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Navigating Breast Cancer: Axon Guidance Molecules as Breast Cancer Tumor Suppressors and Oncogenes

Gwyndolen C. Harburg[1] and **Lindsay Hinck**[1,*]

[1]Department of Molecular, Cell and Developmental Biology University of California, Santa Cruz CA 95064

Abstract

Slit, Netrin, Ephrin, and Semaphorin's roles in development have expanded greatly in the past decade from their original characterization as axon guidance molecules (AGMs) to include roles as regulators of tissue morphogenesis and development in diverse organs. In the mammary gland, AGMs are important for maintaining normal cell proliferation and adhesion during development. The frequent dysregulation of AGM expression during tumorigenesis and tumor progression suggests that AGMs also play a crucial role as tumor suppressors and oncogenes in breast cancer. Moreover, these findings suggest that AGMs may be excellent targets for new breast cancer prognostic tests and more effective therapeutic strategies.

Keywords

Axon Guidance Molecules; Netrin; Semaphorin; Ephrin; Slit; Robo

The Slit, Netrin, Eph/ephrin, and Semaphorin families were originally characterized as axon guidance molecules (AGMs) in the developing nervous system, where they act as repulsive or attractive factors to guide axonal growth and migration [1]. Over the past decade they have also been shown to play roles in other mammalian organs, including the mammary gland, as mediators of tissue morphogenesis, cell adhesion, and proliferation [2]. Dysregulation of AGMs in the mammary gland has been linked to breast cancer initiation and progression, both through autocrine effects on tumor cells as well as paracrine effects on endothelial cells that promote angiogenesis. As the angiogenic role of AGMs has been well reviewed elsewhere, here we focus on the autocrine effects as they pertain to breast cancer [3–5]. In this review, we explore the dual nature of AGMs in breast cancer tumorigenesis and progression and consider their potential in development of new diagnostic markers and therapeutics.

AGMs in the Mammary Gland

It is only in the past fifteen years that researchers have begun exploring the role of AGMs in organs outside the nervous system. AGMs, belonging to each of the four families, are expressed in the mammary gland (Table 1), but for the most part their function is unknown.

*Corresponding Author: hinck@biology.ucsc.edu.

Many AGMs are upregulated during puberty, and are often enriched in the terminal end buds (TEBs), a developmental structure distinctive for its high proliferation levels and invasive behavior. Other AGMs, such as SLIT3 and ROBO2 are only expressed in the mature mammary epithelia, where they may regulate the basal level of proliferation during normal epithelial cell turnover [6]. Taken together, these expression patterns in both normal and diseased mammary gland suggest that AGMs are important for gland morphogenesis, epithelial homeostasis, and breast cancer development or progression.

Although few AGMs have available knockout mouse models, those that exist often have mammary gland phenotypes supporting a role for AGMs in normal gland development. These models indicate that there are three major roles for AGMs in gland development: proliferation, adhesion, and migration. Alterations in proliferation of mammary epithelial cells have been seen in mice with disrupted Eph/ephrin and Slit signaling. For example, *Epha2* deficiency leads to deficient mammogenesis, in which there is a failure of the mammary gland to fill the fat pad, while overexpression of *Ephb4* or *Efnnb2* in a transgenic mouse model leads to growth retardation of the mammary gland, and altered proliferation and apoptosis in mammary epithelial cells [7–9]. Conversely, loss of Slit signaling in *Slit2*;*Slit3* or *Robo1* knockout mice results in increased epithelial proliferation and development of ductal hyperplasias [10]. Mice with disrupted Slit, Netrin, or Eph/ephrin signaling also have altered cell adhesion that results in aberrant mammary gland morphology. Both *Slit/Robo* and *Netrin/Neogenin* knockout mice display disrupted cell adhesion in the TEB [6, 11]. *Ephb4* overexpressing mice have irregular alveolar morphology in which epithelial cells partly lose cell-cell contacts with their neighbors [8]. Thus, AGMs are important factors in normal mammary gland development.

Are AGMs Tumor Suppressors in the Breast?

The link between AGMs and highly proliferative regions of the mammary gland suggest that AGMs may be important in regulating normal epithelial proliferation. This brings up the question of whether AGMs also regulate proliferation during breast tumorigenesis. Although neither loss nor overexpression of AGMs, alone, has been linked to development of mammary tumors in mouse models, perturbation of their expression in a cancer-prone genetic environment has been shown to alter time to tumor development and aggressiveness of resulting tumors. Here, we present evidence supporting a role for AGMs as tumor suppressors in the breast that act by inhibiting proliferation and metastasis.

AGMs in Breast Cancer

Slit/Robo

Slit/Robo signaling acts as both a tumor suppressor and anti-metastatic factor in breast cancer. There are three Slits expressed in mammals – Slit1, Slit2, and Slit3. They act as ligands for Robo receptors, of which there are 4 in mammals, Robo 1–4 (Figure 1). Slits are not freely diffusible due to their association with heparin sulfate proteoglycans, such as glypican and syndecan [3]. *Slit2*, which is broadly expressed in the mammary gland during development and adulthood, is lost in 43–63% of sampled breast cancers, while *Slit3*, expressed only in the adult mammary gland, is lost in 16% (Table 2) [12, 13]. The Slit

receptor, *Robo1*, is also lost in 2–19% of sampled breast tumors and ∼5% of breast cancer cell lines (Table 2) [14], whereas potential roles for *Robo2* or *Robo3* in regulating mammary development and tumorigenesis have not been explored. The primary mechanism for loss of *Slit/Robo* expression in breast cancer is hypermethylation, rather than chromosomal arrangements or deletions [12–14]. Of particular note, both *Slit2* and *Robo1* show hypermethylation and gene silencing at early stages of breast cancer development, with *Slit2* hypermethylation even detected in 8–14% of histologically normal breast tissues [12, 14]. This suggests that loss of SLIT/ROBO signaling is an early event in tumor progression.

Slits may act through the Robo1 receptor to prevent tumor formation. In support of this, *Slit2;Slit3* knockout mouse mammary glands display an identical phenotype to *Robo1* knockout mice in which the glands develop ductal hyperplasias [6, 10]. The ductal hyperplasias are a result of increased proliferation in the ductal epithelia, indicating that SLIT/ROBO1 signaling regulates cell proliferation [10, 15]. In support, breast cancer cell lines MCF-7 or MDA-MB-231 that overexpress SLIT2 or SLIT3, or that are treated with SLIT2 conditioned medium have reduced proliferation and reduced ability to form colonies in Matrigel as indicated by fewer colonies and smaller colony size [10, 12]. *In vivo*, MDA-MB-231 or MCF7 cells overexpressing *Slit2* also give rise to tumors that are significantly smaller than those generated from control cells [10, 12]. Concordantly, knockdown of *Robo1* in MCF7 cells leads to increased proliferation, while knockdown of *Robo1* in MCF7 cells overexpressing *Slit2* returns proliferation to near control levels [16]. These studies support a role for SLIT2/ROBO1 signaling in regulating cancer cell proliferation.

