The Guanine Nucleotide Exchanger Vav2 Interacts with c-ErbB-2 and Induces Alveolar Morphogenesis of Mammary Epithelial Cells

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ABSTRACT

The ErbB receptor tyrosine kinases constitute a subfamily of four structurally related members, the EGF receptor (ErbB-1), ErbB-2, ErbB-3 and ErbB-4. ErbB receptor tyrosine kinases are critical for embryonic development of central and peripheral neural structures and heart. In addition, ErbB receptors play an important role in the postnatal development of the mammary gland. Previous studies showed that activated ErbB-2 receptor induces alveolar morphogenesis of EpH4 mammary epithelial cells that are cultured on a three-dimensional matrix (termed Matrigel). However, the downstream signaling proteins that mediate this biological activity of ErbB-2 were unknown. In this work, Vav2 was identified as a direct interaction partner of tyrosine-phosphorylated ErbB receptors using the yeast two-hybrid system. Vav2 is a member of a family of guanine nucleotide exchange factors that induce cytoskeletal rearrangements, transcriptional alterations, and have oncogenic potential when activated. To test the ability of Vav2 to mediate morphogenic signals of ErbB-2, EpH4 cells overexpressing Vav2 protein were cultured on Matrigel. Indeed, Vav2 induces alveolar morphogenesis of EpH4 cells when activated either by oncogenic mutation or tyrosine phosphorylation by ErbB-2. The morphogenic activity of Vav2 requires the Dbl homology domain, which mediates GDP/GTP exchange. Dominant-negative Vav2 specifically blocks the morphogenic signals of ErbB-2 in EpH4 cells without interfering with ErbB2-induced mitogenesis. Importantly, Vav2 is co-expressed and interacts with ErbB-2 in the mammary glands of pregnant mice. Taken together, these results point to Vav2 as a candidate to mediate ErbB-2 signals for alveolar morphogenesis *in vivo*, which is a relevant step in the development of the mammary gland during pregnancy.

ZUSAMMENFASSUNG

Die Familie der ErbB-Rezeptor-Tyrosinkinasen besteht aus vier Mitgliedern, dem EGF-Rezeptor (ErbB-1), ErbB-2, ErbB-3 und ErbB-4. ErbB-Rezeptoren spielen eine wichtige Rolle bei der Entwicklung des Nervensystems, des Herzens und der Brustdrüsen. Ein Teil dieser Differenzierungsvorgänge läßt sich *in vitro* nachvollziehen: so ist zum Beispiel die Aktivierung des ErbB-2 Rezeptors ausreichend für alveoläre Morphogenese der Brustdrüsenepithelzellinie EpH4. Intrazelluläre Moleküle, die dieses ErbB2-Signal übertragen, sind allerdings noch unbekannt. Mit Hilfe eines neuen, modifizierten Hefe-2-Hybrid-Systems wurde in der vorliegenden Arbeit Vav2 als neuer Interaktionspartner von ErbB-2 identifiziert. Vav2 assoziiert mit aktiviertem ErbB-2 über eine SH2-Domäne. Die Interaktion ist direkt und ist von zwei Phosphotyrosinen in ErbB-2 abhängig. Vav2 kann den GDP/GTP-Austausch bei GTPasen der Rho-Familie vermitteln. Dadurch kann der Umbau des Zytoskeletts und Veränderungen der Transkription sowie Zelltransformation induziert werden. In einem dreidimensionalen Zellkultursystem kann aktiviertes Vav2 in EpH4 Zellen die Bildung von alveolären Zellaggregaten induzieren. In diesen Alveolen umgibt eine Schicht polarisierter milchproduzierender Zellen ein zentrales Lumen. Diese Vav2-vermittelte Morphogenese ist abhängig von der katalytischen GDP/GTP-Austausch Aktivität von Vav2. Katalytisch-inaktives Vav2 kann die morphogenetische Aktivität von ErbB-2 in EpH4-Zellen verhindern, ohne die mitogene Aktivität von ErbB-2 zu beeinflussen. Vav2 ist mit ErbB-2 coexprimiert und interagiert mit dem Rezeptor in Brustdrüsenzellen schwangerer Mäuse. Diese Untersuchungen deuten darauf hin, dass Vav2 eine wichtige Funktion bei der durch ErbB-2 induzierten alveolären Morphogenese der Brustdrüse spielt.

1 INTRODUCTION

Epithelium and mesenchyme are two distinct types of tissues that are present in virtually every organ. Lung, kidney, vascular system and most glands consist of tubular epithelial networks embedded in mesenchymal tissue. The formation of the first rudiments for these tree-like structures is specified during embryogenesis; epithelial buds invade the underlying mesenchyme and ramify to create interconnected tubules, a process known as *branching morphogenesis*. Lungs, salivary glands and mammary glands exhibit further developmental changes, namely *alveolar morphogenesis*, whereby round hollow alveoli arise from the ductal tree and differentiate into the functional units of the organ. Mesenchymal-epithelial interactions are strictly required for patterning morphogenic events (Sawer and Fallon, 1983); mesenchymal soluble factors bind to their epithelial receptors thus activating several signaling pathways which lead to local cellular responses like growth, motility, morphogenesis and differentiation.

The mammary gland is one of the most interesting models to study mesenchymal-epithelial interactions, as they play critical roles not only in embryonic mammary development but also in postnatal growth and differentiation of the gland. The mammary gland is a dynamic organ which has the unique property to undergo main developmental changes after birth. The complex mechanisms supporting mammary ductal branching and alveolar morphogenesis have been subject of extensive research, but are still far from being fully unravelled.

This work characterizes Vav2 as a novel intracellular effector of the receptor tyrosine kinase ErbB-2 that mediates lobulo-alveolar morphogenesis of the mammary gland. The first part of this Introduction reviews the developmental stages of the mammary gland and their regulation. The second part concerns the ErbB family of receptor tyrosine kinases and their role in mammary gland development. The third part describes features of the Vav family of guanine nucleotide exchange factors that may be useful for interpreting the results.

1.1 Development of the Mammary Gland

The development of the murine mammary gland will be here outlined, since the mouse is the most thoroughly studied mammalian model (reviewed in Sakakura, 1987; Silberstein *et al*., 2001). During embryonic life, the mammary anlagen appear between days E10-E12 as an invagination of epidermal cells into the underlying mesenchyme, the fat pad. The mammary fat pad differentiates from deeply-placed mesenchymal cells. At day E16, primary ducts emerge from the epithelial rudiments into the fat pad and undergo an initial round of branching morphogenesis. Later, the nipples derive from epidermal invagination around the primary duct. The infant mammary gland consists of a primitive ductal tree that emanates from the nipple into the proximal fat pad. During puberty (5 to 8 weeks of age), the ducts elongate and branch further into the fat pad. The growing points for ductal growth are structures termed end buds, located at the terminal ends of the ducts. These end buds consist of two distinguishable cell populations: the cap cells, which are the progenitors for myoepithelium, and the body cells, which give rise to mammary epithelium. During the estrous cycle, alveolar buds first emerge from the lateral walls of the ducts, then regress and form again in the next cycle. Alveolar morphogenesis begins early in pregnancy with extensive budding of alveoli from the ducts; later, alveoli cluster to form lobulo-alveolar structures that are the secretory units of milk components throughout late pregnancy and lactation. Following weaning, the glands undergo a remodelling process known as involution (Lund *et al*., 1996; Li *et al*., 1997); during this phase, massive epithelial apoptosis results in destruction of lobulo-alveolar structures and regression of the gland to the pre-pregnancy state.

1.1.1 Regulation by Systemic Hormones

The development and biology of the mammary gland is controlled by a complex interplay of systemic hormones and local growth factors. Hormones are critical regulators of mammary

development both in embryonic and postnatal life. Once the embryonic mammary buds are formed, they stimulate expression of androgen receptors in the surrounding mesenchyme (Kratochwil, 1986). Testicular androgens from male fetuses induce regression of the epithelial mammary rudiment at E14, whereas estrogens are apparently not required for prenatal development (Korach, 1994).

The structure and functionality of the postnatal mammary tissue is orchestrated by hormonal changes occurring with age, estrous cycle and reproductive status. Pubertal growth and secretory differentiation of mammary parenchyme is dependent on ovarian steroids (estrogen, progesterone), prolactin and growth hormone. A role for estrogen and progesterone in ductal growth has been demonstrated by genetic and tissue recombination studies. Estrogen induces ductal dichotomous branching via paracrine stimulation of the stromal receptors, whereas the epithelial receptors are dispensable (Bocchinfuso and Korach, 1997; Cunha *et al*., 1997). Conversely, progesterone targets its epithelial receptors at puberty to promote ductal side-branching (Lydon *et al*., 1995; Brisken *et al*., 1998). Genetic ablation of prolactin or its epithelial receptor revealed that prolactin controls regression of terminal end buds and ductal side-branching during puberty (Ormandy *et al*., 1997; Horseman *et al*., 1997); these effects of prolactin are indirect and may involve regulation of ovarian production of progesterone (Brisken *et al*., 1999). Recent tissue recombination experiments circumvented the sterility of the abovementioned knockout mice and showed that both progesterone and prolactin act directly on their epithelial receptors to stimulate lobulo-alveolar development (Brisken *et al*., 1998; Brisken *et al*., 1999). Ectopic expression of growth hormone in transgenic mice leads to precocious mammary development and epithelial differentiation (Bchini *et al*., 1991), indicating that growth hormone promotes functional differentiation of the gland. This effect may be mediated by insulin-like growth factor-1 (IGF-1), as growth hormone induces synthesis of IGF-1 in stromal mammary cells (Kleinberg, 1997). Moreover, an essential role of IGF-1 in end bud formation was shown by the targeted deletion of IGF-1, which resulted in

retardation of ductal morphogenesis that could be rescued by exogenous IGF-1 (Ruan and Kleinberg, 1999).

1.1.2 Local Effects of Multiple Growth Factors

The complexity of signals that control postnatal mammary growth and differentiation is intriguing. Systemic hormones may synergize with a variety of growth factors that are locally produced either in the mammary mesenchyme (like HGF/SF, FGF-4, FGF-2, KGF, HRG1α, TGF-β, insulin-like growth factors) or in the mammary epithelium (Wnts, EGF, TGF-α, amphiregulin, FGF-1). This section concerns stimulatory and inhibitory growth factors that are implicated in growth and differentiation of the mammary gland. The function of EGF-like growth factors and their receptors in mammary development will be addressed separately.

The role of Wnt signals in mammary development begins in embryonic life. Lef-1 null mice fail to form mammary anlagen (van Genderen *et al*., 1994). Synergism of Wnt and parathyroid hormone-related peptide (PTHrP) signals has been shown to be essential for branching morphogenesis, sexual dimorphism and nipple formation of the mammary gland during embryogenesis (Wysolmerski *et al*., 1998; Dunbar and Wysolmerski, 1999; Foley *et al*., 2001). In postnatal life, the Wnt pathway is essential for mammary growth and differentiation during pregnancy. Wnt-4 and progesterone signals trigger interconnected cascades that control ductal side-branching and alveolar morphogenesis; using genetic and tissue recombination techniques, Brisken *et al*. (2000) showed that progesterone impacts nearby epithelial cells to induce expression of Wnt-4, which in turn synergistically acts in a paracrine fashion to promote ductal side-branching. Further support for a role of Wnt signaling in mammary development at pregnancy comes from genetic experiments with β-catenin, a downstream effector of the Wnt pathway. Mammary glands from virgin mice overexpressing an active β-catenin mutant show pregnancy-like lobulo-alveolar development and differentiation (Imbert *et al*., 2001); however, ductal side-branching is normal in these transgenic mice. These findings suggest that the canonical Wnt signaling pathway contributes to alveolar

morphogenesis, while Wnt control of ductal side-branching during pregnancy is not mediated by β-catenin but by other as yet unknown downstream effectors. Alternative Wnt-induced effectors are reviewed elsewhere (Hülsken and Birchmeier, 2001).

Previous observations suggest a role for hepatocyte growth factor/scatter factor (HGF/SF) and its receptor c-Met in ductal growth of the virgin mammary gland. HGF/SF and its receptor c-Met are coordinately produced in mammary mesenchyme and epithelium, respectively, with highest expression levels throughout puberty and in adult life until mid-pregnancy (Pepper *et al*., 1995; Niranjan *et al*., 1995; Yang *et al*., 1995); HGF/SF is expressed at low levels during late pregnancy and throughout lactation, and expression again increases during involution of the glands. In support of a role in ductal growth, HGF/SF stimulates branching when human mammary organoids are cultured on collagen, resembling the ductal elongation events that are observed in the mammary gland during puberty (Niranjan *et al*., 1995). Similarly, exogenous HGF induces extensive ductal branching in organ culture, whereas antisense HGF oligonucleotides block ductal growth of explanted mouse mammary glands (Yang *et al*., 1995). Branching morphogenesis is also observed in organotypic cell culture experiments whereby primary mammary epithelial cells, or cells from the mammary epithelial cell lines NMuMG and EpH4 are grown on three-dimensional matrices in the presence of HGF/SF (Niranjan *et al*., 1995; Soriano *et al*., 1995; Niemann *et al*., 1998). However, direct proof of a physiological role of HGF/SF signaling in the mammary gland *in vivo* is still awaited.

Factors that regulate differentiation of cells from the mononuclear phagocytic lineage also appear to be involved in mammary development. The colony stimulating factor 1 gene (CSF-1) is disrupted by an inactivating mutation in the recessive osteopetrosis (*op*) allele; homozygous *op/op* mice show a lactational defect due to incomplete mammary ductal side-branching and lactational failure, despite normal lobulo-alveolar morphogenesis and expression of milk proteins (Pollard and Hennighausen., 1994). The TNF family member osteoprotegerin-ligand or osteclast differentiation factor (ODF) synergizes with CSF-1 in

osteoclastogenesis (Lacey *et al*., 1998); absence of ODF or its epithelial receptor RANK (receptor activator of NFκB) impairs formation of lobulo-alveolar structures during pregnancy due to enhanced cell death; however, and in contrast to CSF-1 ablation, it does not affect side-branching (Fata *et al*., 2000). These results suggest a model whereby CSF-1 regulates ductal sprouting, and ODF subsequently promotes lobulo-alveolar development and terminal differentiation.

The formation of the adult mammary gland depends not only on growth stimulation but also on active inhibition, which prevents infilling the extraglandular mesenchyme. There is ample evidence that various TGF-β factors reversibly inhibit growth of the mammary end buds (reviewed in Silberstein, 2001). Overexpression of TGF-β1 in the mammary gland leads to impaired ductal elongation (Pierce *et al*., 1993) and absence of alveolar outgrowth and milk secretion (Jhappan *et al*., 1993), indicating that TGF-β1 negatively regulates both ductal and alveolar morphogenesis. Conversely, inhibition of TGF-β1 signaling promotes excessive ductal branching in mice that overexpress a kinase-deficient TGF-β type II receptor in the mammary stroma (Gorska *et al*., 1998; Joseph *et al*., 1999); this effect may be driven by up-regulation of HGF/SF expression, thus suggesting that the chronic inhibition of ductal growth by TGF-β1 results from its down-regulatory effect on the periductal synthesis of HGF/SF. Transgenic mice and transplantation experiments identified TGFβ-3 as a local factor that is secreted by alveolar cells upon milk stasis, and initiates apoptosis during the first stage of involution (Nguyen *et al*., 2000); the autocrine pro-apoptotic effect of TGFβ-3 involves activation of Stat3 followed by up-regulation of IGF-binding protein-5 (IGFBP-5), which sequesters and inactivates the mitogen IGF-1 (Li *et al*., 1997; Tonner *et al*., 1997; Chapman *et al*., 1999; Nguyen *et al*., 2000).

The role of fibroblast growth factor (FGF) signaling in the mammary gland is still controversial; whereas the different members of the FGF family show a temporally and spatially regulated expression in mammary tissue, genetic experiments suggest that they may

have redundant roles in the development of the gland. Expression of endogenous FGF4 and its receptor, FGFR1, is detected in virgin females, but not during pregnancy and lactation (Coleman-Knarcik *et al*., 1994; Chodosh *et al*., 2000). However, transgenic mice for FGF4 exhibit hyperplastic lobulo-alveolar structures that persist longer after weaning due to impaired apoptosis, and mice expressing an ectopic dominant-negative form of FGFR1 in mammary epithelium surprisingly lack a discernible phenotype (Morini *et al*., 2000). It is possible that FGF4, like TGFβ factors, plays a role in the control of apoptotic remodelling during ductal development and involution. In contrast to the transgenics for inactive FGFR1, mice overexpressing a dominant-negative FGFR2 transgene show an impairment in lobulo-alveolar development by mid-pregnancy (Jackson *et al*., 1997); knockout mice for FGF-7, its putative mammary ligand, show normal mammary development (Guo *et al*., 1996). Future research employing combined FGF-factor knockout mice may help to understand the function of this family in mammary tissue.

To conclude, the mammary development is regulated by multiple growth and differentiation factors and their intracellular signaling cascades. Loss of a single factor is often not compensated by the others, indicating that these factors trigger essential interacting or intersecting signaling cascades. Therefore, integration of these signals and the identification of new mammogenic factors still represent a major challenge.

1.1.3 The Role of Extracellular Matrix

The inducing effects of embryonic mesenchyme or its postnatal counterpart, termed stroma, are partially mediated by components of the extracellular matrix (ECM). In the adult gland, epithelial cells are in direct contact with basal myoepithelial cells and with the ECM structure known as basement membrane. The basement membrane is a complex and organized three-dimensional array of laminin, type IV collagen, heparan sulphate, proteoglycans and other proteins (Timpl, 1996). It is known that cell-ECM interactions influence tissue architecture through modulation of signaling pathways that affect cell growth, differentiation, survival and

morphogenesis (reviewed in Adams and Watt, 1993). The use of reconstituted basement membranes provides an excellent system to study the essential role of ECM for lactogenic differentiation *in vitro*. In organotypic cell culture, synthesis and secretion of milk components by mammary epithelial cells results from the interrelated effects of hormones, cell-cell and cell-ECM contacts (reviewed in Lin and Bissell, 1993). Primary mammary epithelial cells fail to differentiate to a secretory phenotype when cultured on plastic or onto a thin collagen type I layer (Emerman and Pitelka, 1977; Berdichevsky *et al*., 1992); however, these cells undergo alveolar morphogenesis and secrete milk proteins vectorially when cultured on a reconstituted, laminin-rich matrix from Engelbreth-Holm-Swarm (EHS) tumours (termed Matrigel; Kleinman *et al*., 1986; Barcellos-Hoff *et al*., 1989). Indeed, adhesion of mammary epithelial cells to laminin is critical for β-casein gene expression and for activation of Stat5, an essential regulator of milk gene expression (Streuli *et al*., 1995a; Streuli *et al*., 1995b; see also section 2.5 of this Introduction). However, the extent of morphogenic events *in vitro* is limited, indicating that additional signals from living stromal cells are required to support formation of fully-developed ducts or alveoli.

Integrins are cellular receptors for laminin, a major ECM component (Sonnenberg *et al*., 1990). Integrins are expressed at the basal membrane of epithelial cells both in mammary alveolar tissue and in EHS-cultured alveoli (Streuli *et al*., 1991). A role of integrins as physiological receptors for ECM was suggested by cell culture studies in which integrin function was blocked by pan-antibodies (Streuli *et al*., 1991); such blocking antibodies prevented the expression of milk proteins by mammary epithelial cells embedded in EHS matrix, indicating that the ECM induces lactogenic differentiation via integrin signaling. Genetic studies confirmed that integrins are cellular laminin receptors that control mammary functional differentiation; targeted expression of a transgene coding for an inactive β1-integrin subunit impairs lobulo-alveolar development and secretory differentiation during pregnancy (Faraldo *et al*., 1998). Moreover, laminin accumulates at the lateral surface of

luminal cells in the transgenic glands, indicating that integrin-ECM interactions determine baso-apical polarity of alveolar cells.

