Review

Metastasis: tumor cells becoming MENAcing

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During breast cancer metastasis cells emigrate from the primary tumor to the bloodstream, and this carries them to distant sites where they infiltrate and sometimes form metastases within target organs. These cells must penetrate the dense extracellular matrix comprising the basement membrane of the mammary duct/acinus and migrate toward blood and lymphatic vessels, processes that mammary tumor cells execute primarily using epidermal growth factor (EGF)-dependent protrusive and migratory activity. Here, we focus on how the actin regulatory protein Mena affects EGF-elicited movement, invasion and metastasis. Recent findings indicate that, in invasive migratory tumor cells, Mena isoforms that endow heightened sensitivity to EGF and increased protrusive and migratory abilities are upregulated, whereas other isoforms are selectively downregulated. This change in Mena isoform expression enables tumor cells to invade in response to otherwise benign EGF stimulus levels and could offer an opportunity to identify metastatic risk in patients.

Introduction

A traditional view of metastasis holds that metastases result from a process similar to Darwinian evolution involving the natural selection of tumor cells that are capable of migration and survival at distant sites. In this model, tumor cells exhibiting stable genetic changes are selected for; these selected cells are very rare and cause metastasis late in tumor progression [1]. The recent development of new technologies, including high-density microarray-based expression profiling, intravital imaging and the collection of invasive tumor cells from live tumors, has challenged this traditional model of metastasis. These technologies have also supplied new diagnostic and therapeutic markers of metastatic disease. Studies of mammary tumors in mice [2–5], expression profiling of whole human breast tumors [6,7], and collection and profiling of the invasive subpopulation of tumor cells isolated from rat and mouse mammary tumors [8–10] indicate that metastatic ability is acquired at much earlier stages of tumor progression than predicted by the Darwinian model, is encoded throughout the bulk of the primary tumor, and involves transient changes in gene expression.

These results can be reconciled with the Darwinian model if the selection of stable genetic changes in the primary tumor during progression contributes the microenvironments necessary to induce the transient changes in gene expression that support the invasive and metastatic phenotype. The stable genetic changes required for induction of the microenvironments of invasion and cell dissemination could occur early in progression and throughout the tumor. The tumor microenvironment invasion model, which is based on this idea, holds that the tumor microenvironment initiates the expression of genes that induce cell motility, invasion and metastasis [9–11]. In this model, it is proposed that oncogenic mutations in tumor cells in the primary tumor lead to microenvironments that induce cell motility in tumor cells and stromal cells. Examples of such microenvironments are increased microvascular density [12], inflammation [13], and hypoxia [14]. These microenvironments are speculated to elicit transient and epigenetic changes in gene expression in tumor and stromal cells that resemble the programs of gene expression used to drive morphogenetic cell movements in the developing embryonic organ. When the primary tumor is located in an adult organ, tumor microenvironments could trigger the embryonic program of gene expression of this organ, leading to epithelial to mesenchymal transition (EMT) and the morphogenetic-like movements of cells clinically referred to as invasion and metastasis.

The tumor microenvironment invasion model predicts that microenvironments causing invasion and metastasis could appear randomly in time and location in the primary tumor, leading to repeated episodes of invasion and systemic tumor cell dissemination (potentially leading to metastasis) throughout tumor progression [9]. Consistent with this model, intravital imaging of experimental mammary tumors demonstrates that only a small proportion of tumor cells are motile but that they are distributed throughout the tumor and are observed most frequently localized in certain areas of the tumor, particularly around peri-vascular macrophages [15–17]. Furthermore, genes correlating with metastatic outcome in a variety of solid tumors appear to be expressed early and throughout the bulk of the tumor [6,7], and invasive mammary tumor cells can be collected throughout tumors with chemoattractant-containing needles [5,10]. The model is also supported by the observation that micrometastases are often genetically heterogeneous, suggesting that invasive behavior is not stably specified [18]. Finally, the tumor microenvironment invasion model is generally consistent with our current understanding of how the tumor microenvironment contributes to invasion and metastasis [19].

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Expression profiling of invasive tumor cells collected from primary mammary tumors reveal an invasion signature – a list of genes whose expression is uniquely altered in invasive tumor cells – that involves motility pathways accounting for the migratory and chemotactic activity of these cells *in vivo* [8–11]. One of the molecules that was highly upregulated in the invasive mammary tumor cells collected *in vivo* is Mena [10], consistent with observations that high Mena levels are associated with poor clinical outcome in breast cancer patients [20,21]. Mena (from mammalian homolog of *Drosophila* Enabled), an actin regulatory protein, influences several of the motility pathways of the invasion signature by controlling the actin polymerization that is initiated in common by these pathways [10,22]. The frequency of a tripartite structure – comprised of perivascular tumor cells expressing high Mena levels in direct contact with perivascular macrophages and endothelial cells within breast cancer patient samples – correlates well with the likelihood of metastatic disease, suggesting that Mena will be a useful prognostic biomarker for metastasis [23].

The goal of this review is to outline recent approaches developed in mammary tumors to investigate the properties of the tumor microenvironment and how tumor cells in this setting can acquire an invasive, metastatic phenotype by changes in gene expression. We also provide a background on the Mena protein, summarize recent findings indicating that changes in Mena expression promote metastatic progression, and discuss the possible mechanisms that underlie Mena's effects on tumor cell behavior.