SLIT/ROBO signaling is not only important in regulating cell proliferation, but also plays an important role in maintaining proper cell-cell adhesion and preventing tumor metastasis. SLIT2 has been proposed to act as an adhesive factor by binding to ROBO1-expressing myoepithelial cells and mediating their adhesion to luminal cells, perhaps through indirect binding of heparin sulfate proteoglycans [6]. The localization of ROBO1 to the plasma membrane where it could act adhesively is regulated by USP33, a deubiquitinating enzyme of the USP family, which stimulates the redistribution of ROBO1 to the plasma membrane in response to SLIT2 [17]. *Slit2;Slit3* and *Robo1* knockout mice both exhibit defects in cell adhesion between luminal and myoepithelial cells leading to disruptions in ductal architecture [6, 10]. In tumors, SLIT2 may also act adhesively to prevent metastasis by inhibiting detachment of tumor cells.

Overexpression of SLIT2 in MCF7 cells has been shown to reduce the amount of beta-catenin in the nucleus and enhance its co-localization with E-cadherin at the plasma membrane, potentially strengthening cell contacts [16] (Figure 2). In contrast, MCF7 cells in which *Robo1* is knocked down form large disorganized colonies, in comparison to the smooth, well-organized colonies that arise from control cells [10]. These results suggest that SLIT2/ROBO1 signaling is important in the mammary gland for maintaining appropriate cell-cell contacts.

The effect of SLIT2 on subcellular localization of beta-catenin may not only enhance cell adhesion, but can also inhibit cell proliferation by blocking canonical Wnt signaling (Figure 2). During mammary gland development, SLIT2 limits the proliferation of cap cells in the

terminal end bud by increasing the cytoplasmic and membrane pools of beta-catenin at the expense of its nuclear pool, suggesting that subcellular redistribution of beta-catenin is sufficient to inhibit cell proliferation. *In vitro* studies show that SLIT2 antagonizes downstream signaling of pro-proliferative factors, such as EGF, by blocking activation of AKT [16] (Figure 2). This inhibition of AKT, in turn, results in activation of GSK-beta, which inhibits beta-catenin translocation to the nucleus. SLIT2-induced exclusion of beta-catenin from the nucleus results in decreased expression of beta-catenin downstream transcriptional targets, such as *cyclin D1*, which may account for the observed decreases in cell proliferation. *Slit2*-overexpressing cancer cells also show decreased levels of MMP-2 and MMP-9, extracellular matrix proteases implicated in tumor progression and metastasis [16] (Figure 2). Thus, *Slit2* expression results in a change in the subcellular localization of beta-catenin, reducing its nuclear localization and decreasing transcription of pro-proliferative genes while increasing its membrane-association and enhancing cell adhesion.

SLIT/ROBO signaling also regulates cell-cell adhesion and metastasis indirectly by mediating CXCL12/CXCR4 signaling (Figure 2). The CXCL12 receptor, CXCR4, is not expressed in the normal mammary gland, but can be detected in tumor cells during early stages of tumor development [10, 18, 19]. CXCR4 expression is responsible for homing of breast cancer cells to common sites of metastasis, such as bone, lung, and brain, where the CXCL12 ligand is highly expressed [18]. CXCR4 expression is regulated by SLIT2, since *Slit2*-overexpressing cells show reduced levels of *Cxcr4*, while *Slit2;Slit3* knockout tissue shows increased levels of *Cxcr4* [10]. There is also an inverse correlation between *Slit2* and *Cxcr4* levels in breast tumors, suggesting that loss of SLITs plays an important role in tumor progression [10]. One possibility arising from these studies is that SLITs could function therapeutically to inhibit the CXCL12/CXCR4 chemokine axis. Indeed, SLIT has been shown to function as a non-competitive antagonist to block CXCL12-induced chemotaxis and invasion [20, 21]. SLIT also inhibits CXCL12-induced tyrosine phosphorylation of RAFTK, FAK, and paxillin, which maintain focal adhesions and preserve cell-cell contacts [17, 20], while inhibiting Src kinase, phosphatidylinositol 3-kinase (PI3-kinase), and ERK1/2 activation, all signaling mechanisms implicated in regulating cell motility [20]. Together these results show that the regulation of Wnt and CXCL12/CXCR4 pathways by SLIT2 is important for maintaining cell adhesion and preventing tumor cell metastasis.

Netrin

Netrin (*Ntn1*) signaling may also play a role in regulating tumor cell proliferation and metastasis; however, rather than the presence or absence of ligand mediating tumorigenesis, it is primarily the concentration of ligand that determines the outcome of ligand/receptor signaling [3]. The netrin receptors DCC and UNC5H are immunoglobulin superfamily members (Figure 1), and have been identified as “dependence receptors,” because in the absence of netrin-1 ligand these receptors are postulated to induce apoptosis [22]. In breast cancer cell lines, *Ntn1* is often highly expressed, as would be expected if its expression were essential for survival (Table 2) [23]. Furthermore, it was shown that reduction of *Ntn1* expression in these high expressing breast cancer cell lines results in increased apoptosis [23]. These findings support the hypothesis that NTN1 acts as a pro-survival factor in the breast, although the *in vivo* translation of this concept is controversial. *Ntn1* knockout mice

exhibit a small increase in apoptosis, but this only occurs in a select population of cells in the TEB during development and seems to be due to anoikis-induced apoptosis [11]. This suggests that in the mouse mammary gland, NTN1 is not an essential survival factor. Conversely, loss of *Dcc* receptor expression in a knockout model, which would presumably allow a cell to escape from NTN1 regulation, does not result in tumorigenesis [24]. This may be due to the fact that, as with other genes discussed in this review, loss of *Dcc* may increase tumor susceptibility, but is not sufficient to initiate tumorigenesis.