Overall, it is evident that direct cell-ECM contacts contribute to the induction of morphogenic and lactogenic events in mammary development. Future work will help to fully understand the cooperative effects of the ECM and other mitogenic factors in growth, morphogenesis and functional differentiation of the mammary epithelium.

1.2 The ErbB Family of Receptor Tyrosine Kinases

ErbB proteins belong to subclass I of the superfamily of receptor tyrosine kinases. This family has evolved from a single ligand-receptor pair in *Caenorhabditis elegans*, lin-3/let-23 (Aroian *et al*., 1990). *Drosophila melanogaster* also express one ErbB receptor, but three activating ligands and one inhibitor (Freeman, 1998). In vertebrates, the ErbB family comprises four membrane receptors: the epidermal growth factor receptor (EGF receptor; also termed ErbB-1, HER1), ErbB-2 (c-Neu, HER2), ErbB-3 (HER3) and ErbB-4 (HER4) (reviewed in Olayioye *et al*., 2000). These receptors share a glycosylated extracellular ligand-binding region with two cysteine-rich domains, a transmembrane stretch, and an intracellular region that encompasses a tyrosine kinase domain and a C-terminal tail containing the autophosphorylation sites. ErbB-1, ErbB-2 and ErbB-4 encode ligand-activated tyrosine kinases, whereas the corresponding ErbB-3 sequence is apparently devoid of kinase activity (Guy *et al*., 1994). ErbB receptors show different patterns of expression: ErbB-1 is expressed by liver parenchymal cells, fibroblasts, keratinocytes and several epithelial tissues, like the basal layer of the skin (Adamson, 1990; Partanen, 1990); ErbB-2 is expressed in a variety of tissues including nervous system, connective tissue and secretory epithelium (Kokai *et al*., 1987); ErbB-3 is expressed primarily in epithelium of various organs, in peripheral nervous system and in oligodendrocytes, while ErbB-4 is mostly restricted to central nervous system, cardiac muscle and glial cells (Pinkas-Kramarski *et al*., 1997). These differential expression

patterns are consistent with specific biological activities during embryonic life (discussed in Section 2.4 of this Introduction).

The ErbB receptors are activated upon ligand binding, a general mechanism that is shared by various receptor tyrosine kinases. Ligand binding induces formation of receptor dimers; this key step allows each receptor subunit to cross-phosphorylate tyrosine residues in the activation loop of the kinase domain of its partner, thus enhancing the catalytic activity (Hubbard *et al*., 1998). Following activation of the kinases, specific tyrosine residues on the C-terminal tail of the receptors become autophosphorylated; these phosphotyrosine residues are docking sites for intracellular signaling molecules that couple the receptors to signal transduction cascades, thus ultimately resulting in specific cellular responses. The hallmark of the ErbB family is the formation of both homo- and heterodimers following ligand binding. Moreover, each ligand induces the formation of preferential dimers in tissues where more than two ErbB receptors are expressed, leading to signal diversification (see below). The cellular routing of each receptor after ligand binding also differs for each family member: ErbB-1 undergoes rapid internalization and targets EGF for lysosomal degradation, whereas the other ErbBs are slowly internalized and are recycled back to the cell surface without significant degradation of the endocytosed ligand (Baulida *et al*., 1996; Pinkas-Kramarski *et al*., 1996; Lenferink *et al*., 1998).

1.2.1 ErbB Ligands Contain an EGF-like Domain

ErbB receptors are activated upon binding ligands that are known as EGF-related growth factors. These factors are mostly produced as transmembrane precursors, which can be proteolytically cleaved, thus releasing the extracellular, biologically active region. ErbB ligands are characterized by the presence of a conserved EGF-like domain of 35-50 amino acids that is essential for receptor binding. Six cystein residues form three disulfide bonds that hold together the characteristic three-loop structure of this motif. EGF-like ligands can be grouped according to their receptor-binding affinity: EGF, amphiregulin and transforming

growth factor-α (TGF-α) specifically bind to ErbB-1; betacellulin, heparin-binding EGF (HB-EGF) and epiregulin bind both ErbB-1 and ErbB-4; neuregulins are a complex family of proteins that include NRG-1 and NRG-2, which bind to ErbB-3 and ErbB-4, and the recently described NRG-3 and NRG-4, which bind to ErbB-4. So far, no direct ligand for ErbB-2 has been described (Peles *et al*., 1993; Tzahar *et al*., 1994).

NRG-1 (also termed Neu differentiation factor) was first isolated from medium of Ras-transformed rat fibroblasts, and the human counterpart Heregulin was detected in medium of breast cancer cells (reviewed in Peles and Yarden, 1993). Two neuronal factors, termed glial growth factor (GGF) and acetylcholine receptor-inducing activity (ARIA) are alternatively spliced variants of NRG-1. Four different *nrg* genes code for the neuregulin isoforms and their related variants (Holmes *et al*., 1992; Wen *et al*., 1992; Falls *et al*., 1993; Marchionni *et al*., 1993; Carraway *et al*., 1997; Chang *et al*., 1997; Harari *et al*., 1999). NRG-1 shows a wide expression pattern during embryogenesis, being detectable in the central nervous system and in ventricular endothelium (Meyer *et al*., 1995). NRG-2 is also expressed in embryonic neural tissue and heart but otherwise is largely non-overlapping with NRG-1 expression (Carraway *et al*., 1997; Chang *et al*., 1997). NRG-3 expression is restricted to developing and adult nervous tissue (Zhang *et al*., 1997); NRG-4 is highly expressed in adult pancreatic tissue and weakly in muscle, but no data exist about embryonic expression (Harari *et al*., 1999).

1.2.2 Generation of Signal Diversity

It has been proposed that EGF-like ligands are bivalent; in case of NRG-1, an N-terminally located high affinity site with narrow specificity first binds a direct receptor (ErbB-3 or ErbB-4), and then a second C-terminal site recruits a co-receptor with lower affinity but broader specificity, ErbB-2 usually being the preferred one (Tzahar *et al*., 1997). Such a mechanism may account for the diversity of receptor dimers that are observed with a single ligand, as well as for the activation of the orphan receptor ErbB-2 in response to different EGF-like growth factors (Karunagaran *et al*., 1996; Graus-Porta *et al*., 1997). Evidence for the existence of all

ten possible homo- and heterodimers of ErbB proteins has been reported, including the ErbB-2 homodimer that is stabilized by oncogenic mutation or overexpression (Riese *et al*., 1995; Tzahar *et al*., 1996). This network of inter-receptor interactions displays a strict hierarchy rather than a random pattern (Tzahar *et al*., 1996). In fact, ErbB-2 is the preferred heterodimerization partner for all other ErbB family members. A driving force for the preferential binding to ErbB-2 could be that heterodimers containing ErbB-2 have a very low rate of ligand dissociation compared to other receptor pairs; this property of ErbB-2 can significantly prolong cell stimulation by every ErbB ligand (Graus-Porta *et al*., 1995; Karunagaran *et al*., 1996). In the absence of ErbB-2, NRG-1 induces formation of other heterodimers like ErbB-1/ErbB-3 and ErbB-1/ErbB-4 heterodimers, which explains the inhibition *in trans* of EGF binding to ErbB-1 when NRG-1 is present (Karunagaran *et al*., 1995). The existence of several ligands, together with their distinct ability to stabilize preferential homo- and heterodimeric receptor pairs, points to the existence of a hierarchical network of ligand-stimulated receptor dimerization events within the ErbB family (Pinkas-Kramarski *et al*., 1996).

Autophosphorylation of tyrosine residues may also be influenced by the heterodimer combination (Olayioye *et al*., 1998); in this way, each receptor has the ability to interact with distinct sets of intracellular signaling proteins thus increasing the functional versatility of the ErbB family. There are four potential mechanisms which may account for ligand-induced differential phosphorylation of the receptors (reviewed in Sweeney and Carraway, 2000). One ligand may induce receptor dimerization and phosphorylation of a particular subset of tyrosine residues. Binding to a different ligand could influence site usage for phosphorylation by promoting the association of the dimeric receptor complex with other cellular proteins like kinases, phosphatases or even cell surface molecules, which may mediate phosphorylation or dephosphorylation of specific sites. Alternatively, this second ligand could stimulate the assembly of oligomeric receptor complexes, or induce a different conformation of the receptor pair. Taken together, combinatorial dimerization and ligand-induced diversification of

signaling appear to confer ErbB receptors the potential to give rise to a broad range of cellular responses; moreover, because each receptor is unique in terms of catalytic activity, cellular routing and transmodulation, the resulting network allows fine tuning and stringent control of cellular functions.

1.2.3 Intracellular Effectors of ErbB Receptors

As for other receptor tyrosine kinases, ligand-induced autophosphorylation of ErbB receptors on specific tyrosine residues creates docking sites for cytoplasmic signaling proteins containing Src-homology2 (SH2) or phosphotyrosine-binding (PTB) domains (reviewed in Pawson, 1995). The binding specificity of these proteins is determined by the amino acid sequences adjacent to the phosphorylated tyrosines; amino acids located N-terminally determine the binding of specific PTB domains, and amino acids that are C-terminally located select SH2 domains. All ErbB family members, including the *C. elegans* and *D. melanogaster* homologs Let23 and DER, couple via Shc and/or Grb-2 to the mitogen-activated protein kinase (MAPK) pathway (Pinkas-Kramarski *et al*., 1996). However, certain intracellular proteins are preferential substrates of specific ErbB receptors. ErbB-3 is a preferred activator of p85 subunit of phosphatidylinositol-3-kinase (PI-3-K) due to the multiple specific binding motifs present in the ErbB-3 C-terminal tail, which are virtually absent in case of ErbB-2 (Fedi *et al*., 1994; Prigent *et al*., 1994). Similarly, the negative regulator c-Cbl and phospholipase Cγ1 (PLCγ1) couple to both ErbB-1 and ErbB-2 but not to ErbB-3 or ErbB-4 (Fazioli *et al*., 1991; Fedi *et al*., 1994; Cohen *et al*., 1996; Levkowitz *et al*., 1998; Klapper *et al*., 2000; Levkowitz *et al*., 2000). Olayioye *et al*. (2000) offers an excellent review of the present knowledge about the intracellular mediators of ErbB signals. As this work aimed to find novel substrates for the ErbB-2 receptor, its known downstream effectors will be described in detail.

ErbB-2 contains 5 putative autophosphorylation sites in its C-terminal tail, termed here Y1 (the most N-terminal tyrosine residue) to Y5 (the most C-terminal one; Hazan *et al*., 1989;

Segatto *et al*., 1990; Akiyama *et al*., 1991). It has been described that Shc binds to Y4 through its PTB domain (Segatto *et al*., 1993; Ricci *et al*., 1995; Dankort *et al*., 1997). Grb-2 directly binds to Y2 via its SH2 domain, and indirectly via Shc (Ricci *et al*., 1995; Dankort *et al*., 1997). Chk binds to Y5 (Zrihan-Licht *et al*., 1998). Grb-7, c-Src, Ras-GTPase activating protein (Ras-GAP) and the abovementioned PLCγ1 also interact with ErbB-2, though the binding sites are unclear (Fazioli *et al*., 1991; Jallal *et al*., 1992; Stein *et al*., 1994; Muthuswamy and Muller, 1995b).

The functional role of the ErbB-2 autophosphorylation sites in receptor-mediated transformation has been assessed by mutational analysis of the rodent constitutively active ErbB-2, termed Neu (Dankort *et al*., 1997). Absence of all major autophosphorylation sites of Neu dramatically decreases transforming activity upon overexpression in fibroblasts. The C-terminal tyrosine residues Y2 to Y5 can independently mediate transformation, since they fully restore transforming activity when individually added back to the inactive receptor. In contrast, the first tyrosine residue Y1 may not be involved in receptor-mediated transformation, as the resulting add-back mutant lacks transforming potential; moreover, Y1 may represent a negative regulatory site, since it supresses transforming activity when restored in combination with any other single tyrosine residue. Recent studies show that the functionally-redundant add-back mutants containing tyrosines Y2, Y4 and Y5 activate Ras to induce transformation, whereas the add-back mutant containing tyrosine Y3 operates independently of Ras to activate MAPK (Dankort *et al*., 2001).

1.2.4 ErbB Signaling in Embryonic Development

The importance of ErbB signaling in development was revealed by genetic studies in mice. Null mutations of individual ErbB receptor loci are embryonic lethal. Loss of *erbB-1* leads to embryonic or perinatal lethality depending on the genetic background of the host (Miettinen *et al*., 1995; Sibilia *et al*., 1995; Threadgill *et al*., 1995; Sibilia *et al*., 1998); the mice display abnormalities in multiple organs including brain, skin, lung and gastrointestinal tract. Mice

that show spontaneous or targeted mutation of *TGFα*, one of the various ErbB-1 ligands, show only part of the phenotype observed in *erbB-1* null mice, like impaired development of the eyes and hair follicles (Luetteke *et al*., 1993; Mann *et al*., 1993); this partial overlap suggests that each ErbB-1 ligand may play a distinct developmental role. The information gained by targeted mutation of *erbB-2*, *erbB-3* and *erbB-4* receptors and their ligand *NRG-1* clearly demonstrates that distinct receptor heterodimers are essential for different developmental events. *ErbB-2* -/- mice die at midgestation (E10.5) due to malformation of myocardial trabeculae in the heart ventricle (Lee *et al*., 1995), a phenotype that is shared by the *NRG-1* (Meyer *et al*., 1995) and the *erbB-4* null mice (Gassmann *et al*., 1995). These results are consistent with the view that NRG-1, which is expressed in endothelial cells of the endocardium (Meyer and Birchmeier, 1994), is required for activation of myocardial ErbB-2/ErbB-4 heterodimers to promote trabecular formation in the developing heart. In contrast to ErbB-2 and ErbB-4, ErbB-3 is not expressed in myocardium but in mesenchyme of the pre-valvular endocardial cushions. Accordingly, *erbB-3* null mice die at day E13.5 displaying normal heart trabeculation but defective valve formation (Erickson *et al*., 1997; Riethmacher *et al*., 1997).

In addition to cardiac abnormalities, *erbB-3* -/- mice show a generalized neural crest defect that affects both central and peripheral nervous structures. *ErbB-3* mutant mice fail to form cranial sensory ganglia due to impaired migration of neurons from the hindbrain neural crest (Erickson *et al*., 1997; Riethmacher *et al*., 1997). This phenotype is also observed in mice lacking *erbB-2* or *NRG-1* (Lee *et al*., 95; Meyer *et al*., 1995); in contrast, *erbB-4* deficient mice do not exhibit deficient cellularity of cranial ganglia but rather the innervation of these ganglia is disrupted, thus suggesting a unique role for ErbB-4 (Gassmann *et al*., 1995). In the peripheral nervous system, *erbB-3* -/- mice completely lack Schwann cell precursors and Schwann cells that normally accompany nerves (Erickson *et al*., 1997; Riethmacher *et al*., 1997). In addition, degenerative motor and sensory neurons are found in the dorsal root ganglia (Erickson *et al*., 1997; Riethmacher *et al*., 1997), and migration of sympathogenic

neural crest cells is also impaired in *erbB-3* mutants (Britsch *et al*., 1998); similar defects have been observed in *NRG-1* and *erbB-2* deficient mice (Meyer *et al*., 1995; Kramer *et al*., 1996; Britsch *et al*., 1998). It is evident from these studies that ErbB-2/ErbB-3 heterodimers transmit NRG-1 signals for neural crest cells to migrate. Recently, the early mortality of *erbB-2* null mice has been rescued by myocardial expression of an *erbB-2* transgene (Morris *et al*., 1999; Woldeyesus *et al*., 1999); *erbB-2* rescued mice show striking similarities with the *erbB-3* null mice, thus confirming the role of ErbB-2/ErbB-3 complexes in the development of the peripheral nervous system. Data on genetic ablation of the other *NRG* genes are largely missing. Mice carrying a targeted mutation of the *NRG-3* gene, however, do not show an overt phenotype, but may deserve a more detailed analysis (T. Müller and C. Birchmeier, unpublished data). So far, the differential embryonic expression of the various neuregulin proteins and their distinct biological properties *in vitro* suggest different physiological functions (Carraway *et al*., 1997; Chang *et al*., 1997; Crovello *et al*., 1998). Together, these observations show the critical function of ErbB receptors and their ligands during embryogenesis. Moreover, the above genetic studies define distinct developmental roles for certain receptor combinations and are therefore strong support for the occurrence of signal diversification *in vivo*.

1.2.5 ErbB Receptors in Mammary Gland Development

There is considerable evidence that ErbB signaling has important roles in both normal and neoplastic growth of the mammary gland. All four ErbB receptors are found in mammary tissue (Schroeder *et al*., 1998). In prepubescent mammary gland, ErbB-1 and ErbB-2 are widely expressed in epithelium, stroma and mesenchymal fat, with ErbB-1 levels being particularly high in stromal cells. In the mature gland, ErbB-3 and ErbB-4 are also detected; just at this stage, ErbB-1 and ErbB-2 are differentially located: ErbB-1 is present in stroma and adipose compartments, while ErbB-2 is prominent in epithelium. During pregnancy, the four ErbB receptors are coordinately expressed in mammary epithelium; ErbB-1 and ErbB-2 are found at high levels in the alveolar epithelium throughout pregnancy, whereas ErbB-3 and

ErbB-4 levels increase preferentially in the ductal epithelium later in pregnancy. During lactation, receptor levels are low. ErbB-1 and ErbB-2 expression markedly increases during involution, while ErbB-3 expression declines and ErbB-4 expression is not detectable.

Despite expression of all ErbB receptors during puberty, only ErbB-1 and ErbB-2 are tyrosine-phosphorylated while ductal growth occurs (Sebastian *et al*., 1998). ErbB-3 and ErbB-4 seem to be present in a non-phosphorylated, inactive state at this stage, suggesting that none of them is relevant for ductal morphogenesis. Endogenous phosphorylation of all four receptors is detected during late pregnancy and lactation, with increasing levels of tyrosine-phosphorylated ErbB-1, ErbB-2 and ErbB-4 later at lactation. Information of the phosphorylation state of the ErbB receptors during involution is missing.

Like the receptors, the six EGFR ligands and NRG-1 are differentially expressed in mammary tissue (Schroeder *et al*., 1998). TGFα, betacellulin and heparin-binding EGF (HB-EGF) transcripts are found in prepubescent mammary gland and through mid-pregnancy, drop markedly during late pregnancy and lactation and again increase at involution. Amphiregulin and epiregulin transcripts appear in mature virgin glands and early in pregnancy, respectively; transcript levels of both factors decline later in pregnancy, remain low throughout lactation and involution, and reappear as the gland again resembles the mature virgin state. EGF transcripts are present at low levels in the virgin gland, and they dramatically increase during late pregnancy and lactation and return to low levels during involution. The NRG-1α isoform is found in mammary mesenchyme and shows a strongly regulated pattern of expression (Yang *et al*., 1995). NRG-1 is present at low levels in the virgin gland. At mid-pregnancy, NRG-1 exhibits a sudden concentration peak and then rapidly decreases to basal levels, which are constant later in pregnancy, throughout lactation and involution.