Identifying and collecting invasive tumor cells

To detect tumor cell behaviors in primary mammary tumors that resemble 'morphogenetic' cell movements, as described above, much effort has been expended to develop intravital imaging methods that enable detailed characterization of the behavior of carcinoma and stromal cells within intact primary tumors [15,24–28]. The resulting methods yield quantitative information about individual cell behavior *in vivo*, permitting analysis of parameters including directional migration toward histological landmarks such as blood vessels, the frequency, velocity and persistence of cell motility, interactions between tumor cells, extracellular matrix and stromal cells that lead to invasion, and intravasation and extravasation. These imaging methods are valuable in defining cell behaviors necessary for invasion, intravasation and extravasation, phenotypes of cells harboring specific mutations, polarized motility and chemotaxis of cells *in vivo*, and the definition, size and regulation of microenvironments *in vivo*.

In mammary carcinomas, intravital imaging in mice and rats revealed the microenvironments in which tumor cells undergo migration and intravasation, and the importance of macrophages in these events (reviewed in [13,15,29,30]). In particular, chemotaxis of tumor cells toward macrophages was found to be essential for invasion in primary mammary tumors [5,31], whereas chemotaxis of tumor cells toward peri-vascular macrophages was required for intravasation [16]. Furthermore, invasion, intravasation and metastasis all involve a paracrine loop between macrophages and tumor cells which secrete EGF and colony-stimulating factor-1 (CSF1), respectively [5,31].

The point at which tumor cells migrate through the endothelium of blood vessels was identified as the site of blood vessel docking of at least one peri-vascular macrophage [16]. This detailed information about how tumor cells are attracted to blood vessels led to the development of the '*in vivo* invasion assay.' This assay mimics a blood vessel's microenvironment, being comprised of a tube filled with matrigel and EGF or CSF1 to attract invasive tumor cells and their associated macrophages *in vivo* as a migrating population of cells. The *in vivo* invasion assay has enabled the capture of live invasive tumor cells directly from the microenvironment for expression profiling [8,10].

Expression profiling of invasive mammary tumor cells collected *in vivo* defines an invasion signature

Expression profiling of invasive tumor cells obtained from mammary tumors using the *in vivo* invasion assay has revealed genes correlated with survival, adjuvant-resistance and chemotaxis of invasive cancer cells inside living mammary tumors [8,10,32–35]. These genes, known collectively as the invasion signature, fall into well-defined pathways and are coordinately regulated in metastatic tumor cells [9–11] (Figure 1).

The relevance of the invasion signature to the chemotactic migratory behavior of metastatic cancer cells during invasion and intravasation has been examined in a number of studies. A major insight to emerge from this body of work is that the motility pathways of the invasion signature define the mechanisms for tumor cell migration *in vivo* [11]. One of the genes highly upregulated in the motility pathways of the invasion signature of invasive tumor cells collected from rat and mouse mammary tumors is Mena [8,10]. Mena is also upregulated in human breast cancer [20,21,23] as well as pancreatic, colon, gastric and cervical cancers [36–39].

Mena and the tumor microenvironment of metastasis

The above results suggest that the level of Mena expression in tumors will be a useful biomarker for the evaluation of enhanced tumor cell motility, invasion, and metastasis in human tumors. In addition, as summarized above, invasive carcinoma cells in mouse and rat mammary tumors intravasate when associated with perivascular macrophages, thereby identifying a metastasis microenvironment as an anatomical structure in tumors [16,17]. We therefore define the tripartite arrangement identified by triple immunohistochemistry – an invasive carcinoma cell (marked by Mena overexpression), a macrophage, and an endothelial cell – as TMEM, for tumor microenvironment of metastasis. TMEM has been identified in human breast tumors using this technique [23]. In a retrospective study, TMEM density in human breast carcinoma samples was found to predict the development of systemic, hematogenous metastases. In this study, a case–control analysis was performed on 30 patients who developed metastatic breast cancer and 30 patients without metastatic disease. Cases were matched to controls based on currently used prognostic criteria. Primary breast cancer samples were stained using the triple immunohistochemical method to identify and count TMEM density. Two pathologists, blinded to outcome, evaluated the number of TMEM per