Studies performed using human breast cancer cell lines suggest the alternate possibility that NTN1 exerts its pro-survival effect via UNC5H, rather than DCC receptors [23]. In line with this idea, one study reported that *Unc5h* expression is lost in about 50% of breast tumors (Table 2) [25]. These tumors would presumably gain a survival advantage because they would no longer be dependent on NTN1, which is required to prevent receptor-mediated apoptosis. In this circumstance, the remaining 50% of tumors, which still express *Unc5h*, are expected to upregulate *Ntn1*, which would protect the cells from unliganded receptor. Currently, it is unclear when during tumor progression *Ntn1* expression becomes upregulated. A different study has shown that *Ntn1* is rarely upregulated in primary tumors, but does show massive upregulation during tumor progression [23]. This finding suggests that NTN1 pro-survival signaling is important for promotion of more aggressive, metastatic breast cancers, but may not play an important role in the primary tumor during its initiation. The importance of NTN1 in regulating tumor progression is highlighted by the findings that breast cancer cell lines, expressing high levels of *Ntn1*, also tend to be highly aggressive and form metastases in mice, while knockdown of *Ntn1* by injection of *Ntn1* siRNA into mice reduces the formation of lung metastases by 4T1 cells [23]. Taken together, these studies suggest that in metastases that retain UNC5H expression, high levels of NTN1 promote further tumor progression and metastasis by conferring survival advantage; however, more studies will be required to confirm this hypothesis.

The consequences of NTN1 signaling through the neogenin (*Neo1*) receptor are not well described in breast cancer, but indicate that NEO1 may play a tumor suppressor role. *Neo1* expression is lost in almost 95% of invasive ductal carcinomas (Table 2) [26]. Although loss of expression is not commonly seen in breast cancer cell lines, one study found that expression was lost in a breast cancer cell model where progressive environmental insults result in incremental increases in tumorigenicity and corresponding progressive decreases in *Neo1* expression [26]. Knockout mouse studies have shown that NTN1/NEO1 interaction is necessary for the appropriate adhesion and stabilization of the highly proliferative cap cells of the TEB, and that loss of *Ntn1* and *Neo1* leads to breaks in the basal lamina, a phenomenon necessary for tumor progression [11]. These findings suggest that NTN1/NEO1 signaling may be an important in preventing tumor metastases, but warrants further exploration.

Netrin-4 (*Ntn4*) may also act as a ligand for NEO1 and UNC5H, but there is little indication that it is necessary to prevent dependence-mediated apoptosis like NTN1 [27–29]. In fact, while *Ntn4* is normally expressed by epithelial cells of the breast, its expression is often suppressed by hypermethylation during tumorigenesis [27, 30, 31]. In matched normal and tumor samples from breast cancer patients, *Ntn4* is expressed at lower levels or lost entirely

in tumors, and expression is often lost in breast cancer cell lines (Table 2) [27, 31]. Interestingly, NTN4 has a biphasic effect on cell proliferation, where low levels stimulate cell growth, and high concentrations inhibit cell growth [27]. The downstream pathways regulating this biphasic effect have not been explored, but work done in pancreatic cells shows that NTN4 can inhibit the Jnk pathway leading to decreased phosphorylation of JNK2, AKT, and JUN, and presumably decreased tumor cell proliferation and survival [27]. Thus, loss of *Ntn4* during tumor progression would be presumed to confer survival advantage on tumor cells. Much research still needs to be done to determine the role of NTN4 in breast cancer development.

Eph/Ephrin

Unlike the other AGMs, that have clear ligand and receptor categories, Ephs and ephrins can signal bidirectionally, with the potential to behave as both ligand and receptor. Forward signaling, which we will address in this review, is dependent on Eph tyrosine kinase activity and propagates in the Eph-expressing cell. Reverse signaling, which may play a role in breast cancer angiogenesis, depends on Src-family kinases and propagates in the ephrin-expressing cell [5, 32]. Ephs, the tyrosine kinase receptors for ephrins, are classified into EphA and EphB subtypes, which generally correspond to their ligand preference (Figure 1). The Ephrin ligands are classified as members of the ephrin-A or ephrin-B families based on their plasma membrane association, which is either GPI-anchored or transmembrane, respectively (Figure 1). Although a number of Ephs and ephrins are expressed in the mammary gland, the majority of breast cancer research has focused on ephrin-A1 (EFNA1)/EPHA2 and ephrin-B2 (EFNB2)/EPHB4. In both of these Eph/ephrin pairs, the ephrins play a tumor suppressor role by regulating the expression and activity of the Eph receptors. In the absence of EFNA1 or EFNB2, the activity of EPHA2 and EPHB4 is oncogenic and promotes both cell proliferation and metastasis.

EFNA1 acts as a tumor suppressor by initiating EPHA2 forward signaling, as well as by triggering the ligand-dependent phosphorylation, internalization, and degradation of EPHA2, which can otherwise act oncogenically [33]. EPHA2 is normally expressed at low levels in the mammary gland, but its expression is significantly increased in 40% of breast cancers (Table 2) [34]. Overexpression of *EphA2* in MCF10A cells, a non-tumorigenic epithelial cell line, confers the ability to give rise to colonies *in vitro*, as well as tumors *in vivo* [7, 34]. In contrast, high expression levels of the EFNA1 ligand correlate with a more “normal” epithelial-like phenotype in breast cancer cells and can inhibit colony formation in MDA-MB-231 and MCF10A cells that overexpress EPHA2 [34, 35]. EFNA1 initiation of EPHA2 forward signaling in breast cancer cells results in increased caspase-3 activity, reduced ERK activation and attenuated Ras/MAPK pathway activation in response to EGF (Figure 2) [35, 36]. Thus, EFNA1 signaling normally prevents tumor formation by inhibiting proliferation and promoting apoptosis of EPHA2-expressing cells. When the ratio of EFNA1:EPHA2 becomes unbalanced during tumorigenesis, EFNA1 no longer regulates EPHA2-expressing cells, and EPHA2 ligand-independent signaling is able to promote tumor progression.