Several lines of evidence support a role of ErbB signaling in the development of the mammary gland. In early studies using mice, pellets containing EGF-like factors were

surgically implanted into the mammary glands to allow the slow local release of these factors. Implants of EGF, TGFα and NRG-1α stimulate ductal side-branching and lobulo-alveolar morphogenesis in virgin glands (Vonderhaar *et al*., 1987; Jones *et al*., 1996). EGF- or TGFα-induced alveoli lack secretory activity; in contrast, alveoli that derive from NRG-1α treatment are differentiated into secretory structures, which accumulate secreted milk proteins in their luminal compartment. These observations indicate that all factors can promote formation of alveoli but only NRG-1α stimulates their terminal differentiation. In other experiments, EGF-like growth factors were directly injected into the mammary glands to study their ability to induce tyrosine phosphorylation of ErbB receptors. Treatment of prepubescent glands with EGF stimulates tyrosine phosphorylation of stromal ErbB-1 (the EGF receptor) and ErbB-2 (Schroeder *et al*., 1998; Sebastian *et al*., 1998); as above mentioned, endogenous phosphorylation of ErbB-1 and ErbB-2 is observed in mammary tissue during puberty (Sebastian *et al*., 1998). Therefore, it is likely that locally-produced EGF induces at puberty the formation of active ErbB-1/ErbB-2 heterodimers, which may be essential for mammary ductal growth. Exogenous EGF induces phosphorylation of ErbB-1 and ErbB-2 at pregnancy, despite all ErbB receptors being present; in contrast, administration of NRG-1β results in trans-phosphorylation of all ErbB receptors, clearly indicating that neuregulin can induce the formation of combinatorial receptor complexes at pregnancy. Together with the pregnancy-restricted expression of neuregulin in mammary mesenchyme (Yang *et al*., 1995), these findings strongly suggest that NRG-1 may play a major role in the mammary gland during pregnancy to promote alveolar morphogenesis via trans-activation of ErbB heterodimeric receptors.

Convincing evidence of the physiological role of ErbB signals in mammary development has been supplied by genetic studies in mice; moreover, the phenotypes of knockout or transgenic mice support the differential roles of the ErbB receptors and their ligands that could be expected from the abovementioned stimulation studies, and from expression and activation patterns *in vivo*. Mammary glands from mice carrying a targeted mutation of the *amphiregulin*

gene show impaired ductal growth, whereas *TGFα/EGF* double null mice show normal mammary development at this stage (Luetteke *et al*., 1999). *Amphiregulin* null mammary glands are competent for lobuloalveolar differentiation; however, additional loss of *TGFα* and/or *EGF* severely compromises lactogenesis. Coherent with these findings, expression of a *TGFα* transgene in mammary tissue induces precocious alveolar development in virgin females, alveolar hyperplasia during pregnancy and delayed involution (Matsui *et al*., 1990; Sandgren *et al*., 1995). Together, these results suggest distinct functions of the various ErbB-1 ligands in the mammary gland: amphiregulin may be critical for ductal growth during puberty, while TGFα or EGF may be involved in alveolar differentiation during pregnancy. ErbB-1 is activated in virgin tissue; mammary expression of a transgene encoding a dominant-negative ErbB-1 receptor (the EGF receptor) inhibits ductal branching in the glands of virgin mice thus showing a role for ErbB-1 signaling in pubertal mammary development (Xie *et al*., 1997); similarly, female *waved-2* mice, which carry a spontaneous inactivating mutation of the *erbB-1* gene, display impaired glandular development (Fowler *et al*., 1995). Transplantation and tissue recombination experiments further support a role for ErbB-1 in ductal morphogenesis. Mammary gland grafts from neonatal *erbB-1* -/- mice fail to undergo ductal growth (Sebastian *et al*., 1998); however, they develop lobulo-alveolar structures when stimulated by prolactin, indicating that the EGF receptor is essential for ductal branching but not for alveolar morphogenesis (Wiesen *et al*., 1999). Moreover, tissue recombinants revealed that wild-type fat pad supports outgrowth of *erbB-1* -/- epithelium whereas the -/- fat pad does not, thus clearly showing the relevant role of stromal ErbB-1 in mammary ductal growth.

A role for NRG-1 in mammary gland development during pregnancy is substantiated by its restricted expression and pan-activating effect on ErbB receptors at this stage (see above). Indeed, mammary glands from mice that lack *NRG-1α* fail to undergo lobulo-alveolar morphogenesis at pregnancy (Li *et al*., submitted); NRG-1α is the isoform that is normally expressed in the glands. In contrast, *NRG-1β* null mice die during embryogenesis; the NRG-1β isoform accounts for the cardiac and neural crest phenotypes that were described in section

2.4 of this Introduction (C. Birchmeier, unpublished data). These results definitely demonstrate that NRG-1α is the naturally occurring isoform in mammary tissue, where it functions during pregnancy as a critical growth factor that promotes alveolar morphogenesis and secretory differentiation. Further insights into the mechanisms of neuregulin-induced morphogenesis have been gained from genetic studies with ErbB receptors. Expression of dominant-negative forms of *erbB-2* and *erbB-4* in the mammary gland of transgenic mice revealed a physiological role for both receptors in lobulo-alveolar development at late pregnancy and lactation (Jones *et al*., 1999; Jones and Stern, 1999). Moreover, there is genetic and biochemical evidence that Stat5a mediates morphogenic signals of ErbB-4 in the mammary gland during lactation (Liu *et al*., 1997; Jones *et al*., 1999). According to this data, it can be speculated that NRG-1α induces alveolar morphogenesis via signaling pathways that involve trans-activation of ErbB-2 and ErbB-4 and transcriptional regulation by Stat5a.

As already mentioned in Section 1.3 of this Introduction, the physiological processes that prepare the mammary gland for lactogenesis can be mimicked *in vitro* using organ culture and organotypic assays. By these means, our group has extensively contributed to understand the function of neuregulin in the mammary gland. The pioneering work of Yang *et al*. (1995) has shown that NRG promotes lobulo-alveolar differentiation of mammary gland explants in organ culture. The same morphogenic effect of neuregulin has been observed by Niemann *et al*. (1998) in organotypic cell culture. Treatment with neuregulin induces EpH4 mammary epithelial cells to form alveoli-like structures when cultured on a Matrigel reconstituted matrix; moreover, alveolar cells functionally differentiate and secrete milk components into a luminal compartment, thus reproducing *in vitro* the physiological responses of mammary epithelium to neuregulin. In addition, these studies show that activation of exogenous ErbB-2 receptor tyrosine kinase is sufficient for EpH4 cells to undergo alveolar morphogenesis in the Matrigel system. The present work contributes to understand the intracellular mechanisms underlying the morphogenic events of neuregulin-stimulated EpH4 cells in organotypic

culture, for it identifies Vav2 as an essential mediator of ErbB-2 specific signals leading to alveolar morphogenesis.

1.3 Vav Family of Guanine Nucleotide Exchange Factors

Vav proteins constitute a family of structurally related guanine nucleotide exchange factors that are involved in signaling pathways leading to cytoskeletal rearrangements and changes in gene expression (reviewed in Bustelo, 2000). *Vav*, the first member of the family, was identified as an oncogene encoding a constitutively active protein which lacks 67 amino acids at the N-terminus and induces transformation of NIH 3T3 fibroblasts (Katzav *et al*., 1989; Katzav *et al*., 1991). *Vav* expression and function is restricted to the hematopoietic system, and T and B lymphocytes derived from v*av* -/- mice reveal defects in antigen-receptor induced proliferation (Fischer *et al*., 1995; Tarakhovsky *et al*., 1995; Zhang *et al*., 1995; Fischer *et al*., 1998). The recently discovered *vav2* and *vav3* are also expressed in tissues of non-hematopoietic origin, among them epithelia (Schuebel *et al*., 1996; Movilla *et al*., 1999). Transient expression of N-terminally truncated Vav2 and Vav3 proteins in fibroblasts results in morphological changes such as membrane ruffling and formation of lamellipodia, and N-terminally truncated Vav2 is transforming in fibroblasts (Schuebel *et al*., 1996; Schuebel *et al*., 1998; Movilla *et al*., 1999). Vav2 has been implicated in cell-mediated killing by cytotoxic lymphocytes and in cellular responses following activation of B cell receptor and CD19 (Billadeau *et al*., 2000; Doody *et al*., 2000; Doody *et al*., 2001; Tedford *et al*., 2001). Vav and Vav3 can enhance NFκB-dependent transcription after TCR engagement (Moores *et al*., 2000). No biological function has as yet been assigned to Vav2 and Vav3 in epithelial tissues. Vav proteins share many different structural domains, among them a Dbl-homology domain that is responsible for their GDP/GTP exchange activity, and an SH2 domain that may link them to receptor tyrosine kinases (Bustelo, 2000). Indeed, tyrosine phosphorylation is required for the activation of full-size but not of N-terminally truncated forms of Vav proteins (Bustelo, 2000). Recently, crystallographic analysis revealed that the N-terminus of Vav acts

as an autoinhibitory site, and that phosphorylation of one specific tyrosine residue within this region relieves autoinhibition (Aghazadeh *et al*., 2000).

2 MATERIALS AND METHODS

Standard protocols for various techniques in molecular biology, like preparation and analysis of DNA and RNA, enzymatic manipulation, transfection of DNA into mammalian cells, protein analysis and setup of polymease chain reaction (PCR) were performed according to Current Protocols in Molecular Biology (Ausubel *et al*., 1994).

2.1 Amplification of the Hollenberg cDNA Yeast Expression Library

The Hollenberg mouse embryo cDNA library (Behrens *et al*., 1996) was amplified to gain enough plasmid for yeast transformation. E.coli library culture was thawed on ice, and 10^3 and 10^6 dilutions were made in LB-ampicillin selective medium. One µl of the 10^3 dilution, and 50-100 µl of the 10^6 dilution was mixed with LB-ampicillin medium, spread onto LB-ampicillin plates and incubated at 37°C overnight. On the next day, colonies were counted to calculate the library titer (number of colony forming units per ml of bacterial suspension). A volume of this suspension containing twice as much cells as the number of independent clones of the library ($5x10^6$) was diluted in LB-ampicillin, plated at 200,000 colonies per 15-cm dish (nearly confluent) and incubated at 37°C overnight. Colonies were scraped into 1.5-liter selective medium; this suspension was incubated at 37°C with shaking for a further 2 h, bacteria were pelleted and large-scale plasmid isolation was performed using Megaplasmid columns 2500 (Qiagen).

2.2 Yeast Plasmids and Strains

Bait proteins for the yeast two-hybrid system are encoded in the pBTM116 vector (Bartel and Fields, 1995). This vector carries the *TRP1* gene and has a polylinker downstream of LexA coding sequences. The E. coli repressor LexA consists of two domains: a C-terminal domain, which is responsible for dimerization prior to DNA binding, and an N-terminal domain responsible for specific binding to a palindromic operator containing the CTGTNNNN consensus half-site. The Hollenberg library is inserted in the VP16 vector, which carries the

LEU2 gene and contains a nuclear localization signal-VP16 activation domain sequence upstream of the multiple cloning site. Both pBTM116 and VP16 yeast expression vectors bear a bacterial origin of replication and an ampicillin resistance gene, which allow plasmid amplification in bacteria.

To generate TprMet-ErbB2 yeast baits, a cDNA fragment encoding amino acids 1005-1260 of rodent ErbB-2 harbouring a Y1253F mutation was inserted into the Not I/Sal I sites of pSK^+ (Stratagene) This region stretches over Y1028 (Y1), Y1144 (Y2), 1201 (Y3) and 1226/7 (Y4) of ErbB-2. A cDNA sequence coding for dimerization and kinase domains of TprMet (amino acids 1-481; Park 86 cell) was generated by PCR; this fragment, flanked by 5´ Not I/EcoR I and 3´ Not I sites, was inserted into the Not I site at 5´ end of ErbB-2 in pSK^+, thus rendering the hybrid cDNA for the TprMet-ErbB2(Y1-4) bait. The complete cDNA sequence was finally subcloned downstream of LexA into EcoR I/Sal I sites of pBTM116 yeast expression vector. A new TprMet hybrid protein was constructed to include Y1253 (Y5) of ErbB-2 in the bait. An ErbB-2 sequence encoding amino acids 1197-1260 was fused to TprMet as above to generate the TprMet-ErbB2(Y3-5) bait; thus, Y1201 (Y3), 1226/1227 (Y4) and 1253 (Y5) of ErbB-2 were present in this bait. A BTM-TprMet-Δtail control plasmid lacking the Met C-terminal multiple docking site was constructed by inserting a PCR fragment encoding amino acids 1-479 of TprMet into the EcoR I site of pBTM116. To generate kinase-deficient bait proteins, the wild type sequences of TprMet were excised by EcoR I/Not I and replaced by a PCR fragment containing the inactivating mutation K243A (Rodrigues and Park, 1993).

Both TprMet-ErbB2(Y1-4) and TprMet-ErbB2(Y3-5) bait proteins were used to screen the Hollenberg cDNA library from E10.5 mouse embryos. *Saccharomyces cerevisiae* strain L40 was used as host. This yeast strain carries two reporter genes, *HIS3* and *lacZ*, whose expression is driven by minimal GAL1 promoters fused to multimerized LexA binding sites; therefore, yeast expressing LexA activators can be detected as histidine prototrophs or by measurable β-galactosidase activity. A pBTM116-lamin plasmid was used as a further control

to eliminate false positive clones. Reagents and methods for yeast two-hybrid analyses were adapted from MATCHMAKER™ Handbook (PT1265-1, Clontech).

2.3 Yeast Two-Hybrid Screen

Bait plasmids and library were sequentially introduced into host L40 yeast strain to improve the efficiency of transformation. Briefly, yeast was initially transformed with bait plasmid in a small-scale procedure, and then library screens were performed (Fields and Song, 1989). To prepare competent yeast, a single colony of L40 yeast was inoculated into 20 ml of YPD (10 g/l yeast extract, Difco; 20 g/l peptone, Difco; 2% dextrose) medium and incubated at 30°C overnight. On the next day, culture was diluted 10-fold and further incubated until OD_{600} was 0.5. Cells were pelleted, washed with water and resuspended in 1.5 ml of sterile 1X TE/LiAc (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM lithium acetate). Competent L40 yeast was then transformed with bait plasmid; 0.5 µg of plasmid DNA was mixed with 50 µg of salmon sperm carrier DNA, 50 µl of competent yeast and 300 µl PEG/TE/LiAc (40% polyethylene glycol, 1X TE/LiAc), vortexed and incubated at 30°C for 30 min. DMSO was added to 10%, and heat-shock transformation was performed at 42°C for 15 min. Mixture was chilled on ice, cells were pelleted, resuspended in 250 µl water and spread onto selection agar plates lacking tryptophan. Colonies appear after 2 or 3 days. For the library screen, one single colony of the bait transformants was grown to obtain 200 ml of a saturated cell suspension (OD_{600} greater than 1) in medium without tryptophan. This culture was added to 800 ml YPD medium to prepare 20 ml of competent yeast as above. Yeast suspension was incubated at room temperature for 10 min, and 10 mg carrier DNA, 250 µg library DNA and 140 ml PEG/TE/LiAc was addded. Mixture was incubated at 30°C for 30 min, 17.6 ml of DMSO was added, and heat-shock transformation was performed as above. Co-transformed yeast cells were resuspended in 1 liter YPD and incubated at 30°C for 1 h. Cells were pelleted, resuspended in selection medium lacking tryptophan and leucine and further incubated at 30°C for 8 h. Finally, cells were resuspended in 10 ml water and 200 µl of a dilution series was plated onto 50 selection agar plates without tryptophan, leucine and histidine in the

presence of 20 mM 3-aminotriazole and incubated at 30°C. Double transformants that express interacting proteins rendered colonies within 8-10 days, which were re-plated onto fresh selection agar plates for further analysis.

2.4 Verification of Interacting Clones

Interaction between bait and preys was confirmed by β-galactosidase activity filter assay and by re-transfection of bait and prey plasmids back into yeast. For filter assay, colonies from selection plates (without tryptophan and leucine) were replica-transferred onto a Whatman Nr.1 filter. The replica filter was submerged in liquid nitrogen to permeabilize cells, and then allowed to thaw. The filter was placed (colonies facing up) onto another filter presoaked in Z buffer/X-gal solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0, 0.27% β-mercaptoethanol and 0.334 g/l X-gal) and incubated at 30°C. Colonies expressing β-galactosidase appeared blue within 1-12 h.

For yeast re-transformation, library plasmids encoding interacting proteins were isolated from individual positive colonies. To remove the bait plasmid from double transformant yeast, colonies were inoculated into medium lacking leucine and cultured at 30°C for 1 day. Growth in the absence of tryptophan selection allows survival of yeast segregants without bait plasmid, which is randomly lost. To isolate the library plasmid from yeast segregants, cells from 1 ml of the above culture were pelleted, lysed in 200 µl of lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 1 mM EDTA, 10 mM Tris pH 8.0) and disrupted in the presence of 200 µl phenol/chloroform/isoamyl alcohol (25:24:1) and 0.3 g of acid-washed glass beads by vortexing for 2 min; suspension was clarified by centrifugation and plasmid was recovered from the supernatant by standard ethanol precipitation. Library plasmid was amplified in E. coli HB101 strain, which is leucine auxotroph due to a *leuB* mutation. HB101 cells were electroporated with the isolated library plasmid and plated onto selection agar plates without leucine; therefore, transformants can be selected by complementation with the yeast LEU2 gene from the VP16 library plasmid. Bait and prey plasmids were re-transformed into yeast

according to the small-scale transformation protocol previously described. True positive interacting clones grew again on selective agar plates without tryptophan, leucine and histidine and were positive in β-galactosidase assays.

2.5 Mutagenesis of the Yeast TprMet-ErbB2 Baits

Mutational analysis of the bait proteins was performed to characterize the ErbB-2 binding sites for each interacting protein found in the screen. Deletion mutants bearing single or tandem tyrosine residues of the ErbB-2 multiple docking region were generated by standard PCR. The PCR fragments were flanked by 5´ Not I and 3´ Sal I sites, and were used to replace the ErbB-2 wild type sequence of the hybrid TprMet-ErbB2 baits. The ErbB-2 deletion mutants fused to TprMet were: Y1 (amino acids 1014-1081) includes Y1028; Y1-2 (amino acids 1014-1160) includes Y1028 and Y1144; Y2 (amino acids 1138-1160) includes Y1144; Y2-3 (amino acids 1138-1221) includes Y1144 and Y1201; Y2F-3 is a variant of Y2-3 in which Y2 was mutated to F using a commercial kit (Clontech); Y3 (amino acids 1194-1221) includes Y1201; Y3-4 (amino acids 1194-1244) includes Y1201 and Y1226/1227; Y4 (amino acids 1220-1244) includes Y1226/1227; Y4-5 (amino acids 1220-1260) includes Y1226/1227 and Y1253; Y5 (amino acids 1248-1260) includes Y1253.

Expression of the various TprMet-ErbB2 proteins in the yeast was checked by Western blot analysis using anti-LexA antibodies (Clontech). L40 yeast transformants were grown in selective medium without tryptophan at 30°C. During exponential growth phase, OD_{600} was measured for 1 ml suspension; cells were pelleted from an aliquot corresponding to OD_{600}= 0.5 and resuspended in 100 µl 2X denaturing buffer for SDS-PAGE (100 mM Tris base, pH 6.8, 20% glycerol, 4% SDS, 2% β-mercaptoethanol, 0.2% bromphenol blue). Twenty µl of this suspension was loaded per slot onto an 8% polyacrylamide gel, proteins were resolved, blotted in a PVDF membrane and Western Blotting was performed according to standard protocols.

2.6 Mammalian Expression Plasmids

Full-length cDNA of Vav2 (Δ29 isoform; Schuebel *et al*., 1996) was isolated from a λgt11 E17.5 mouse embryo cDNA library, using a cDNA fragment of Vav2 from a clone found in the yeast two-hybrid screen. To generate Flag-tagged Vav2 proteins, the corresponding cDNA sequences were inserted in frame in the Not I site downstream of a Flag epitope tag in pcDNA 3.1(+) (Invitrogen). The cDNAs of ΔN-Vav2 (encoding amino acids 189-839 of full-sizeVav2) and ΔN-Vav2-ΔC (encoding amino acids 189-595) were amplified by PCR using full-size Vav2 as template. To generate ΔN-Vav2-dbl^{mut} and ΔN-Vav2-ΔCΔPH, the sequences encoding amino acids 336-342 and 383-507, respectively, were further deleted by overlapping PCR. To generate the cDNA of TrkErbB2-Δtail, an Nco I/Not I fragment encoding the ErbB-2 C-terminal tail (amino acids 1006-1260) was removed from a pSK^{+}/TrkErbB2 plasmid (Sachs *et al*., 1996) and replaced by a triple HA epitope tag flanked by Nco I and compatible EagI sites; then, the sequence coding for HA-tagged TrkErbB2-Δtail was excised EcoR I/Eag I and subcloned into pcDNA 3.1(+) using EcoR I/Not I sites. The cDNA of HA-tagged TrkErbB2-Vav2 was constructed by inserting the full-size sequence of Vav2 (encoding residues 2-839) into the Eag I site downstream from triple HA. To generate the kinase-deficient mutant TrkErbB2-Kin^{-}, a 951 bp-cDNA fragment containing the inactivating K758A mutation was excised by Nco I/Mro I from the pLSV-K758A plasmid (Ben-Levy *et al*., 1994) and inserted into pUC118-TrkErbB2 (Sachs *et al*., 1996) in place of the wild-type sequence; the cDNA of TrkErbB2-Kin^{-} was excised by Acc65 I/Spe I from pUC118 and inserted into Acc65 I/Xba I sites of pcDNA 3.1(+). In TrkErbB2^{mut}, Y1028 and Y1201 of ErbB-2 C-terminal tail were mutated to phenylalanine using a commercial kit (Clontech).

