

# Numb

## A new player in EMT

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**E**pithelial to mesenchymal transition (EMT) is a critical event in embryogenesis and plays a fundamental role in cancer progression and metastasis.<sup>1</sup> Numb has been shown to play an important role in the proper functions of Par protein complex and in cell-cell junctions,<sup>2,3</sup> both of which are associated with EMT.<sup>4,5</sup> However, the role of Numb in EMT has not been fully elucidated. Recently, we showed that Numb is capable of binding to both Par3 and E-cadherin. Intriguingly, the interaction of Numb with E-cadherin or the Par protein complex is dynamically regulated by tyrosine phosphorylation induced by HGF or Src. Knockdown of Numb by shRNA in MDCK cells led to a lateral to apical translocation of E-cadherin and  $\beta$ -catenin, active F-actin polymerization, mis-localization of Par3 and aPKC, a decrease in cell-cell adhesion and an increase in cell migration and proliferation. These data suggest a diverse role for Numb in regulating cell-cell adhesion, polarity and migration during EMT.<sup>6</sup>

Numb was originally identified as a gene required for cell fate determination during neuroblast division and sensory organogenesis.<sup>7</sup> Recently, a number of proteins involved in cell polarity, cell-cell adhesion and tumorigenesis have been identified as binding partners for Numb. These include the Par3-Par6-aPKC polarity complex, E-cadherin, integrin, Notch, WNT and p53.<sup>8-10</sup> Although these new data implicate Numb in multiple signaling pathways, questions remain as to how the various interactions are regulated and

in which biological context they occur. Interestingly, most binding partners of Numb are involved in one way or another in the onset and/or progression of cancer. For instance, the Par complex is involved in regulating the formation and stability of tight junction whereas E-cadherin is a key component of the adherens junction in epithelial cells. Understandably, deregulation of the Par protein complex and/or E-cadherin is implicated in EMT. A variety of stimuli have been identified to induce EMT,<sup>1</sup> including transforming growth factor- $\beta$  (TGF $\beta$ ), hepatocyte growth factor (HGF), fibroblast growth factor (FGF) and activation of tyrosine kinase Src. During the progression of EMT, non-motile epithelial cells gradually lose their apical-basal polarity and cell-cell junctions and become mesenchymal cells with an ability to migrate away from the primary site to surrounding tissues.<sup>1</sup> Therefore, the study of Numb interactions with the Par protein complex and E-cadherin in the context of EMT provides a good point of entry to decode the complex signaling network mediated by Numb.

Based on data obtained from Madin-Darby canine kidney (MDCK) cells,<sup>6</sup> we propose a model in which Numb regulates epithelial polarity and cell-cell adhesion in EMT (Fig. 1). In epithelial cells, Numb binds to E-cadherin or the Par protein complex via Par3 under a normal physiological condition to stabilize adherens and tight junctions. Under the influence of an extracellular cue, such as HGF, the Met receptor recruits and activates one of its downstream components, c-Src.