EFNA1 can also inhibit EPHA2-mediated breast cancer metastasis. In non-metastatic cells, EPHA2 co-localizes with E-cadherin at the cell membrane at points of cell-cell contact where contact with EFNA1 maintains forward signaling and promotes cell adhesion [37]. In metastatic cells, EPHA2 expression decreases at points of cell-cell contact, instead becoming diffuse or enriched within membrane ruffles at the leading edge of migrating metastatic cells, where it colocalizes with F-actin. EFNA1-mediated tyrosine phosphorylation of EPHA2 is also decreased in metastatic cells, however EPHA2 remains active through gain of ligand-independent oncogenic signaling [37]. If overexpression of EPHA2 occurs in “normal” MCF10A cells, it leads to their malignant transformation, allowing them to rapidly form tumors that show invasive characteristics, including loss of cell-cell contact and decreased cell-ECM adhesion [34]. This transformation can be reversed by treatment with EFNA1, which impairs cell migration and anchorage-dependent growth in breast cancer cells [38]. Concordantly, loss of *EphA2* expression in *MMTV-Neu;EphA2*-/- mice or knockdown of its expression in 4T1 cells results in impaired lung metastasis and decreased motility in transwell migration assays [7, 39]. These data suggest that in *Efna1*-expressing tumors, EFNA1-mediated degradation of EPHA2 prevents metastasis. The loss of *Efna1* expression during tumor progression leads to overexpression of EPHA2 and, consequently, a more invasive, metastatic phenotype.

Several mechanisms may be involved in mediating EPHA2-induced migration. First, RhoA activation appears to play a role. Loss of *EphA2* in *MMTV-Neu;EphA2*-/- mice, decreases levels of both total and active-GTP-bound RHOA, and inhibits cell migration. Overexpression of activated RHOA restores cell motility, supporting the notion that RHOA activation contributes to EPHA2-mediated migration [7]. Second, EPHA2 may also promote migration through activation of the non-canonical Wnt pathway. Overexpression of *EphA2* in breast cancer cells results in upregulation of genes associated with the non-canonical Wnt pathway - four and a half LIM domains 2 (*Fhl2*) and *Wnt6*, both associated with the promotion of tumor invasiveness [40]. Third, *EphA2* overexpression increases FAK phosphorylation at tyrosine 925, which is associated with integrin adhesion and E-cadherin downregulation [40]. Lastly, EPHA2 also interacts with Ephexin4, a Dbl family GEF, leading to local activation of Rac by DOCK4, formation of cortactin-rich protrusions, and promotion of ligand-independent cell polarization and migration [41]. Thus, *EphA2* overexpression in breast cancer results in the activation of a number of pathways involved in promoting migration and invasiveness.

Estrogen appears to play a significant role in mediating EPHA2 signaling. An inverse correlation between estrogen receptor (ER) status and EPHA2 expression exists in which ER-overexpressing tumors show little or no EPHA2 expression, and ER-negative tumors show high levels of EPHA2 expression [42]. Furthermore, estradiol treatment of non-transformed mammary epithelial cells decreases EPHA2 expression in a dose-dependent manner, an effect that is reversible by tamoxifen. This suggests that one of the consequences of losing normal ER signaling during cancer progression is increased EPHA2 expression, which contributes to an increasing aggressive phenotype. It also appears that EPHA2 desensitizes breast cancer cells to the effects of estrogen because tumors derived from *EphA2*-overexpressing ER+ MCF-7 cells increase in size in response to estrogen, but retain

their tumorigenic potential in the absence of supplemental estrogen and are less sensitive to tamoxifen [43]. Further studies have shown that a monoclonal antibody, which mimics the binding of EFNA1 to EPHA2, reverses this effect of *EphA2* overexpression and restores tamoxifen sensitivity [40]. Thus a potentially promising therapeutic strategy could involve dual targeting of EPHA2 and ER, with the goal of re-sensitizing breast cancer cells to tamoxifen by restoring the normal regulation of EPHA2 in breast tumors overexpressing this AGM.

The relationship of EFNB2 to EPHB4 is similar to that just described for EFNA1/EPHA2 in which binding of EFNB2 to EPHB4 leads to tumor suppression through ligand-activation of forward signaling, involving EPHB4 phosphorylation and degradation [44]. Like EPHA2, ligand-stimulated EPHB4 forward signaling results in tumor suppression [44], and when EFNB2/EPHB4 forward signaling becomes perturbed, due to changes in expression levels during tumorigenesis, kinase-independent EPHB4 signaling can promote tumor progression [32].

An imbalance in EFNB2 and EPHB4 expression occurs during tumorigenesis, which may promote ligand-independent EPHB4 signaling. EFNB2, like EFNA1, is also lost during cancer progression, with only weak expression of EFNB2 observed in some invasive ductal carcinoma cells and with weak-to-absent EFNB2 expression in 75% of sampled breast cancer cell lines, while non-transformed cells show high EFNB2 expression (Table 2) [44, 45]. EFNB2 expression is also absent in two cancer models, Wap-*ras* and Wap-*myc* tumors [46]. The loss of EFNB2 in breast tumors correlates with increased EPHB4 expression, affirming the ligand/receptor relationship whereby EFNB2 ligand keeps EPHB4 receptor expression in check in normal tissue. EPHB4 expression, similar to EPHA2, is increased in a large proportion of breast cancers, with one study showing that 65% of breast cancers had moderate to strong straining of EPHB4, usually with cytoplasmic localization (Table 2) [44, 47, 48]. Moreover, expression of EPHB4 is increased with clinical stage and histological grade of the tumor and positively correlates with DNA aneuploidy and S-phase fraction; however, there is no association with patient survival [48]. These studies suggest that in the absence of EFNB2, EPHB4 may provide a survival advantage, and in fact, overexpression of EPHB4, alone, may be sufficient for its activation [44]. Short-term activation of EPHB4 forward signaling in breast cancer cells using clustered EFNB2, which allows for EPHB4 activation but not its internalization and degradation, results in increased phosphorylated-AKT [44]. This suggests that in the absence of EFNB2, which terminates EPHB4 forward signaling, EPHB4 may promote constitutive pro-survival AKT signaling (Figure 2). Furthermore, this PI3K–AKT pathway may also regulate EPHB4 expression in a feed-forward loop as treatment with PI3K or AKT inhibitors leads to complete loss of EPHB4 expression in SK-BR-3 cells. Thus, one way EFN2B inhibits the oncogenic activity of EPHB4 is by preventing its constitutive, pro-survival signaling, which can occur upon its overexpression.

A second way that EFNB2 appears to function as a tumor suppressor is by signaling through EPHB4 to actively inhibit proliferation. This has been demonstrated in breast cancer cell lines by treating them with EFNB2-Fc that mimics ligand binding and inhibits spheroid growth of MCF-7, MDA-MB-231, and MDA-MB-453 cells by reducing proliferation and

enhancing apoptosis [45]. Other studies have shown that this EFNB2/EPHB4 forward signaling is through the Abl-Arg tyrosine kinase family and ultimately acts to inhibit Rac (Figure 2) [45]. Rac has been implicated in breast cancer cell proliferation and may also promote metastasis by upregulating MMP expression. The notion that Abl/Arg is downstream of EFNB2 is supported by studies in which the Abl-inhibitor, Gleevec, blocks the effects of EFNB2 on cell growth and survival, and also abolishes the inhibition of tumor growth that can be achieved *in vivo* using EFNB2-Fc [45]. Taken together, these studies show that EFNB2 functions as a tumor suppressor by both actively engaging EPHB4 in anti-proliferative signaling through Ras and by promoting the degradation of EPHB4, which, as a result, prevents its constitutive oncogenic signaling.