2.7 Site-Directed Mutagenesis

The Transformer^{TM} Site-Directed Mutagenesis kit (Clontech) was used to generate Trk-ErbB2 variants in which single or pairs of tyrosine residues of the ErbB-2 C-terminal tail were

mutated to phenylalanine. Two oligonucleotide primers were used to introduce a base change in double-stranded DNA. One primer (referred to as the mutagenic primer) introduced the desired mutation. The second primer (referred to as the selection primer) disrupted a unique restriction site to facilitate the ultimate selection of the mutated plasmid. A unique Sca I of pUC118-Trk-ErbB2 was mutated to a Hpa I site with the selection primer 5' CAGAATGACTTGGTTAA**C**TACTCACCAGTC 3' (highlighted is the mutated base). Mutagenic primers were Y2F (5' CCCCAGCCCGAGT**T**TGTGAACCAA 3'), Y3F (5' GGAG AACCCTGAAT**T**CTTAGTACCGAGAGAAGGC 3') and Y4F (5' GCCCAGCCTTTGACA ACCTCT**T**TTT**T**CTGGGACCAG 3'). The two primers were simultaneously annealed to one DNA strand of the target plasmid. After standard elongation, ligation and a primary selection by digestion with Sca I and Hpa I, the mixture of wild-type and mutated plasmid was transformed into BMH 71-18 *mutS* E. coli strain, which contains a DNA mismatch repair deficiency mutation. Plasmid DNA was prepared from the mixed bacterial population and isolated DNA was again digested by Sca I and Hpa I. Mutated DNA was resistant to digestion with Sca I but sensitive to Hpa I, whereas parental DNA was linearized by Sca I. A new transformation of E. coli with the Sca I restriction mixture rendered high recovery of the mutated plasmid. Protocols were carried out according to Clontech's user manual. Mutated Trk-ErbB-2 DNAs were digested by Acc65 I/Spe I from pUC118 and inserted into the Kpn I/Xba I sites of pcDNA3.1(+). A TrkErbB2 variant without both Vav2 binding sites (termed TrkErbB2^{mut}) was generated by Y1F mutation of pcDNA-TrkErbB2-Y3F, using a unique Nde I site for the selection primer (5' TACATCAAGTGTATC**C**T**C**TGCCAAGTACGCCCC CTA 3') and a Y1F mutagenic primer (5' CTGGTAGACGCTGAAGAGT**T**TCTGGTGCCCC AGC 3').

2.8 Mammalian Cell Lines

Human embryo kidney 293T cells were used for transient transfections. For morphogenesis assays on matrigel, EpH4 mouse mammary epithelial cells were used. EpH4 cells (Lopez-Barahona *et al*., 1995) are a clonal epithelial derivative of IM-2 mouse mammary gland

epithelial cells, which were originally isolated from mammary tissue of a mid-pregnant mouse (Reichmann *et al*., 1989). In our lab, the variant EpH4 K6 was subcloned (Niemann *et al*., 1998), which exhibits a pronounced morphogenic potential on matrigel. The K6 subclone was used in this work.

2.9 Immunoprecipitation and Western Blotting

$3x10^6$ 293T cells were plated per 15-cm dish and co-transfected with 4 µg Flag-tagged Vav2 and 200 ng TrkErbB2, TrkErbB2-Kin^- or $TrkErbB2^{mut}$ expression vectors by calcium/phosphate precipitation. Twelve hours after transfection, cells were starved in serum-free DMEM medium for 36 h. Prior to lysis, cells were stimulated with 50 ng/ml NGF (Promega) in the presence of 1 µM phenylarsine oxide at 37°C for 10 min, and then washed with ice-cold PBS (Sachs *et al*., 1996). Cells were harvested in 800 µl lysis buffer supplemented with phosphatase and protease inhibitors (50 mM HEPES, 50 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, pH 7.5; inhibitors were added at the following end concentrations: 10 mM NaF, 1 mM Na orthovanadate, 10 mM Na pyrophosphate, 1 mM PMSF, 0.5 µg/ml aprotinin) and incubated on ice for 15 min. Lysates were clarified by centrifugation at 14,000 rpm for 10 min at 4°C. For immunoprecipitations, 300 µl cell lysate was mixed with an equal volume of HNTG buffer (20 mM HEPES, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, pH 7.5; inhibitors were used as for lysis buffer) and incubated with primary antibodies at 4°C overnight. Immunoprecipitates were resuspended in 70 µl denaturing loading buffer and heated at 95°C for 3 min. Proteins were resolved by SDS-PAGE, and transferred to PVDF membranes for 1 h at 100 mAmp per blot using a Trans-Blot SD semi-dry electroblotter (BioRad). Membranes were blocked with 5% low-fat milk powder in PBS at 25°C for 3 h, or with 0.5% Tween 20 and 10% FCS in PBS for anti-phosphotyrosine primary antibodies. Membranes were incubated with primary antibodies in blocking solution at 4°C overnight. Following 3 washes with PBS containing either 5% milk or 0.05% Tween (for anti-phosphotyrosine Western blots), membranes were incubated with peroxidase-conjugated secondary antibodies at 25°C for 45 min. After thorough washing,

proteins were detected by enhanced chemiluminescence (Amersham). For protein interaction studies in mammary epithelial cells, EpH4 cells were serum-starved for 1 day, stimulated with 2 ng/ml neuregulin-β1 (R&D Systems) at 37°C for 10 min and lysed as for 293T cells. Immunoprecipitation followed by SDS-PAGE and Western blotting was performed as above. For detection of Vav2/ErbB-2 complexes in mammary tissue, mammary glands from 17.5-day pregnant mice were solubilized in lysis buffer and incubated with anti-Vav2 antibodies at 4°C for 7 h. Co-immunoprecipitated ErbB-2 was detected as described above.

Antibodies used for immunoprecipitation and Western blotting were: anti-Flag (affinity beads, Sigma), anti-Flag Octapeptide (Zymed), anti-Vav2 (DPH, Calbiochem), anti-ErbB-2 (Ab-8, NeoMarkers) and anti-PY antibodies (PY20, Transduction Laboratories). Peroxidase-conjugated secondary antibodies were: goat anti-rabbit (Calbiochem), goat anti-mouse (Jackson ImmunoResearch) and rabbit anti-sheep, Upstate Biotechnology). Protein G Sepharose 4 FAST FLOW (Amersham Pharmacia Biotech) was used for immuno-precipitation.

2.10 Matrigel Assays

EpH4 cells were transfected with Flag-tagged ΔN-Vav2, Flag-tagged ΔN-Vav2 mutants, HA-tagged TrkErbB2-Vav2 or TrkErbB2-Δtail expression vectors by standard calcium/phosphate method and selected for neomycin resistance in the presence of 800 µg/ml G418. Individual clones stably expressing the exogenous proteins were expanded and tested for morphogenesis on Matrigel (basement membrane from Engelbreth-Holm-Swarm murine tumor, Sigma; Niemann et al., 1998). 24-well plates were cooled on ice, and each well was coated with 70 µl Matrigel solution. Plates were incubated at 37°C for 30-60 min until Matrigel solidified. EpH4 cells were plated dropwise as a suspension containing approximately 300-500 cell clusters/ml in DMEM supplemented with 10% FCS and the following hormones: bovine prolactin (at 3 µg/ml; Sigma), hydrocortisone (at 1 µg/ml; Merck) and insulin (at 5 µg/ml; Sigma). After one day of culture at 37°C, medium was replaced by serum-free DMEM

containing hormones. Neuregulin-β1 (at 1 ng/ml) or NGF (at 100 ng/ml) were added to medium, which was changed daily. Assays were terminated after 5 days of culture. For branching morphogenesis assays, 24-well plates were coated with a 5 mm-thick layer of collagen; then, matrigel was added on top and cells were plated as above. Assays were performed in the presence of DMEM medium supplemented with 10% FCS, hormones and HGF/SF (at 10 units/ml).

2.11 Light and Electron Microscopy

For light and electron microscopy studies, matrigel specimens were fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer and 0.18 M sucrose at 4°C for 2 days, postfixed with 1% OsO4 in 0.1 M phosphate buffer for 2 h, dehydrated in a graded ethanol series and embedded in Poly/BedR 812 (Polysciences, Inc). For light microscopy, semithin section (1 μm) were stained with toluidine blue and analyzed in a Zeiss Axioplan II imaging microscope. Ultrathin sections (70 nm) were contrasted with 2% uranyl acetate and lead citrate (Merck) and analyzed in a Philips EM 400T electron microscope.

2.12 In Situ Hybridization Analysis

For in situ hybridization (Hülsken *et al*., 2001), sections of third thoracic glands of 16-day pregnant mice were used. Digoxygenin-labelled RNA probes were synthesized with T3 or T7 RNA polymerase using DIG RNA kit (Boehringer Mannheim). Probes were: neuregulin, nucleotides 391-1458 (Yang *et al*., 1995); ErbB-2, nucleotides 1746-3780; Vav2, nucleotides 1-2232 plus 504 nucleotides 5´ from start codon. Pyrogenin was used for red counterstaining.

3 RESULTS

3.1 Yeast Two-Hybrid Screen with ErbB-2 Bait Proteins

Previous studies implicate the receptor tyrosine kinase ErbB-2 in lobulo-alveolar development of the mammary gland (Niemann *et al*., 1998; Jones and Stern, 1999). In search for new substrates of ErbB-2 that mediate its function in mammary alveolar morphogenesis, yeast two-hybrid screens were performed with ErbB-2 baits (Fields and Song, 1989). The classical baits for receptor tyrosine kinases are hybrid proteins, which consist of an E.coli LexA DNA-binding and dimerization domain followed by the intracellular region of the receptor tyrosine kinase (O'Neill *et al*., 1994; Weidner *et al*., 1996). However, baits spanning the full-length cytoplasmic region of the ErbB-2 receptor were highly unstable in the yeast (my own observation; data not shown). Therefore, modified yeast baits were conceived on the basis of previous studies with chimeric receptors; when C-terminal sequences of c-Met are fused to the kinase of TrkA (the nerve growth factor receptor), the hybrid receptor elicits Met-specific morphogenic responses upon activation with NGF (Sachs *et al*., 1996). Thus, the C-terminal docking region of a receptor is sufficient to determine its signaling specificity; this region contains tyrosine residues that, when phosphorylated, become docking sites for receptor-specific downstream effectors. In view of these results, chimeric ErbB-2 bait proteins were generated: the tyrosine kinase TprMet was fused to LexA, and the C-terminal tail of Met was replaced by the C-terminal tail of ErbB-2 (Figure 1). TprMet was chosen as heterologous bait kinase since it is strongly active when expressed in the yeast (my own observation; data not shown). TprMet is the human oncogenic counterpart of c-Met (Park *et al*., 1986). Tpr (translocated promoter region) codes for two putative dimerization domains, and is a movable DNA element that has spontaneously translocated into a genomic region upstream of the Met kinase; the resulting TprMet protein dimerizes in a ligand-independent fashion through its newly-acquired Tpr sequences, and is therefore a cytoplasmic, constitutively active tyrosine kinase protein with the biological activity of c-Met. It is therefore likely that the presence of a C-terminal tail of ErbB-2 in the modified TprMet-ErbB2 baits bestows ErbB-2 signaling

properties on the chimeric proteins (see below). Two ErbB-2 baits, TprMet-ErbB2(Y1-4) and TprMet-ErbB2(Y3-5), were generated to cover the five tyrosine residues that are putative autophosphorylation sites of ErbB-2 (Fig. 1).

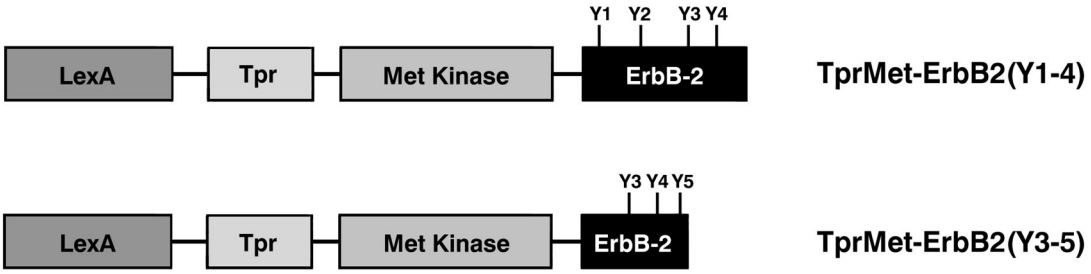


Figure 1. Structure of the chimeric ErbB-2 baits used in yeast two-hybrid screens.

The baits consist of a LexA DNA-binding and dimerization region, followed by the kinase domain of TprMet; the C-terminal tail of TprMet is substituted for sequences of the ErbB-2 multiple docking region. The TprMet-ErbB2(Y1-4) bait stretches over tyrosines Y1028 (for simplicity Y1 in the scheme), Y1144 (Y2), Y1201 (Y3) and Y1226/7 (Y4) of ErbB-2, with Y1253 (Y5) being mutated to F; the TprMet-ErbB2(Y3-5) bait includes tyrosines Y1201 (Y3), Y1226/7 (Y4) and Y1253 (Y5).

TprMet-ErbB2(Y1-4) contains the four N-terminal tyrosines of c-ErbB-2 (Y1 to Y4); TprMet-ErbB2(Y3-5) includes the three most C-terminal tyrosines (Y3 to Y5). Both baits were efficiently expressed in the yeast and were constitutively phosphorylated on tyrosine residues (data not shown). In preliminary tests, these baits exhibited ErbB-2 selectivity for binding downstream signaling molecules: TprMet-ErbB2(Y1-4) interacted with Grb-2 and Shc, while TprMet-ErbB2(Y3-5) interacted with Shc (data not shown); it has already been described that Grb-2 and Shc directly interact with phosphorylated Y2 and Y4 residues of ErbB-2, respectively (Dankort *et al*., 1997).

Both chimeric ErbB-2 baits were used to screen the Hollenberg library (see Materials and Methods). The library consists of mouse E10.5 embryonic cDNAs that are fused to the VP16 activation domain, and has extensively been used in our group to find novel interaction

partners of receptor tyrosine kinases (Weidner *et al*., 1996; Grimm *et al*., 2001). In several independent screens, clones encoding the SH2 domains of known and novel interaction partners of ErbB-2 were isolated. Table 1 summarizes the results of screens for each ErbB-2 bait protein. Fig. 2 indicates the amino acid region encoded in the interacting clones. Src and PLCγ1 have previously been described as substrates of c-ErbB-2 (Fazioli *et al*., 1991; Muthuswamy and Muller, 1995b); their isolation in the yeast screen corroborates ErbB-2 binding specificity of the chimeric baits. Vav2, Nck and Grb10 were here identified as novel interaction partners of the ErbB-2 receptor.

Table 1. Interaction partners of ErbB-2 isolated in independent yeast two-hybrid screens using different baits.

Clone	TprMet-ErbB2(Y1-4)	TprMet-ErbB2(Y3-5)
Vav2	2	1
PLCγ1	6	7
Nck	-	1
Src	-	2
Grb10	-	34

Numbers indicate frequency of each isolated interaction partner, including overlapping clones.

Interaction of the prey proteins with the baits was confirmed by co-transfection into yeast cells, followed by analysis of yeast growth and β-galactosidase activity assays (data not shown). Sequence comparison analyses revealed that the interacting regions spanned the SH2 domain of each ErbB-2 partner (Fig. 2), strongly suggesting that interaction involved phosphotyrosine residues of the baits. This hypothesis was confirmed by using kinase-defective baits; a point mutation of lysine 243 to alanine (K243A) was introduced in the ATP-

binding site of the TprMet kinase, which completely disrupts the catalytic activity (Rodrigues and Park, 1993). The inactivating mutation of the bait kinases abolished interaction, confirming that the isolated SH2 domains bound to phosphotyrosine residues (Fig. 3, middle panel; compare to interaction with wild-type bait, upper panel). Furthermore, deletion of the ErbB-2 C-terminal tail also impaired binding (Fig. 3, lower panel), clearly indicating that the clones interacted with phosphotyrosines that were located on the ErbB-2 tail and not within the TprMet sequences of the baits.

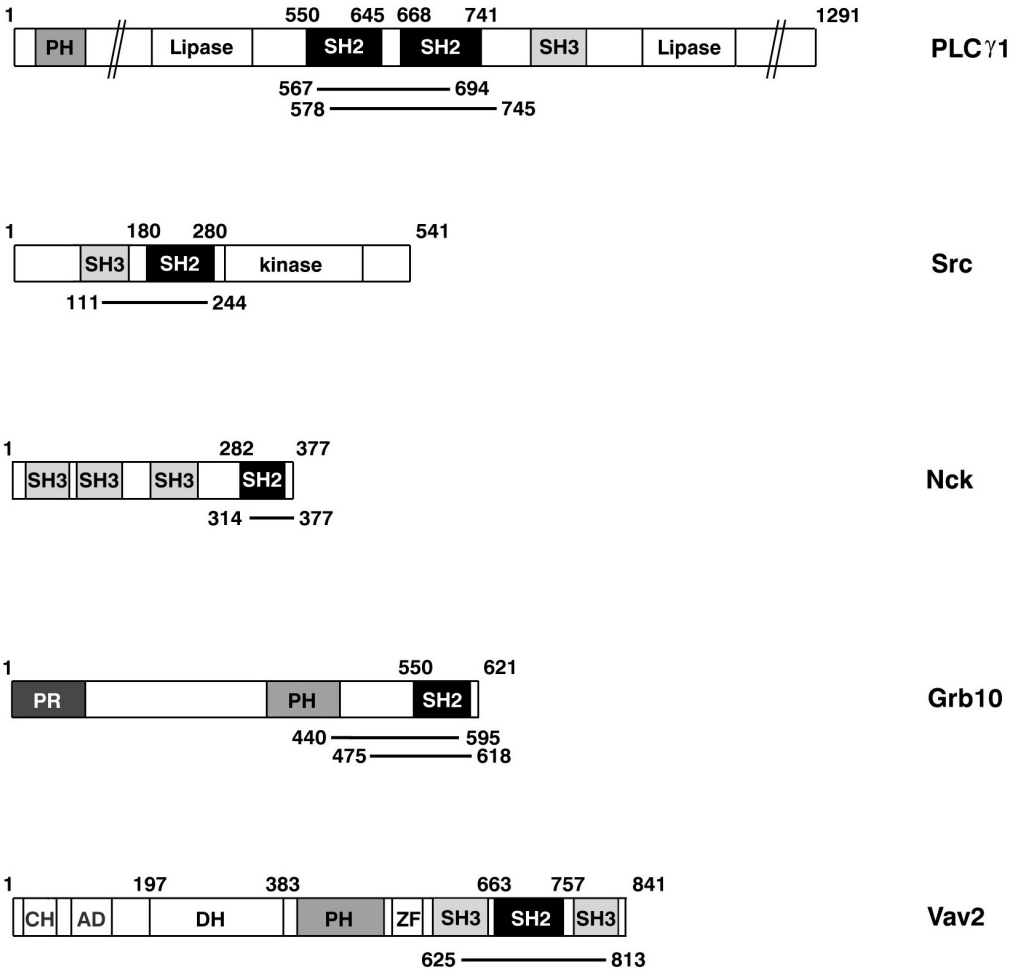


Figure 2. Interaction partners of ErbB-2 in the yeast system are SH2 domains.