**Key words:** Numb, E-cadherin, tyrosine kinase, cell polarity, adhesion, EMT

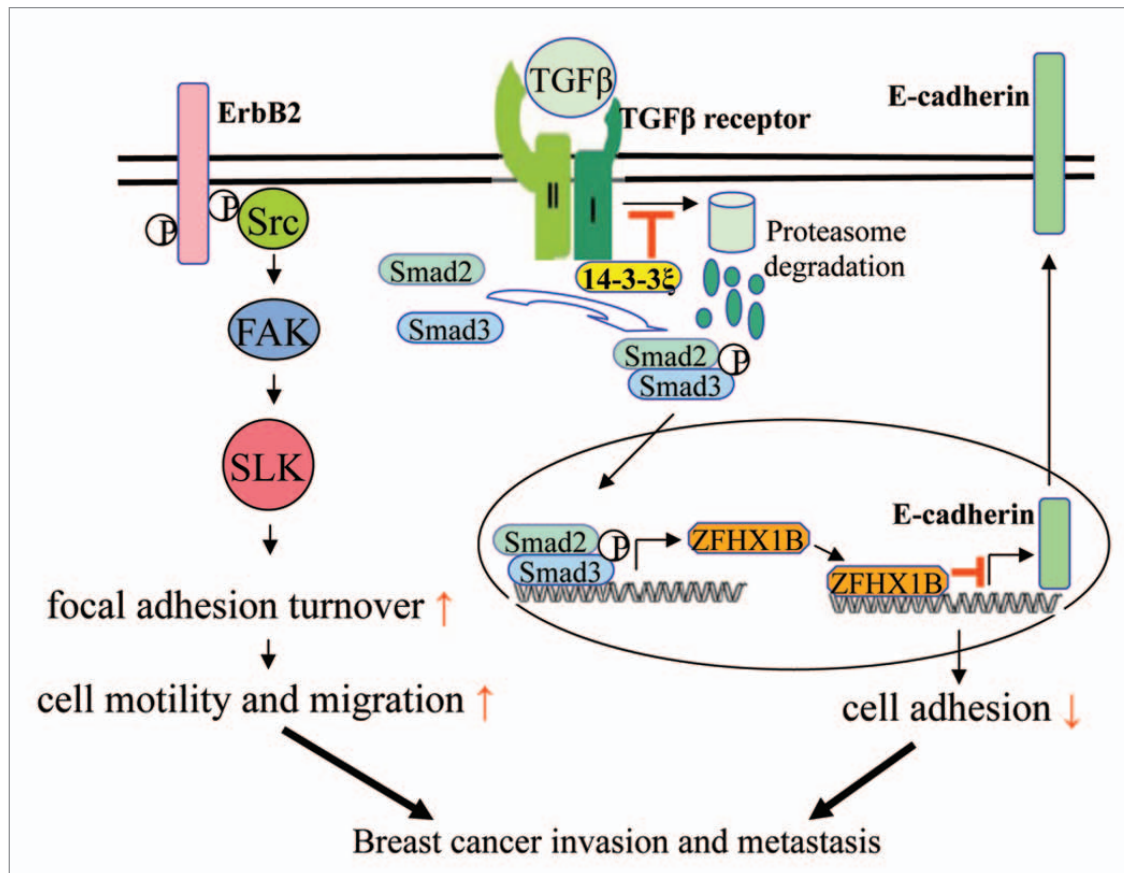
**Abbreviations:** sh-RNA, small hairpin RNA; MDCK, madin-darby canine kidney; aPKC, atypical protein kinase C; Par3, partitioning defective 3; Par6, partitioning defective 6; ts-src, temperature sensitive src mutant; Cdc42, cell division cycle 42; GDP, guanosine diphosphate; GTP, guanosine triphosphate

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**Figure 1.** A model depicting the role of Numb in epithelial-mesenchymal transition (EMT). In epithelial cells, Numb stabilizes both E-cadherin based adherens junctions through a PTB domain binding to NVYY motif on E-cadherin, and Par protein complex on tight junctions by interacting with Par3. During the early stage of EMT, c-Met recruits the tyrosine kinase c-Src upon its activation by HGF. C-Src phosphorylates E-cadherin on the NVYY motif, which leads to dissociation of Numb from E-cadherin and the translocation of E-cadherin to an apical domain. Numb forms a complex with phosphorylated aPKC and Par6, whereas phosphorylated Par3 is transported into the nucleus. Enhanced F-actin polymerization, together with reduced cell-cell adhesions, promotes the transition to mesenchymal cells.

C-Src subsequently phosphorylates the DNVYYY motif on E-cadherin, resulting in Numb dissociation from phosphorylated E-cadherin. Numb then binds and sequesters phosphorylated aPKC and Par6, while phosphorylated Par3 is released from the Par complex. The Numb-aPKC-Par6 complex remains on the plasma membrane or in the cytoplasm, whereas Par3 is transported into the nucleus (by an unknown mechanism). Phosphorylated E-cadherin is relocated to an apico-lateral domain accompanied with active F-actin polymerization. Enhanced F-actin polymerization, together with reduced cell-cell adhesion and increased cell proliferation, promotes cell migration (Fig. 1).