EFNB2 also inhibits breast cancer cell migration, probably through bi-directional signaling. In MCF10A cells, EFNB2/EPHB4 is concentrated at cell-cell junctions. Blocking the ligand/receptor association using an antagonist peptide is sufficient to disturb the integrity of the junctions [45]. Studies show that activation of EPHB4 forward signaling with EFNB2-Fc reduces cell migration, decreases Crk activation and inhibits MMP-2 expression and these effects in turn restrict cell motility and invasion. Conversely, knockdown of *EphB4* in breast cancer cells, in which EPHB4 has presumably gained ligand-independent activity, leads to similar decreases in MMP-9 and MMP-2 activity as well as uPA levels, and reduced breast cancer cell migration and invasion [44]. *In vivo*, there is evidence that bi-directional or reverse signaling is important for metastasis prevention. Overexpression of either a mutant *Efnb2* that is unable to reverse signal or *EphB4* under an MMTV-NeuT background increases incidence of metastasis [8, 9, 49]. The mechanism by which EFNB2 reverse signaling controls metastasis is unknown, but one possibility is that as demonstrated with EFNB1 in *Xenopus*, tyrosine phosphorylation of EFNB2 may disrupt its association with the Par polarity complex member, PAR6, allowing PAR6 to interact with CDC42-GTP, inducing aPKC, and establishing tight junctions [50, 51]. Loss of EFNB2 or loss of reverse signaling would thus lead to disruption of tight junctions, which might account for the increased incidence of metastasis. Together these studies provide further evidence that EFNB2 ligand binding of EPHB4 is necessary to maintain normal cellular adhesion and inhibit inappropriate cell migration [45].

Semaphorins

Semaphorins are unique among the AGMs both in their expression and the manner in which they act as tumor suppressors. There are 21 semaphorins expressed in vertebrates that are divided into 8 classes, with only classes 3–7 expressed in vertebrates. The primary focus of this review is Class 3 semaphorins and they are secreted proteins, whereas class 4–7 semaphorins are membrane-anchored (Figure 1). Semaphorin receptors are plexins, which consists of 4 subfamilies (types A-D), and neuropilins 1 (NP1) and 2 (NP2) (Figure 1). Class 4–7 semaphorins and SEMA3E bind directly to specific plexins and activate plexin-mediated signal transduction, while the remainder of Class 3 semaphorins bind to neuropilins, which act as the binding receptor, and then associate with type A plexins or plexinD1 (PLXND1) to mediate signal transduction [4]. A number of semaphorins are upregulated in tumors, suggesting that they are important players in tumorigenesis (Table 2).

The ratio of VEGF to SEMA3 expression may be a key determinant in tumor progression. Several studies show that $VEGF_{165}$ and a subset of Class-3 semaphorins, SEMA3A, 3B, and 3F, both bind to the b1 domain of neuropilins, and thus may act as competitive inhibitors to each other [52, 53], while an alternative view is that both ligands can bind to neuropilins at independent binding sites to initiate antagonistic signaling pathways [54]. When $VEGF_{165}$ levels are higher than SEMA3A, 3B, and 3F, VEGF165 binding to NP1 enhances breast cancer cell survival by maintaining constitutive elevation of PI3K activity [55, 56]. This effect is independent of VEGFR signaling, as $VEGF_{165}$ acts as a pro-survival factor in breast cancer cells, such as MDA-MB-231, which express NP1 and NP2, but not VEGFR1 or VEGFR2 [55, 57]. High levels of SEMA3A, 3B, or 3F block $VEGF_{165}$ binding to NP1, resulting in the inhibition of the PI3K/AKT pathway and promotion of apoptosis (Figure 2) [55]. Reducing the activity of the PI3/AKT pathway can also have consequences for cell migration through downstream stimulation of GSK-3beta activity and inhibition of pro-proliferative beta-catenin signaling. SEMA3A induction of GSK-3beta activity in a breast cancer cell line has been linked to increased expression of alpha2beta1 integrin, leading to increased adhesion, and decreased migration and invasion [58]. Thus, some members of the SEMA3 family function to inhibit breast cancer cell migration and promote their apoptosis, by inhibiting the binding of $VEGF_{165}$ to neuropilin, thereby blocking PI3/AKT activation in the mammary gland.

In contrast to the clear pro-apoptotic role of the previously described subset of class-3 semaphorins, the effect of other semaphorins on cell migration and metastasis is less well defined. SEMA3F repulses cell migration in NP2-expressing breast cancer cells, but does not alter motility in cells that only express NP1 [59, 60]. Instead, in NP1-expressing cells, SEMA3F reduces the levels of membrane-associated E-cadherin and beta-catenin, leading to a corresponding decrease in cell adhesion and eventual cell detachment from the tissue culture plate [59, 60]. These findings suggest that SEMA3F may play a pro-metastatic role by promoting tumor cell detachment, however the authors interpreted the results differently, proposing that SEMA3F may be upregulated in normal tumor-adjacent mammary epithelia during early tumorigenesis in an attempt to prevent tumor cells from spreading and attaching to stroma during extravasation [60]. Clearly, the *in vivo* implications of these *in vitro* studies merits attention to determine how SEMA3F affects breast cancer metastasis. In contrast, SEMA3B, previously described as a pro-apoptotic factor, promotes migration in breast cancer cells, suggesting that its expression may be beneficial during early tumorigenesis by inhibiting tumor growth, but could promote metastasis during later stages of cancer progression [61]. SEMA3C, another class-3 semaphorin has no reported effects on cell proliferation, but acts as a pro-metastatic AGM. Studies show that overexpression of SEMA3C in breast cancer cell lines results in increased migration, but whether this corresponds to increased metastasis *in vivo* has not been explored [62]. Instead, *in vitro* studies have shown that SEMA3C activity is regulated by ADAMTS1 cleavage, increasing its availability to tumor cells. ADAMTS1 is acutely upregulated in metastatic breast cancer cells, suggesting that co-expression of ADAMTS1 and SEMA3C in tumors may drive metastasis [62]. These studies demonstrate that, while class-3 semaphorins often act as tumor suppressors by suppressing cell proliferation during early tumorigenesis, they may switch to an oncogenic role during tumor progression by promoting tumor metastasis.