The overall structure of the interacting proteins is schematically shown, relevant domains are highlighted. The amino acid stretch encoded in the isolated clones is indicated by the bars below the protein structures, overlapping clones are shown. AD: acidic domain; CH: calponin-homology domain; DH: Dbl-homology domain; PH: pleckstrin-homology domain; PR: proline-rich region; SH2: Src-homology-2 domain; SH3: Src-homology-3 domain; ZF: zinc finger.

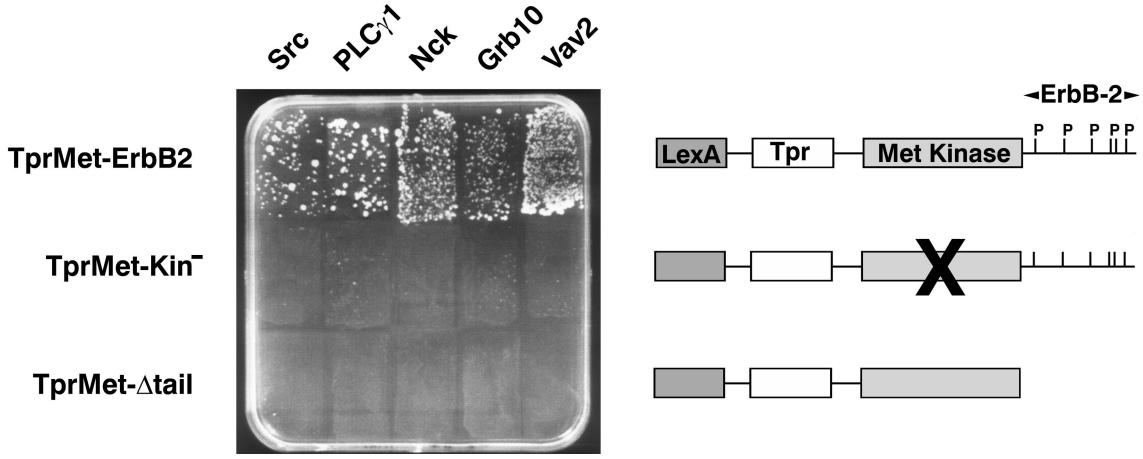


Figure 3. The SH2 domains of substrates isolated in yeast two-hybrid screens interact with phosphotyrosine residues of the ErbB-2 C-terminal tail.

Preys were co-transfected into yeast together with wild-type TprMet-ErbB2(Y3-5) (TprMet-ErbB2 for simplicity), kinase-defective (TprMet-ErbB2-Kin$^-$) or C-terminally deleted (TprMet-Δtail) baits (schematic structures are on the right), and interaction was tested in a yeast colony-growth assay on selection plates. The various preys interacted with the wild-type TprMet-ErbB2 bait, but not with the kinase-defective or the deletion mutant without ErbB-2 C-terminal tail.

3.2 Distinct Phosphotyrosine Residues of ErbB-2 Bind to Various Interaction Partners

The phosphotyrosine residues of ErbB-2 that are direct binding sites for its interaction partners were mapped by mutational analysis of the bait proteins. Sequences of the C-terminal tail of ErbB-2 containing single tyrosines or pairs of consecutive tyrosine residues were fused to TprMet to generate bait deletion mutants (see Materials and Methods); these new baits were tested with the library clones for interaction in the yeast, which was evaluated by colony-growth on selection plates and β-galactosidase activity. Grb-2 and Shc were included in these

experiments as controls. The results of yeast colony-growth and β-galactosidase assays are summarized in Table 2. Nck interacted with baits containing Y3 (Y1201) and Grb10 with baits containing Y4 (Y1226/7); Src required simultaneously both phosphorylated Y3 and Y4 (Y1201 and Y1226/7); PLCγ1 interacted with every tyrosine-phosphorylated bait mutant (data not shown). Vav2 directly bound to phosphorylated Y1 (Y1028) and Y3 (Y1201) of ErbB-2 (see also Fig. 4). Interestingly, both tyrosines that bound Vav2 are located within a pYLVP motif, which apparently constitutes the consensus binding sequence for the SH2 domain of Vav2. These results again validate the reliability of this modified yeast two-hybrid approach to identify phosphotyrosine binding proteins and to map their direct binding motifs.

Table 2. Characterization of specific binding sites of ErbB-2 for its interaction partners.

Bait*	Vav2	Nck	Src	Grb10	Grb2	Shc
Y1	+	-	-	-	-	-
Y1-2	+	-	-	-	+	-
Y2	-	-	-	-	+	-
Y2-3	+	+	-	-	+	-
Y3	+	+	-	-	-	-
Y3-4	+	+	+	+	-	+
Y3-5§	+	+	-	+	-	+
Y4-5	-	-	-	+	-	+
Y4	-	-	-	+	-	+
Y5	-	-	-	-	-	+

Plus (+) indicates interaction, minus (-) indicates no interaction.

*Baits are TprMet-ErbB2 deletion mutants; numbers indicate single or pairs of tyrosine residues in the C-terminal tail of ErbB-2.

§TprMet-ErbB2(Y3-5) bait was included as internal control.

Figure 4. Vav2 directly interacts with phosphotyrosines Y1 and Y3 of c-ErbB-2.

The full-size cDNA of Vav2 was isolated from a phage library (see Materials and Methods) and inserted in VP16 yeast vector. To characterize the Vav2 binding sites on c-ErbB-2, the full-length Vav2 prey protein was tested with various ErbB-2 bait mutants for interaction in the yeast. The results from colony-growth (left panel) and β-galactosidase assays (right panel) are shown. ErbB-2 bait mutants are those from Table 2; additionally, Y2 and Y3 are mutated to phenylalanine in the TprMet-ErbB2(Y2F-3) and TprMet-ErbB2(Y2-3F) baits, respectively. TprMet-Δtail lacks an ErbB-2 C-terminal tail. Kin⁻ denotes kinase-deficient bait mutants. Hybrid TprMet bait proteins for the other ErbB receptors (EGF receptor, ErbB-3 and ErbB-4) were simultaneously tested (see below).

3.3 Vav2 Interacts with Receptor Tyrosine Kinases of the ErbB Subfamily

Next, the receptor specificity of each ErbB-2 interaction partner for other RTKs was tested in the yeast system. Src, Nck, PLCγ1 and Grb10 interacted with various receptor tyrosine kinases from unrelated subfamilies (Table 3). In contrast, Vav2 appears to preferentially bind receptors of the ErbB subfamily; Vav2 bound to baits encoding TprMet fusion proteins with

the C-terminal tails of EGF receptor, ErbB-3 and ErbB-4 (Table 3 and Fig. 4); it additionally interacted with a constitutively active PDGFβ receptor (Table 3).

Table 3. Interaction of the various ErbB-2 partners with different receptor tyrosine kinases.

Receptor*	**PLCγ1**	**Src**	**Nck**	**Grb10**	**Vav2**
TprMet-ErbB2	+	+	+	+	+
TprMet-EGFR	+	+	+	+	+
TprMet-ErbB3	-	+	+	+	+
TprMet-ErbB4	-	-	-	+	+
TprMet-Δtail	-	-	-	-	-
Met	+	+	+	+	-
Insulin Receptor	+	-	+	+	-
Ret	+	+	-	+	-
Kit	-	-	-	-	-
KGF Receptor	-	-	-	-	-
Ros	+	+	-	+	-
Sea	+	-	-	-	-
PDGFβ Receptor	+	+	+	+	+

Plus (+) indicates interaction.

*Baits are LexA-receptor fusion proteins (Weidner *et al*., 1996). Baits containing TprMet sequences are indicated. EGFR: epidermal growth factor receptor; KGF: keratinocyte growth factor; PDGF: platelet-derived growth factor.

Together, these results point to Vav2 as a putative candidate to mediate ErbB signals in mammary gland development. First, Vav2 specifically interacts with all the ErbB receptors; second, all ErbB receptors are essential at different stages of mammary development (see section 2.5 of the Introduction). Lastly, activated Vav2 induces cytoskeletal rearrangements, which are a critical step for morphogenic events. Therefore, the potential of Vav2 to mediate ErbB-2 signals in alveolar morphogenesis was next tested in organotypic cell culture experiments.

3.4 Vav2 Induces Alveolar Morphogenesis of EpH4 Mammary Epithelial Cells

Activation of endogenous or ectopic ErbB-2 receptor instructs EpH4 mammary epithelial cells to form functional alveoli-like structures when these cells are cultured on EHS matrix (termed Matrigel; Niemann *et al*., 1998). To examine whether Vav2 mediates the morphogenic effect of ErbB-2 in organotypic culture, EpH4 mammary epithelial cells were stably transfected with a cDNA encoding N-terminally truncated Vav2, the oncogenic, constitutively active form of Vav2 (termed ΔN-Vav2; structures are shown in Fig. 5; see Schuebel *et al*., 1996); afterwards, transfectants were cultured on Matrigel to test their ability to undergo alveolar morphogenesis. ΔN-Vav2 was preferred to full-size Vav2 for the Matrigel assay in view of its functional properties: ΔN-Vav2 is constitutively active as guanine nucleotide exchange factor and induces cytoskeletal rearrangements when overexpressed in fibroblasts (Schuebel *et al*., 1996); in contrast, full-size Vav2 requires tyrosine phosphorylation to elicit such responses (Schuebel *et al*., 1998). Remarkably, overexpression of ΔN-Vav2 induced the arrangement of the transfected EpH4 cells into large alveolar structures within two days of culture on Matrigel (Fig. 5B, compare with control in A). The alveoli consisted of monolayers of tightly associated cells facing a lumen (Fig. 5D). Ultrastructural studies revealed that the alveolar cells were polarized; microvilli were scattered at the luminal side, and tight junctions were observed at apical cell-cell borders (Fig. 6B, C). Polarization of alveolar cells in the mammary gland during pregnancy and lactation has been described (Nemanic *et al*., 1971). Control

EpH4 cells formed small solid aggregates, and cells were non-polarized and were only loosely connected (Fig. 6A).

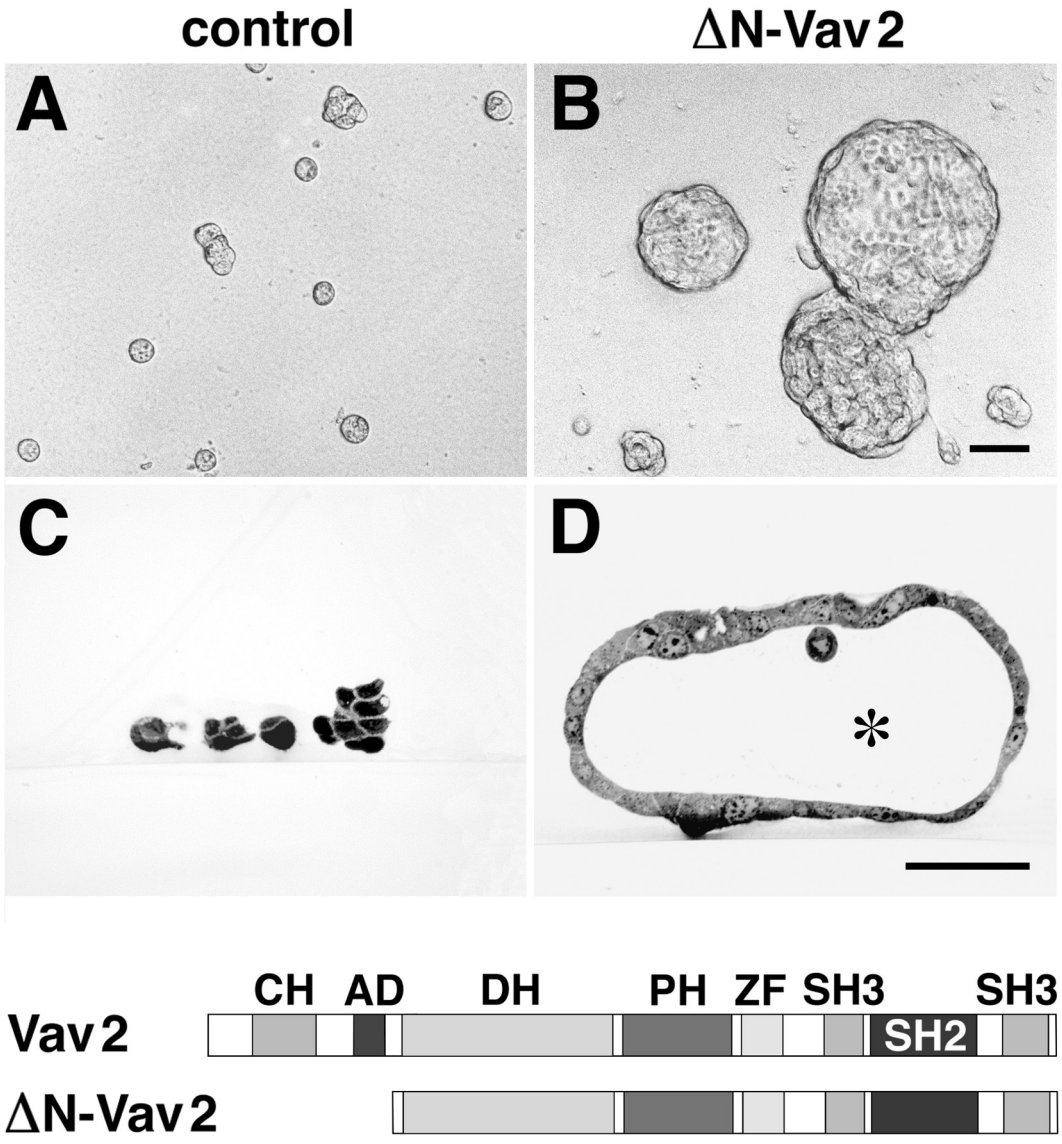


Figure 5. Constitutively active Vav2 induces alveolar morphogenesis of EpH4 mammary epithelial cells.

(**B**) Cells expressing ΔN-Vav2 (see schematic structures below) form large alveoli-like structures on matrigel. (**D**) Alveolar cells are facing a lumen (asterisk), as observed in semithin sections. (**A**, **C**) Mock-transfected controls form small aggregates. Bar: 50 µm. CH: calponin-homology domain; AD: acidic domain; DH: Dbl-homology domain; PH: pleckstrin-homology domain; ZF: zinc finger.

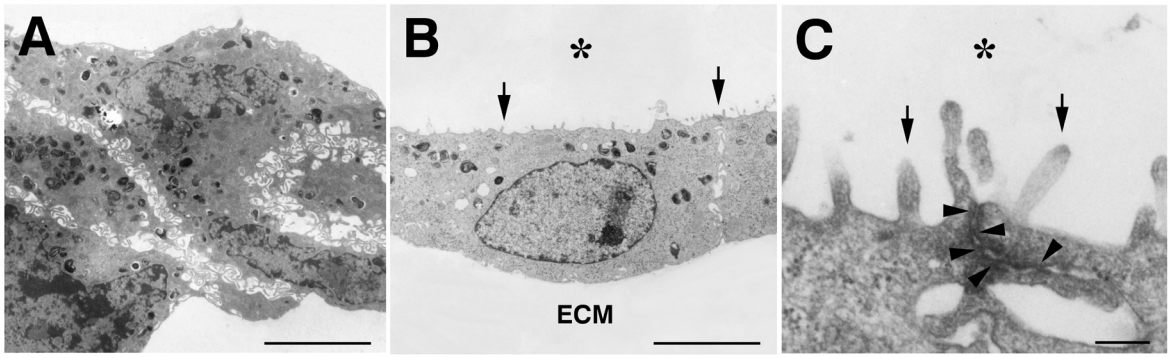
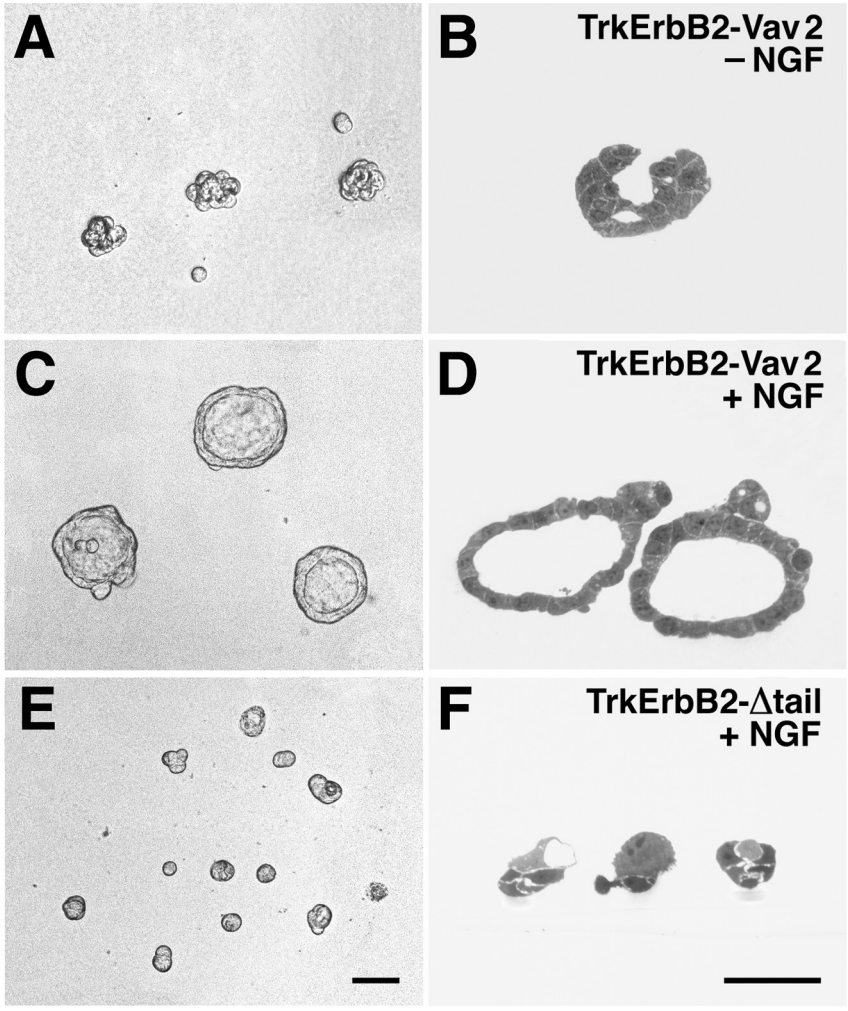


Figure 6. Constitutively active Vav2 induces polarization of alveolar EpH4 mammary epithelial cells.

(**B, C**) Microvilli (arrows) are present at the luminal side (asterisk) of alveolar EpH4 cells expressing ΔN-Vav2; apical tight junctions (arrowheads) are revealed by electron-microscopy of ultrathin sections. (**A**) Mock-transfected controls form small clumps of loosely associated cells. Bars: (A, B) 5 μm; (C) 0.5 μm. ECM: extracellular matrix.

Full-size Vav2 is inactive as GDP/GTP exchanger in a non-phosphorylated state (Schuebel *et al*., 1998). Not surprisingly, EpH4 cells overexpressing full-size Vav2 did not produce alveoli (data not shown), suggesting that full-size Vav2 may also require tyrosine-phosphorylation to become morphogenic. It has previously been shown that C-terminal fusion of a substrate to its receptor results in efficient tyrosine phosphorylation of the substrate (Schaeper *et al*., 2000); moreover, the insulin receptor substrate sequences are fused to the insulin receptor in *D. melanogaster* (Yenush *et al*., 1996). Therefore, the fusion protein TrkErbB2-Vav2 was generated by replacing the C-terminal tail of ErbB-2 with full-length Vav2 in a TrkErbB2 receptor (Sachs *et al*., 1996; structures are shown in Fig. 7). The resulting protein lacks the docking sites of ErbB-2 and hence could only signal through the coupled substrate when activated by nerve growth factor (NGF). Indeed, EpH4 cells expressing TrkErbB2-Vav2 formed large alveoli following stimulation with NGF (Fig. 7C, D). In the absence of NGF or in transfectants lacking Vav2 in the fusion protein, respectively, none or only rudimentary structures were seen (Fig. 7A, B and E, F). Taken together, these findings demonstrate that

Vav2 induces alveolar morphogenesis of EpH4 cells when activated either by N-terminal truncation or upon tyrosine phosphorylation by ErbB-2.