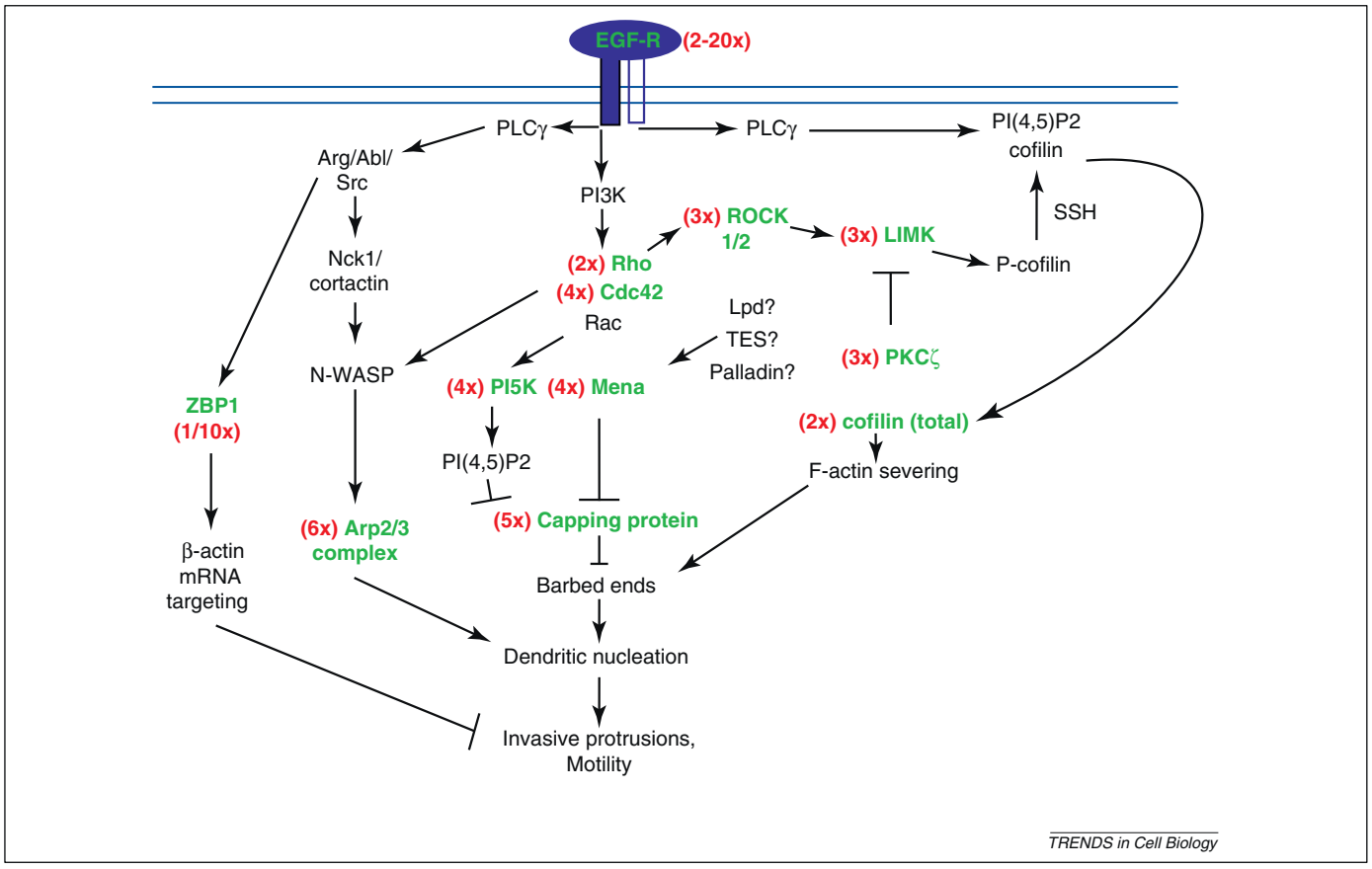

Figure 1. The motility and chemotaxis pathways of the invasion signature. Expression profiling of invasive tumor cells obtained using the *in vivo* invasion assay revealed the identities of the genes correlated with the survival, adjuvant-resistance and chemotaxis of invasive cancer cells inside living tumors. These genes fall into well-defined pathways and are coordinately regulated in metastatic tumor cells. These pathways are collectively called the invasion signature [8–11]. The motility and chemotaxis pathways of the invasion signature are shown here. Genes highlighted in green are those whose expression is altered in invasive tumor cells. The extents of over- and underexpression are indicated next to each (red text) as fold change at the mRNA level. Mena is upregulated in rat, mouse and human invasive mammary carcinoma cells [68]. Mena regulates cell motility by increasing the elongation of actin filament barbed ends produced by the cofilin and N-WASP pathways by antagonizing the ability of capping protein to bind barbed ends, increasing filament elongation rates, or both [58]. Mena function can be modulated by interactions with its binding partners, including Lpd [101], TES [111] and Palladin [108].

20 high-power fields. TMEM density was not correlated with tumor size, lymph node metastasis, lymphovascular invasion, or hormone receptor status. However, TMEM density was greater in patients who developed systemic metastases compared to the patients with only localized breast cancer. In addition, for every tenfold increase in TMEM density the risk of systemic metastasis increased by 90%. TMEM is therefore a novel prognostic marker for hematogenous metastasis of human breast tumors [23]. This work illustrates the power of combining multiphoton imaging with mouse models of breast cancer in the development of new insights into, and markers for predicting, metastasis and the microenvironments essential for the dissemination of tumor cells *in vivo*. The insights into metastasis provided by multiphoton imaging also help to refine or challenge existing models for the molecular mechanisms underlying metastatic progression and to develop hypotheses to be tested using cell biological and molecular approaches *in vitro*.

Mena and the Ena/VASP family in actin dynamics

As noted above, Mena [also referred to as 'ENAH' (Enabled homolog) by the Human Genome Organisation nomenclature committee database] is upregulated in human breast cancer and is a part of the cell motility pathways identified in the mammary tumor invasion signature. Mena, the mammalian ortholog of *Drosphila* Enabled (Ena), was first identified as a genetic suppressor of phenotypes caused by mutations in the *Drosophila* homolog of the c-Abl tyrosine kinase gene [40]. Mena, along with the highly-related VASP (vasodilator-stimulated phosphoprotein) and EVL (Ena/VASP-like) proteins, comprise the vertebrate members of the Ena/VASP family, molecules that regulate cell movement, shape and adhesion [41,42], processes required during invasion and metastasis. *Caenorhabditis elegans* and *Drosophila melanogaster* each contain a single Ena/VASP ortholog [42]; genetic analysis in both systems revealed roles for Ena/VASP in neural development [43] and epithelial morphogenesis [44,45]. The vertebrate Ena/VASP proteins play pivotal roles in controlling the movement and morphology [42] of a variety of cell types including fibroblasts [46], endothelial cells [47], epithelial cells [44,48,49] and neurons [47,49,50–53]. Ena/VASP proteins are also required for a variety of chemotactic responses [22,54,55], such as to the axon guidance factors Netrin (a chemoattractant) and Slit (a repulsive cue) [56]. Subsequent work showed that Ena/VASP proteins are required in the early stages of neurite formation to generate filopodia. Unexpectedly, Ena/VASP proteins enable exocytosis, mediated specifically by VAMP2

(vesicle-associated membrane protein 2) which delivers membrane needed for the massive increase in surface area that accompanies neurite formation [57]. Interestingly, one way in which Mena expression drives metastasis is by increasing the amount of secreted protease activity by carcinoma cells [22].