In contrast to class-3 semaphorins, SEMA4D regulates cell migration by mediating plexin binding to tyrosine kinases. SEMA4D is highly expressed in invading tumor epithelial cells, where it is can be diffusely detected in the cytoplasm or robustly on the cell-surface [63]. As described earlier, class-4 semaphorins bind directly to plexins to initiate plexin-mediated signaling. In breast cancer cells, SEMA4D activates PlexinB1 (PLXNB1) to promote or inhibit metastasis in a context dependent manner. Binding of SEMA4D to PLXNB1 can lead to stable association of PLXNB1 and activation of receptor tyrosine kinases MET or ERBB2, resulting in tyrosine phosphorylation of both receptors. Again, this appears to be a situation where the relative expression of receptors determines the activity of a Semaphorin [64]. In the presence of ERBB2, SEMA4D increases migration by activating the PI3K/AKT pathway, resulting in pro-migratory RHOA-mediated signaling (Figure 2). In contrast, in the presence of MET, SEMA4D inhibits migration through inhibition of integrin function, a process that involves R-RasGAP activity or P190RhoGAP-dependent RHO inhibition [64]. Thus, SEMA4D has opposing effects on RHO activity and cell migration, mediated by PLXNB1 interaction with either MET or ERBB2.

Putting AGMs into Context

SEMA4D's ability to act as both a pro-migratory and anti-migratory factor depending on expression of ERBB2 and MET underscores the importance of cellular context in ascribing tumor suppressor or oncogene labels to some AGMs [64]. EPHA2 also exhibits a context dependent oncogenic effect in which its loss only inhibits tumorigenesis under an *MMTV-Neu* background, which overexpresses ERBB2, but not in *MMTV-PyV-mT* transgenic mice, which expresses only moderate levels of ERBB2 [7]. This study also shows that EPHA2 physically interacts with and is phosphorylated by activated ERBB2 to promote tumor progression [7]. It is interesting that SEMA4D and EPHA2 both acquire oncogenic activity only in the context of ERBB2 overexpression. This suggest that blockade of ERBB2 overexpression during cancer treatment may have a secondary effect on these AGMs by "deactivating" their oncogenic activity.

Another principle that is repeated in most of the AGM families is the importance of relative ratios of ligands and receptors in determining oncogenic or tumor suppressor activities. This is the basic concept of NTN1 function, where in the presence of DCC or UNC5H, high levels of NTN1 are thought to be pro-survival, and thus oncogenic, while loss of NTN1 expression leads to induction of apoptotic signaling. In contrast, NTN4 appears to act in a converse manner where low levels of NTN4 promote proliferation, while high levels inhibit cell growth – reinforcing the concept that relative ligand/receptor levels determine function. This same concept has been echoed in Eph/ephrin signaling, wherein ephrins act to suppress Eph forward signaling, thus acquiring a role as tumor suppressors. When the balance of Eph/ephrin signaling is perturbed, either by loss of ephrin expression or Eph overexpression, EPHA4 and EPHB2 signaling is no longer suppressed, and they become oncogenic. Thus, changes in the relative expression of these ligand/receptor pairs during tumorigenesis can have a profound outcome on the role of these signaling pathways in promoting or inhibiting tumor progression.

Use of AGMs in Cancer Diagnosis and as Therapeutic Targets

AGMs show promise as breast cancer diagnostic/prognostic markers as well as potential therapeutic targets. We have already discussed the prognostic value of AGM expression in tumor samples. What is of even greater interest is that these changes can often be detected in patient blood plasma samples. Recent studies have shown that plasma NTN1 is increased in breast cancer patients [65]. *Slit2* methylation is also increased in breast cancer patients, with a complete concordance between tumor and paired sera [66]. These findings may form the foundation for the development of quick, non-invasive breast cancer prognostic tests, and in the case of *Slit2*, which appears to be methylated early during cancer progression, may lead to more effective early diagnostic tests. Targeting of pro-oncogenic or pro-metastatic AGMs using siRNA or cytotoxin-conjugated ligands, may also be an effective strategy for treating breast cancer. For example, injection of antisense-EphB4 oligo (siRNA) into mice that had been inoculated with tumor cells led to a reduction in tumor growth and smaller tumor size, with a corresponding decrease in proliferation and increase in apoptosis [44]. Similarly, injection of a cancer xenograft model with EFNB2-Fc, which like the antisense-EPHB4 oligo inhibits EphB4 forward signaling, results in decreased tumor growth [45]. As mentioned previously, treatment with a monoclonal antibody that mimics the binding of EFNA1 to EPHA2, inhibits EPHA2 oncogenic activity and restores tamoxifen sensitivity in breast cancer cells [40]. Likewise, treatment of EPHA2-overexpressing breast cancer cells with cytotoxin-conjugated EFNA1 induced apoptosis [67]. Targeting of NTN1 using siRNA or inducing its multimerization using a recombinant soluble fifth fibronectin domain of DCC also may be a potential therapy for inhibition of metastasis [23, 68]. These studies may pave the way for development of more effective breast cancer therapeutics in the future.

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Abbreviations

AGM	Axon Guidance Molecule
CUB	Complement-Homology Domain
EGF	Epidermal Growth Factor
ER	Estrogen Receptor
Ig	Immunoglobulin
MAM	Meptin/A5/Mu-Phosphatase Homology Domain
TEB	Terminal End Bud