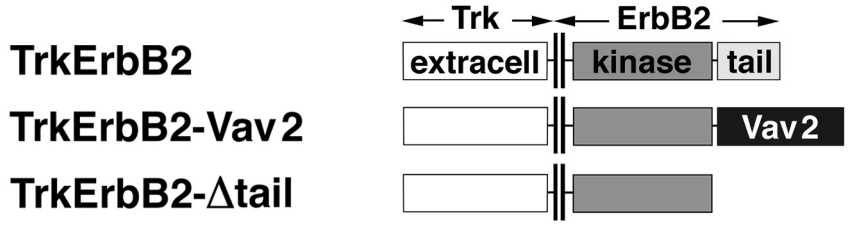


Figure 7. Tyrosine phosphorylation of full-size Vav2 by ErbB-2 activates its morphogenic potential.

Left panel: overviews by light microscopy; right panel: semithin sections. (**A**, **B**) EpH4 transfectants expressing the TrkErbB2-Vav2 hybrid (see structure below) form alveolar structures on matrigel upon NGF stimulation. (**C**,

D) Non-stimulated transfectants and (**E**, **F**) cells expressing a TrkErbB2 protein without the C-terminal tail of ErbB-2 (TrkErbB2-Δtail) lack morphogenic activity. Bars: 50 μm.

3.5 Vav2 and ErbB-2 Can Both Directly and Indirectly Associate in Mammalian Cells

The interaction between Vav2 and ErbB-2 was also studied in cultured mammalian cells. Human epithelial kidney 293T cells were transiently co-transfected with Vav2 and wild-type or mutated TrkErbB2 receptors, and interaction after stimulation with NGF was examined by Western blotting. Exogenous Vav2 co-immunoprecipitated with a TrkErbB2 hybrid receptor in lysates of transfected 293T cells, but not with kinase-deficient receptor TrkErbB2-Kin$^-$ (Fig. 8A, upper panel). These results indicate that association of Vav2 with ErbB-2 requires tyrosine phosphorylation of the receptor, as previously observed in the yeast system. Surprisingly, Vav2 still interacted with TrkErbB2^{mut}, which lacks the Vav2 binding sites of ErbB-2 (Y1028 and Y1201) identified in the yeast two-hybrid system. This suggests that Vav2 may bind directly and indirectly to ErbB-2. However, interaction of Vav2 with TrkErbB2 or TrkErbB2^{mut} depends on activation of the hybrid receptors by NGF (Fig. 8A, upper panel). Increased tyrosine phosphorylation of Vav2 was observed upon binding to ErbB-2 proteins that contain an activated kinase (Fig. 8A, middle panel).

Interaction between Vav2 and ErbB-2 was also tested in EpH4 cells that are cultured under standard conditions. Subconfluent EpH4 cells were treated shortly with neuregulin-1 (see Materials and Methods) to activate the endogenous ErbB-2 receptor (Niemann *et al*., 1998) and then lysed. Immunoprecipitation of endogenous Vav2 from cell lysates followed by Western blotting revealed that Vav2 associated with ErbB-2 in lysates of neuregulin-treated EpH4 cells (Fig. 8B, upper left panel). In addition, increased tyrosine phosphorylation of ErbB-2 and Vav2 was observed in lysates of stimulated EpH4 cells (Fig. 8B, upper right panel). These findings indicate that interaction between Vav2 and ErbB-2 occur in EpH4 cells after activation of ErbB-2 by neuregulin. Moreover, activated ErbB-2 may further

phosphorylate Vav2 on tyrosine residues. The interaction between Vav2 and ErbB-2 in EpH4 cells may therefore be an essential step in neuregulin-induced morphogenesis of these cells.

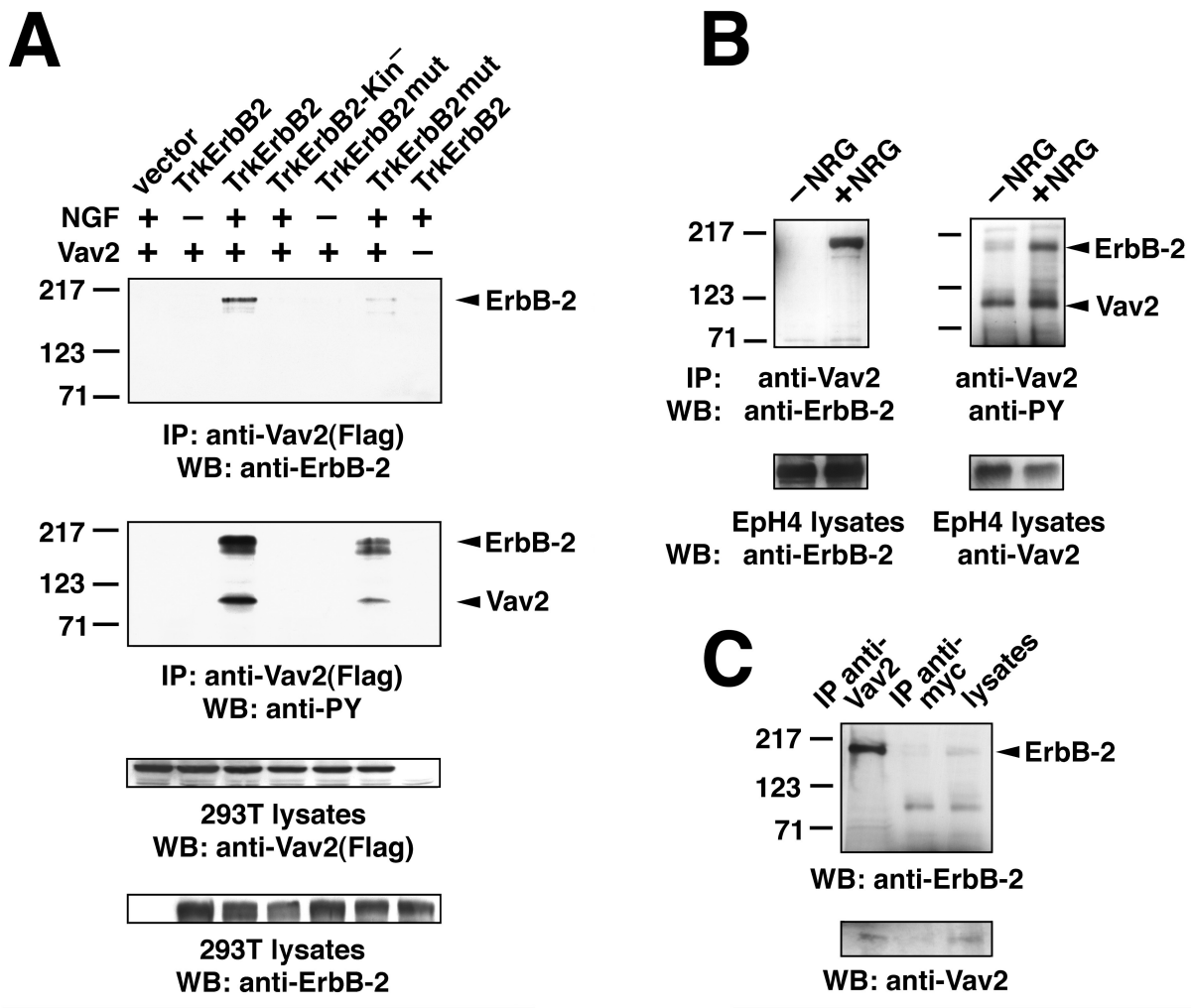


Figure 8. Analysis of the interaction between Vav2 and ErbB-2 in mammalian cells and in mammary tissue.

(**A**) Co-immunoprecipitation of Vav2 with various TrkErbB2 receptors in transfected 293T cells. (Top) Vav2 co-immunoprecipitates with TrkErbB2 and TrkErbB2^{mut}, when these hybrid receptors are activated by NGF. Vav2 does not associate with kinase-defective TrkErbB2-Kin^{-}. (Middle) Binding of Vav2 to activated TrkErbB-2 receptors results in increased tyrosine phosphorylation of Vav2. (Bottom) Control immunoblots of total cell lysates. Molecular weight markers on the left are in kDa. (**B**) Interaction between Vav2 and ErbB-2 in EpH4 mammary epithelial cells. (Top) Vav2 co-immunoprecipitates with ErbB-2 in lysates of neuregulin-treated cells (left panel); association of Vav2 with activated ErbB-2 results in increased tyrosine phosphorylation of Vav2 (right panel). (Bottom) Control immunoblots of total cell lysates. (**C**) *In vivo* association between Vav2 and ErbB-2 in mammary glands during pregnancy. Vav2 co-immunoprecipitates with ErbB-2 in mammary gland lysates from pregnant mice. Anti-c-Myc antibody was used as non-specific control; Vav2 immuno-precipitation is shown below.

3.6 Vav2 and ErbB-2 Are Associated in Mammary Epithelium During Pregnancy

Interaction between Vav2 and ErbB-2 was also studied in mammary glands from pregnant mice. Lysis of all mammary glands from a female mouse at day 17.5 of pregnancy was followed by immunoprecipitation of endogenous Vav2 and Western blotting as above. Indeed, Vav2 co-immunoprecipitated with ErbB-2 from the mammary lysates (Fig. 8C), suggesting the presence of endogenous complexes between Vav2 and ErbB-2 in mammary tissue during pregnancy.

Cellular localization of Vav2 and ErbB-2 was studied in mammary tissue from pregnant mice by in situ hybridization. Importantly, Vav2 and ErbB-2 are co-expressed in the epithelial cell layer that lines alveoli and ducts of mammary glands during pregnancy (Fig. 9; Schroeder *et al*., 1998). The co-localization of Vav2 and ErbB-2 in mammary epithelium supports the presence of interaction *in vivo* during pregnancy, as above suggested by biochemical analysis (see Fig. 8C); moreover, it rules out the possibility of association during the immuno-precipitation of Vav2. Taken together, these observations indicate that ErbB-2 and Vav2 can functionally associate in the mammary gland while lobulo-alveolar development takes place.

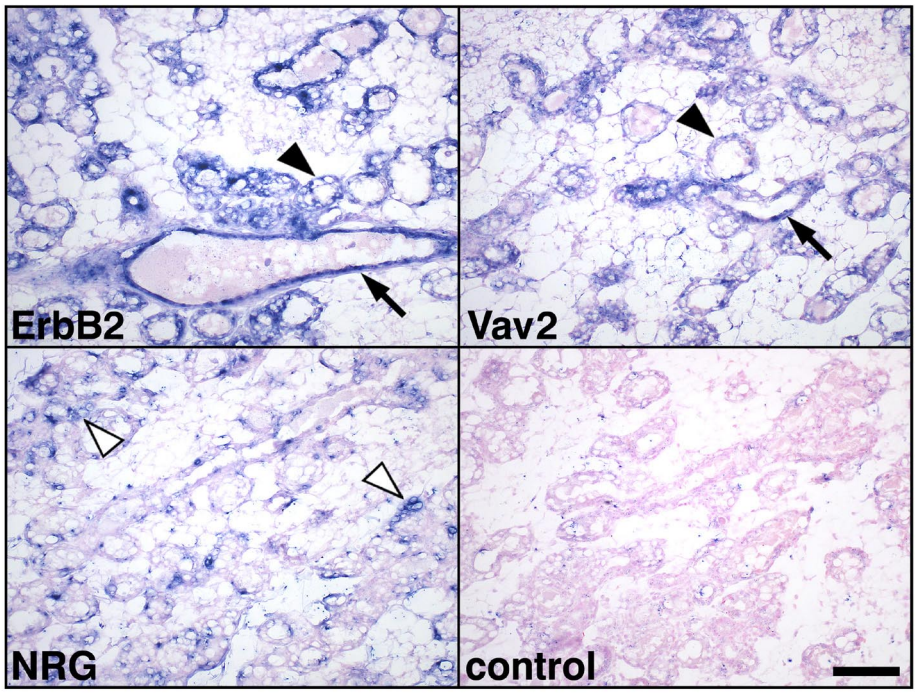


Figure 9. Vav2 and ErbB-2 are co-expressed in mammary alveolar epithelium during pregnancy.

(Top) Vav2 and ErbB-2 co-localize in alveolar (black arrowheads) and ductal (arrows) epithelia of the mammary gland at late pregnancy. (Bottom) Neuregulin (NRG) is located in the mesenchyme surrounding alveoli and ducts (white arrows); a control section (control) was hybridized with a Vav2 sense probe. Bar: 100 µm.

3.7 The Dbl-Homology Domain of Vav2 Is Required for Its Morphogenic Activity in EpH4 Cells

The structural domains of Vav2 confer the protein two interesting features: first, Vav2 acts as a GDP/GTP exchange factor for Rho/Rac GTPases via its catalytic Dbl-homology domain; second, Vav2 has the capability to participate in signal transduction cascades by binding further downstream proteins via its C-terminal SH2 and SH3 domains (Bustelo, 2000). To define the functional role of the various domains of Vav2 in alveolar morphogenesis, mutational analyses of constitutively active Vav2 were performed. ΔN-Vav2-ΔC encodes an active protein which lacks SH2 and both SH3 domains (structures are shown in Fig. 10). ΔN-Vav2-dbl^{mut} lacks the GTP/GDP exchange activity due to a deletion of seven conserved amino acids within the Dbl homology domain ($_{336}$LLLKELL$_{342}$), which has been reported

to inactivate Dbl, GRF1 and Sos1 (Hart *et al*., 1994; Freshney *et al*., 1997; Qian *et al*., 1998). EpH4 cells were stably transfected with plasmids encoding Vav2 mutant proteins and examined in Matrigel assays. Clearly, the Dbl homology domain is required for the biological function of Vav2 whereas the SH2/SH3 domains are dispensable (Fig. 10B, C). Overexpression of a minimal Vav2 protein which additionally lacks the pleckstrin homology domain (ΔN-Vav2-ΔCΔPH) was also sufficient to elicit alveolar morphogenesis (Fig. 10D).

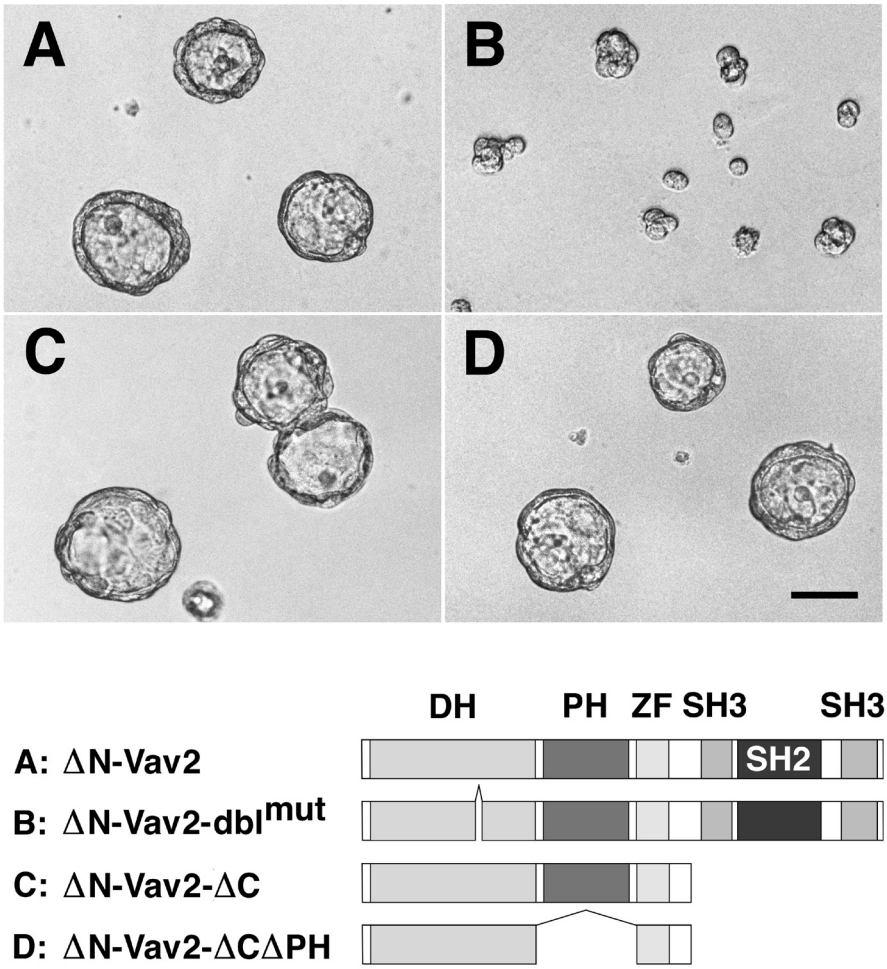


Figure 10. The Dbl homology domain of Vav2 is essential to promote alveolar morphogenesis of EpH4 mammary epithelial cells.

(**C**) C-terminally truncated Vav2 and (**D**) a minimal protein encompassing the DH and ZF domains, respectively, are as efficient as constitutively active Vav2 (in **A**) to induce morphogenesis. (**B**) Catalytically inactive Vav2 carrying a mutation in the Dbl homology domain does not promote formation of alveoli. Bar: 50 μm. DH: Dbl-homology domain; PH: pleckstrin-homology domain; ZF: zinc finger.

3.8 Catalytically Inactive Vav2 Blocks Neuregulin-Mediated Morphogenesis of EpH4 Cells

EpH4 cells that express catalytically inactive Vav2 (ΔN-Vav2-dbl^{mut}) were tested for alveolar morphogenesis following neuregulin treatment. Importantly, ΔN-Vav2-dbl^{mut} interferred with the formation of alveoli when cells were treated with neuregulin: instead of large hollow structures (Fig. 11B, C), cell aggregates without lumina were observed (Fig. 11E, F), indicating that alveolar morphogenesis is prevented while cell growth still occurs. Only small cell groups were formed in absence of neuregulin treatment (Fig. 11A, D).

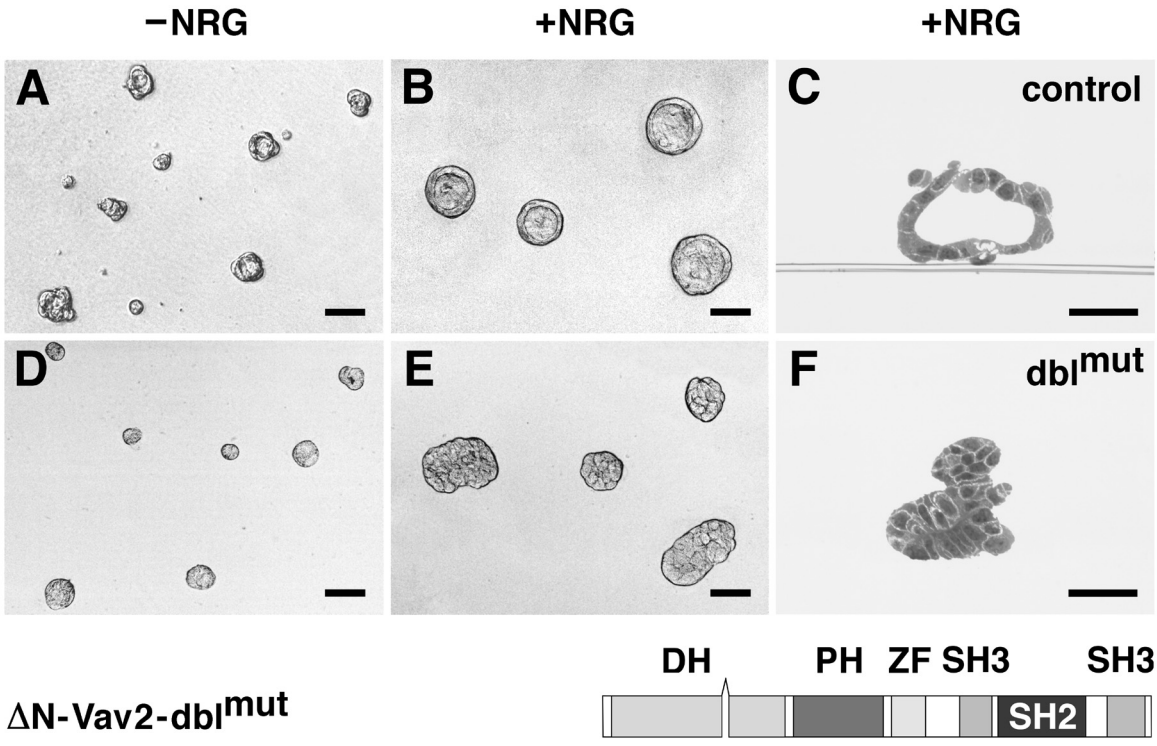


Figure 11. Catalytically inactive Vav2 harbouring a mutation in the Dbl homology domain blocks the morphogenic signals of neuregulin in EpH4 mammary epithelial cells.