While our work in MDCK epithelial cells identifies a key role for Numb in regulating the sub-cellular localizations

and functions of E-cadherin and the Par protein complex, it raises a number of unaddressed questions. First, biochemical data obtained from temperature sensitive src mutant (ts-src) MDCK cell line suggest that tyrosine phosphorylation plays a critical role in regulating the dynamic interactions of Numb with E-cadherin or the Par protein complex. Using a peptide array screen, we found that a conserved DNVYYY motif in E-cadherin is the binding site for phosphotyrosine binding (PTB) domain of Numb. Interestingly, peptide analogs in which a Tyr residue in the YYY triad is replaced by a pTyr were deficient in binding. This suggests that the Numb PTB domain may act in a phosphotyrosine-independent manner in EMT signal transduction events, and that the interaction of Numb with

E-cadherin is negatively regulated by tyrosine kinase signaling. An intriguing question is why E-cadherin harbors a conserved YYY triad<sup>6</sup> when phosphorylation of one Tyr is sufficient to eliminate Numb binding. Furthermore, when and where are the YYY triad phosphorylated during EMT? It is likely that one or more tyrosine residues are phosphorylated by Src, depending on the duration of stimulus that a cell receives during EMT. Phosphorylated E-cadherin may be targeted to an alternative location in the cell by the E3 ligase Hakai which mediates the endocytosis and degradation of E-cadherin<sup>11</sup> after its phosphorylation and dissociation from Numb. To complicate the issue, Par3 and aPKC themselves are also shown to be tyrosine phosphorylated by HGF treatment or Src activation in our studies, although the precise

phosphorylation sites on either protein remain to be determined. In a previous report, a high-throughput phosphoproteomic screen has identified multiple tyrosine phosphorylation sites in the carboxyl terminus of Par-3.<sup>12</sup> Phosphorylation of Y1127 in Par-3 reduced its interaction with LIMK2 and delayed tight junction assembly in mammalian epithelial cells with a constitutive Src activation or under EGF treatment.<sup>12</sup> A recent study, which shows that the SH2 domain of C-terminal Src kinase (Csk) binds to the Y1127 site on Par3, adds another layer of complexity to the scenario.<sup>13</sup> In the same vein, Src has been shown to associate with aPKC and phosphorylate the latter in PC12 cells.<sup>14</sup> Par6 appears also to be subjected to Src regulation as recruitment of Src by Pals leads to Pals1 and Par6 to be sequestered away from JAM-C of desmosome adhesions in the blood-testis-barrier, causing a disruption in cell adhesion.<sup>15</sup> It will be of interest to determine whether Par6 is directly phosphorylated by Src in this case.<sup>15</sup> Together, these studies suggest a role for tyrosine phosphorylation, besides serine/threonine phosphorylation, in regulating the functions of various polarity proteins.

Second, our biochemical and immunofluorescence data suggest that the interactions of Numb with E-cad/Par complex are spatially and temporally regulated in response to HGF treatment. However, the molecular mechanisms for some remarkable phenotypes remain poorly defined. For instance, shRNA-mediated Numb knockdown caused a dramatic apicolateral mis-localization of E-cadherin and  $\beta$ -catenin. Dynamic cellular localization may be regulated by endocytosis and post-translational modifications such as phosphorylation. In keeping with this, Numb can directly bind to several endocytic proteins, including AP2 and Eps15, through its conserved DPF and NPF motifs.<sup>16</sup> Our data suggest that Numb is responsible for targeting E-cadherin to correct localization on the basolateral membrane, but we cannot rule out the possibility that other proteins may be involved in this process. One candidate that may facilitate E-cadherin localization is Rab11. Previous studies revealed that in newly polarized MDCKII cells, Rab11

mutation causes an apical mis-localization of E-cadherin and aberrant actin localization, while leaving ZO-1 localization unchanged. This mirrors the phenotype we observed in the Numb shRNA cells.<sup>17</sup> In support of the notion that Rab11 and Numb may be functionally related, Rab11 and Numb are segregated selectively in the p11b daughter cell during the division of a sensory organ precursor (SOP).<sup>18</sup> It is likely that the mis-localization of E-cadherin is caused by an abnormal endocytosis of E-cadherin in the absence of Numb.