Most cell types express one or more of the Ena/VASP proteins, and these in turn localize to the leading edges of lamellipodia, the tips of filopodia, focal adhesions, cell–cell junctions and, in some cell types, in a sarcomeric pattern along stress fibers [42] (Figure 2). Ena/VASP proteins promote the formation of long sparsely-branched actin filament networks [42,46,56–60] that modulate the morphology and dynamics of membrane protrusions and ultimately affect cell shape and motility [46,50,61]. The number of free barbed ends detected by incubating permeabilized cells with labeled actin correlates directly with Ena/VASP levels [22,47]. Because Ena/VASP proteins do not create new barbed ends by nucleating new actin filaments under physiological conditions, these findings point to a role for Ena/VASP in maintaining polymerization-competent barbed ends *in vivo* [58,62]. The molecular mechanism underlying the ability of Ena/VASP to regulate the geometry of actin network assembly has been recently reviewed (Box 1).

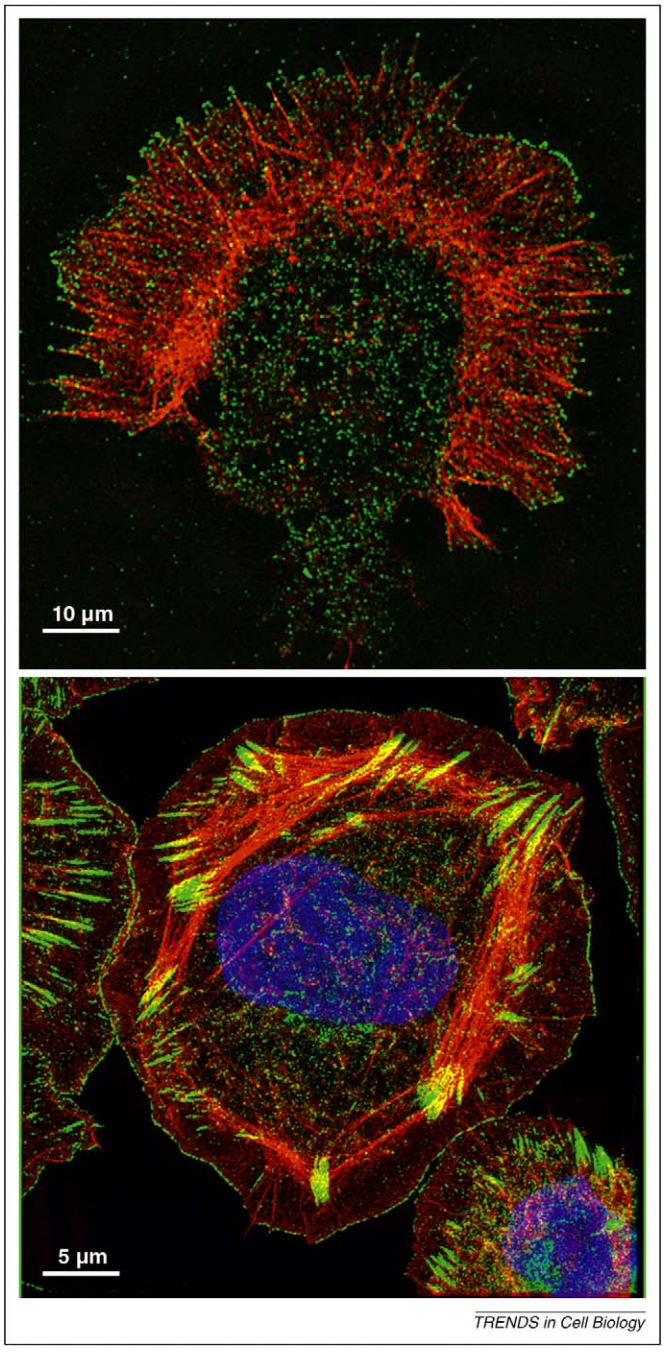

Figure 2. Distribution of Mena in a primary hippocampal growth cone (upper panel) and an MTLn3 carcinoma cell stimulated with EGF (lower panel). Mena signal is in green, F-actin in red, and nuclei are labeled in blue. Mena is concentrated in filopodial tips, lamellipodia and focal adhesions.

Given the effects of Ena/VASP on actin networks it is not surprising that one major function of Ena/VASP is to regulate the dynamics of the unbranched, parallel and bundled actin filaments that comprise filopodia, and that loss of Ena/VASP function greatly impairs or eliminates filopodia formation in neurons and a variety of other cell types [43,51,52,54,63,64]. In some cell types, such as fibroblasts, elevated Ena/VASP activity leads to frequent failure in effective lamellipodial protrusion due to the relatively long sparsely-branched actin networks that buckle in response to the countervailing forces of membrane tension [46]. Other cell types, including carcinoma cells, are equipped to translate the effects of elevated Ena/VASP activity into productive protrusions that lead to cell translocation [22].

Mena isoforms in motility and invasion

Mena contains, as do other Ena/VASP proteins, two conserved domains called EVH1 and EVH2 (for Ena/VASP homology domains 1 and 2) and a central unstructured proline-rich region (Figure 3, Box 2). The EVH1 domain mediates protein–protein interactions important for Ena/VASP localization and regulation (Box 3). The polyproline-rich region and EVH2 interact with the actin monomer binding protein profilin and directly with G- and F-actin, respectively [58].

