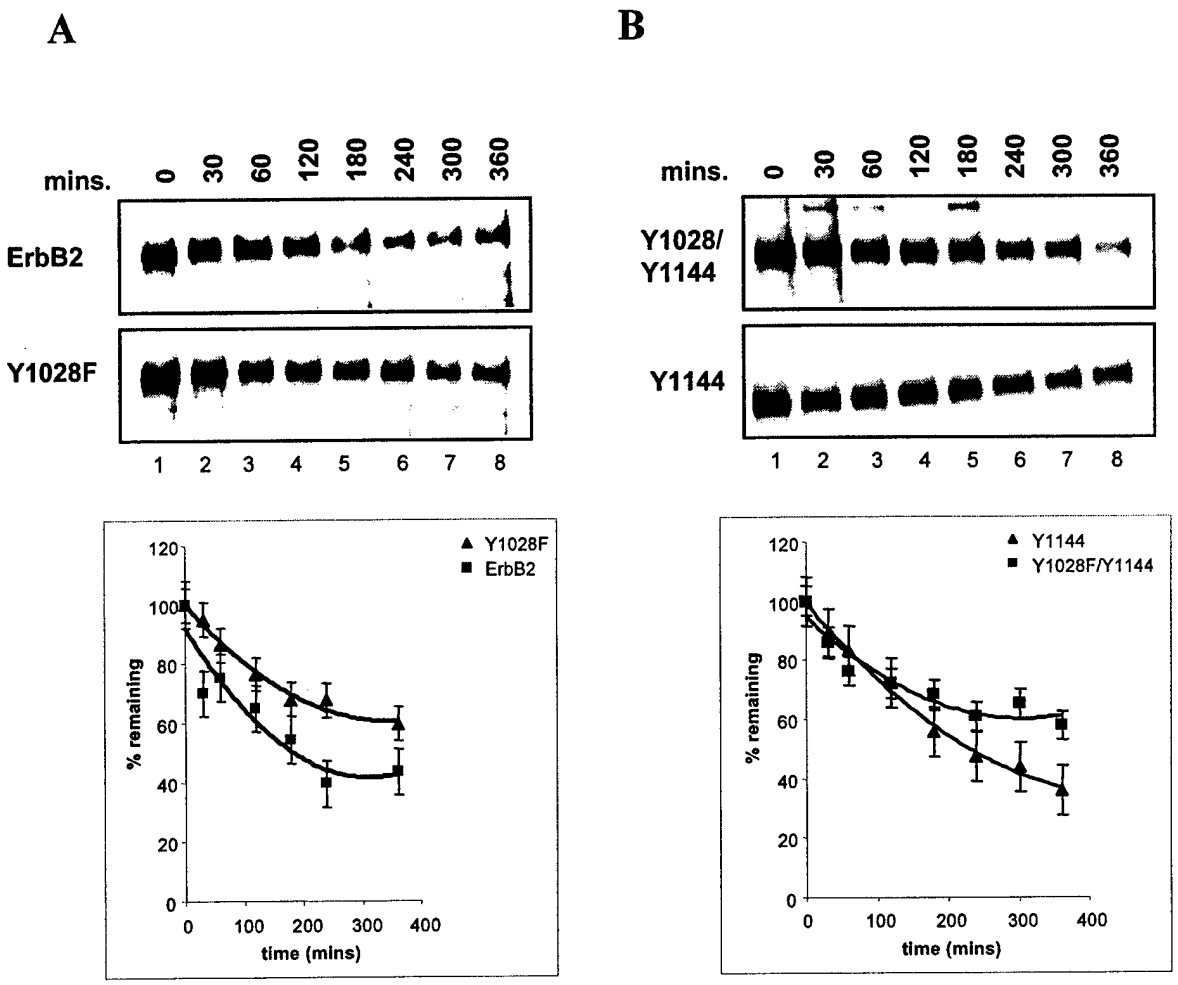
Figure

Figure 5, *Chan et al.*



Figure

Figure 6, *Chan et al.*



Figure

Figure 7, *Chan et al.*