In contrast, the Par protein complex provides critical spatial information in the formation of tight junctions. Treatment of MDCK cells by HGF caused Par3 to dissociate from Par6-aPKC, suggesting that the Par3-Par6-aPKC complex<sup>19</sup> is dynamically regulated. Additionally, both Par3 and aPKC are found to translocate to the nucleus following HGF treatment. A key question, therefore, is how the localizations of these polarity proteins themselves are regulated during EMT. Through a PB1-PB1 domain heterodimerization,<sup>20</sup> Par-6 binds to aPKC and thereby inhibits its catalytic. Moreover, Par6 also helps recruit substrates to aPKC,<sup>20</sup> one of which is Par3. Par6 binds to Par3, through a PDZ-PDZ interaction, but aPKC can also bind directly to Par3 through its kinase domain, and phosphorylates a Ser residue on Par3.<sup>21</sup> Importantly, depending on the cellular context, Par3, Par6 and aPKC may not form a constitutive complex.<sup>20</sup> For example, in *Drosophila* neuroblasts and embryonic epithelial cells, Par3 apical localization is independent of aPKC,<sup>22</sup> while in mammalian epithelial cells, Par3 is not apical but is associated with tight junctions.<sup>23</sup> Interactions of these polarity proteins are dynamically regulated by multiple protein kinases, small GTPases, or competition from other binding partners.<sup>19</sup> The dynamic change of components of the Par complex leads to the different sub-cellular distribution of these individual polarity proteins, and thereby alters their functions.<sup>19</sup> We showed that activation of Src kinase or HGF treatment reduces the association of Par3 with aPKC, but does not change the binding between aPKC and Par6. In support of this, a similar observation is made in MDCK cells with activation of tyrosine kinase ErbB2,

although neither Par3 nor Par6 is a substrate of ErbB2.<sup>24</sup> The dynamic assembly and dissolution of the Par3-Par6-aPKC complex in response to intracellular cues or extracellular stimuli may be a general mechanism used by metazoan to control cell polarity and movement.

Lastly, active F-actin polymerization is one of most striking morphologies we observed in the Numb-shRNA cells. Given that the Numb-shRNA cells exhibit a faster rate of migration and wound healing than control cells, it is likely that the active F-actin polymerization is caused by aberrant activation of the Rho family GTPase activity. Small GTPases of the Rho family control organization of cytoskeleton, cell motility, cell growth, morphogenesis, cytokinesis and trafficking.<sup>25</sup> The most common members of small Rho GTPases are RhoA, Rac1 and Cdc42. RhoA is responsible for the activation of stress fibers and cell contractility.<sup>25</sup> Rac1 activation leads to polymerization of filamentous actin, which results in lamellipodium formation and membrane ruffling at the leading edge of migrating cells.<sup>25</sup> Cdc42 activation causes the formation of filopodia, long finger-like protrusions at the edges of lamellipodia.<sup>25</sup> A Rho family of GTPase exists in two states: a GDP-bound inactive state and a GTP-bound active state. The switch between the two states is regulated by a large group of guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs).<sup>25</sup> GEFs and GAPs typically contain interaction domains which direct the enzymes to specific sub-cellular locations and help recruit upstream/downstream partners to affect processes such as actin cytoskeleton, cell polarity, microtubule dynamics and membrane transport.<sup>25</sup> It is often difficult to pinpoint exactly which member of the Rho family GTPases leads to a specific phenotype due to the overlapping functions of the Rho GTPase members. However, several observations have suggested that certain small Rho GTPases are essential for the establishment of the apico-basal polarity and likely interplay with Numb. Deletion of Cdc42 abolished normal localization of aPKC, Par6 and Numb in neuroepithelium.<sup>26</sup> Par6 binds to GTP-bound Cdc42 through a Rho GTPase-binding

Cdc42/Rac-interactive binding (CRIB) domain.<sup>23,27</sup> Another line of evidence is that Numb binds to intersectin, a Cdc42 GEF, and enhances the GEF activity of intersectin, leading to activation of Cdc42 in vivo.<sup>28</sup> It appears that Numb both regulates and is regulated by Cdc42 temporally and spatially. Par3 has also been reported to sequester Tiam1, a Rac GEF, to inhibit Rac activation in hippocampal neurons.<sup>29</sup> Nevertheless, Par3 recruits Tiam1 to activate Rac in keratinocytes.<sup>30</sup> Thus, the dynamic interplay between Rho GTPases and the Par protein complex may be cell type-dependent. It will also be of interest in the future to determine whether other GEFs/GAPs interact with the Par protein complex, and whether and how these interactions are modulated by Numb.

A more detailed understanding of the role of Numb in EMT will undoubtedly provide valuable insights into the molecular basis of cancer metastasis and suggest novel therapeutic strategies for cancer.

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