Mena has several unique features not found in the other Ena/VASP proteins that endow it with the ability to potentiate carcinoma metastasis dramatically. Importantly, alternative splicing produces distinct Mena protein isoforms including an invasion-specific isoform, Mena^{INV} (discussed further below) that has no counterpart in VASP or EVL, and which is found exclusively in invasive tumor cells.

Analysis of the invasion signature of mammary carcinoma cells revealed that Mena expression is upregulated in invasive cells compared to average primary tumor cells [8,10]. Increased Mena levels were also observed in invasive human breast cancers compared to normal mammary tissue [20]. As noted above, perivascular tumor cells expressing high Mena levels are a component of TMEM, a structure whose density in clinical samples correlates with increased risk of metastatic outcome in breast cancer patients [23]. In addition to breast cancer, Mena upregulation has been observed in advanced pancreatic, colon and cervical carcinomas [36–38,65].

Mena has a number of features that its paralogs VASP and EVL do not share. The first is an extended repeat region spanning 70 residues with most of the repeats containing the consensus L/M-E-Q/R-E/Q-Q/R (abbreviated as 'LERER' repeat) which is predicted to form a coiled-coil structure [66].

Box 1. Ena/VASP and the regulation of actin dynamics

How Ena/VASP proteins regulate actin dynamics has been debated in the literature and, because this topic has been reviewed recently [58,74], we will present only a brief overview of the subject. Multiple distinct models for Ena/VASP function have been proposed over the past five years. One model proposes that Ena/VASP interacts with the barbed ends of F-actin filaments and enhances the rate of F-actin polymerization, and delays capping by barbed end capping proteins ('anti-capping') [46,62,75]. An extension of this model suggests that the Ena/VASP interaction with profilin:actin complexes facilitates direct monomer transfer to the barbed ends, increases the rate of filament elongation and enhances the anti-capping activity [62,75,76]. Another study proposed that Ena/VASP has no effect on filament elongation, suggesting instead that Ena/VASP acts solely to bundle filaments that are nucleated by formins, which nucleate linear actin filaments and act as processive (remaining attached to the filament) barbed end elongating factors [77]. However, a third model proposed that Ena/VASP enhances filament elongation but does not have anti-capping activity in solution or utilize profilin for monomer transfer to filaments [78]. In addition, this study suggested that Ena/VASP could block capping of filaments, but only upon dense clustering on beads that also induced a shift to processive filament elongation [78].

All of the studies listed above used either bulk polymerization assays or visual assays in which the actin, but not the Ena/VASP, was labeled. Direct insight into Ena/VASP function by visual, single-molecule assays has been missing from the field. A recent study employing a visual assay with labeled VASP found that VASP binding to the barbed ends of filaments is strongly enhanced by the presence of actin monomer, suggesting that the F-actin binding activity in VASP combined with monomer binding impaired all F-actin binding except to the barbed end, which could accommodate the monomer. Labeled VASP was observed at the tips of elongating filaments and enhanced the rate of filament growth [79]. Therefore, VASP is in fact a processive actin polymerase. VASP also enhances the rate of filament elongation in the presence of profilin, supporting the direct monomer transfer model. Importantly, VASP reduced by sixfold the rate of filament capping by capping protein, proving definitively that it has anti-capping activity [79]. The development of a visual assay for Ena/VASP activity will allow the field to move beyond this debate over whether VASP has anti-capping activity, whether it can utilize profilin-actin for polymerization, and whether it is a processive elongation factor. It will be interesting to see how the other Ena/VASP proteins, and in particular the various Mena isoforms, behave in similar assays.

A further interesting twist to the study of Ena/VASP function comes from several studies suggesting that various Ena/VASP family members interact genetically with members of the formin family of actin nucleation/elongation factors. In some cases Ena/VASP proteins can be co-immunoprecipitated with formin family proteins including mDia1 [80], DdDia2 [77] and *Drosophila* Diaphonous [81]. It is not clear what proportion of the total pools of Ena/VASP and the various formins are in complex with each other, but this is likely to be only a relatively small fraction of each. Although it seems unlikely that Ena/VASP proteins act simply to bundle filaments behind formins, it will be interesting to determine the functional role of these Ena/VASP:formin complexes.

The repeat is located between the EVH1 domain and the proline-rich region. In addition to this unique feature, the Mena message undergoes extensive alternative splicing to give rise to multiple protein isoforms that are expressed in specific tissues and cell-types [41] (Figure 3). By contrast, the *EVL* gene contains one alternatively spliced exon and *VASP* has none. There are 14 constitutively included exons in Mena mRNA and five alternatively spliced exons that can all encode protein sequence in frame. There has not been a comprehensive analysis of which of the possible combinations of alternatively included exons are actually produced as mRNA, nor do we know all of the cell types which produce the various Mena isoforms.