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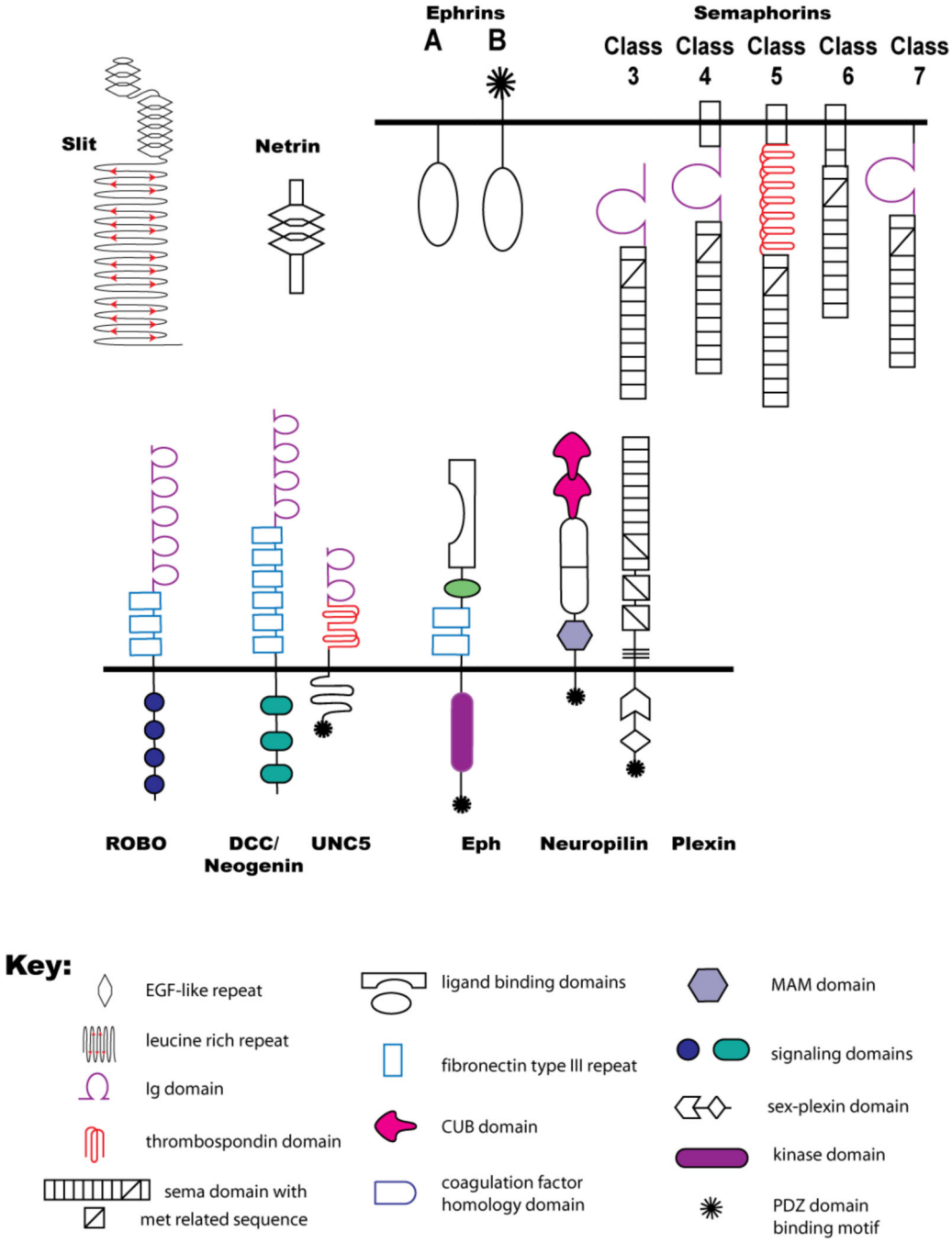

Figure 1. Axon Guidance Molecule Ligands and Receptors

Top: AGM ligands Slits, Netrins, and Class 3 Semaphorins are all secreted proteins, whereas Class 4–7 semaphorins and ephrins-A and B are tethered to the membrane via GPI or transmembrane linkages.

Bottom: AGM receptors are all single-pass transmembrane proteins.

Domain structures are schematically represented.

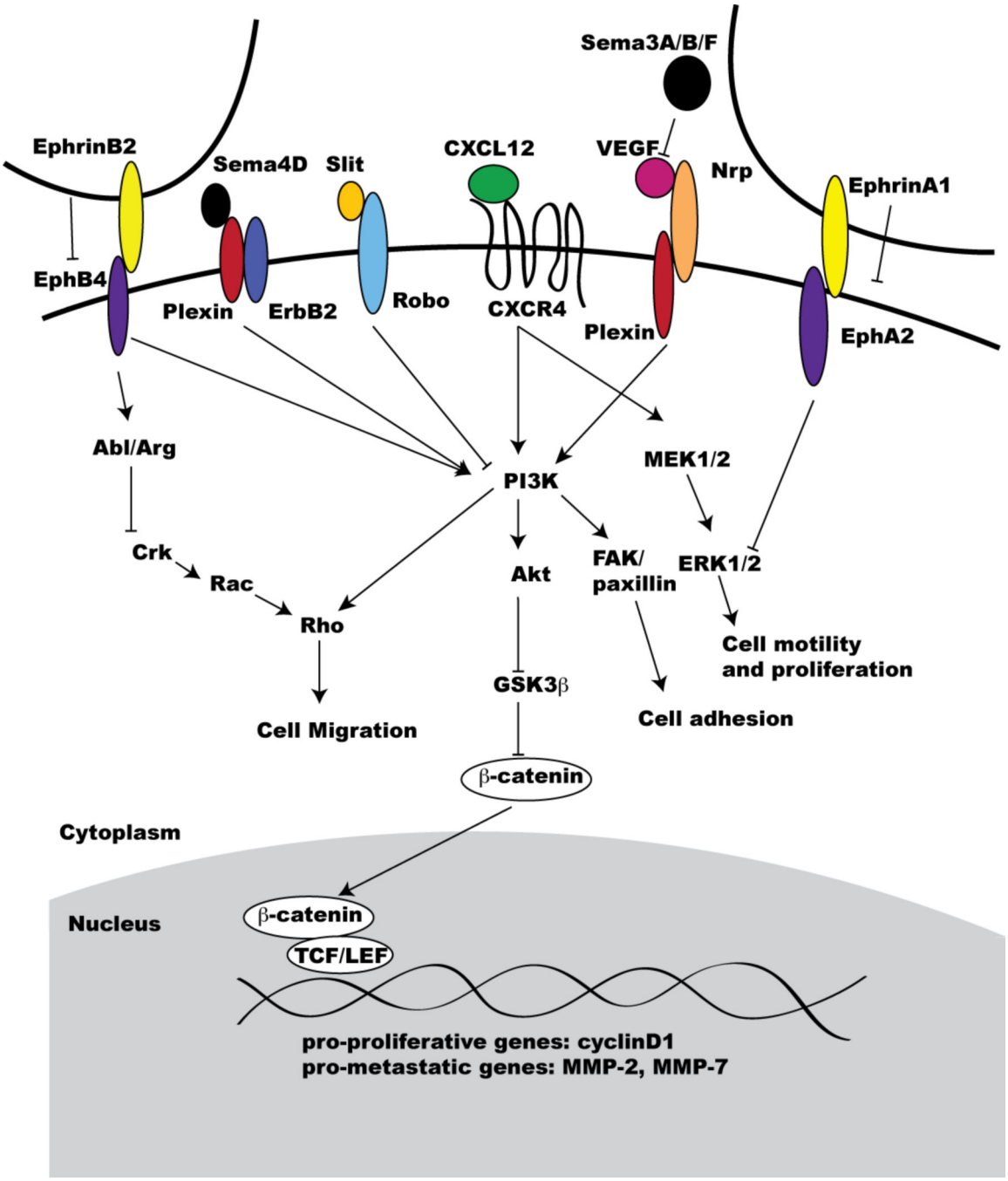

Figure 2. Axon Guidance Molecules Regulate Key Pathways involved in Cell Proliferation and Migration