(**E**, **F**) EpH4 cells expressing ΔN-Vav2-dbl^{mut} (see structure below) do not form hollow alveoli but rather large cell aggregates upon stimulation with neuregulin. (**B**, **C**) Mock-transfected cells form alveoli-like structures in

response to neuregulin, as previously described (Niemann *et al*., 1998). (**A**, **D**) Non-stimulated controls. Bars: 50 µm. DH: Dbl-homology domain; PH: pleckstrin-homology domain; ZF: zinc finger.

Interestingly, ΔN-Vav2-dbl^{mut} did not interfere with tubular branching when EpH4 cells were treated with hepatocyte growth factor/scatter factor (Fig. 12D, compare to control cells in B; Niemann *et al*., 1998). Thus, catalytically inactive Vav2 blocks neuregulin-specific signals for alveolar morphogenesis of EpH4 cells, while it does not affect cellular responses to other morphogenic stimuli.

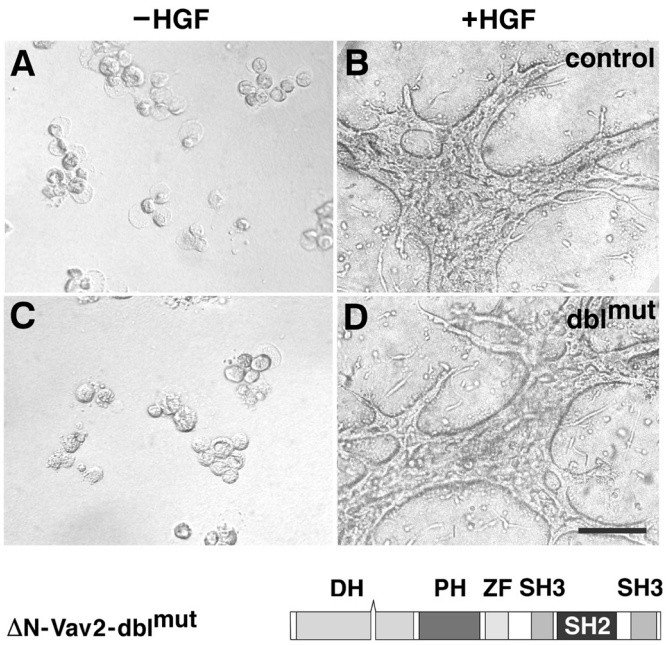


Figure 12. Catalytically inactive Vav2 does not interfere with branching morphogenesis of EpH4 mammary epithelial cells.

(**D**) EpH4 cells expressing ΔN-Vav2-dbl^{mut} (see structure in Fig. 11) form tubular structures upon stimulation with HGF/SF. (**B**) Mock-transfected cells exhibit branching morphogenesis in response to HGF/SF, as previously described (Niemann *et al*., 1998). (**A**, **C**) Non-stimulated controls. Bars: 50 µm. DH: Dbl-homology domain; PH: pleckstrin-homology domain; ZF: zinc finger.

4 DISCUSSION

Previous results from our and other groups suggest that neuregulin activates the receptor c-ErbB-2 to promote lobulo-alveolar development of the mammary gland (Yang *et al*., 1995; Jones *et al*., 1996; Niemann *et al*., 1998; Jones and Stern, 1999). However, little was known about the intracellular effectors that mediate this morphogenic effect. Here, a modified yeast two-hybrid screen was developed to search for new substrates of ErbB-2 that are involved in alveolar morphogenesis of the mammary gland. The guanine nucleotide exchange factor Vav2 was identified among other proteins as a novel interaction partner of ErbB-2. A full characterization of the ErbB-2 docking sites for these interacting proteins was performed in the yeast system, thus adding complexity to the previous knowledge on intracellular effectors of ErbB-2. Next, the potential function of Vav2 in ErbB2-induced morphogenesis was tested in EpH4 mammary gland epithelial cells that were cultured on a reconstituted basement membrane (Matrigel). These studies show two main points: first, Vav2 indeed promotes alveoli-like growth and reorganization of EpH4 cells, either when constitutively active or upon activation by ErbB-2; second, co-expression of Vav2 and ErbB-2 in mammary alveolar epithelium is observed in pregnant mice. These findings suggest a functional association between Vav2 and ErbB-2 during alveolar morphogenesis *in vivo*. Together, this work supports a model for neuregulin-induced mammary alveolar morphogenesis *in vivo*, in which Vav2 is a physiological target downstream of ErbB-2 that plays a crucial role in transduction of signals for lobulo-alveolar morphogenesis.

4.1 Modified Yeast Two-Hybrid System: A Powerful Tool to Search for Phosphotyrosine-Interacting Proteins

The commonly-used yeast two-hybrid baits to search for interaction partners of receptor tyrosine kinases are hybrid proteins, which consist of a LexA DNA-binding and dimerization sequence fused to the intracellular region of the receptors (O'Neill *et al*., 1994; Weidner *et al*., 1996; Grimm *et al*., 2001). Bait molecules dimerize via LexA when expressed in yeast cells,

thus enabling the activation of receptor tyrosine kinases without requirement of a ligand. This approach could not be used in case of the receptor ErbB-2, since the resulting hybrid proteins were rapidly degraded in the yeast. Therefore, modified yeast baits were generated: C-terminal sequences of ErbB-2 containing the autophosphorylation sites were fused to an oncogenic TprMet kinase in order to obtain constitutively active TprMet-ErbB2 baits. TprMet encodes the intracellular region of c-Met in frame with a leucin-zipper dimerization domain derived from a different locus (Park *et al*., 1986). The heterologous TprMet kinase efficiently phosphorylates tyrosine residues of ErbB-2, thus creating docking sites for signaling proteins with SH2 and PTB domains. By this means, known and novel interacting proteins for ErbB-2 were identified in a cDNA library screen in yeast.

This modified yeast two-hybrid system has proven to be useful for standard search of proteins that interact with peptides and proteins containing phosphorylated tyrosine residues; even unrelated protein sequences become tyrosine-phosphorylated when fused to the TprMet kinase and can be used as yeast baits. For example, coupling Gab1 to TprMet enabled the analysis of the interaction between phosphorylated Gab1 and the SH2 domains of PI-3-K, PLCγ, Shp2 and CRKL (Schaeper *et al*., 2000). Thus, the new method presented here offers an easy alternative for yeast screens as opposed to a tribrid approach, which includes an additional kinase (Licitra and Liu, 1996). The main advantage of the here described modified two-hybrid over a tribrid system is that only two selectable markers instead of three markers are necessary; therefore, the efficiency of yeast transformation is improved, and protein expression levels in the yeast are higher than those of the tribrid method.

4.2 New Insights into the Intracellular Signaling Pathways of ErbB-2

In independent cDNA library screens with tyrosine phosphorylated ErbB-2 baits, novel ErbB2-interacting proteins like Nck, Grb-10 and Vav2 were identified, along with the known partners Src and PLCγ1 (Muthuswamy and Muller, 1995b; Fazioli *et al*., 1991). These proteins belong to the group of multiadaptor signaling molecules; the most conspicuous

feature of such proteins is the presence of several specialized regions that allow protein-protein interactions (reviewed in Pawson and Scott, 1997). The ErbB-2 partners identified in the present workt contain SH2 domains; indeed, these SH2 domains were always present in the interacting clones and therefore mediate the association with phosphotyrosine residues of ErbB-2. The direct binding sites of ErbB-2 for these proteins were mapped by mutational analysis of the baits in the yeast system. So far, tyrosines Y2 and Y4 of the ErbB-2 multidocking site were known to directly bind Grb-2 and Shc, respectively (Ricci *et al*., 1995; Dankort *et al*., 1997). Here, direct binding of Vav2 to Y1 and Y3, Grb-10 to Y4, Nck to Y3 and Src to complexed Y3-Y4 were characterized. The overlap of Vav2, Nck and Src for binding to Y3 suggests that these proteins may represent alternative effectors of ErbB-2. Alternatively, these proteins may assemble to form a hierarchy of different protein multicomplexes; any of such supramolecular complexes may lead to a unique outcoming signal, regardless of the protein-protein interaction pattern within the complexes. The formation of functionally redundant protein alignments between Src, Nck and Vav2 (possibly together with some other proteins) is indeed conceivable. Preliminary data indicate that Vav2 can directly bind Src or Nck; using the modified yeast two-hybrid method, a TprMet-Vav2 fusion bait was recently tested with several SH2 domains for interaction in the yeast. Indeed, tyrosine-phosphorylated Vav2 interacted with the SH2 domains of Src and Nck but not with that of Grb2 (data not shown). A mechanism whereby Vav2 is ultimately recruited and activated via different protein-protein associations may account for the direct and indirect binding of Vav2 to ErbB-2 (see also below).

4.3 Vav2 Couples to Receptor Tyrosine Kinases via Its SH2 Domain

Direct association of the SH2 domain of Vav2 with ErbB-2 involves recognition of the motif pYLVP, present at Y1 (Y1028) and Y3 (Y1201). Using an entirely different selection procedure, the sequence pYXEP (where X is L, M or E) has been identified to bind the SH2 domain of Vav (Songyang *et al*., 1994). The SH2 domains of Vav and Vav2 share 55% identity (Schuebel *et al*., 1996), and it is therefore possible that they have distinct binding

preferences. From all the ErbB-2 partners that were found in the yeast screens, only Vav2 showed a particular affinity for binding to the various ErbB receptors. Vav2 also interacts with the PDGFβ receptor the yeast system (Table 3). In fact, recent biochemical and mass spectrometry analyses have shown that all three Vav proteins can form complexes with EGF or PDGFβ receptors and become phosphorylated on tyrosine residues following stimulation with EGF or PDGF (Bustelo *et al*., 1992; Margolis *et al*., 1992; Lopez-Lago *et al*., 2000; Moores *et al*., 2000; Pandey *et al*., 2000). However, it was not clear from these studies whether Vav proteins couple directly to the receptors or indirectly via binding to adaptor proteins. The results from yeast two-hybrid analyses that are here presented do not rule out the possibility of indirect interaction (see below); however, they provide the first line of evidence that indeed Vav2 can directly bind to receptor tyrosine kinases via its SH2 domain, a mechanism that may be shared by the other Vav proteins.

The above identified pYLVP consensus motif for binding to Vav2 is conserved in the C-terminal region of ErB-4 (Y1022) but not in the EGF receptor, ErbB-3 or the PDGFβ receptor; however, the rather similar sequences pYLIP in the EGF receptor (at Y1012), pYLMP in ErbB-3 (at Y1159) and pYIIP in PDGFβ receptor (at Y1021) may be predicted as Vav2 binding sites. This suggests that positions 1+ and 2+ of the consensus motif for Vav2 are flexible but, in contrast to the pYXEP binding motif of Vav, they may selectively be occupied by amino acids with a non-polar side chain.

The examination of ErbB-2/Vav2 interactions in mammalian cells rendered an unexpected result: in 293T cells, a mutant ErbB-2 receptor that lacks both Y1 and Y3, the direct docking sites for Vav2 identified in the yeast system, still binds Vav2. This indicates that, in addition to direct binding, Vav2 may also interact indirectly with ErbB-2 via a further adaptor protein. Similarly, direct and indirect binding has been reported for recruitment of the adaptor Gab1 to the receptor tyrosine kinase c-Met (Fixman *et al*., 1995; Holgado-Madruga *et al*., 1996; Weidner *et al*., 1996; Bardelli *et al*., 1997; Nguyen *et al*., 1997; Schaeper *et al*., 2000). ErbB-2

binds to Grb2, Shc, the Csk-homologous kinase, PLCγ1, Nck, Grb10 and Src (Fazioli *et al*., 1991; Ricci *et al*., 1995; Muthuswamy and Muller, 1995; Dankort *et al*., 1997; Zrihan-Licht *et al*., 1998; this work), which may indirectly recruit Vav2 to the receptor. Sequence analysis of Vav2 reveals the presence of putative consensus motifs for binding the SH2 domains of Src, Lck (a Src-related tyrosine kinase) and Shc (Songyang *et al.*, 1994). Ongoing studies suggest that Vav2 may directly interact with the SH2 domains of Src and Nck.

4.4 Vav2 Mediates ErbB-2 Signals for Alveolar Morphogenesis of EpH4 Mammary Epithelial Cells

Recent work has shown that normal development of the mammary gland during pregnancy to prepare lactogenesis can be mimicked *in vitro*. The reorganization of mammary epithelial cells to form lobulo-alveolar structures is markedly influenced by the matrix on which these cells are cultured. Barcellos-Hoff *et al*. (1989) demonstrated that primary mammary epithelial cells form functional, albeit rudimentary, alveoli-like structures when cultured on a reconstituted three-dimensional matrix, in the presence of lactogenic hormones and absence of serum. Within the first days of culture, cells remodel the exogenous basement membrane and form matrix-ensheated aggregates, which subsequently cavitate; by day 6 of culture, cells are reorganized into hollow spheres composed of morphologically polarized cells facing a lumen. These cells are functionally differentiated and secrete milk proteins vectorially into the luminal compartment. A reconstituted basement membrane alone, however, does not account for the complex epithelial-mesenchymal interactions that are linked to mammary development. The spontaneously immortalized, non tumorigenic mouse mammary cell line IM-2 consists of both epithelial and fibroblastic cell populations (Reichmann *et al*., 1989); it has been shown that the fibroblastic cells render the epithelial cells competent to undergo cytoskeletal rearrangements and to functionally differentiate on Matrigel. The physiological signals from the mammary mesenchyme can alternatively be supplied by addition of growth factors to mammary epithelial cells in organotypic culture. Previous studies pointed to neuregulin as a mesenchymal growth factor that stimulates alveolar morphogenesis of

mammary glands in organ culture (Yang *et al.*, 1995). In view of these results, Niemann *et al*. (1998) used EpH4 mammary cells (an epithelial subclone derived from the abovementioned IM-2 cell line) to test the effect of neuregulin in a Matrigel system; EpH4 cells indeed form large alveoli-like structures when cultured on Matrigel in the presence of neuregulin. Additionally, biochemical studies revealed tyrosine phosphorylation of endogenous ErbB-2 in EpH4 cell lysates following neuregulin treatment (Fig. 8). Formation of similar alveolar structures is observed when EpH4 transfectants that stably overexpress a TrkErbB2 chimeric receptor are cultured on Matrigel in the presence of nerve growth factor (Niemann *et al*., 1998). These results indicate that activation of the overexpressed ErbB-2 receptor is sufficient to elicit alveolar morphogenesis in organotypic cultures of mammary epithelial cells. Together with the mesenchymal localization of neuregulin transcripts and the epithelial expression of ErbB-2 in mammary tissue at mid-pregnancy, these findings strongly suggest that stromal neuregulin activates epithelial ErbB-2 to induce alveolar morphogenesis of the mammary gland *in vivo*. Moreover, this model of neuregulin signaling supports the increasing evidence of the role of epithelial–mesenchymal interactions in postnatal growth and differentiation of the mammary gland (reviewed in Cunha and Hom, 1996; Robinson *et al.*, 1999; Silberstein, 2001).

To understand the molecular mechanisms of neuregulin/ErbB signaling in mammary alveolar morphogenesis, this work aimed at the identification of intracellular effectors of ErbB-2 that mediate these morphogenic effects. In yeast two-hybrid screens with ErbB-2 baits, Vav2 was identified as a novel partner of ErbB-2. Vav2 belongs to a family of guanine nucleotide exchange factors for small GTPases of the Rho superfamily, like Rho, Rac and Cdc42 (reviewed in Bustelo, 2000). Vav proteins contain a common array of domains, which include an N-terminal regulatory acidic region, a catalytic Dbl-homology domain and several C-terminal SH2 and SH3 domains (see Fig. 2). Unlike Vav, Vav2 expression is not restricted to the hematopoietic system, but is also found in several epithelia, for example in the mammary gland epithelium (Fig. 9). Though recent reports involve Vav2 in immune responses of certain

hematopoietic lineages (Billadeau *et al*., 2000; Doody *et al*., 2000), a function of Vav2 in epithelial tissues is also possible. Upon phosphorylation of a single regulatory tyrosine residue in the N-terminus, Vav proteins gain catalytic activity towards Rho GTPases, thus leading to changes in the actin cytoskeleton and gene transcription (reviewed in Bustelo, 2000). Vav2 associates not only with ErbB-2 in the yeast system, but also with all other ErbB receptors (Fig. 4), which are known to be involved at different stages of mammary development (Xie *et al*., 1997; Jones and Stern, 1999; Jones *et al*., 1999). The capability of Vav2 to induce cytoskeletal reorganization, together with its high affinity for ErbB receptors, favored the choice of Vav2 as candidate to mediate morphogenic signals of ErbB-2.

The morphogenic potential of Vav2 was here tested in a Matrigel assay. It was known that truncation of the N-terminal region of Vav proteins eliminates an autoinhibitory loop and exposes the catalytic site to small GTPases, thus enhancing GDP/GTP exchange (Aghazadeh *et al*., 2000). Therefore, EpH4 mammary epithelial cells were stably transfected with a cDNA encoding an N-truncated, constitutively active Vav2 protein (Schuebel *et al*., 1996), and transfectants were cultured on Matrigel under serum-free conditions and in the absence of growth factors. In this organotypic system, catalytically active Vav2 was sufficient to induce formation of large alveolar structures that resembled those that are observed following activation of ErbB-2 by neuregulin (see Niemann *et al*., 1998). Alveoli consisted of a monolayer of polarized epithelial cells, which enclose a luminal compartment by means of apical tight junctions. These results represent the first evidence of a biological response elicited by overexpression of active Vav2 in epithelial cells. Constitutively active but not wild-type Vav2 elicited these morphogenic events in EpH4 cells, indicating that GDP/GTP exchanger activity, and therefore cytoskeletal reorganization, may be essential in these processes. In line with these findings, it has been reported that truncated forms of all Vav family members have a deregulated activity as guanine nucleotide exchange factors *in vitro*, are highly transforming in focus formation assays, and elicit changes in cell morphology upon transient transfection into NIH 3T3 fibroblasts (Bustelo, 2000 and references therein).

Activation of an overexpressed TrkErbB2-Vav2 fusion protein by nerve growth factor also induced alveolar morphogenesis of EpH4 transfectants. This finding suggests that the activated ErbB-2 kinase of the fusion protein efficiently phosphorylated wild-type Vav2. Phosphorylation may occur on the putative regulatory tyrosine Y172 of Vav2; thus, the autoinhibitory loop of wild-type Vav2 is disrupted and therefore, phosphorylated full-size Vav2 can now behave as its oncogenic truncated counterpart to elicit morphogenesis.