Cloning Mena cDNA from a breast cancer cell line identified the Mena11a isoform [67]. Analysis of RNA from primary mammary tumor cells collected by fluorescence-activated cell sorting, compared to that expressed in invasive mammary tumor cells collected using the *in vivo* invasion assay, revealed that the 11a exon is expressed in tumor cells making up the bulk of the primary tumor, but this exon is essentially undetectable in the mRNA from invasive tumor cells [68]. Consistent with this finding, the 11a exon is specific to Mena isoforms expressed in epithelial cell lines and is not found in mesenchymal cells [65,67]. In fact, the 11a exon becomes excluded in human mammary epithelial cells that are driven to undergo epithelial to mesenchymal transition (EMT) by expression of the EMT-inducing transcription factor Twist [69]. The retention of exon 11a in epithelial cell mRNA is driven in part by the activity of the recently identified epithelial-specific splicing factors ESRP1 and ESRP2 [70]. Mena11a is also expressed in normal ovarian tissue where its inclusion is promoted by the Fox2 splicing factor [71]. Interestingly, analysis of 21 aggressive ovarian tumors revealed a reduction in Fox2 levels compared to normal tissue and a concomitant loss of 11a inclusion in Mena [71]. Therefore, Mena11a appears to be included in epithelial cell and primary carcinoma mRNA but is excluded in mesenchymal cells as well as in invasive/aggressive tumor cells.

The alternatively spliced 11a exon encodes 21 amino acids that are inserted into the EVH2 domain between the FAB sequence and the coiled-coil tetramerization domain. The Mena paralog EVL also has an alternatively spliced 21 amino acid insertion (EVL-I) in a relative location identical to that of the 11a insertion site, but the sequences share no similarity [72]. The site of the 11a insertion is adjacent to the F- and G-actin binding sites, and the 11a insertion can be phosphorylated [67], potentially disrupting actin binding. Therefore, it is possible that the 11a inclusion affects the way in which Mena interacts with barbed ends and adds an extra site for phosphoregulation of Mena function.

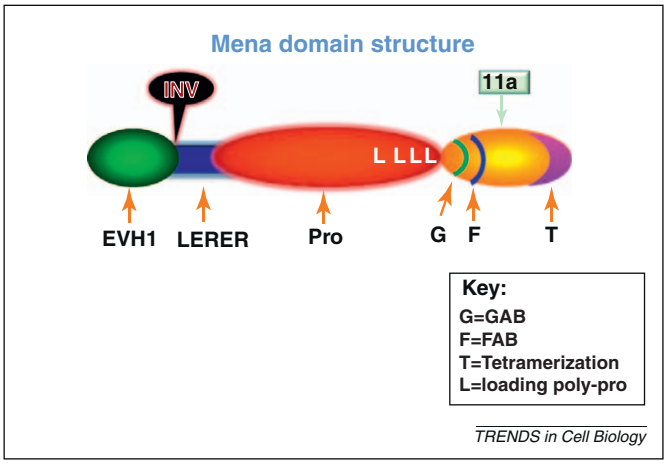

Figure 3. Mena domain structure. A schematic of the Mena domain organization and motif locations as described in the text is depicted. The insertion sites of the alternatively included sequences INV and 11a are indicated.

Box 2. Organization of Ena/VASP proteins

The N-terminal EVH1 domain (for Ena/VASP homology) binds to proteins containing a specific proline-rich motif that helps localize Ena/VASP proteins and recruit them into complexes with signaling proteins.

The middle portion of Ena/VASP proteins consists of a proline-rich region that binds to several SH3- and WW-domain-containing proteins including IRSp53, an I-Bar protein, and the Cdc42 effector that promotes filopodial formation [82,83]. The proline-rich region also binds to the actin-monomer-binding profilin proteins, which play diverse roles in regulating actin dynamics (recently reviewed in [84,85]), including the ability to transfer bound monomer onto free F-actin barbed ends. Profilin can bind actin monomer and interact simultaneously with Ena/VASP through a high-affinity profilin binding-site (termed 'loading site' [76]) with the consensus PPP[A/P]PPLP [76,84,86,87]. Importantly, profilin:actin complexes have a higher affinity for the loading site than does profilin alone, suggesting that once actin monomer is transferred from profilin to a barbed end, exchange of the profilin bound to the loading site for a new profilin:actin complex would be favored [87]. Interestingly, whereas VASP and Evl each have a single loading site, Mena contains four, suggesting that it could have a higher capacity for forming profilin:actin complexes than its paralogs. Importantly, the poly-Pro loading sites in Ena/VASP proteins are located adjacent to actin-binding motifs contained in the EVH2 domain.

The C-terminal EVH2 domain of Ena/VASP contains binding sites for G- and F-actin [88,89], termed GAB and FAB, respectively. The proximity of a poly-Pro loading site permits Ena/VASP to bind profilin+G-actin complexes through two interfaces simultaneously: profilin PPP[A/P]PPLP and the adjacent G-actin+GAB. The G-actin in this complex is oriented towards the FAB motif of Ena/VASP, presumably positioned to be added to growing filaments [76]. The organization of binding sites for profilin, actin monomer and F-actin lead to a model in which profilin:actin binding to the loading site+GAB is followed by direct transfer of the monomer onto the adjacent F-actin barbed end and subsequent exchange of profilin for profilin:actin [84,90].

EVH2-mediated interactions with the growing ends of actin filaments are required for stable targeting of Ena/VASP to the leading edge of lamellipodia [46,63,91]. The GAB motif stabilizes Ena/VASP at the tips of filopodia, suggesting that it plays a role in recognizing barbed ends analogous to the barbed-end capture activity in the highly-related WH2 domain within N-WASP [63]. Both G- and F-actin interactions are disrupted by phosphorylation at sites within the EVH2 domain [62,92], including a protein kinase G site found in both Mena and VASP [41,91].

At the very C-terminus of EVH2, a right-handed coiled-coil mediates both homo-tetramerization and the formation of mixed tetramers containing different family members [93,94]. The combination of tetramerization and F-actin binding allows Ena/VASP to bundle actin filaments [88]; this bundling activity acts to cluster the tips of elongating filaments during filopodial formation and extension [63], however, a physiological role for Ena/VASP bundling along the length of filaments in cells has not been demonstrated.