Many AGMs regulate cell proliferation and migration through activation or inhibition of PI3K signaling. EPHB4 and SEMA4D are both positive regulators of PI3K signaling, leading to enhanced proliferation and migration, while EFNB2, SLITs, and SEMA3A, B, and F all inhibit PI3K signaling, by inhibiting EPHB4 kinase-independent signaling, CXCL12/CXCR4 signaling, and VEGF signaling respectively.

Table 1

Expression of Slit/Robo, Netrins, and Eph/Ephrins in the Mammary Gland

	Time of Expression		
	Puberty	**Adult**	**Cellular Localization**
Slit/Robo			
Slit2	+	+	epithelia [6]
Slit3	-	+	epithelia [6]
Robo1	+	+	puberty: cap cells of the TEB and myoepithelial cells, adult: some luminal cells, stroma [6]
Robo2	-	+	subset of myoepithelial cells [6]
Netrins			
Netrin-1	+	?	prelumenal epithelial cells during development and stroma [11]
Netrin-4	?	+	epithelia and basal lamina [30]
DCC	−	+	epithelia [11, 69]
Un5H	+	+	only expressed in fibroblasts during puberty [11, 25]
Neogenin	+	+	cap cells and subset of prelumenal cells during development [11]
Ephrins			
EphrinA1	?	+	luminal cells, stroma, and fat [70]
EphrinB1	+	?	enriched in TEB, but also expressed at low levels in ducts [71]
EphrinB2	+	+	ducts and end buds, luminal, absent during lactation [8, 46]
EphrinB3	?	?	[72]
EphA2	+	+	enriched in TEBs during puberty [71], expressed in luminal cells in adult [70]
EphA7	?	+	upregulated in the mammary gland during early pregnancy [72]
EphB3	?	?	[72]
EphB4	+	+	myoepithelia of ducts and alveoli [46]
EphB6	?	+	[73]
Semaphorins			
Sema3A	−	−	[74]
Sema3B	+	+	in TEB, but not ducts during development [74]
Sema3C	+	+	fat and stroma [74]
Sema3E	−	−	[74]
Sema3F	−	+/−	detected in human mammary gland, but not mouse [74, 75]
Sema4A	+	+	fat, stroma, and epithlia (Morris, 2006) Upregulated during lactation and involution [76]
Sema4B	−	+	upregulated during involution [76]
Sema4D	+	+	expressed in TEB, ducts, and stroma during development in mouse. [74] upregulated during lactation [76]
Sema4F	+	+	epithelia, fat, and stromal expression [74]

	Time of Expression		
	Puberty	**Adult**	**Cellular Localization**
Sema6B	−	?	[74]
Sema6C	−	+	[77]
Sema6D	−	+	[77]
Sema7A	−	+	Upregulated during involution [76]
Neuropilin1	+	+	epithelia, fat, and stromal expression [74]
Neuropilin2	−	+	expressed in adult epithelia, but absent during development [74]
PlexinA1	?	?	expressed in tumors, but normal expression unknown [78]
PlexinA2	+	?	expressed in epithelia [74]
PlexinA3	+	?	expressed in epithelia [74]
PlexinB1	?	+	[79]
PlexinB2	+	?	expressed in epithelia; enriched in TEB [74]
PlexinD1	+	?	epithelia, fat, and stromal expression [74]

Table 2

AGM Expression in Breast Cancer and Breast Cancer Cell Lines

Slit/Robo	
Slit2	Lost in 43–63% of breast cancers (methylation) [10, 12, 66]; reduced expression in breast cancer cell lines [12, 80]
Slit3	Lost in 16% of breast cancers (methylation) [10, 13]; reduced expression in breast cancer cell lines [13]
Robo1	Lost in 2–19% of breast cancers (methylation) [14]; Rarely lost in breast cancer cell lines (exon 2 deletion) [14, 80, 81]
Netrins	
Netrin-1	Increased in metastatic breast cancer [23]; Highly expressed in many breast cancer cell lines [23]
Netrin-4	Lost in breast cancer, particularly in ER- tumors [27, 30]; Not expressed in breast cancer cell lines [27]
DCC	Lost in breast cancer (LOH); Expression also lost in breast cancer cell lines [23, 69, 82–85]
Unc5H	Lost in ∼50% of breast cancers [25]
Neogenin	Lost in ∼95% of invasive ductal carcinomas; No loss seen in breast cancer cell lines [26]
Ephrins	
EphrinA1	No correlation between expression and breast cancer malignancy [86]
EphrinB2	Expression is lost in breast cancer cell lines [45]
EphA2	Increased in 40% of breast cancers [34]; Overexpressed in ER- breast cancer cell lines [36, 42]
EphB4	Increased in 23–65% of breast cancers (amplification of 7q.22 in 29% of cases) [44, 47, 48, 87]; Increased expression in breast cancer cell lines (amplification) [44, 47]
EphB6	Lost in metastatic breast cancer [73, 88]; Lost in invasive breast cancer cell lines (methylation) [73, 88, 89]
Semaphorins	
Sema3A	Expressed in breast cancer and breast cancer cell lines [78]
Sema3B	Unknown, but Sema3B is located at 3p21.3, a site of frequent allele loss and methylation in breast cancer [57]
Sema3E	Increased in 69% breast cancers (Christensen, 2005); Increased expression in breast cancer cell lines [90]
Sema3F	Unknown, but Sema3F is located at 3p21.3, a site of frequent allele loss and methylation in breast cancer [57]
Sema4D	Increased in breast cancers [63]
PlexinA1	Is expressed in cancers, although whether there are changes in expression is unknown [78]
PlexinB1	Increased expression in ER+ breast cancer [91] Lost in ER- breast cancers [79]