Biochemical studies revealed association between Vav2 and ErbB-2 in lysates from EpH4 cells following neuregulin treatment (Fig. 8B, left panel). Activation of ErbB-2 was observed after stimulation with neuregulin as an increase in phosphotyrosine content, despite high basal phosphorylation levels (Fig. 8B, right panel). Importantly, endogenous complexes between Vav2 and ErbB2 were found in lysates of mammary glands from pregnant mice (Fig. 8C). In addition, in situ hybridization studies showed spatial co-localization of Vav2 and ErbB-2 in mammary alveolar epithelium (Fig. 9), which allows the physical interaction between the receptor and its putative effector in the mammary gland during pregnancy.

Taken together, Vav2 can induce alveolar morphogenesis of EpH4 cells, both in its constitutively active state or when activated by ErbB-2. This places Vav2 as a downstream effector of ErbB-2 for this biological response. In both cases, alveolar structures were similar to those that are formed upon neuregulin treatment or activation of ectopic ErbB-2 (Niemann *et al*., 1998); along with the functional link between neuregulin and ErbB-2, and with the physical association of ErbB-2 and Vav2 in mammary tissue, these findings support a relevant role of Vav2 as a downstream effector of neuregulin/ErbB-2 signals for alveolar morphogenesis of the mammary gland *in vivo*. Furthermore, they provide clear evidence of the as yet suggested function of Vav2 in signaling of receptor tyrosine kinases that lead to a concrete biological response.

4.5 The Morphogenic Activity of Vav2 on Mammary Epithelial Cells May Involve Changes in Actin Cytoskeleton

Mutational analysis revealed that a functional Dbl-homology domain is necessary and sufficient for Vav2 to elicit alveolar morphogenesis. The Dbl-homology domain enables Vav2 to function as guanine nucleotide exchange factor towards small GTPases of the Rho family with an as yet unclear specificity (Schuebel *et al*., 1998; Abe *et al*., 2000). Disruption of this domain in a mutant Vav2 protein completely abolished its ability to promote alveolar morphogenesis of EpH4 mammary epithelial cells. Moreover, the isolated Dbl-homology domain of Vav2 still retained morphogenic properties, suggesting that the GDP/GTP exchange activity is critical in this process, whereas the SH2/SH3 adaptor domains are dispensable. Similarly, integrity of the Dbl-homology domain in the absence of SH2/SH3 modules is required by Vav3 to promote formation of lamellipodia and membrane ruffling in transfected NIH3T3 fibroblasts (Movilla *et al*., 1999). In view of these results, it is evident that the Dbl-homology domain of Vav proteins is sufficient to reorganize the actin cytoskeleton, a process that may be required for Vav2-mediated alveolar morphogenesis.

Rho proteins constitute a subgroup of the Ras superfamily of small GTPases that are key regulators of the actin cytoskeleton and of several cellular processes like gene transcription, membrane trafficking, growth, movement and morphogenesis (reviewed in Van Aelst and D'Souza-Chorey, 1997; Hall, 1998; Mackay and Hall, 1998). Rho small GTPases switch from an inactive GDP-bound state to an active GTP-bound state; this process is accelerated by guanine nucleotide exchange factors containing a Dbl-homology domain. Activated small GTPases trigger actin polymerization to form stress fibers, focal adhesion complexes, lamellipodia, membranes ruffles and filopodia. An increasing amount of genetic studies in invertebrates provide ample evidence that GEFs and Rho proteins play a central role in several morphogenic events during the development of a multicellular organism. It has been shown that the putative GDP/GTP exchanger DRhoGEF2 and the small GTPase DRho1 are part of a signaling pathway that determines cell shape changes during gastrulation of *Drosophila*

(Barrett *et al*., 1997; Häcker *et al*., 1998). The recently described *Drosophila* Trio GEF or its *C. elegans* homolog UNC-73 have been shown to regulate axon guidance and cell migration in the central nervous system (Steven *et al*., 1998; Awasaki *et al*., 2000; Liebl *et al*., 2000; Newsome *et al*., 2000; Bateman *et al*., 2000). Still life, another *Drosophila* RhoGEF, has been identified in a mutational screen for motor activity defects, and its absence determines reduced locomotion as well as male infertility (Sone *et al*., 1997). Furthermore, Rho proteins have also been shown to control dorsal closure at late stages of *Drosophila* embryogenesis (Magie *et al*., 1999), directed cell movement in the *Drosophila* ovary (Murphy *et al*., 1999) and planar polarity in eyes and wing hair (Eaton *et al*., 1995; Eaton *et al*., 1996; Strutt *et al*., 1997). In mammals, the complexity of the small GTPase superfamily, and therefore the potential redundancy of its members, has made the study of their role during embryogenesis difficult. In humans, mutations of the *FGD1 (faciogenital dysplasia)* gene, which encodes the Vav-related guanine nucleotide exchanger FGD1, cause an X-linked autosomal developmental disorder (known as Aarskog-Scott syndrome or faciogenital dysplasia) involving skeletal and genito-urinary anomalies (Pasteris *et al*., 1994; Olson *et al*., 1996; Zheng *et al*., 1996). In view of the accumulating evidence of a role for GEFs and Rho proteins in epithelial development, it is conceivable that Rho small GTPases are downstream effectors of Vav2 in the mammary gland. Matrigel assays with EpH4 cells overexpressing constitutively active or dominant negative forms of Rho, Rac and Cdc42 small GTPases may elucidate their contribution to alveolar morphogenesis.

4.6 Vav2 Is a Specific Effector of Neuregulin Signals for Alveolar Morphogenesis

EpH4 mammary epithelial cells express endogenous c-Met and ErbB receptors and are competent to elicit biological responses following stimulation by HGF/SF or neuregulin, the ligands for such receptors (Niemann *et al*., 1998). EpH4 cells exhibit ductal growth when cultured on Matrigel in the presence of HGF/SF, while they form alveoli-like structures upon neuregulin treatment. Therefore, they provide a versatile system to study different developmental events of the mammary gland *in vitro.* A Dbl-defective mutant of Vav2 did not

affect cell proliferation but completely blocked neuregulin-induced morphogenesis in a dominant-negative manner (Fig. 11). In contrast, this catalytically-inactive Vav2 mutant did not interfere with the morphogenic stimulus of HGF/SF for ductal growth (Fig. 12). These results suggest that Vav2 is specifically involved in the morphogenic but not the mitogenic signaling cascades of neuregulin/ErbB-2, whereas other intracellular effectors may mediate the stimulatory effects of HGF/SF and c-Met. It is known that one such effector is the multiadaptor protein Gab1 (Grb2-associated binder; Holgado-Madruga *et al*., 1996). Gab1 specifically couples to activated c-Met (Weidner *et al*., 96; Schaeper *et al*., 2000), thus transmitting unique HGF/SF signals for essential developmental processes during embryonic life; genetic analysis revealed that both *Gab1*- and *c-Met*-deficient mice exhibit similar phenotypes (Bladt *et al*., 1996; Sachs *et al*., 2000). In the last years, several multiadaptor proteins have been described as specific substrates of receptor tyrosine kinases and key transducers of their biological function. DOS, a Gab1-related multiadaptor protein of *Drosophila*, mediates retinal development downstream of the receptor tyrosine kinase Sevenless (Herbst *et al*., 1996; Raabe *et al*., 1996). IRS-1 and IRS-2 are major substrates of the insulin and insulin-like growth factor receptors (Sun *et al*., 1991; Sun *et al*., 1995). Mice lacking IRS-1 are mildly insulin-resistant due to compensation by IRS-2, but show a dramatic impairment of glucose uptake in skeletal muscle (Araki *et al*., 1994; Tamemoto *et al*., 1994; Yamauchi *et al*., 1996; Bruning *et al*., 1997). Disruption of the *IRS-2* gene results in diabetes (Withers *et al*., 1998), thus clearly showing distinct and critical roles for IRS-1 and IRS-2 in different tissues that are targeted by insulin. Recently, novel Dok proteins have been characterized as interaction partners of c-Ret and, like the receptor, induce neurite outgrowth in PC12 cells (Grimm *et al*., 2001). The present work supports a putative role of Vav2 in mediating neuregulin signals for mammary alveolar morphogenesis. Moreover, all these studies provide substantial evidence that specific biological responses may result from specific signaling pathways involving particular adaptors and hence, they put forward an alternative mechanism for the generation of the unique developmental responses of receptor tyrosine kinases.

Recent studies show that activation of Vav2 and RhoA also inhibits HGF/SF-induced cell scattering upon overexpression in Madin-Darby canine kidney cells (MDCK; Kodama *et al*., 2000). This observation suggests a negative role of Vav2 in branching morphogenesis and raises the possibility of a dual role *in vivo*. Vav2 may therefore mediate neuregulin-induced alveolar morphogenesis while simultaneously repressing HGF/SF-induced ductal growth. Other groups, in contrast, report that activation of RhoG is essential for differentiation and neurite outgrowth in PC12 cells following NGF treatment (Katoh *et al*., 2000). In the present work, catalytically active Vav2 as GDP/GTP exchanger is strictly required for neuregulin-induced alveolar morphogenesis. Future experiments in more physiological systems are required to clarify the regulatory role of GEF factors and their targets in different biological responses.

4.7 Possible Molecular Mechanisms Involving Vav2 as Critical Effector of Alveolar Morphogenesis

This work provides evidence that Vav2 and ErbB-2 may be functionally associated *in vivo* to promote alveolar morphogenesis of mammary epithelium. However, it does not resolve the issue whether Vav2 is a direct or indirect substrate of the ErbB-2 kinase. A TrkErbB2-Vav2 fusion protein becomes morphogenic upon NGF stimulation (Fig. 7); nevertheless, it is possible that an additional kinase is recruited to this hybrid protein via Vav2. Indeed, activation of all Vav proteins by phosphorylation through Src tyrosine kinases has been reported (Crespo *et al*., 1997; Schuebel *et al*., 1998; Movilla *et al*., 1999). In addition, recent studies show that the PDGF receptor stimulates Vav2 through tyrosine phosphorylation by Src (Chiariello *et al*., 2001). Recently, *in vitro* kinase assays were here performed with immunoprecipitated TrkErbB-2 receptor from lysates of stimulated cells, together with recombinant Vav2 protein as substrate. In line with the aforementioned report, these data suggests that Vav2 is not directly phosphorylated by ErbB-2 *in vitro* (data not shown). It is thus possible that the recruitment and activation of Src by ErbB-2 represents an intermediate step resulting in the engagement of Vav2 for morphogenesis. However, activation of Src has

largely been implicated in the etiology of ErbB2-overexpressing breast tumors but not in normal mammary development (Muthuswamy and Muller, 1994; Muthuswamy and Muller, 1995a); in fact, targeted overexpression of activated c-Src in the mammary epithelium results in epithelial hyperplasia and impaired lobulo-alveolar development followed by severe lactational failure (Webster *et al*., 1995). Latest experiments show that phosphorylated Vav2 interacts with the SH2 domain of Src (data not shown). This interaction is indeed likely, since Vav2 contains a Src-binding consensus motif involving the regulatory tyrosine Y172; however, it requires previous phosphorylation of Vav2. It is therefore possible that ErbB-2 phosphorylates Vav2 first on some of its various, non-regulatory tyrosine residues, thus creating docking sites for Src other than the predicted consensus motif, and then Src futher phosphorylates and activates Vav2. In support of this hypothesis, none of the known autophosphorylation sites of ErbB-2 corresponds to the optimal Src consensus binding motif (Songyang *et al*., 1993), though direct interaction between Src and ErbB-2 was here observed in the yeast system and has also been reported by others (Muthuswamy and Muller, 1995). Similarly, the direct binding site for Src on the PDGFβ receptor (DGHEYpYIpYVDP; Mori *et al*., 1993) does not match the predicted motif; instead, it resembles the amino acid sequence of ErbB-2 encompassing tyrosine Y3 (pYLVP), which was mapped in the yeast system as part of the Src binding site (Table 2). However, such sequence is not present in Vav2, thus raising the possibility of a broader range of phosphotyrosine-containing sequences that may be recognized by the SH2 domain of Src. Alternatively, it has been proposed that Src phosphorylates its own binding sites on the EGF receptor (Olayioye *et al*., 1999); such a model would account for simultaneous binding of Src and activation of Vav2. Taken together, these latest experiments favor that the indirect recruitment of Vav2 to ErbB-2 is of physiological relevance. Nevertheless, further research that addresses the role of direct and indirect binding of Vav2 to ErbB-2 may contribute to understand the mechanisms of Vav2 recruitment and activation in neuregulin signaling.

Previous studies suggest that activation of the MAPK/ERK (mitogen-activated protein kinase/extracellular signal regulated protein kinase) pathway is a necessary step in neuregulin-induced alveolar morphogenesis (Niemann *et al*., 1998). Indeed, MAP kinases are stimulated by all ligand-activated combinations of ErbB receptors (Ben-Levy *et al*., 1992; Graus-Porta *et al*., 1995; Karunagaran *et al*., 1996; Pinkas-Kramarski *et al*., 1996; Dankort *et al*., 1997; Pinkas-Kramarski *et al*., 1998). The MAPK pathway is triggered via recruitment of Grb2 or Shc/Grb2 complexes by the activated receptors (reviewed in Schlessinger and Bar-Sagi, 1994 and references therein). It has been shown that Grb2 constitutively binds Sos (Son of Sevenless) via its SH3 domains. Association of the SH2 domain of Grb2 with activated receptor tyrosine kinases leads to recruitment of the GDP/GTP exhanger Sos to the membrane, where it can switch the membrane-anchored Ras GTPase to the active GTP-bound form. Activation of Ras triggers a signaling cascade of kinases involving Raf (also termed MAP/ERK kinase kinase MEKK, or MAPKKK), MAPK/ERK kinase (MEK, also termed MAPKK) and finally the MAPK, which ultimately regulates transcription (reviewed in Schaeffer and Weber, 1999). Tyrosines Y2 and Y4 of the ErbB-2 multidocking site directly bind Grb-2 and Shc, respectively (Ricci *et al*., 1995; Dankort *et al*., 1997), and Grb2/Sos/ErbB-2 complexes have been detected in breast cancer cells (Janes *et al*., 1994). Moreover, different patterns of MAPK activation by α- and β-isoforms of NRG-1 and NRG-2 were reported (Pinkas-Kramarski *et al*., 1998); it has been suggested that duration of coupling to MAPK pathway contributes to the signaling specificity by several ErbB heterodimers, and the presence of ErbB-2 in receptor combinations contributes to prolong MAPK activation (Karunagaran *et al*., 1996). Overexpression of constitutively active Vav proteins does not activate MAPK; instead, Vav proteins strongly activate the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK; Crespo *et al*., 1996; Abe *et al*., 2000; data not shown). Activation of JNK/SAPK by Vav proteins depends on the integrity of the Dbl-homology domain, as it requires activation of the small GTPase Rac1. Nevertheless, a connection between Vav2 and Ras has been suggested by synergism for cellular transformation (Schuebel *et al*., 1998). It has been shown that Vav indirectly enhances Ras signaling; Vav-mediated

activation of small GTPases leads to subsequent activation of p21-activated protein kinases (PAKs), which in turn activate Raf and MEK (Bustelo, 2000 and references therein). It is still unclear whether Vav2 engages similar phosphorylation cascades. The possible requirement of JNK/SAPK activation in neuregulin-induced alveolar morphogenesis therefore requires attention.

It is clear that activation of the transcription factor Stat5a (signal transducer and activator of transcription 5a) is critical in lobulo-alveolar morphogenesis. High levels of activated Stat5a are found in the mammary gland at late pregnancy and during lactation. Inactivation of the *stat5a* gene is accompanied by failure in terminal mammary differentiation but normal production of milk proteins during pregnancy (Liu *et al*., 1997). Moreover, activation and function of Stat5a during alveolar morphogenesis seems to be located downstream of ErbB-4 and prolactin signaling (Jones *et al*., 1999; Ihle and Kerr, 1995). Phosphorylation and activation of Stat5 following neuregulin treatment has been observed in NIH3T3 fibroblasts overexpressing both ErbB-2 and ErbB-4 (Olayioye *et al*., 1999); this observation indicates that either heterodimers of ErbB2/erbB4, homodimers of ErbB-4 or both are responsible for Stat5 activation. Since transgenic mice overexpressing dominant-negative ErbB-2 and ErbB-4 receptors in the mammary gland also show lactational failure, it is likely that heterodimers of ErbB-2 and ErbB-4 control the activity of Stat5a *in vivo*. The activity of Stat5a in EpH4 cells that overexpress Vav2 proteins has not been evaluated.

4.8 Conclusions

The results presented in this work suggest that Vav2 is a specific interacting partner of the ErbB subgroup of receptor tyrosine kinases. This particular affinity of Vav2 for ErbB receptors has a functional outcome: Vav2 can mediate specific signals from these receptors to elicit alveolar morphogenesis of mammary epithelial cells. The enzymatic activity of Vav proteins as GDP/GTP exchangers may provide a direct link to cytoskeletal rearrangements, an essential step in morphogenic events. *In vivo*, Vav2 and ErbB-2 co-localize and are associated

in mammary alveolar epithelium during pregnancy, while neuregulin is simultaneously synthesized in the mammary stroma. Therefore, this work supports a model whereby neuregulin activates ErbB-2 in mammary epithelium, which then recruits Vav2 to trigger unique signaling cascades that lead to lobulo-alveolar morphogenesis of the gland during pregnancy.

As discussed above, there are some mechanistic points of ErbB-2/Vav2 signaling that still need to be clarified. In addition, data on the function of Vav2 in mammary development *in vivo* are required. Genetic ablation of the *vav2* gene in mice revealed defective immune response to thymus-independent antigens, and the additional loss of *vav* led to a severe defect in B cell maturation (Doody *et al*., 2001; Tedford *et al*., 2001); however, *vav2*-deficient mice apparently lack an overt epithelial phenotype, and it is not clear whether the mammary glands from *vav2* null mice undergo normal alveolar morphogenesis during pregnancy. An explanation for this lack of an evident phenotype in epithelia is that Vav3, the other epithelial Vav protein, may compensate for Vav2 function in tissues where both Vav2 and Vav3 are co-expressed, as is the case of the mammary epithelium. Thus, generation of *vav2/vav3* double knockout mice may shed light on the physiological function of these guanine nucleotide exchange factors in mammalian epithelial morphogenesis.

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ABBREVIATIONS

ATP	adenosine 5' triphosphate
cDNA	complementary DNA
C-terminus	carboxyl terminus
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E	day of embryonic development
ECM	extracellular matrix
EDTA	ethylendiaminotetraacetic acid
EGF	epidermal growth factor
EHS	Engelbreth-Holm-Swarm murine tumor
FCS	fetal calf serum
FGF	fibroblast growth factor
GDP	guanosine 5' diphosphate
GTP	guanosine 5' triphosphate
HA	hemagglutinin epitope
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HGF/SF	hepatocyte growth factor/scatter factor
IgG	Immunoglobulin Class G
JNK/SAPK	c-Jun N-terminal kinase/stress-activated protein kinase
kDa	kiloDalton
LB	Luria-Bertani medium
M	mol/l
MAPK	mitogen-activated protein kinase
Matrigel	reconstituted basement membrane from EHS
mRNA	messenger RNA

NGF	nerve growth factor
NP-40	Nonidet P-40
NRG	neuregulin
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
PLCγ1	phospholipase C gamma1
PMSF	phenylmethylsulfonylfluoride
Y	tyrosine residue
RNA	ribonucleic acid
SDS	sodium dodecylsulphate
TE	tris-EDTA solution
TGF	transforming growth factor
Tpr	Translocated promoter region
Tris	tris(hydroxymethyl)-aminomethane hydrochloride
x-Gal	5-chlor-4-brom-3-indolyl-β-galactoside
YPD	yeast extract/peptone/dextrose

ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig und nur unter Verwendung der angegebenen Hilfsmittel erarbeitet und verfasst habe. Diese Arbeit wurde keiner anderen Prüfungsbehörde vorgelegt.

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