Three alternatively included Mena exons were identified by screening a mouse brain cDNA library [40]. The largest exon, denoted as '+', falls adjacent to the proline-rich region and the encoded polypeptide is itself quite rich in proline. Mena^{+} is a 798 residue protein (the most widely expressed form of Mena, denoted Mena^{classic}, is 541 amino acids in length); however, owing to their high proline content, both Mena^{+} and Mena^{classic} migrate aberrantly on SDS-PAGE gels at approximately 140 kDa and 80 kDa, respectively. Western blot analysis of adult tissues has shown that the 140 kDa isoform is only readily detected in the brain compared to other organs and tissues [52]. Two other short exons, denoted '++' and '+++' and encoding 4 and 19 residues, respectively, were identified in brain cDNAs containing the '+' exon. Both ++ and +++ are inserted at the same site between the EVH1 domain and the LERER repeat. No tissue-specific expression has been identified for Mena^{++} and Mena^{+++}. Interestingly, the +++ exon is highly conserved in mammals but is not found in other vertebrates.

Box 3. EVH1-mediated interactions

EVH1 domains bind proteins that contain the consensus: [FL]PXφP, where φ is any hydrophobic residue [95,96]. There are a growing number of proteins with EVH1-binding sites and a full discussion of all such molecules is beyond the scope of this review, therefore only a few examples will be presented. The first characterized EVH1-ligand was ActA, a protein found on the surface of the intracellular bacterial pathogen *Listeria monocytogenes* that contains four EVH1-binding motifs; these motifs recruit host cell Ena/VASP proteins to the bacterial surface [96]. *Listeria* employ host-cell proteins to trigger actin polymerization on the bacterial surface to produce a propulsive force that drives their movement [97], and Ena/VASP recruitment by ActA greatly enhances actin polymerization and bacterial movement [86,98]. Zyxin, which helps to recruit Ena/VASP to focal adhesions and stress fibers, contains four EVH1-binding sites [99]. Lamellipodin (Lpd), an adaptor protein containing RA and PH domains that bind Ras and PI(3,4)P2, respectively, harbors six EVH1-binding sites and plays an important role in recruiting Ena/VASP to lamellipodia [100,101]. Silencing Lpd in B16 cells produces a dramatic reduction in F-actin content, thereby eliminating normal lamellipodial protrusion. Lpd is a target for Abl/Arg tyrosine kinases and is required along with Ena/VASP for PDGF-induced dorsal ruffling in fibroblasts [102]; *Drosophila* Lpd is required for normal epithelial morphogenesis [103]. Mig-10, the *C.elegans* Lpd ortholog, is required for cell polarization in response to Netrin and for axon guidance responses to Netrin and Slit [104–106]. The Slit receptor, Robo, binds to Ena/VASP through EVH1-binding sites in its cytoplasmic tail [56]. Palladin, an actin-binding protein and EVH1 ligand [107], has been implicated in metastatic progression; it is upregulated 3.2-fold in the invasion signature [11], contributes to breast cancer cell invasion [108], and is a target for the anti-metastatic kinase Akt-1 – which blocks Palladin-driven invasion [109]. Finally, the putative tumor suppressor TES is an unconventional Mena-specific EVH1 ligand that binds via a LIM domain to a region that overlaps with the [FL]PXφP binding pocket [110].

The majority of Mena mRNA upregulated in the invasive subpopulation of tumor cells isolated from rat, mouse and human mammary tumors using the *in vivo* invasion assay contains either the ++ or +++ exon, whereas strong downregulation of Mena 11a occurs in the same invasive tumor cells. The upregulation of the ++ or +++ exons persists in circulating tumor cells isolated from blood [68]. These results suggest that Mena^{+++} and Mena^{++} are the isoforms that function in metastatic progression.

This prediction has recently been tested [22] (Roussos, X., personal communication) and findings suggest that expression of Mena^{classic}, and Mena^{+++} (referred to as the 'invasion isoform' or Mena^{INV}) in particular, promotes carcinoma cell invasion in 3D collagen gels and increases carcinoma cell motility *in vivo* [22]. Mena^{classic} and Mena^{INV} localize to and stabilize invadopodia, actin-rich protrusions required for degradation and movement through the extracellular matrix and possibly for invasion across basement membranes, thereby increasing the invasive and metastatic potential of tumor cells.

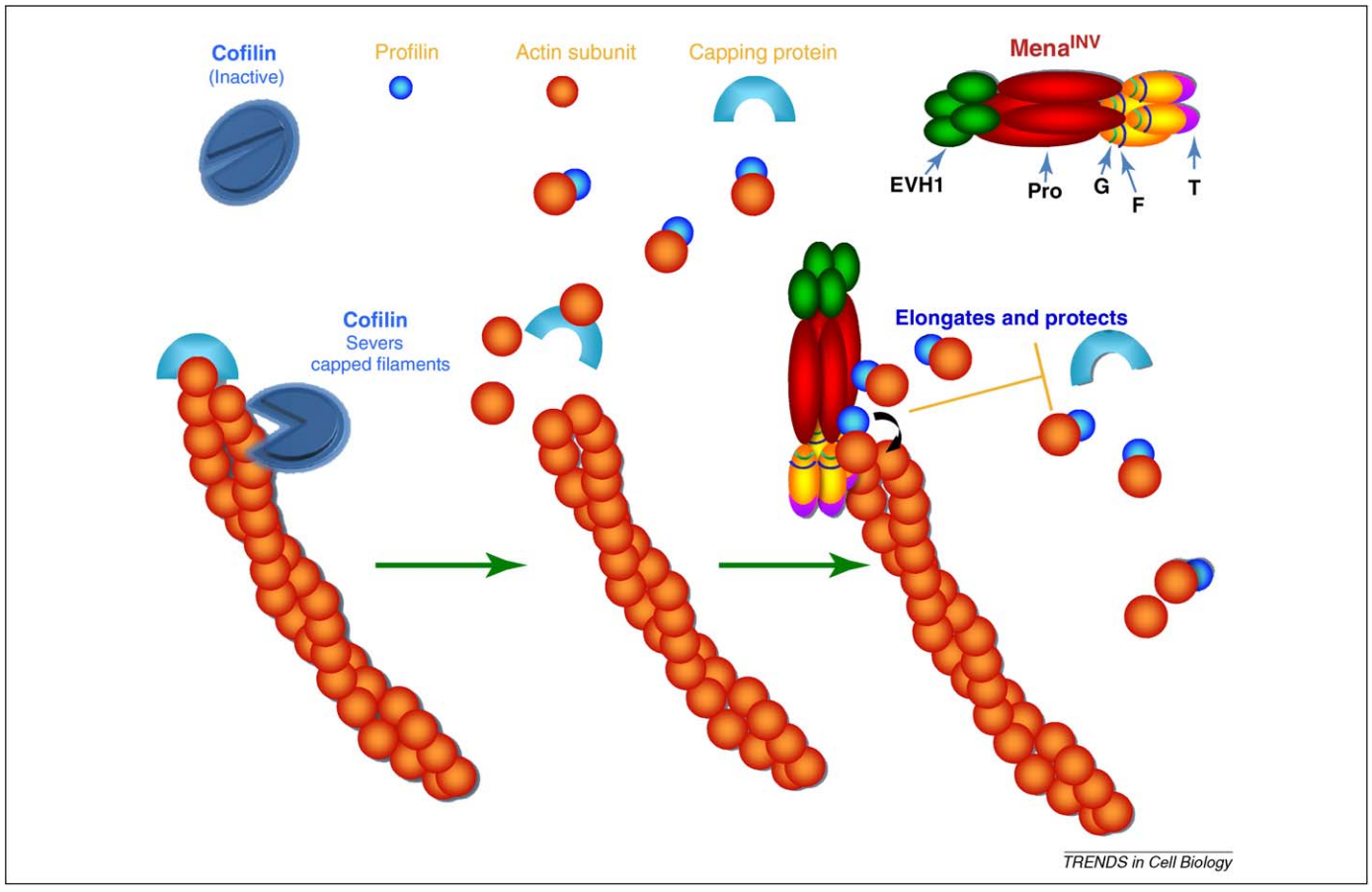

Figure 4. Proposed model for Mena anti-capping/elongation activity in carcinoma cell invasion. Growth factor stimulation leads to activation of cofilin near the plasma membrane. Activated cofilin severs capped filaments to generate free barbed ends. Mena^{INV} binds to the free barbed ends and promotes filament elongation through a combination of direct actin monomer transfer from bound profilin:actin to the filament ends and protection from capping by capping proteins.

Finally, Mena^{INV} plays a sensitizing role in the chemotactic and motility responses of tumor cells to EGF because expression of Mena^{INV} sensitizes mammary tumor cells to EGF signals by at least 25- to 50-fold, causing tumor cells to respond to otherwise undetectable EGF levels [22] (Roussos, X., personal communication). Mena^{INV} regulates the lifetime of actin filament barbed ends produced by EGF-elicited protrusion; within as little as 20 s of stimulation, cells expressing Mena^{INV} have 80% more free barbed ends than control cells or cells expressing Mena^{classic} [22]. The stimulatory effect of Mena^{INV} requires cofilin severing but precedes the accumulation of Arp2/3 in lamellipodia, indicating that Mena^{INV} acts directly on barbed ends generated by cofilin severing. Therefore, we propose that Mena^{INV} exerts this stimulatory effect by delaying barbed end capping (Figure 4). This is an important finding because cofilin-generated barbed ends of actin filaments are needed to initiate invasive protrusions during chemotaxis and maintain the motility of crawling tumor cells [10,22,73]. The mechanisms underlying the effect of the additional 19 amino acids in the Mena^{INV} isoform, and the ability of this isoform to potentiate EGF-dependent motility responses, are under investigation. The present findings, however, indicate that we have identified a master gene that makes breast cancer cells aMENAble to metastasis.

Concluding remarks and future directions

The identification of Mena^{INV} and direct observation of its effects on tumor cell invasion and metastasis were made possible through the use of multiphoton imaging and the *in vivo* invasion assay. The next challenge is to turn these new insights into tools that can be used to diagnose, and potentially treat, metastatic disease. As a component of TMEM, Mena expression is already being used to develop prognostic tests. The development of new probes to the INV and 11a sequences could prove to be even more powerful and straightforward predictors of metastatic spread. Furthermore, because Mena deficiency in mice is compatible with viability, inhibitors of Mena function could be useful tools to prevent metastatic disease. Finally, given the powerful effects of alternative splicing on Mena, it is likely that regulation by splicing will alter the properties of many molecules relevant to morphogenetic cell movements and to cancer onset and progression. Regulation by splicing could be as, or even more, functionally significant than regulation at the level of gene expression. Through the use of new sequencing technologies it should be possible to use the *in vivo* invasion assay to identify the entire repertoire of invasion isoforms.

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